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Exogenous Fatty Acid Metabolism in Bacteria

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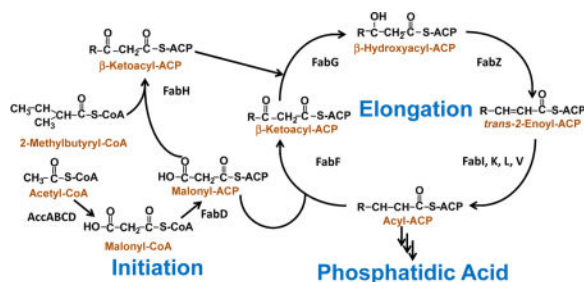
Abstract

Bacterial type II fatty acid synthesis (FASII) is a target for novel antibiotic development. All bacteria encode for mechanisms to incorporate exogenous fatty acids, and some bacteria can use exogenous fatty acids to bypass FASII inhibition. Bacteria encode three different mechanisms for activating exogenous fatty acids for incorporation into phospholipid synthesis. Exogenous fatty acids are converted into acyl-CoA in Gammaproteobacteria such as *E. coli*. Acyl-CoA molecules constitute a separate pool from endogenously synthesized acyl-ACP. Acyl-CoA can be used for phospholipid synthesis or broken down by β -oxidation, but cannot be used for lipopolysaccharide synthesis. Exogenous fatty acids are converted into acyl-ACP in some Gram-negative bacteria. The resulting acyl-ACP undergoes the same fates as endogenously synthesized acyl-ACP. Exogenous fatty acids are converted into acyl-phosphates in Gram-positive bacteria, and can be used for phospholipid synthesis or become acyl-ACP. Only the order Lactobacillales can use exogenous fatty acids to bypass FASII inhibition. FASII shuts down completely in presence of exogenous fatty acids in Lactobacillales, allowing Lactobacillales to synthesize phospholipids entirely from exogenous fatty acids. Inhibition of FASII cannot be bypassed in other bacteria because FASII is only partially down-regulated in presence of exogenous fatty acid or FASII is required to synthesize essential metabolites such as β -hydroxyacyl-ACP. Certain selective pressures such as FASII inhibition or growth in biofilms can select for naturally occurring one step mutations that attenuate endogenous fatty acid synthesis. Although attempts have been made to estimate the natural prevalence of these mutants, culture-independent metagenomic methods would provide a better estimate.

Graphical abstract

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1. INTRODUCTION

Phospholipids are major components making the essential cellular membrane that forms the boundary between the organism and the environment [1]. Phosphatidic acid, the precursor to all phospholipid species, is synthesized through two successive acylation reactions of glycerol-3-phosphate (G3P) using acyl-acyl carrier protein (ACP) synthesized by the bacterial type II fatty acid synthesis system (FASII) (Fig. 1) [2]. Due to differences between FASII and the monofunctional mammalian type I fatty acid synthase [3], targeting FASII enzymes for novel antibiotic therapeutics is an active area of research [4–7]. However, all bacteria characterized to date can assimilate exogenous fatty acids [8–12], but only some species can bypass inhibition of FASII by incorporating exogenous fatty acids [13]. Several FASII inhibitors are currently in clinical development [14–20], making understanding the mechanism of exogenous fatty acid activation for phospholipid synthesis and whether exogenous fatty acids can bypass FASII inhibition in pathogenic bacterial species an important area of research. This review will summarize the pathways for the incorporation of exogenous fatty acids into phospholipids and whether exogenous fatty acids can bypass the inhibition of endogenous fatty acid synthesis in those species.

2. PATHWAYS FOR EXOGENOUS FATTY ACID INCORPORATION

Fatty acid synthesis is an energy and material intensive process (Fig. 1) in phospholipid synthesis and the incorporation of useable exogenous fatty acids saves energy and metabolic building materials. The synthesis of the 16 carbon, saturated palmitoyl-CoA requires 8 acetyl-CoA molecules, 14 NADPH reducing equivalents, and the hydrolysis of 7 ATP molecules [1]. Two acyl-ACP molecules are used in successive acylation reactions to synthesize phosphatidic acid, the precursor to all bacterial phospholipid species [2]. There are two major types of acyltransferase systems found in bacteria. The PlsB pathway found in *E. coli* and other Gammaproteobacteria acylates the G3P using acyl-ACP or acyl-CoA [21–23]. The PlsX/PlsY pathway found in all other characterized bacterial taxa acylates G3P using an acyl-phosphate derived from acyl-ACP via PlsX [24,25]. PlsC acylates the resulting lysophosphatidic acid using acyl-ACP in both pathways [26,27]. Further details of these two pathways can be found in a recent review [2]. This review will focus on how the exogenous fatty acid activation systems present fatty acids to the acyltransferases.

The energy and material savings gained from being able to use exogenous fatty acids (when the correct acyl chain species are available) in lieu of endogenous synthesis suggests why all bacteria characterized to date encode for some mechanism to incorporate exogenous fatty

acids [2]. However, the acyl chain composition of the phospholipid controls the membrane fluidity and function [28–32], so bacteria must balance the energy savings from incorporating exogenous fatty acids with synthesizing enough fatty acids endogenously to ensure the proper membrane biophysical properties. There are three characterized mechanisms for incorporating exogenous fatty acids: acyl-CoA synthetase, acyl-ACP synthetase, and fatty acid kinase. The acyl-CoA synthetase was first discovered in *E. coli* and found in Gammaproteobacteria bacteria [12]. Acyl-CoA synthetases are also found in mammalian lipid synthesis, and is an essential component because the free fatty acids generated by mammalian fatty acid synthase must be converted into acyl-CoA for phospholipid synthesis [33]. Acyl-ACP synthetase was the second mechanism discovered [34,35]. Acyl-ACP synthetases belong in the same structural family as acyl-CoA synthetases and are also found in Gram-negative bacteria [9]. Acyl-CoA synthetase and acyl-ACP synthetase are found in bacteria encoding either the PlsB or the PlsX/PlsY acyltransferase system [8,9]. Biochemical validation is needed to identify acyl-ACP synthetase versus acyl-CoA synthetase. Finally, the fatty acid kinase system is found in Gram-positive bacteria that exclusively encode the PlsX/PlsY acyltransferase system [36,37]. The fatty acid kinase generates an acyl-phosphate molecule which can enter into phospholipid synthesis in bacteria encoding the PlsX/PlsY acyltransferase pathway.

The following sections describe four characterized pathways to activate exogenous fatty acids for incorporation into phospholipid synthesis. Exogenous fatty acids are converted into acyl-CoA, which can be used by the PlsB/PlsC acyltransferase system in *E. coli* and other Gammaproteobacteria. Exogenous fatty acids are converted into acyl-ACP, which can enter into FASII and become indistinguishable from endogenously synthesized fatty acids in *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. In *C. trachomatis*, the acyl-ACP is used directly by the PlsE/PlsC pathway for phosphatidic acid synthesis. In *N. gonorrhoeae*, the acyl-ACP is converted into acyl-phosphate by PlsX to initiate phosphatidic acid synthesis by PlsY. Finally, exogenous fatty acids are converted into acyl-phosphate by the fatty acid kinase system in Gram-positive bacteria. The resulting acyl-phosphate can be used by PlsY in the first step of phosphatidic acid synthesis, or converted back into acyl-ACP by PlsX. The resulting acyl-ACP can be elongated by FASII or used by PlsC in the second step of phosphatidic acid synthesis. The following section also notes other instances where fatty acid activation is needed such as β -oxidation and recycling free fatty acids generated in other processes.

