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Theoretical study of lipid biosynthesis in wild-type *Escherichia coli* and in a protoplast-type L-form using elementary flux mode analysis

Dimitar Kenanov[1,*,†], Christoph Kaleta[1,*], Andreas Petzold[2], Christian Hoischen[3], Stephan Diekmann[3], Roman A. Siddiqui[2,‡] and Stefan Schuster[1]

1 Department of Bioinformatics, Friedrich-Schiller University, Jena, Germany
2 Department of Genome Analysis, Fritz Lipmann Institute, Jena, Germany
3 Department of Molecular Biology, Fritz Lipmann Institute, Jena, Germany

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Correspondence
S. Schuster, Department of Bioinformatics, Friedrich-Schiller University, Ernst-Abbe-Platz 2, 07743 Jena, Germany
Fax: +49 3641 946452
Tel: +49 3641 949580
E-mail: stefan.schu@uni-jena.de

*These authors contributed equally to this work

Present address
†Bioinformatics Institute, A*STAR, Matrix, Singapore
‡Department of Infection Biology, Leibniz Institute for Primate Research, Göttingen, Germany

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In the present study, we investigated lipid biosynthesis in the bacterium *Escherichia coli* by mathematical modeling. In particular, we studied the interaction between the subsystems producing unsaturated and saturated fatty acids, phospholipids, lipid A, and cardiolipin. The present analysis was carried out both for the wild-type and for several *in silico* knockout mutants, using the concept of elementary flux modes. Our results confirm that, in the wild type, there are four main products: L1-phosphatidylethanolamine, lipid A, lipid A (cold-adapted), and cardiolipin. We found that each of these compounds is produced on several different routes, indicating a high redundancy of the system under study. By analysis of the elementary flux modes remaining after the knockout of genes of lipid biosynthesis, and comparison with publicly available data on single-gene knockouts *in vivo*, we were able to determine the metabolites essential for the survival of the cell. Furthermore, we analyzed a set of mutations that occur in a cell wall-free mutant of *Escherichia coli* W1655F+. We postulate that the mutant is not capable of producing both forms of lipid A, when the combination of mutations is considered to make a nonfunctional pathway. This is in contrast to gene essentiality data showing that lipid A synthesis is indispensable for the survival of the cell. The loss of the outer membrane in the cell wall-free mutant, however, shows that lipid A is dispensable as the main component of the outer surface structure in this particular *E. coli* strain.

Abbreviations
AccA, AccC, AccD, acetyl CoA carboxylase; CdsA, CDP-diglyceride synthetase; Cl, cardiolipin; Cls, cardiolipin synthase; EFM, elementary flux mode; FabA_1, beta-hydroxyacyl-ACP dehydratase; FabA_2, beta-hydroxydecanoyl-ACP dehydratase; FabA_3, trans-2-decenoyl-ACP isomerase; FabB_1, FabB_2, FabB_4, beta-ketoacyl-ACP synthase I; FabB_3, malonyl-ACP decarboxylase; FabD, malonyl-CoA-ACP transacylase; FabF_1, FabF_2, beta-ketoacyl-ACP synthase II; FabG_1, FabG_2, beta-ketoacyl-ACP reductase; FabH_1, beta-ketoacyl-ACP synthase III; FabH_2, acetyl-CoA:ACP transacylase; FabI, enoyl-ACP reductase (NAD[P]H); FabZ_1, FabZ_2, beta-hydroxyacyl-ACP dehydratase; GpsA, glycerol-3-phosphate-dehydrogenase; GutQ, arabinose 5-phosphate isomerase; KdsA, 3-deoxy-D-manno-octulosonic acid 8-phosphate synthase; KdsB, 3-deoxy-D-manno-octulosonatecytidylyltransferase; KdsC, 3-deoxy-D-manno-octulosonate 8-phosphate phosphatise; KdsD, arabinose 5-phosphate isomerase; KdtA_1, KdtA_2, KDO transferase; PEA, L1-P-EtAmine, L1-phosphatidylethanolamine; lipid A (ca), lipid A cold-adapted form; LpxA, UDP-*N*-acetylglucosamine acyltransferase; LpxB, lipid A disaccharide synthase; LpxC, UDP-3-O-acyl-*N*-acetylglucosamine deacetylase; LpxD, UDP-3-*O*-[3-hydroxymyristoyl]-glucosamine *N*-acetyltransferase; LpxH, UDP-2,3-diacylglucosamine hydrolase; LpxK, tetraacyldisaccharide 4′-kinase; LpxL, lauroyl acyltransferase; LpxM_1, LpxM_2, myristoyl acyltransferase; LpxP, palmitoleoyl acyltransferase; PgpA, phosphatidylglycerophosphatase A; PgpB, phosphatidylglycerophosphatase B; PgsA, phosphatidylglycerophosphate synthase; PlsB, glycerol-3-phosphate acyltransferase; PlsC, 1-acylglycerol-3-phosphate acyltransferase; Psd, phosphatidylserine decarboxylase; PssA, phosphatidylserine synthase.

Introduction

Lipid biosynthesis is a complex subsystem of metabolism, because of the chain elongation reactions of fatty acids and the combinatorial complexity in the composition of different phospholipids, triglycerides, and other lipid species. Understanding this complex network is of practical relevance in view of medical, pharmaceutical and biotechnological applications [1,2]. Here, we analyze lipid biosynthesis in the bacterium *Escherichia coli*. We elucidate the interaction between the subsystems involved in the synthesis of unsaturated and saturated fatty acids, phospholipids (including cardiolipin), and lipid A. It is important to study the metabolism of the glucosamine-based lipid A, because it is a major constituent of the outer membrane of the cell wall of so-called Gram-negative bacteria, and helps them to survive during environmental stress [3]. Moreover, lipid A also plays a crucial role in sepsis, because it is the glycolipid core of lipopolysaccharide, also known as endotoxin [4,5]. Although lipid A was previously believed to exist in prokaryotes only, there is recent evidence that it also occurs in the chloroplasts of several plants [6].