2.1. Acyl-CoA Synthetase and Acyl-ACP Synthetase in *E. coli*

E. coli utilizes the acyl-CoA synthetase (FadD) to generate acyl-CoA from exogenous fatty acids (Fig. 2) [12]. Acyl-CoA synthetase catalyzes the formation of acyl-CoA from a fatty acid and a CoA using the energy generated from the hydrolysis of an ATP into AMP and pyrophosphate [38]. Members of this superfamily of proteins contain the AFD_class_I domain, which corresponds to the binding site for the acyl-adenylate intermediate. Acyl-CoA molecules cannot enter FASII and become elongated in *E. coli* [39], but undergoes two other fates. The *E. coli* acyltransferases PlsB and PlsC both use acyl-CoA molecules of the appropriate acyl chain length to acylate G3P and synthesize phosphatidic acid [26,40]. Acyl-CoA molecules are also broken down via β -oxidation to generate acetyl-CoA molecules and

ATP [41,42]. *E. coli* also encodes for a short chain acyl-CoA synthetase (FadK) that is expressed during anaerobic conditions and in presence of the terminal electron acceptor fumarate [43]. Acyl-CoA generated through this pathway cannot enter phosphatidic acid synthesis due to the acyl chain length, and can only be broken down via β -oxidation. It is important to note that the *E. coli* FASII generates β -hydroxyacyl-ACP for lipopolysaccharide synthesis in addition to acyl-ACP [44]. There is no known mechanism for converting exogenous fatty acids into β -hydroxyacyl-ACP in *E. coli*. Because lipopolysaccharide synthesis is essential in *E. coli* [45], exogenous fatty acids cannot bypass genetic or biochemical inhibition of FASII in *E. coli*. The *E. coli* model is predicted to be representative of other Gammaproteobacteria.

E. coli also encodes for a bifunctional 2-acylglycerophosphoethanolamine acyltransferase [23]. The carboxy-terminus of this bifunctional protein contains the acyl-ACP synthetase (Aas) domain that generates the acyl-ACP from ACP and exogenous fatty acids, while the amino-terminus of this protein catalyzes the acylation of lysophospholipids using acyl-ACP [46]. Lysophospholipids are generated from the synthesis of lipoproteins, where the 1-acyl chain of phospholipids is used to acylate the lipoprotein [47]. The lysophospholipid is reacylated into a phospholipid by the 2-acylglycerophosphoethanolamine acyltransferase [48]. The acyl-ACP generated by the carboxy-terminal Aas cannot dissociate from the enzyme complex, and therefore the acyl-ACP can only be used for acylating lysophospholipids [49,50]. The FadD is responsible for the bulk of exogenous fatty acid incorporation in *E. coli*, while the bifunctional 2-acylglycerophosphoethanolamine acyltransferase pathway is only involved in recycling lysophospholipids.

2.2. Acyl-ACP Synthetase in *Vibrio harveyi*

The first described soluble acyl-ACP synthetase is from *Vibrio harveyi* (*VhAas*) [34]. The expression of *VhAas* in *E. coli* allows the elongation of medium chain exogenous fatty acids and their subsequent incorporation into the membrane phospholipids [35,51]. *VhAas* accepts a broad array of acyl chains including unnatural fatty acid analogues, and has been extensively used as a chemical biology tool to generate unnatural acyl-ACP molecules [52]. However, the function of *VhAas* in the parent organism has been poorly characterized. The low acyl chain selectivity of the *VhAas* suggests that it plays a role other than incorporating exogenous fatty acids into the phospholipids because bacteria have high acyl chain selectivity for their phospholipids [53]. Bacterial bioluminescence is generated from the conversion of acyl aldehyde, O₂, and reduced flavin mononucleotide into free fatty acids, H₂O and oxidized flavin mononucleotide. This acyl-ACP synthetase could be involved in recycling the free fatty acid generated by the bacterial luciferase.

2.3. Acyl-ACP Synthetase in *Chlamydia trachomatis*

The obligate intracellular parasite *C. trachomatis* utilizes an acyl-ACP synthetase to incorporate exogenous fatty acids from the host into its phospholipid (Fig. 3) [8,54]. Isotopic labeling experiments found that deuterium labeled, exogenous palmitic acids were incorporated into both host and chlamydial synthesized phospholipids in a *C. trachomatis* infected HeLa cell culture. In contrast, deuterium labeled, exogenous lauric acid was incorporated only into chlamydial synthesized phospholipids as elongated palmitic acid.

These labeling experiments show that *C. trachomatis* is able to elongate exogenous fatty acids before using them for phospholipids synthesis. Bioinformatic analysis found that *C. trachomatis* encodes for 2 separate genes homologous to the two separate domains of the bifunctional *E. coli* acyl-ACP synthetase/2-acylglycerophosphoethanolamine acyltransferase [8]. The independent encoding of the acyl-ACP synthetase domain (AasC) in *C. trachomatis* suggests that acyl-ACP generated from exogenous fatty acids can dissociate from the AasC and offers a mechanism by which exogenous fatty acids are elongated in *C. trachomatis*. Biochemical analysis confirmed that the AasC can catalyze the formation of acyl-ACP with preference for the saturated 16:0 fatty acid over the unsaturated 18:1 fatty acid. Expression of AasC in *E. coli* allowed elongation of exogenous fatty acids prior to incorporation into phospholipids. The function of the 2-acylglycerophosphoethanolamine acyltransferase was also confirmed using biochemical and genetic complementation experiments. Together, these experiments show that exogenous fatty acids are converted into acyl-ACP by the *C. trachomatis* AasC. The acyl-ACP synthesized from AasC undergoes the same fate as endogenously synthesized acyl-ACP: elongation by FASII or acylation by the acyltransferase system [54]. The most abundant fatty acids synthesized by the mammalian host are 16:0 and 18:1 fatty acids. AasC has high preference for 16:0 over 18:1 and 16:0 is the major constituent of the 1-position of *C. trachomatis* phospholipid. Therefore, the purpose of AasC is to selectively scavenge saturated fatty acids from the host.

An alternative model proposed that *C. trachomatis* also synthesized phospholipids by deacylating the 2-position of host phospholipids using the host cytosolic phospholipase A2 and reacylating it with endogenously synthesized branched-chain fatty acids [55,56]. Additional reports propose that endogenously synthesized fatty acids are exported out of the *C. trachomatis* cell and into the chlamydial inclusion, converted to acyl-CoA by the host acyl-CoA synthetase, and used to acylate the deacylated host phospholipids in the inclusion [57,58]. These models for phospholipid synthesis are unprecedented in literature and are not consistent with other experimental results. Molecular species analysis by mass spectrometry found no evidence for the products of the proposed deacylation/reacylation in *C. trachomatis* infected HeLa cell cultures [54]. The deacylation/reacylation model also proposes the extracellular synthesis of phospholipids, which has never been described and would seem to violate the principle of thermodynamics. It is unclear why *C. trachomatis* would encode for such a convoluted mechanism for synthesizing phospholipids when it has a vastly reduced genome [59] and already encodes for all the normal bacterial enzymes required for phospholipid synthesis and exogenous fatty acid incorporation [8,54].

2.4. Acyl-ACP and Acyl-CoA Synthetase in *Neisseria gonorrhoeae*

Isotopic labeling experiments found that exogenous lauric acids are also elongated before being incorporated into the phospholipids of *N. gonorrhoeae* (Fig. 4) [9]. Bioinformatic analysis found 2 predicted acyl-CoA/ACP synthetase genes in the *N. gonorrhoeae* genome. Genetic complementation studies identified NGO0530 as an acyl-ACP synthetase and NGO1213 as an acyl-CoA synthetase [9]. The elongation of exogenous fatty acids by *N. gonorrhoeae* suggests that exogenous fatty acids are converted into acyl-ACP by NGO0530. The acyl-ACP is subsequently used by the PlsX/PlsY acyltransferase system to make phosphatidic acid. How the acyl-CoA synthetase, NGO1213, functions in *N. gonorrhoeae*

biology has yet to be determined. The synthesis of acyl-CoA molecules in *N. gonorrhoeae* is unexpected because *N. gonorrhoeae* lacks a β -oxidation system to breakdown acyl-CoA [60]. It is unknown if the *N. gonorrhoeae* acyltransferases can use acyl-CoA as substrates. PlsB and PlsC acyltransferases from species such as *E. coli* can use acyl-CoA as the acyl donor. However, PlsX cannot function as an acyl-CoA/phosphate transacylase meaning that acyl-CoA cannot be converted to form acyl-phosphate or acyl-ACP in the cases where the acyltransferases are specific for this acyl donors.

2.5. Exogenous Fatty Acids Cannot Bypass the Inhibition of Endogenous Fatty Acid Synthesis in *C. trachomatis* and *N. gonorrhoeae*

Experiments show that exogenous fatty acids cannot bypass inhibition of endogenous fatty acid synthesis in either *C. trachomatis* or *N. gonorrhoeae*. Despite actively incorporating exogenous fatty acids synthesized from the host and available from the growth media, inhibition of FabI blocks the replication of the metabolically active *C. trachomatis* reticulate body over the entire growth phase [61]. The essentiality of endogenous fatty acid synthesis is likely due to a combination of the selectivity of the chlamydial PlsC for endogenously synthesized *anteiso*15:0 fatty acid and the essentiality of β -hydroxyacyl-ACP for lipopolysaccharide synthesis.

Similarly, addition of the fatty acid species found in *N. gonorrhoeae* phospholipids cannot rescue FabI inhibition [9]. Why exogenous fatty acids cannot rescue the inhibition of FASII in *N. gonorrhoeae* is unclear. Lipopolysaccharides are not essential for *N. gonorrhoeae* so FASII is not required to generate β -hydroxyacyl-ACP [62,63]. The same fatty acid species found in the phospholipids were supplemented so the lack of bypass is not due to the incorrect acyl chain species. These results highlight the need to experimentally verify the efficacy of FASII inhibition against distinct bacterial groups because bypass cannot be reliably predicted by the existence of an acyl-ACP synthetase in the genome.

2.6. Fatty Acid Kinase in Gram-Positive Bacteria

Gram-positive bacteria use a fatty acid kinase system for the incorporation of exogenous fatty acids (Fig. 5) [36,37,64]. The exogenous fatty acids are bound by the fatty acid binding protein, FakB, and then phosphorylated by the fatty acid kinase, FakA. FakB can deliver the resulting acyl-phosphate to the membrane where it is used by the acyltransferase PlsY to acylate G3P in the first step of phosphatidic acid synthesis. The acyl-phosphate can also be converted into acyl-ACP by the acyl-ACP/phosphate transacylase PlsX [36]. The resulting acyl-ACP undergoes the same fate as the endogenously synthesized acyl-ACP—it can be elongated by FASII and converted back into acyl-phosphate or used for phosphatidic acid synthesis by PlsC. Gram-positive bacteria do not have an outer membrane and fatty acids derived from FASII are used for phospholipid and glycolipid synthesis. One might think that providing the correct species of exogenous fatty acids should be able to bypass the need for endogenous fatty acid synthesis. However, whether exogenous fatty acids can actually bypass endogenous synthesis varies by bacterial taxa and how exogenous fatty acids regulate endogenous synthesis [11,65,66].

2.7. Fatty Acid Kinase in Lactobacillales Allows Bypass of Endogenous Synthesis

Members of the order Lactobacillales, which includes *Streptococcus*, *Lactococcus*, *Lactobacillus*, and *Clostridia*, can bypass endogenous fatty acid synthesis using exogenous fatty acids [65]. FASII can be completely shut down in presence of exogenous fatty acids, allowing the synthesis of phospholipids entirely from exogenous fatty acids. In the most extreme example, certain *Lactobacillus* species don't encode the genes for FASII and synthesize phospholipids entirely from exogenous fatty acids [67]. Clearly, FASII inhibition would be ineffective in such bacteria. Exogenous fatty acids regulate FASII activity via both transcriptional and biochemical mechanisms. First, FASII genes are encoded in a single locus in these bacteria, and are coordinately regulated by the transcriptional repressor FabT [68]. FabT binding to long-chain acyl-ACP increases the FabT affinity for the repressor binding sites and can completely down-regulate the expression of the FASII genes [69]. Second, exogenous fatty acids cause a dose dependent repression of endogenous fatty acid synthesis in these bacteria, including production of malonyl-CoA, the rate-determining intermediate required for endogenous fatty acid synthesis [11]. The metabolite(s) and enzyme(s) responsible for this biochemical feedback is unknown, but the complete inhibition of fatty acid synthesis and the reduction of malonyl-CoA points to acyl-phosphate or acyl-ACP inhibition of the acetyl-CoA carboxylase complex, the initiator of FASII [70].

2.8. Fatty Acid Kinase in Bacillales Cannot Bypass Endogenous Synthesis

In contrast, members of the order Bacillales, which includes *Staphylococcus* and *Listeria*, cannot bypass FASII inhibition using exogenous fatty acids. No combination of exogenous fatty acids can rescue FASII inhibition in *S. aureus* without genetic modification to the reference organism [11,16]. Host fatty acids cannot rescue the FabI inhibition of intracellular *Listeria monocytogenes*, and exogenous fatty acid combinations cannot replace endogenous synthesis in extracellular cultures [10]. Exogenous fatty acids cause only a dose dependent, partial inhibition of fatty acid synthesis in these bacteria [11]. Malonyl-CoA levels remain elevated and endogenous fatty acids made up half or more of the acyl chains in phospholipid synthesis even in presence of exogenous fatty acids [10,11]. The inhibition of the remaining FASII activity causes accumulation of short chain acyl-ACP and depletion of free ACP [16]. The lack of free ACP prevents the synthesis of acyl-ACP from exogenous fatty acid. Because the PlsC acyltransferase requires acyl-ACP of the appropriate chain length in the second step of phosphatidic acid synthesis, phospholipid synthesis is halted by FASII inhibitors even in presence of exogenous fatty acids. Exogenous fatty acids also do not down-regulate the expression of FASII genes. The FASII genes are spread throughout the genome, and regulated by the FapR repressor [71]. The FapR repressor is released from the DNA binding site after binding to malonyl-CoA to allow for the expression of the FASII genes [72]. Because malonyl-CoA levels remain high in presence of exogenous fatty acids, FASII genes continue to be transcribed.