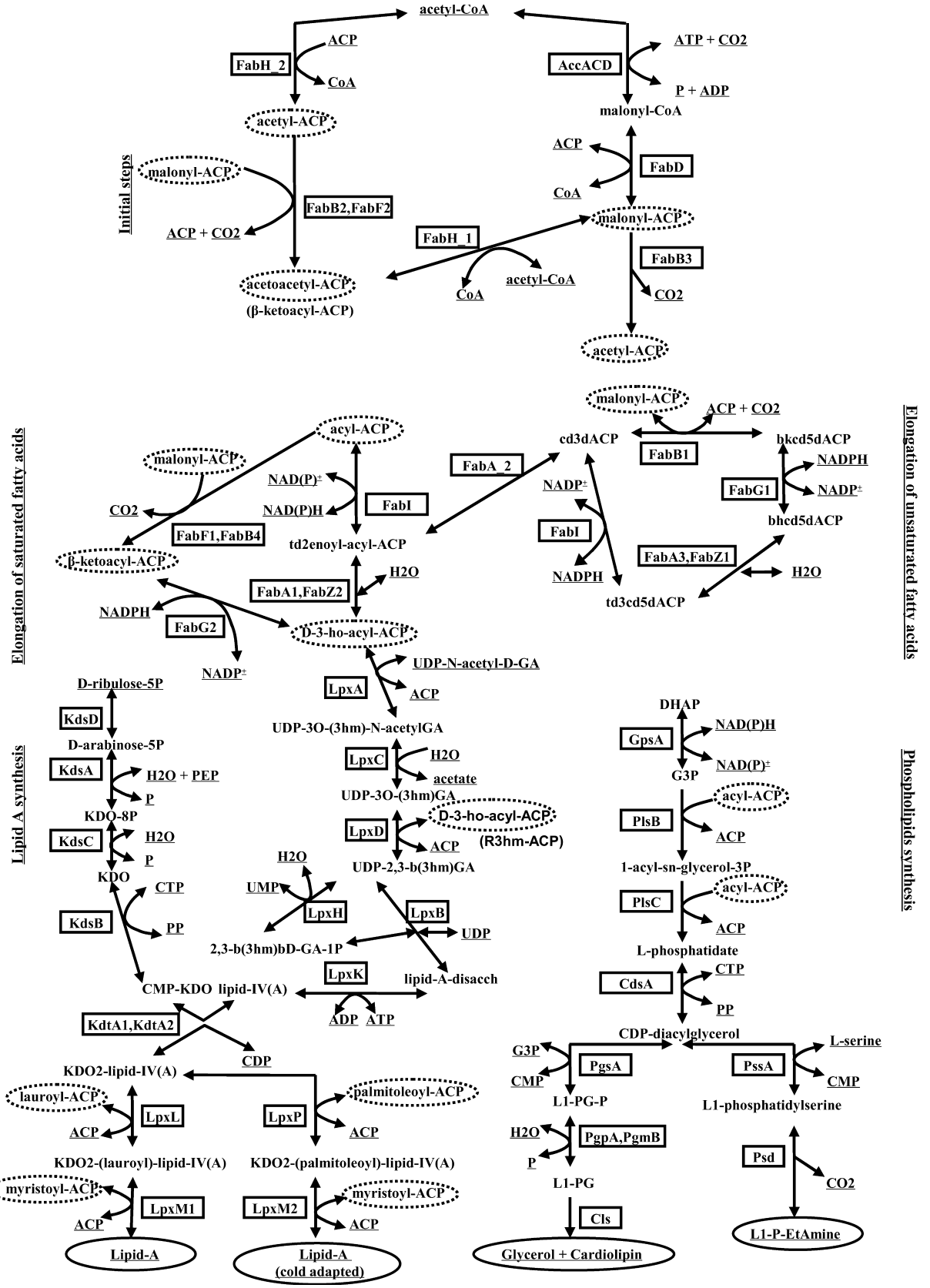
Cell wall-free bacteria, with the exception of *Mycoplasma*, are rather uncommon in the prokaryotic tree of life. Interestingly, however, experimental findings have shown that several other bacterial species can grow without a protecting cell wall, and these have been collectively termed 'stable L-forms' [7–9]. Such L-form mutants are also known from a Gram-negative *E. coli* laboratory strain showing no outer membrane structures (strain LW1655F+ [10]), which are typical for this model bacterium [11–13]. In particular, it has remained elusive how *E. coli* may have been able to shut off the biosynthesis of this essential cell structure. In the context of our study on lipid biosynthesis, we aimed at determining which of the membrane constituents and products may still be produced by such an L-form mutant, and investigated whether hitherto unknown bypass mechanisms exist. This should prove useful for understanding such morphogenetic changes in more detail.

Our theoretical study is based on the concept of 'elementary flux modes' (EFMs). An EFM corresponds to a minimal set of enzymes that can operate at stationary state with all of the irreversible reactions carrying flux only in the thermodynamically feasible direction [14–16]. Thus, all intermediates, called internal metabolites, are balanced with respect to production and consumption. In contrast, source and sink compounds, called external metabolites, are considered to have buffered concentrations and need not to be balanced. If only the enzymes belonging to one EFM are operative and, thereafter, one of the enzymes is completely inhibited, then the remaining enzymes can no longer function, because the system can no longer maintain a steady state. Thus, EFMs represent a formal definition of the concept of 'metabolic pathway' used in biochemistry on an intuitive basis.

EFM analysis opens up the possibility of studying the various modes of behavior of a biochemical system, and allows the detection of possible bypasses. It gives an idea of how redundant or, in other words, how flexible the biochemical system is, and in what molar yields the products of interest are synthesized. This tool enables us to study the interaction between several subsystems, utilizing substrates of interest or systems with enzyme deficiencies or knockouts. Thus, it can be used in the investigation of diseases caused by these deficiencies [17]. EFM analysis has been employed on various organisms [17–19]. For example, a catabolic pathway that is an alternative to the Krebs cycle was predicted by EFM analysis in [14], and found later by experiment [20]. The elementary modes in nucleotide metabolism in *Mycoplasma pneumoniae*, which does not have a cell wall, have also been analyzed [21]. Moreover, the complexity of the computation of elementary modes has been analyzed recently [22].

In the present work, we studied the metabolic capabilities of lipid metabolism in the wild type in comparison to several '*in silico* mutants' of *E. coli*. These mutants are characterized by various enzyme deficiencies, with one of these corresponding to the above-mentioned cell wall-free *E. coli* L-form. This allowed us to estimate the significance of enzymes of lipid biosynthesis and to deduce the metabolic capabilities of the L-form.

Fig. 1. Lipid biosynthesis in *E. coli*. Symbols in boxes represent enzymes. Underlined metabolites are set to external status. Products of interest are indicated by ellipses. In the elongation of saturated and unsaturated fatty acids, metabolites correspond to fatty acids of different chain lengths: lauroyl-ACP corresponds to an acyl-ACP of length 12, myristoyl-ACP corresponds to an acyl-ACP of length 14, and palmitoleoyl-ACP corresponds to a cd3dACP of length 16. Metabolites encircled by dashed lines appear several times in the representation. For symbols, see list of abbreviations and Tables 5 and 6. Bidirectional arrows indicate reversible reactions.


acetyl-CoA
ACP
FabH_2
CoA
acetyl-ACP
Initial steps
malonyl-ACP
FabB2,FabF2
ACP + CO2
acetoacetyl-ACP
(β-ketoacyl-ACP)
AccACD
ATP + CO2
P + ADP
malonyl-CoA
ACP
FabD
CoA
malonyl-ACP
FabH_1
CoA
acetyl-CoA
FabB3
CO2
acetyl-ACP
Elongation of saturated fatty acids
acyl-ACP
malonyl-ACP
NAD(P)±
NAD(P)H
FabI
CO2
FabF1,FabB4
β-ketoacyl-ACP
td2enoyl-acyl-ACP
H2O
FabA1,FabZ2
NADPH
FabG2
D-3-ho-acyl-ACP
NADP±
malonyl-ACP
ACP + CO2
cd3dACP
FabB1
bkcd5dACP
FabA_2
NADPH
NADP±
FabG1
bhcd5dACP
NADP±
FabI
NADPH
FabA3,FabZ1
H2O
td3cd5dACP
Elongation of unsaturated fatty acids
Lipid A synthesis
D-ribulose-5P
KdsD
D-arabinose-5P
KdsA
H2O + PEP
P
KDO-8P
KdsC
H2O
P
KDO
CTP
KdsB
PP
CMP-KDO lipid-IV(A)
KdtA1,KdtA2
CDP
KDO2-lipid-IV(A)
lauroyl-ACP
LpxL
ACP
KDO2-(lauroyl)-lipid-IV(A)
myristoyl-ACP
LpxM1
ACP
Lipid-A
palmitoleoyl-ACP
LpxP
ACP
KDO2-(palmitoleoyl)-lipid-IV(A)
LpxM2
myristoyl-ACP
ACP
Lipid-A
(cold adapted)
D-3-ho-acyl-ACP
UDP-N-acetyl-D-GA
LpxA
ACP
UDP-3O-(3hm)-N-acetylGA
LpxC
H2O
acetate
UDP-3O-(3hm)GA
LpxD
D-3-ho-acyl-ACP
(R3hm-ACP)
ACP
UDP-2,3-b(3hm)GA
H2O
UMP
LpxH
LpxB
UDP
2,3-b(3hm)bD-GA-1P
LpxK
lipid-A-disacch
ADP ATP
Phospholipids synthesis
DHAP
GpsA
NAD(P)H
NAD(P)±
G3P
acyl-ACP
PlsB
ACP
1-acyl-sn-glycerol-3P
acyl-ACP
PlsC
ACP
L-phosphatidate
CdsA
CTP
PP
CDP-diacylglycerol
G3P
CMP
PgsA
L1-PG-P
H2O
P
PgpA,PgmB
L1-PG
Cls
Glycerol + Cardiolipin
L-serine
CMP
PssA
L1-phosphatidylserine
Psd
CO2
L1-P-EtAmine

Results

Lipid biosynthesis in *E. coli*

The part of lipid metabolism studied here is depicted in Fig. 1. An SBML model of the system can be found in Doc. S3. Here, we introduce the terms 'core' and 'side' elementary modes. A core mode is defined as a mode that leads to a desired product, but uses no other products of interest as substrates. By the terms 'desired product', 'product of interest', or 'metabolite of interest', we refer to lipid A, lipid A cold-adapted form [lipid A (ca)], L1-phosphatidylethanolamine (L1-P-EtAmine), and cardiolipin. An example of a core mode is an EFM that produces lipid A without using any of the other products of interest, namely lipid A (ca), L1-P-EtAmine, or cardiolipin, as substrates. In contrast, side modes use products of interest as substrates. An example of such a side mode is an EFM that converts lipid A into lipid A (ca).