One major difference between Bacillales and Lactobacillales that might explain the difference in the regulation of FASII in presence of exogenous fatty acids is their respective acyl-chain composition. Membrane fluidity is controlled by the ratio of saturated to unsaturated fatty acids in the phospholipids of mammals and bacteria such as order Lactobacillales [73]. The fatty acid species produced by Lactobacillales FASII can be

salvaged from the mammalian host, meaning that exogenous fatty acids from the host could fully replace endogenous synthesis. In contrast, *anteiso*15:0 fatty acid is found at the 2-position of *Staphylococcus* and *Listeria* phospholipids [11,65]. Because branched-chain *anteiso*15:0 fatty acids are not made by the host, endogenous synthesis of branched-chain fatty acids must continue even in presence of exogenous host fatty acids to ensure proper membrane function. Decreasing the ratio of branched-chain fatty acids severely decreased the extracellular growth rate of *Staphylococcus* and *Listeria*, while restoring the ratio of branched-chain fatty acids restored the growth rate [31,32]. Decreasing the content of branched-chain fatty acids also inhibited intracellular pathogenesis and proliferation in a mouse peritonitis model [10,74,75]. Branched-chain amino acids are precursors to branched-chain fatty acids, and the increased import of branched-chain amino acids correlated with increased virulence while the decreased import of branched-chain amino acids correlated with decreased virulence [76]. Existing data in literature strongly support the importance of *anteiso*15:0 fatty acids in Bacillale phospholipids.

There are disagreements about what fraction of the 2-position of *S. aureus* phospholipids can be occupied by mammalian fatty acids such as 16:0 or 18:1 when endogenous fatty acid synthesis is functional. A paper in 2011 [11] reported that the 2-position is exclusively occupied by 15:0 fatty acids even in presence of millimolar concentrations of 18:1, while a paper in 2016 [77] reported that branched-chain fatty acids were reduced to less than 40% of the total cellular acyl chain composition so 16:0 and 18:1 is assumed to make up at least 10% of the 2-position composition. The difference between these two papers is what was measured and how it was measured. Parsons and coworkers [11] used molecular species analysis via mass spectrometry to look specifically at the acyl chain position of phosphatidylglycerol, the major phospholipid class in *S. aureus*. It was found that all phosphatidylglycerol species contained 15:0 fatty acids. In contrast, Sen and coworkers [77] performed gas chromatography analysis on fatty acid methyl esters generated from hydrolyzing the total lipid extracts. Because lipid extracts contain non-phospholipid components like staphyloxanthin and media components such as free fatty acids and lipoproteins adsorbed to the bacterial cell surface, this form of analysis is less accurate at measuring phospholipid acyl chain composition. In our experience, free fatty acids from the media associate with the bacterial cell wall and are difficult to remove by washing with buffers.

3. EVOLUTIONARY CHANGES IN BACTERIAL PHOSPHOLIPID SYNTHESIS AND EXOGENOUS FATTY ACID INCORPORATION

Bacteria evolve rapidly through genetic alterations in response to changes in their environment. The models of exogenous fatty acid incorporation described in the previous section hold true to the particular reference strain in the specific experimental context. However, evolution is an ongoing process, and this section discusses some of the recently characterized changes that can occur to the bacterial exogenous fatty acid incorporation and phospholipid synthesis systems, as well as their biological consequences. Potential alterations to how bacteria use exogenous fatty acids have profound consequences on biology and medicine.

3.1. Bypassing FASII Inhibition through Deactivating Endogenous Fatty Acid Synthesis in *S. aureus*

A current topic is whether *S. aureus* can become functional, obligate fatty acid auxotrophs through genetic mutations in FASII to bypass the inhibition of bacterial fatty acid synthesis inhibitors [11,65,66]. With the anti-staphylococcal FabI inhibitor AFN-1252 in late phase clinical trials [14,78–80], this debate takes on a greater significance. AFN-1252 targets the enoyl-ACP reductase (FabI) of *S. aureus*. FabI, along with another FASII enzyme FabH, was validated as an antibiotic target by the combined genetic and high-throughput screens conducted by GlaxoSmithKline [81,82]. Lead compounds against FabI and FabH generated by the GlaxoSmithKline efforts was demonstrated to work in the animal model against *S. aureus* [83,84]. AFN-1252 was derived from the scaffold generated by the GlaxoSmithKline lead through rounds of structure based optimization [15].

In 2009, Brinster et al argued that fatty acid synthesis enzymes are not valid antibiotic targets in Gram-positive bacteria because Gram-positive bacteria are able to bypass FASII inhibition by incorporating exogenous fatty acids when endogenous synthesis is inhibited [65]. They demonstrated that *Streptococcus agalactiae* shuts down endogenous fatty acid synthesis in presence of human serum fatty acids, and can grow with deletions in the endogenous fatty acid synthesis genes in presence of human serum fatty acids. This result was generalized to all Gram-positive bacteria and used to argue against fatty acid synthesis as a drug target in Gram-positive bacteria including *S. aureus* because exogenous fatty acids are abundant in animal models. This study was met with intense skepticism by several research groups because a variety of FASII inhibitors have been demonstrated to be effective against *S. aureus* in mouse models and in human clinical trials [14,79,84]. Two papers comparing fatty acid metabolism between *Streptococcus* and *Staphylococcus* found that while exogenous fatty acids can bypass FASII inhibition in *Streptococcus*, exogenous fatty acids cannot bypass FASII inhibition in *Staphylococcus* [11,66]. This differential susceptibility is due to differences in the regulation of endogenous fatty acid synthesis by exogenous fatty acids as described in sections 1.2.6–1.2.8. *S. aureus* could bypass FASII inhibition using exogenous fatty acids if the acetyl-CoA carboxylase (*acc*) or malonyl CoA-acyl carrier protein transacylase (*fabD*) genes were inactivated, and such a strain has been created [11]. However, this strain experienced significant growth defects in planktonic culture even when supplemented with human serum, and failed to proliferate in a mouse peritonitis model [74].

A recent paper proposed that exogenous fatty acids can enable the emergence of infectious, fatty acid auxotrophic *S. aureus* resistant to FASII inhibitors [85]. *S. aureus* was selected on triclosan and a mixture of mammalian fatty acids. Mutants with defects in the FabD gene were found in the selection. Two classes of FabD mutations were found - point mutations in the 196 position (G196V, G196R) of FabD and truncations of the C-terminal of the FabD protein. The FabD truncation mutants were fatty acid auxotrophs, while the point mutants could grow without exogenous fatty acids. These mutants synthesized their phospholipids using exogenous fatty acids in lieu of endogenously synthesized branched-chain fatty acids when grown under exogenous fatty acid supplementation, and are resistant to FASII inhibition when supplemented with exogenous fatty acids. The fitness of these mutants was

evaluated in a mouse bacteremia model by injecting the bacteria (10^6 CFU) into the tail vein of mice. The FabD point mutant had minimal fitness cost in this model, while the FabD truncation mutant had up to a 10^4 decrease in growth. Bioinformatic analysis found that 3 strains of the ~ 4000 strains of sequenced *S. aureus* in the NCBI database harbored mutations in the 196 position of FabD. Two strains encoded the FabD[G196R] mutation, while one strain harbored the FabD[G196S] mutation. From these results, the authors suggested that FASII inhibitors will make poor therapy in the clinic due to the quick emergence of mutants refractory to FASII inhibition by incorporating exogenous fatty acids. It is unclear if this conclusion is warranted based on the experimental results. The FabD truncation mutant had a significant growth defect ($\sim 10^4$ decrease in the liver), demonstrating that endogenous fatty acid synthesis in *S. aureus* is important for fitness within the mouse model. The FabD point mutant does not have an apparent fitness cost, but would not be expected to because it can synthesize fatty acids endogenously. The clearest experiment to test the conclusion of the authors would be to directly test the susceptibility of the two classes of FabD mutants against a FASII inhibitor using a previously described mouse model [14,74]. This experiment would clearly show whether the two classes of mutants could bypass the effects of a FASII inhibitor.

The key point of disagreement between this paper and previous literature is the fitness of *S. aureus* with altered acyl chain composition. Previous work showed that *S. aureus* with decreased proportion of branched-chain fatty acids have significant growth defects in planktonic culture even when supplemented with human serum and in mouse models [32,74]. This branched-chain fatty acid dependency is also observed in the related *Listeria* bacteria family [31,86]. One possible explanation of the discrepancy is the mouse model used for the experiment [87]. It is plausible that endogenous fatty acid synthesis is not required in the bacteremia model, but is required for the peritonitis model. It is also important to note that *S. aureus* lineages are largely host species specific, and mice are not natural hosts for many of the *S. aureus* strains derived from human infections [88]. Because there is no gold standard mouse model for mimicking *Staphylococcus* infection in humans, the FabD mutant should be tested in other models simulating other phases of infection beyond blood growth to determine if FASII inhibitors are effective therapeutics. In particular, *S. aureus* predominantly causes skin and soft tissue infections in humans rather than sepsis and bacteremia, so it is important to evaluate the fitness of the fatty acid auxotroph within a skin and soft tissue infection model [89]. Furthermore, selection screens can introduce other mutations in the genome, making it difficult to assign a specific mutated gene to an altered phenotype without extensively characterizing each mutation. Recapitulating the FASII bypass phenotype by knocking out the *fabD* in a reference *Staphylococcus* strain is a classic microbiology experiment that would give greater confidence that FASII inhibitor resistance is due to *fabD* mutations alone.

3.2. Prevalence of *S. aureus* fatty acid auxotrophs in nature

The question of whether fatty acid auxotrophs thrive in infected tissue is important to evaluating the potential of FASII therapeutics. Recently, Gloux et al. [90] addressed this question by collecting *S. aureus* samples for clinical and veterinary settings and plating them on selective media containing exogenous fatty acids and/or triclosan. They report 12% of

695 the isolates had modification in FASII genes. A major challenge with interpreting these results is determining whether the *S. aureus* FASII mutations existed prior to the selection. Missense mutations at a particular base pair occur at frequencies approximating the error rate in DNA replication [91], and mutations causing inactivation of a protein function occur at even higher frequencies (~1 in 10⁶). Given the number of bacteria spotted on selective media, mutants will arise from a population of wild-type organisms [11,85]. Thus, it is not possible to determine for this approach whether the mutants were selected or whether they existed prior to the experiment. What is required to answer this important question is to use deep sequencing of infected samples to determine the frequency of FASII mutants without amplification or selection of the cells.

Determining the rate and mechanism of resistance mutations against an antibiotic candidate by plating the susceptible bacteria against the antibiotic candidate is a well described and routine step in antibiotic discovery [4,92,93]. Resistant mutants against single-target antibiotics are practically guaranteed to occur and are often used to determine/validate the mechanism of the antibiotic. Extensive literature exists showing the rise of *Staphylococcal* resistance to the FASII/FabI inhibitor triclosan [5,94,95]. Amino acid mutations to the *Staphylococcal*/FabI can cause significant increase in resistance against triclosan (or AFN-1252) with minimal reduction in fitness [5,16]. FabI mutants pose the greatest threat to FabI therapeutics because these mutants are resistant and grow without exogenous fatty acid supplementation. Significant reservoirs of such mutants can accumulate in environmental *S. aureus* populations, unlike the fatty acid auxotrophs which can only grow in environments with sufficient exogenous fatty acids. Whether single step mutations leading to high level resistance affect the efficacy of single-target antibiotics is under active discussion and play into the strategy of developing novel antibiotics [4,92,96]. Well-designed and comprehensive experiments characterizing the fitness consequences of these mutations will be essential to interpret their importance to successful antibiotic therapy.

3.3. Fatty Acid Metabolism Alterations in Biofilm Synthesis

Biofilms are bacteria produced matrices of extracellular polymers that allow the surface adherence of normally planktonic bacteria [97,98]. Biofilms are made of primarily polysaccharides, DNA, and peptides. Biofilm formation promotes bacterial persistence and antibiotic resistance by forming dense matrices that allow the cells in the outer layer to protect the cells in the matrix [99]. Biofilm formation also promotes lateral gene transfer and cooperation between different bacterial species within the matrix [100]. Fatty acid derived quorum sensing molecules are known as communication molecules within the biofilm [101,102], but recent research demonstrates that alterations of fatty acid and phospholipid metabolism are also associated with the formation of biofilms.

A transposon insertion library of *S. aureus* strain USA300 was screened for change in biofilm production [103]. A mutant bearing a transposon insertion in the *fakA* gene had a 1.9–4.6 fold increase in biofilm formation. Transduction of the transposon and complementation with the wild type *fakA* gene confirmed that FakA alone is responsible for the change in biofilm production. This experiment is the first to demonstrate that biofilm production is linked to the molecular machinery for incorporating exogenous fatty acids in

S. aureus, and offers a molecular link for biofilm formation and fatty acid metabolism. However, further research is necessary to elucidate how these two processes are connected.

Mutations to fatty acid and phospholipid synthesis genes were observed in the evolution of *Burkholderia multivorans* during a chronic cystic fibrosis infection [104]. *B. multivorans* is an opportunistic pathogen responsible for chronic lung infections in cystic fibrosis patients. The evolution of a *B. multivorans* infection in a cystic fibrosis patient was documented over 20 years. Ten gene loci acquired three or more independent mutations over the infection, and three of these gene loci map to key steps in fatty acid and phospholipid metabolism (*fabD*, *accD*, and *plsX*). The other seven loci mapped to gene regulation proteins or membrane transporters. Nonsynonymous substitutions in the *fabD* gene and ABC transport permease were the first to arise, and these mutants had decreased growth rate, decreased biomass, but no change in the production of biofilms. Additional and subsequent nonsynonymous substitutions in *accD*, *plsX*, and OmpR-like response regulator caused increase in biofilm production. Bioinformatic analysis performed for this review found that the nonsynonymous amino acid mutations in the *fabD* and *accD* genes do not map to essential catalytic residues. However, some of the mutations such as H89Y in FabD do occur in active site residues, suggesting that these mutations decrease the activity of the enzymes rather than completely abolish the activity. One blind spot in this experiment is that the selection media was not supplemented with exogenous fatty acids, and therefore mutants with severely decreased fatty acid synthesis could never be isolated. Again, this work shows a clear link between fatty acid metabolism and biofilm production, although the underlying mechanism is unclear.