Redundancy as a main characteristic of the wild-type system

First, we performed an *in silico* study of the normal (wild-type) system (Fig. 1). In the wild-type system, we found 168 EFMs (Doc. S1). In Doc. S2, they are described briefly with respect to substrates, products, and ATP and NAD(P)H requirements. One of the 168 EFMs in the intact system represents a futile cycle, composed of the enzymes FabD, FabH_2, FabB_3, and AccACD. In this cycle, acetyl-CoA is carboxylated (driven by ATP hydrolysis) and decarboxylated again (Fig. 2). The remaining EFMs are capable of producing all of the main metabolites that we are interested in.

Obviously, in order to produce one of the forms of lipid A or phospholipids, the production of fatty acids must be intact, because the anabolism of both types of compounds requires products from both saturated and unsaturated fatty acid biosynthesis. Interestingly, the results show a relatively high degree of redundancy in the synthetic pathways; that is, each end-product is synthesized by more than one route. All of the four products under consideration can be produced by at least 24 core EFMs. For example, the core EFMs producing one of the two forms of lipid A comprise 24 EFMs in the case of lipid A and 51 for lipid A (ca). In addition, there are a number of side EFMs forming lipid A [or lipid A (ca)] and, simultaneously, other products of interest.

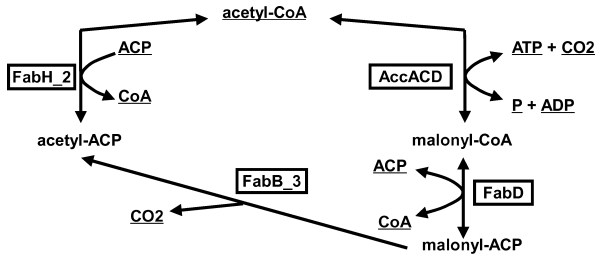


Fig. 2. Futile cycle in the lipid biosynthesis of *E. coli*. Symbols in boxes represent enzymes. Underlined metabolites are set to external status. For symbols, see list of abbreviations and Tables 5 and 6. Bidirectional arrows indicate reversible reactions.

Different, parallel EFMs forming the same product need not have the same molar yield (product/substrate ratio) [14,15]. Indeed, the mole number of ATP needed for one mole of lipid A in the core EFMs varies between 36 and 42. In contrast, the amount of NAD(P)H is 54 per mole of lipid A in all of these EFMs. In this context, we also found several EFMs producing lipid A (ca) from lipid A with a net gain; that is, more moles of lipid A (ca) are produced than moles of lipid A are consumed (see Doc. S2 for more details). This is reminiscent of the ATP production and ATP consumption with a net gain observed in nucleotide salvage pathways [17]. However, these pathways would only be of significance if each of the forms of lipid A could be reimported from the outer membrane into the cytosol, as most of the lipid A is found in the former compartment. According to the EcoCyc database [23], such a transport pathway does not exist.

There are 36 EFMs in total that are able to produce cardiolipin, 24 of which are core EFMs. The same numbers are found for the EFMs producing L1-P-EtAmine. An overview of the detected EFMs and their energetic requirements in terms of moles of ATP hydrolyzed and NADPH oxidized per mole of product of interest produced is given in Table 1.

Key enzymes of lipid biosynthesis and contribution to metabolic capacity

Next, we analyzed in detail the effects of the knockout of several key enzymes of lipid biosynthesis on the

Table 1. Overview of EFMs in the wild-type system. The energetic requirements are given for the core modes only. For further details, see Doc. S1 and Doc. S2.

	EFMs		Energetic requirements [moles]	
Product	Core	Side	ATP	NADPH
Lipid A	24	12	36–42	54
Lipid A (ca)	51	32	38–44	57
Cardiolipin	24	12	28–32	52
L1-P-EtAmine	24	12	14–16	25

metabolic capacity of the cell. These knockouts were simulated by removing those elementary modes from the wild-type system that contained the reactions that were no longer available after the *in silico* knockout of the respective genes. Several of the studied enzymes contain mutations in the cell wall-free mutant. By examining the mutations in the corresponding genes, we were able to deduce which proteins might still be functional in the mutant. Subsequently, we extended this analysis to the study of the effect of every possible *in silico* single-gene knockout on lipid biosynthesis. In comparison with *in vivo* data from the Keio collection [24], this allowed us to estimate the essentiality of the membrane constituents produced in the cell. An overview of the different scenarios analyzed here is given in Table 2, and in Table 4 below.

CDP-diglyceride synthetase (CdsA)/glycerol-3-phosphate dehydrogenase (PlsB) deficiency

The enzymes CdsA and PlsB occupy a central position in phospholipid metabolism. The metabolites produced by both enzymes are converted into either glycerol and cardiolipin, or L1-P-EtAmine. Eliminating either of the two enzymes reduces the possible pathways by $\sim 50\%$ (95 EFMs remain), and only the two forms of lipid A are still produced. According to the Keio collection, these enzymes are essential.

Malonyl-CoA-ACP transacylase (FabD) deficiency

According to our analysis, FabD is an essential enzyme for lipid metabolism - after it is removed from the system, there is no EFM left. This can be seen from Fig. 1: malonyl-ACP, which is produced by FabD only, is used by FabB_3 and FabH_1, and in the combined reaction 'FabB_2, FabF_2', so that no branch of the system can operate after knockout of FabD. This is also corroborated by data from the Keio collection indicating that FabD is essential for *E. coli*.

Table 2. Number of EFMs for the metabolites of interest appearing in the different simulations performed. The last column indicates whether the organism is still viable after an *in vivo* knockout of the corresponding genes.