4. Perspectives

Fatty acid synthesis is the most energy and material intensive part of phospholipid synthesis [1], and bacteria have long been recognized to incorporate exogenous fatty acids from the environment [13]. With the discovery of the fatty acid kinase system [37] and the characterization of acyl-ACP synthetase in pathogenic Gram-negative bacteria species [8,9], the molecular mechanisms for the incorporation of exogenous fatty acids in bacteria are largely characterized. However, more research is required to understand how exogenous fatty acid incorporation effects bacterial physiology. One current area of research focuses on understanding whether exogenous fatty acids can bypass the inhibition of endogenous fatty acid synthesis [11,65,66]. The result of this research will determine whether FASII inhibitors will make therapeutically successful antibiotics for the targeted bacterial species. Only Lactobacillales encode for the ability to bypass the inhibition of endogenous fatty acid synthesis using exogenous fatty acids, but can single genetic mutations allow other pathogenic species to bypass FASII inhibition? Another important question is what are the fitness implications of these mutations and can the mutants persist in the environment? More work is required to characterize the fitness of the *S. aureus fabD* mutants generated from the selection experiments and the putative biochemical defects in the mutant proteins [85]. These questions are also relevant for pathogens like *N. gonorrhoeae* that could scavenge all the essential FASII products from the host [9].

Recent research shows that the fatty acid and phospholipid biosynthetic genes in *B. multivorans* are undergoing long term evolutionary changes in cystic fibrosis patients [104]. The mutations that are predicted to decrease FASII activity are associated with increased biofilm formation. FakA inactivation mutants in *S. aureus* also have increased biofilm production and decreased virulence [37,103], providing another link between phospholipid metabolism and virulence in bacteria. Further work is required to characterize the molecular mechanism linking phospholipid metabolism, biofilm formation, and virulence. Another key question is whether these mutants are able to persist in the environment or if they are dead end mutations that evolve to optimize growth in a specific host niche. Applying culture-independent metagenomic sequencing methods would give a better understanding of the extent of FASII mutations that persist in nature.

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Highlights

- Bacterial fatty acid synthesis is a target for antibiotic development
- Bacteria use one of three enzymes for the activation of exogenous fatty acid for incorporation into phospholipid
- Lactobacillales are the only bacterial order where exogenous fatty acid lead to a shutdown of endogenous fatty acid synthesis
- Fatty acid synthesis cannot be bypassed by exogenous fatty acid in other bacteria
- Culture-independent metagenomic methods are needed to assess the prevalence of fatty acid synthesis mutations in bacterial infections.

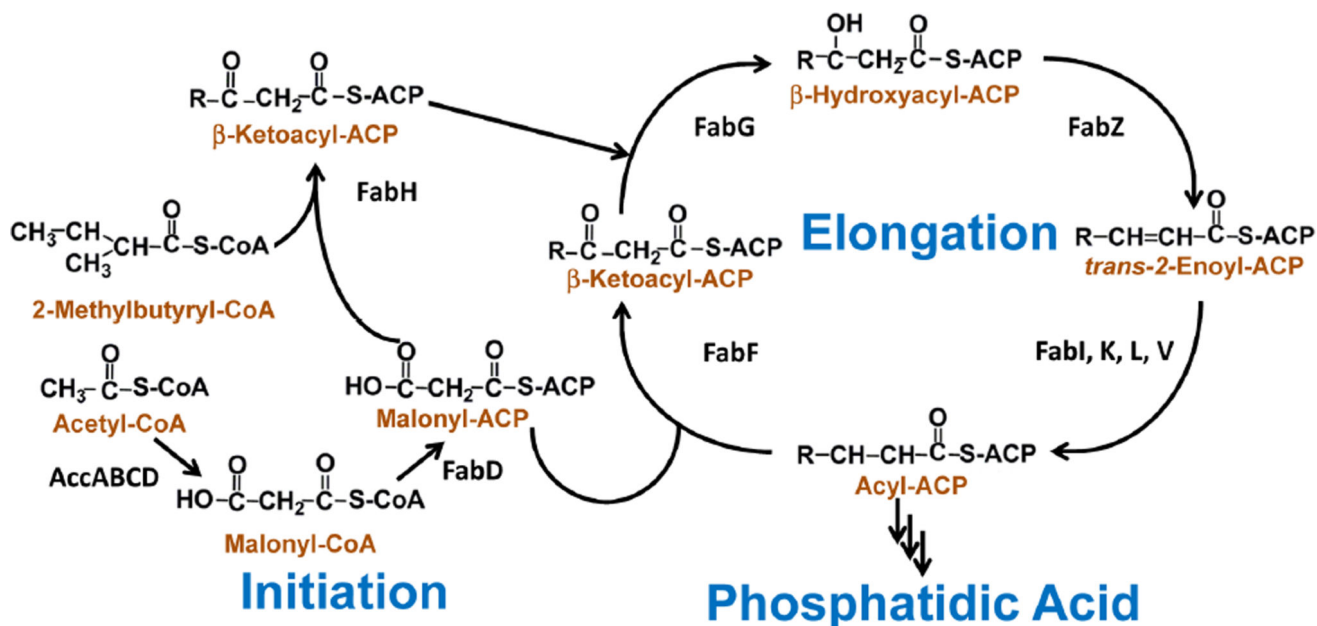


Fig. 1. The core enzymes in bacterial type II fatty acid synthesis

The acetyl-CoA carboxylase enzyme complex (AccABCD) converts acetyl-CoA into malonyl-CoA, which is in turn converted into malonyl-ACP by the malonyl-CoA:ACP transacylase (FabD). The β -ketoacyl-ACP synthase III (FabH) catalyzes the condensation of malonyl-ACP with either acetyl-CoA or 2-methylbutyryl-CoA to form β -ketoacyl-ACP and initiate straight- or branched-chain *anteiso* fatty acid synthesis. The β -ketoacyl-ACP is reduced by β -ketoacyl-ACP reductase (FabG) to make β -hydroxyacyl-ACP. β -hydroxyacyl-ACP of the appropriate length is used for lipopolysaccharide synthesis in Gram-negative bacteria. β -hydroxyacyl-ACP is dehydrated by β -hydroxyacyl-ACP dehydratase (FabZ) to make *trans*-2-enoyl-ACP. The *trans*-2-enoyl-ACP is reduced by enoyl-ACP reductase (FabI) into acyl-ACP. Acyl-ACP of the appropriate length can be used for phospholipid synthesis. Acyl-ACP can also be lengthened by two carbons through condensation with malonyl-ACP by β -ketoacyl-ACP synthase II (FabF) to make β -ketoacyl-ACP, which can undergo another elongation cycle by FabG, FabZ, FabI, and FabF. The bacterial FASII enzymes are soluble, discrete, and monofunctional, in contrast to the multifunctional mammalian fatty acid synthase. There are multiple isoforms of the enoyl-ACP reductase enzyme (FabK, FabL, and FabV). Additional enzymes interact with the FASII intermediates to synthesize unsaturated fatty acid and lipoic acid. Please refer to reviews for more details in FASII operation [1,105].