	Lipid A		Lipid A (ca)		Cardiolipin		L1-P-EtAmine		
Deficiency	Core	Side	Core	Side	Core	Side	Core	Side	Viable
No deficiency	24	12	51	32	24	12	24	12	Yes
CdsA	24	–	51	20	–	–	–	–	No
Cls	24	6	51	26	–	–	24	12	Yes
FabD	–	–	–	–	–	–	–	–	No
KdsA	–	12	–	14	24	12	24	12	No
KdtA	–	12	–	14	24	12	24	12	No
LpxA	–	12	–	14	24	12	24	12	No
LpxL	–	–	51	–	24	–	24	–	Yes
LpxP	24	–	–	–	24	–	24	–	Yes
PlsB	24	–	51	20	–	–	–	–	No
Psd	24	6	51	26	24	12	–	–	No

3-Deoxy-d-manno-octulosonic acid-8-phosphate synthase (KdsA)/KDO transferase (KdtA)/UDP-*N*-acetylglucosamine acyltransferase (LpxA) deficiency

With deficiency of either KdsA, KdtA or LpxA in the system, the calculation resulted in 75 modes in total. There are 12 modes for producing lipid A and 14 for the cold-adapted form. We found that six of the former 12 modes and six of the latter 14 modes produced L1-P-EtAmine as well. The rest of the modes from both groups coproduced cardiolipin. The analysis shows that there is not a single EFM producing lipid A without using its cold-adapted form as initial substrate and vice versa. This implies that, with either of these enzymes missing, lipid A synthesis is no longer feasible, as intermediates that are essential in the biosynthesis of both lipid A forms can no longer be produced. In the Keio collection, *kdsA*, *kdtA* and *lpxA* are noted as essential genes.

Palmitoleoyl acyltransferase (LpxP) deficiency

Removing LpxP prevents the production of lipid A (ca). It also blocks the use of LpxM_2 in its reverse mode. The other products of interest can still be synthesized. This gene is noted as nonessential in the Keio collection.

Lauroyl acyltransferase (LpxL) deficiency

Deleting LpxL totally eliminates the production of the 'normal' form of lipid A. Similar to the case of LpxP, even the reverse mode of the enzymatic reaction LpxM_1 is blocked. The other products of interest can still be synthesized. This deficiency redirects the production to lipid A (ca). The simulation revealed that this system preserved the 51 EFMs for the production of lipid A (ca) present in the intact system. The requirements for ATP and NAD(P)H of these modes are the same as in the unperturbed system. Data from the Keio collection indicate that *lpxL* is also nonessential.

Cardiolipin synthase (Cls) deficiency

For the deficiency of Cls, 132 modes were found in total. The calculation for this system demonstrated

that the production of metabolites of interest is not blocked, except for cardiolipin. Furthermore, this deficiency affected the number of modes that exist for producing both forms of lipid A and L1-P-EtAmine. As already noted in [25] and according to the Keio collection, the knockout of *cls* is nonlethal. Thus, the production of cardiolipin is not required for the survival of *E. coli* [25]. However, cardiolipin synthesis in the L-form might be of more importance, as higher concentrations of this compound were found in the mutant than in the wild type [26]. As cardiolipin was found to have a stabilizing effect on membranes [27], the higher concentrations of this compound might be necessary to partially compensate for the instabilities in the inner membrane caused by the loss of the cell wall and the outer membrane.

Impact of the deficiencies in the cell wall-free mutant

In the cell wall-free mutant, two genes of lipid biosynthesis contain synonymous mutations, and an additional four genes contain nonsynonymous mutations (Table 3). Even though synonymous, the two mutations in *kdsA* and *kdtA* might have an impact on the expression of the encoded proteins, owing to a changed codon bias [28]. In the case of the nonsynonymous mutations in *cls*, *fabD*, *lpxB*, and *plsB*, further clues about the effects of the mutations can be obtained from the analysis of the sequence and the structure of the corresponding proteins. Whereas there is no resolved structure for Cls and PlsB, those of LpxA and FabD bound to their substrates are known [29,30]. Furthermore, putative active sites have been determined for all four proteins [29,31–34]. At the sequence level, the mutations in PlsB, Cls and FabD appear to be far away from the putative active sites. In LpxA, which catalyzes the first committed step in lipid A biosynthesis, a methionine is exchanged for an isoleucine at position 118, which is close to a known active site at positions 122 and 125. Of these, the latter is the catalytic residue, and the former is involved in substrate binding [34]. Examination of the structure of LpxA bound to its substrate substantiates the close proximity of the methionine to the substrate. Thus, this residue is probably involved in substrate binding. Hence, the mutation might have abolished the catalytic activity of LpxA, which leads to the inability of the L-form to produce lipid A, as indicated by our analysis of the EFMs in the *in silico* knockout mutant. These results are in agreement with the finding that lipid A is no longer detectable in the L-form (Siddiqui *et al.*, unpublished results). Furthermore, electronmicrographs indicate the absence of any outer membrane in the mutant (Siddiqui *et al.*, unpublished results). Normally, lipid A accumulates to toxic concentrations if it cannot be exported into the outer membrane [35]. Thus, the impairment in lipid A production could partially explain why the L-form cannot form an outer membrane like wild-type *E. coli*.

In FabD, a glutamate is replaced by an alanine at position 35. Although this position is far away from the active site, the replacement of the negatively charged amino acid could have implications for the folding of the molecule, and thus influence the activity of the enzyme.

Large-scale analysis of substrate production and residual metabolic capacity in single-gene knockout mutants

We analyzed the complete set of single-gene knockouts of the system, and compared our results with data available from the Keio collection (Table 4). The aim of this analysis was to identify which metabolites can still be produced after a knockout and the relation of this to the viability of the organism.

No coherent picture can be drawn at first glance. For instance, suppressing the production of both forms of lipid A is predicted to be lethal in nine cases and nonlethal in two cases. The two contradictory cases are the knockout of *kdsC* and *lpxM*. The encoded enzymes catalyze essential steps in the formation of both forms of lipid A. However, the step catalyzed by

Table 3. Mutations in the cell wall-free mutant affecting enzymes of the system analyzed here. For synonymous mutations, the codon that has been exchanged is indicated.

Protein	Position	Exchange	Protein	Position	Exchange
Cls	13	Ile → Thr	PlsB	265	Arg → Ser
	32	Arg → Cyt		277	Arg → Leu
	305	Gly → Ser			
FabD	35	Glu → Ala	LpxA	118	Met → Ile
KdsA (synonymous)	831	GCG → GCT	KdtA (synonymous)	219	GGC → GGU

Table 4. Metabolites of interest still producible by core EFMs after single-gene knockouts, and comparison with *in vivo* viability data from the Keio collection.

Deficiency	Lipid A	Lipid A (ca)	PEA	CL	Viable	Deficiency	Lipid A	Lipid A (ca)	PEA	CL	Viable
AccACD					No	KdtA			x	x	No
CdsA	x	x			No	LpxA			x	x	No
Cls	x	x	x		Yes	LpxB			x	x	No
FabA	x		x	x	No	LpxC			x	x	No
FabB	x		x	x	No	LpxD			x	x	No
FabD					No	LpxH			x	x	No
FabF	x	x	x	x	Yes	LpxK			x	x	No
FabG					No	LpxL		x	x	x	Yes
FabH	x	x	x	x	Yes	LpxM			x	x	Yes
FabI					No	LpxP	x		x	x	Yes
FabZ	x	x	x	x	No	PgpA	x	x	x	x	Yes
GpsA	x	x			No	PgpB	x	x	x	x	Yes
GutQ	x	x	x	x	Yes	PgsA	x	x	x		No
KdsA			x	x	No	PlsB	x	x			No
KdsB			x	x	No	PlsC	x	x			No
KdsC			x	x	Yes	Psd	x	x		x	No
KdsD	x	x	x	x	Yes	PssA	x	x		x	No

KdsC might also be performed by an unspecific phosphatase, and thus limited lipid A production might still be possible [36]. LpxM, in contrast, catalyzes the final step of the incorporation of myristoate into both forms of lipid A. *In vivo* data suggest that this step is not crucial, and that the cell can also survive with lipid A lacking the myristoyl side chain, even though it is more susceptible to antibiotics [37]. Thus, the terminal products of the biosynthesis of both forms of lipid A are not required for survival of the cell.

Another interesting case can be found in the knockout of *fabZ*, the protein product of which catalyzes several steps in the unsaturated and saturated branches of fatty acid chain elongation. Here, our model predicts that all metabolites of interest are still producible. However, the knockout is found to be lethal *in vivo*. This is interesting, insofar as *fabA* encodes another protein (FabA) that can perform the same functions as FabZ [38], and hence all metabolites should still be producible *in vivo* according to our model. An explanation for the difference between the *in silico* predictions and the *in vivo* data can be found in the different affinities of the proteins for their substrates. Thus, FabZ is more efficient in the elongation of unsaturated fatty acids, and a knockout might result in overproduction of saturated fatty acids and reduced production of unsaturated fatty acids by FabA, leading to the lethality of the knockout [38].

As noted above, cardiolipin is not essential for the survival of the cell. Nevertheless, the knockout of *pgsA* is predicted to be lethal, even though cardiolipin is the only metabolite of interest that is not produced. However, the knockout of *pgsA* additionally prevents the production of phosphatidylglycerol, which is an essential membrane lipid in *E. coli*.

A clear picture can be derived from the cases in which the synthesis of L1-P-EtAmine is prevented. As all corresponding knockouts are lethal, this metabolite is essential for the survival of the cell. This is especially apparent from the lethal knockouts of *psd* or *pssA*. In both cases, only the production of L1-P-EtAmine is suppressed.

It is known that *E. coli* can survive even if only one form of lipid A can be produced [37]. However, in two cases in which only lipid A (ca) production is prevented, the corresponding knockout is found to be lethal *in vivo*. These cases are the knockout of *fabA* and *fabB*. The reason for this discrepancy is that both enzymes are essential in the production of unsaturated fatty acids [39]. Unsaturated fatty acids are also essential for processes not present in our model. Hence, the lethality of the knockouts is due not to the absence of lipid A (ca), but to other processes beyond the scope of our model.

Discussion

In the present theoretical study, we have established a network model of lipid biosynthesis in *E. coli*. We applied metabolic pathway analysis to this model. In an earlier study by Stelling *et al.* [40], lipid metabolism was included in a general, overall model of central metabolism in a simplified way. *E. coli* metabolism has been investigated [41–43] in several studies using flux

balance analysis [44]. However, to our knowledge, metabolic pathway analysis has not been used specifically for lipid biosynthesis in *E. coli* before in so much detail. Nevertheless, an analysis of pathways in a large-scale network using elementary flux patterns [45], an extension of the concept of EFMs to genome-scale metabolic networks, is an interesting possibility for further work.

For the full lipid system in the wild type, we have found 168 EFMs. One of these is a futile cycle. It has been shown previously that EFM analysis is a suitable tool for finding all futile cycles [15]. Several hypotheses concerning the physiological significance of such cycles have been proposed [46].

We studied the system's behavior after *in silico* deletion of enzymes that we considered to be important for the network. Among these were also enzymes that were found to contain mutations in a cell wall-free mutant. Examination of the EFMs remaining in the deficient system allowed us to estimate the significance of those enzymes. The investigation also gave an idea of how redundant or, in other words, how flexible the biochemical system is. Furthermore, we determined the metabolites of interest that could still be produced after knockout of each of the genes concerned with lipid biosynthesis, and compared our results with *in vivo* viability data. This allowed us to determine which metabolites are essential for the survival of the cell. Thus, we found that, whereas cardiolipin is dispensable, L1-P-EtAmine is essential. In the case of lipid A, at least one form is required while it can lack the myristoyl side chain.