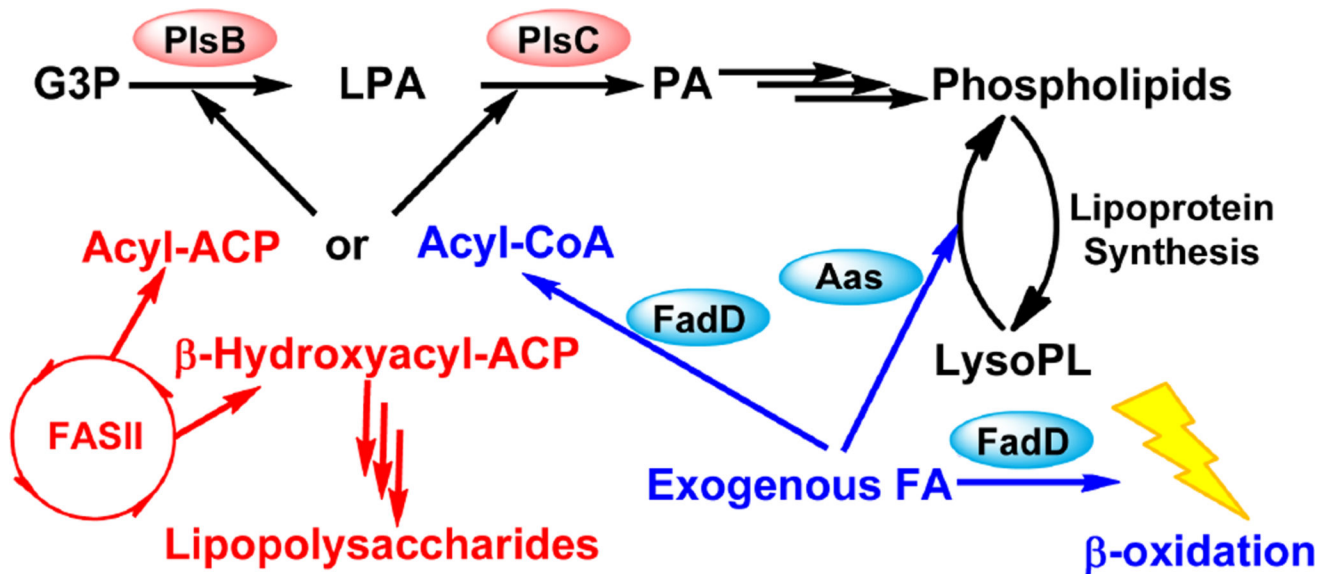


Fig. 2. Phosphatidic acid synthesis and exogenous fatty acid activation in *E. coli*
E. coli and other Gammaproteobacteria utilize the PlsB pathway for the synthesis of phosphatidic acid. Phosphatidic acid is synthesized from the successive acylation of G3P by the *sn*-glycerol-3-phosphate acyltransferase PlsB and 1-acyl-*sn*-glycerol-3-phosphate acyltransferase PlsC in *E. coli*. Both PlsB and PlsC are integral membrane enzymes. Exogenous fatty acids are converted into acyl-CoA by the acyl-CoA synthetase (FadD) and metabolically compartmentalized from endogenously synthesized acyl-ACP. Exogenous fatty acid is used for phospholipid synthesis because both acyltransferases in *E. coli* use either acyl-ACP or acyl-CoA of the appropriate length as acyl donors. Acyl-CoA can also be broken down via β -oxidation to generate acetyl-CoA and energy. Exogenous fatty acid is also used by the 2-acylglycerophosphoethanolamine acyltransferase (Aas) to reacylate lysophospholipids generated by lipoprotein synthesis. This reacylation reaction proceeds through an acyl-ACP intermediate, but the acyl-ACP cannot dissociate from the Aas complex and is used only for the reacylation reaction. Exogenous fatty acids cannot rescue inhibition of endogenous fatty acid synthesis in *E. coli* because β -hydroxyacyl-ACP generated by FASII is required for the synthesis of essential lipopolysaccharides. A pathway to generate β -hydroxyacyl-ACP from exogenous fatty acids does not exist.

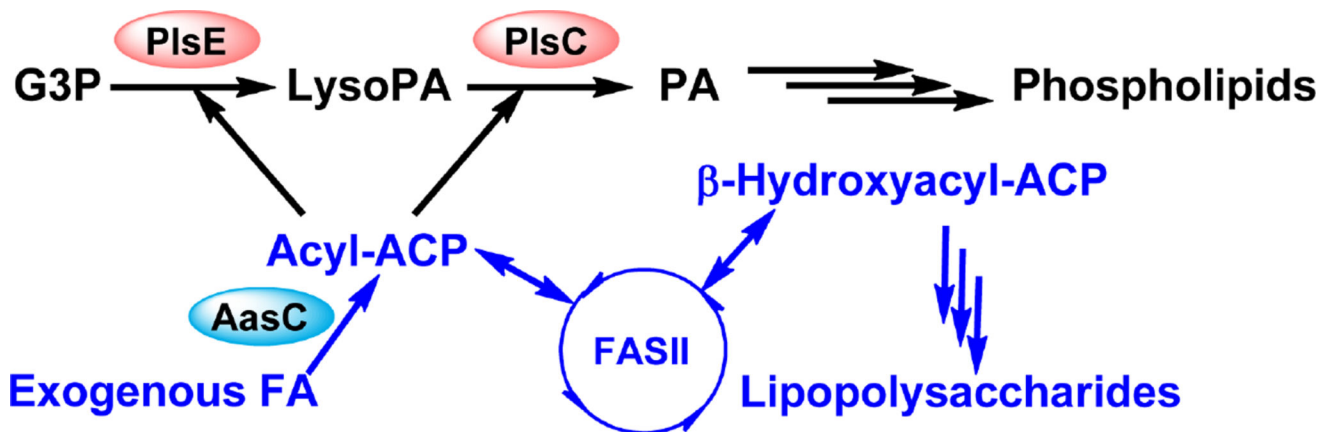


Fig. 3. Phosphatidic acid synthesis and exogenous fatty acid incorporation in *C. trachomatis* Phosphatidic acid is synthesized from the successive acylation of G3P by the G3P acyltransferase PlsE and 1-acyl-*sn*-glycerol-3-phosphate acyltransferase PlsC in *C. trachomatis*. The *C. trachomatis* PlsE is a soluble G3P acyltransferase. Host fatty acids are converted into acyl-ACP by the *C. trachomatis* acyl-ACP synthetase (AasC). The *C. trachomatis* acyl-ACP synthetase and lysophospholipid acyltransferase are encoded as separate enzymes rather than a bifunctional enzyme as found in *E. coli*. This organizational change allows the resulting acyl-ACP to undergo similar fates as endogenously synthesized acyl-ACP: elongated by the FASII or used as an acyl-donor by the acyltransferases. Despite actively incorporating host fatty acids for phospholipid synthesis, *C. trachomatis* cannot bypass FabI inhibition. The 2-position of *C. trachomatis* phospholipids has high preference for *anteiso*15:0 fatty acid, which cannot be obtained from the host.