We focused on EFMs that can produce metabolites of interest without using other such metabolites as substrates. We call those EFMs core modes, in contrast to the side modes. Considering that our main interest lies in the production of some end-products, we could regard the core modes as the main pathways. The side modes, in contrast, give some additional flexibility to the system, as they are able to interconvert the end-products that we are interested in. In the case when only side modes remain, they can usually work only when there is a reserve of a particular metabolite or when this metabolite can be fed to the system externally. This is the case for the KdtA deficiency, where lipid A (ca) production depends solely on the presence of lipid A in the cell. It might be possible to introduce lipid A to the cell in its lamellar form, as Sekimizu *et al.* [47] did with cardiolipin for *E. coli*. However, under normal conditions, reimport of both forms of lipid A from the outer membrane into the cytosol is not possible, reducing the significance of those side modes that use lipid A or lipid A (ca) as substrates.

Interestingly, our theoretical results correspond to an observation made *in vivo*. Wild-type *E. coli* under the appropriate condition (cold shock) produced lipid A (ca) to lipid A in the ratio 2 : 1 [37,48]. As our model includes all of the enzymes involved in lipid A metabolism, our simulation corresponds to a cold-adapted *E. coli*. Our results confirm that the system does indeed produce two-thirds lipid A (ca). In this case, we calculated that for production of lipid A, 24 EFMs exist, whereas for lipid A (ca), the number is 51, which is about two-thirds of all modes producing one form of lipid A. Every EFM leading to one of the forms of lipid A produces one mole of lipid A or lipid A (ca). Thus, assuming that all EFMs carry about the same flux, it could be argued that the fractional number of possible EFMs corresponds to the possible fractional quantity of lipid A produced in the studied system. Although perhaps questionable, it is the most straightforward assumption as long as we do not have any other information about fluxes.

The simulation of enzyme deficiencies revealed a particular behavior of the subsystem responsible for the production of the two forms of lipid A. This behavior is caused by the relative linearity of this subsystem. That is why some deficiencies are either redirecting the production towards one of the lipid A forms (LpxP or LpxL) or suppress the production of both forms totally (KdtA, KdsA, and LpxA). These enzymes prevent the core modes from functioning, and there are other enzymes that disturb the side modes. An example of such an enzyme is CdsA. Removing this enzyme reduced the number of side modes producing lipid A (ca) and suppressed all side modes producing lipid A. Another enzyme of this kind is Cls. According to [25], Cls is a dispensable enzyme. Our analysis reveals that Cls deficiency has a negative effect on the modes producing lipid A. This deficiency removes one-half of the side modes for lipid A. Thus, we can speculate that the side modes do not strongly affect the viability of *E. coli*, and might therefore be dispensable.

Our analysis also demonstrates the interactions of the different subsystems in lipid biosynthesis. Some of them can be observed in Table 2. For example, both Psd and Cls deficiencies have the same effect on lipid A metabolism. On the other hand, deficiencies in lipid A metabolism affect the metabolism of phospholipids as well. Both LpxL and LpxP deficiencies disallow any side modes for production of cardiolipin and L1-P-EtAmine in the system. Deficiencies of KdtA, KdsA and LpxA do not have any effect on the metabolism of phospholipids.

Our results show that lipid biosynthesis in *E. coli* contains much redundancy. Each of the considered

products can, in the wild type, be produced by at least 36 pathways. This redundancy is in agreement with biochemical knowledge implying that *E. coli* has a very complex metabolism. An earlier metabolic pathway analysis of amino acid metabolism in *E. coli* was also indicative of high redundancy [49]. For analysis of robustness, rather than of redundancy, the number of EFMs remaining after knockouts is relevant. As seen in Table 2, 25 of 36 single-gene knockouts are lethal. Thus, lipid biosynthesis in *E. coli* appears to be somewhat less robust than amino acid metabolism. Furthermore, an analysis similar to the one applied in [50] could help in the examination of the general susceptibility of the network to knockouts, as multiple knockouts are also considered to determine the robustness of the network.

For the cell wall-free mutant, we found that no EFM is left in the metabolic network under study if all mutations present in the corresponding genes are assumed to render the encoded enzymes nonfunctional. Analyzing the mutations that occurred in the enzymes of lipid biosynthesis in detail, we found that probably only LpxA is affected. We drew this conclusion from a residue close to a known active site that is mutated in this protein. In the resolved structure of LpxA bound to its substrate, this residue is indeed found in close proximity to the substrate. These results are further corroborated by the finding that lipid A is no longer detectable in the cell wall-free mutant. These findings stand in contrast to a subsequent analysis of single-gene knockout data indicating that *E. coli* can only survive if at least L1-P-EtAmine and a lipid A form lacking the myristoyl side chain is present. However, the loss of the outer membrane in the L-form, as indicated by electron microscopy, might have made lipid A non-essential.

As the biosynthesis of fatty acids in higher organisms is very much like that in bacteria, except for the synthesis of lipid A [51], our analysis is also generally relevant for higher organisms. As there is recent evidence that lipid A also occurs in the chloroplasts of *Arabidopsis thaliana* and some other eukaryotic plants [6], application of our analysis to those organelles could be worthwhile.

Experimental procedures

In the model of *E. coli* lipid biosynthesis, we included the synthesis reactions of unsaturated/saturated fatty acids, phospholipids, and lipid A. The reaction scheme is presented in Fig. 1. The reaction equations and information about reversibility for the lipid biosynthesis model were taken from the EcoCyc database [23] (http://www.ecocyc.org/). For some enzymes, more detailed information about reversibility was taken from a textbook [51] and the KEGG database (http://www.genome.jp/kegg) [52]. The enzymes are here represented by their gene names as given in the EcoCyc database. Many of the enzymes considered are multifunctional. The names of the enzymes together with their gene names and EC numbers are shown in Table S5. In the case of multifunctional enzymes, we denote each function by the gene name augmented by a number. The numbers are given by us and are not part of the official gene name. Table 5 gives the abbreviations of metabolites used in this study.