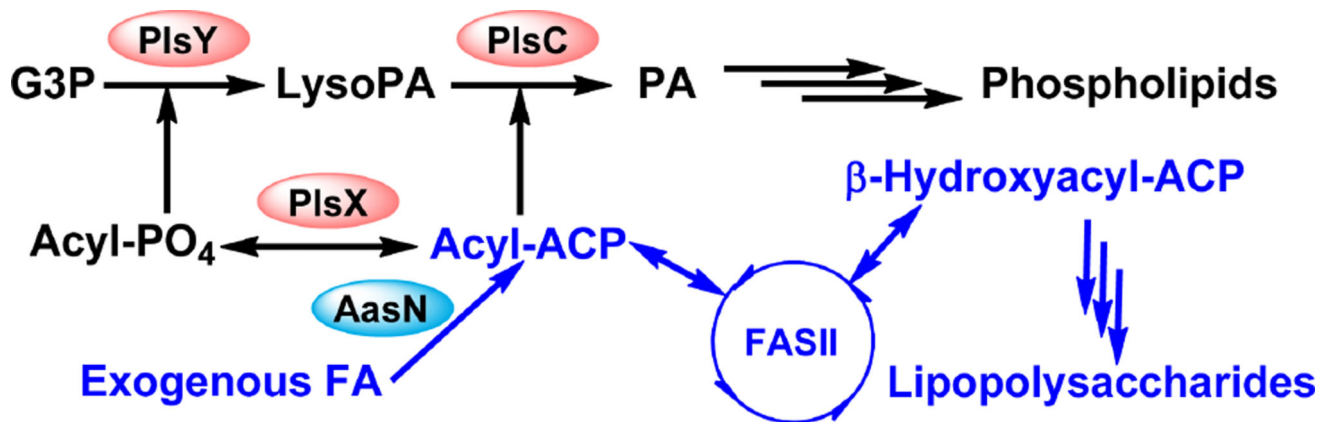
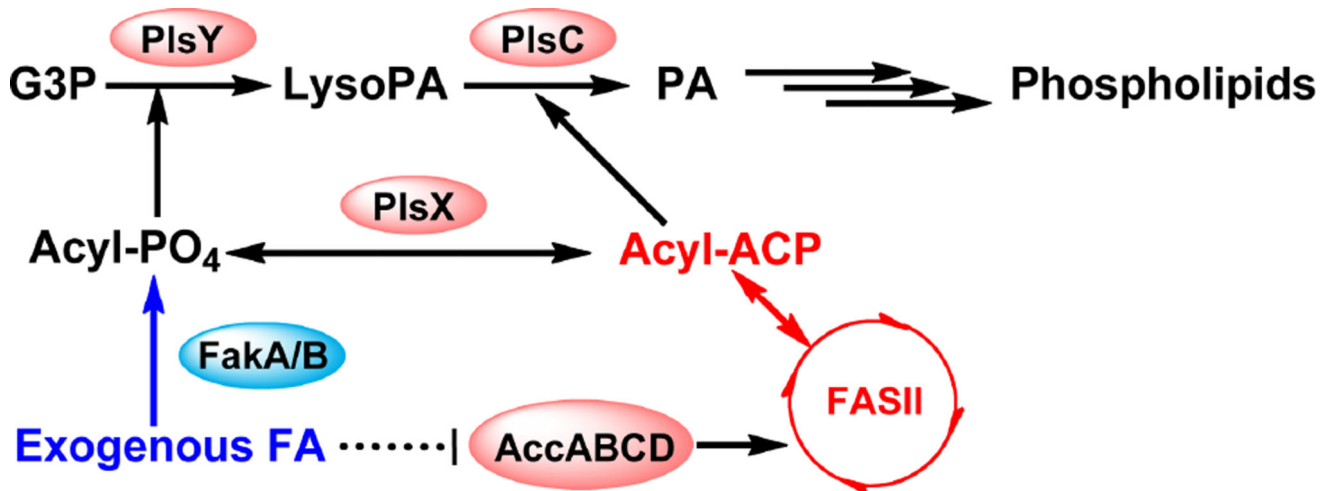


Fig. 4. Phosphatidic acid synthesis and exogenous fatty acid incorporation in *Neisseria*
Neisseria utilize the PlsX/PlsY pathway for the synthesis of phosphatidic acid. Acyl-ACP is converted into acyl-phosphate by the acyl-ACP:phosphate transacylase PlsX. The G3P acyltransferase PlsY utilizes the acyl-phosphate to acylate G3P in the first acylation step of phosphatidic acid synthesis. The acyltransferase PlsC acylates the resulting lysophosphatidic acid using acyl-ACP in the second step to generate phosphatidic acid. Exogenous fatty acids are introduced into phosphatidic acid synthesis via conversion into acyl-ACP by the acyl-ACP synthetase in *Neisseria*. The resulting acyl-ACP undergoes the same fate as endogenously synthesized acyl-ACP. Lipopolysaccharides are not essential in *Neisseria*, and the major acyl chains found in *Neisseria* are 16:0 and 16:1 fatty acids. However, 16:0 and 16:1 fatty acid complementation cannot bypass the inhibition of endogenous fatty acid synthesis. It is unclear why this is the case.



Exogenous fatty acids shut down endogenous fatty acid synthesis in some Gram-positive bacteria, but not others.

Fig. 5. Phosphatidic acid synthesis and exogenous fatty acid incorporation in Gram-positive bacteria

Gram-positive bacteria utilize the PlsX/PlsY pathway for the synthesis of phosphatidic acid. PlsX converts acyl-ACP into acyl-phosphate, which is used as the acyl donor by the acyltransferase PlsY in the synthesis of lysophosphatidic acid. PlsC utilizes acyl-ACP as the acyl donor to generate phosphatidic acid. Exogenous fatty acids are converted into acyl-phosphates by the fatty acid kinase system FakA/B. The resulting acyl-phosphate can be converted into acyl-ACP by the acyl-ACP:phosphate transacylase PlsX. The resulting acyl-ACP can be elongated by the FASII, used by PlsC, or converted back into acyl-phosphate. Exogenous fatty acids can bypass the inhibition of FASII in Lactobacillales but not Bacillales. This difference is due to how exogenous fatty acids regulate endogenous fatty acid synthesis in these two bacterial orders. In Lactobacillales, exogenous fatty acids cause a dose dependent and complete inhibition of the endogenous synthesis of fatty acids, effectively rendering FASII inactive and bypassed. In Bacillales, exogenous fatty acids cause a dose dependent, but only partial inhibition of the endogenous synthesis of fatty acids. Inhibition of FASII in Bacillales causes depletion of the free ACP pool by tying them up as short chain acyl-ACP. Exogenous fatty acids cannot be converted into acyl-ACP, and phospholipid synthesis is halted.