It is interesting to investigate how the fatty acid elongation subsystems interact, considering the exchange of substrates at different levels (chain lengths) and the supply of substrates for the synthesis of lipid A and phospholipids. During the elongation process, fatty acids with different chain lengths are produced. For the production of lipid A, several fatty acids with specific chain lengths are needed – laurate (saturated C12, i.e. 12 carbon atoms), hydroxymyristoate (saturated C14), and palmitoleate (unsaturated C16). For the synthesis of phospholipids, the following fatty acids are needed: palmitate (saturated C16) and palmitoleate (unsaturated C16) [53–55]. The described pathways result in the formation of several end-products: lipid A, lipid A (ca), cardiolipin, and L1-P-EtAmine. For simplicity's sake, we only considered incorporation of palmitate into phospholipids. Alternatively, palmitoleate could be

Table 5. List of abbreviations for names of the metabolites presented in Fig. 1. The names are consistent with the EcoCyc database.

Abbreviation	Name
2,3-b(3hm) bD-GA-1P	2,3-Bis(3-hydroxymyristoyl)-β-D-glucosamine-1-phosphate
ACP	Acyl carrier protein
bhcd5dACP	β-Hydroxy-*cis*-Δ5-decenoyl-ACP
bkcd5d-ACP	β-Keto-*cis*-Δ^5-decenoyl-ACP
cd3dACP	*Cis*-Δ^3-decenoyl-ACP
cd5dACP	*Cis*-Δ^5-decenoyl-ACP
D-3-ho-acyl-ACP	D-3-Hydroxy-acyl-ACP
DHAP	Dihydroxyacetone phosphate
G3P	Glycerol 3-phosphate
KDO	3-Deoxy-D-manno-octulosonate
L1-P-EtAmine	L1-phosphatidylethanolamine
lipid A-disacch	Lipid A disaccharide
td2enoyl-acyl-ACP	*Trans*-Δ^2-enoyl-acyl-ACP
td3cd5dACP	*Trans*-Δ^3-*cis*-Δ^5-decenoyl-ACP
UDP-2,3-b(3hm)GA	UDP-2,3-bis(3-hydroxymyristoyl) glucosamine
UDP-3O-(3hm)GA	UDP-3*O*-(3-hydroxymyristoyl) glucosamine
UDP-3O-(3hm)-N-acetylGA	UDP-3*O*-(3-hydroxymyristoyl)-*N*-acetylglucosamine
UDP-N-acetyl-D-GA	UDP-*N*-acetyl-D-glucosamine

Table 6. Combined enzymes.

Combined reaction	Constituent reactions
AccACD	AccA, AccC, AccD
FabBF2	FabB_2, FabF_2
GpsA	GpsA_1, GpsA_2
J_(10s-14s)_to_(12u-16u)	FabF_1, FabB_4, FabG_2
J_(10u-14u)_to_(12u-16u)	FabB_1, FabG_1, FabZ_1, FabA_3, FabI_1
J_4s_to_10u	FabF_1, FabB_4, FabG_2, FabZ_2, FabA_1, FabI_2, FabI_3
PgpAB	PgpAB

incorporated. However, this would just yield additional pathways in which palmitate-producing subpathways are replaced by palmitoleate-producing subpathways, without providing any new information.

Another important case is where two or more different enzymes catalyze the same reaction (isoenzymes). An example is provided by FabB_2 and FabF_2, which catalyze the condensation of acetyl-ACP and malonyl-ACP. In our model, we grouped those enzymes into one, FabBF_2, and treated other isoenzymes analogously (Table 6). Moreover, we lumped sequential reactions together in order to represent the cycles of the fatty acid elongation more conveniently (Table 6). In the case of elongation of fatty acids, we decided to split the cycles into several parts and combined some of the reactions in these parts. We combined most of the enzymes operating on the same substrates. For example, the enzymes FabF_1, FabB_4 and FabG_2 are united in the reaction named J_10s_to_12u. This reaction represents the first half of the saturated fatty acid elongation, after which the product can be further processed or passed to the unsaturated fatty acid elongation cycle (Fig. 1). In such a manner, we have split the cycles into two parts each.

We did not include in the system the protein encoded by the gene *ybhO*, which is homologous to Cls [56]. YbhO lacks a part of the sequence of Cls, and was found to exhibit only weak activity *in vivo*, even though a cardiolipin synthase activity could be observed *in vitro* [56].

For calculating EFMs, we used the program METATOOL [57], which is freely available from http://pinguin.biologie.uni-jena.de/bioinformatik/networks/index.html. For additional information on how to use EFM analysis, see [14–16,57].

Isolation and analysis of genes of the L-form mutant strain *E. coli* LWF1655F+

Amplification of the genes of interest (Table S6), mutation detection and analysis were essentially performed as previously described [11]. All DNA sequences obtained in this study are deposited at the NCBI within GenBank (for accession numbers, see Table S6).

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Supporting information

The following supplementary material is available:
Doc. S1. List of elementary modes producing the different metabolites of interest.
Table S1. Lipid A-producing elementary modes.
Table S2. Lipid A (cold-adapted)-producing elementary modes.
Table S3. Cardiolipin-producing elementary modes.
Table S4. L1-phosphatidylethanolamine-producing elementary modes.
Doc. S2. Analysis of elementary modes.
Table S5. Protein names, symbols for function used in this study, EC numbers and enzyme names in the lipid metabolism network under study.
Table S6. Genes, Blattner Identifiers and accession numbers of GenBank entries.
Doc. S3. Model of the system studied here in SBML format.

This supplementary material can be found in the online version of this article.

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