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Bacterial Lipids: Metabolism and Membrane Homeostasis

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1. Introduction

Bacterial lipid metabolism has long had a significant impact on the understanding of the basic lipid metabolic pathways, enzyme mechanisms and transcriptional regulation. The early work in the *Escherichia coli* system jump-started the investigation of fatty acid and phospholipid synthesis. A recent review by Dowhan [1] recounts these early days of discovery in bacterial lipid metabolism. For decades, *E. coli* was considered the paradigm for bacterial metabolism; however, the advent of the genomic era revealed that genes and enzymes of lipid metabolism painstakingly investigated in *E. coli* are not common to all bacteria. This realization has accelerated research into the great diversity in pathways, and fatty acid and phospholipid structures that occur in nature. This review attempts to capture and organize this diversity to provide an overview of lipid metabolism in prokaryotes as it stands today.

2.1 The FASII initiation module

The function of the initiation module of FASII is to produce the primer and the building blocks to feed the elongation module (Fig. 1). The acetyl-CoA carboxylase (ACC) performs the first committed step in bacterial phospholipid synthesis to generate malonyl-coenzyme A (malonyl-CoA) through the carboxylation of acetyl-CoA [2–4]. In order to be recognized by the FASII enzymes, the malonate group from malonyl-CoA must be transferred to acyl carrier protein (ACP) by FabD [5]. The condensation of malonyl-ACP with a short-chain acyl-CoA (C₂-C₅) by FabH initiates the elongation cycle [6–8]. The malonyl-ACP generated by the ACC and FabD is also used by the elongation cycle to extend the growing fatty acid chain, illustrating how crucial ACC activity is to maintaining the optimum rate of membrane phospholipid synthesis. Every condensation reaction performed by FabH will result in the production of a new fatty acid to expand the membrane. Consequently, the initiation module is ideally positioned for biochemical and genetic regulation of the amount of fatty acids produced and speed at which they are manufactured.

2.1.1 Acetyl-CoA Carboxylase—The acetyl-CoA carboxylase enzyme is present in animals, plants, fungi and bacteria. In bacteria, the ACC is a multisubunit complex consisting of a biotin carboxylase (AccC), biotin carboxyl carrier protein (AccB, also known as BCCP) and a carboxyltransferase (AccAD). AccB and AccC exist as homodimers while AccAD is a heterotetramer [3,9,10]. In the cell, ACC is likely a multimeric complex of these four subunits; however, the complex readily dissociates following cell disruption leaving only the two ACC half reactions that can be measured. The first half reaction catalyzes the

ATP-dependent carboxylation of biotin on AccB by AccC [11,12]. The carboxylation of free biotin or biotin analogs is an extremely inefficient reaction, and maximum rates required biotin to be covalently attached to a lysine residue of biotin carboxyl carrier protein (AccB) [11,13]. In the second half reaction, the carboxyl group is transferred from carboxyl-AccB to acetyl-CoA by AccAD. The two half-reactions can be assayed individually but reconstituting the complete ACC reaction is more challenging [14,15]. High concentrations of each purified subunit are required to reach a threshold concentration for the catalytically active complex to form in vitro [11,12,16]. The genetic organization of the four *acc* genes differs between organisms. In *Escherichia coli* and *Staphylococcus aureus*, the *accAD* and *accBC* genes are organized in operons located at different regions of the chromosome [9]. In *Streptococcus pneumoniae*, the *acc* genes are located adjacent to each other in a transcriptional unit that also contains the other FASII genes [17]. Evidence that the ACC governs the quantity of fatty acid produced by the cell was provided by a study involving the overexpression of *accABCD* in *E. coli*. Maintaining a normal lipid to protein ratio is critical for homeostasis, therefore the authors devised a means to uncouple FASII from phospholipid synthesis and excrete the excess fatty acids from the cell. The *acc* genes were overexpressed in combination with a soluble acyl-ACP thioesterase to uncouple FASII from membrane synthesis resulting in 5–6 fold increase in fatty acid synthesis [18]. The influence of ACC activity on the rate of fatty acid synthesis underlines the importance of stringent regulation of the ACC activity.

2.1.2 Role of FabH in determining fatty acid structure—There is remarkable diversity in the fatty acid structures produced by different bacteria. Like humans, *E. coli* and *S. pneumoniae* produce even-number straight-chain saturated and unsaturated fatty acids [19]. Many Gram-positive bacteria, such as *B. subtilis* and *S. aureus*, produce predominantly odd-numbered branched-chain fatty acids [20]. The branched/straight and even/odd characteristics are determined by the substrate specificity of FabH, the initiation condensing enzyme [21]. This enzyme catalyzes the first condensation reaction that initiates the fatty acid elongation cycle [22] (Fig. 1). *E. coli* FabH (EcFabH) selectively utilizes acetyl-CoA derived from intermediary metabolism, whereas FabH enzymes from *B. subtilis* and *S. aureus* preferentially utilize the bulkier branched-chain acyl-CoA primers derived from amino acids [20,21,23,24]. The branched-chain acyl-CoA substrates lead to the production of *anteiso* and *iso* fatty acids. The selectivity of *E. coli* FabH (EcFabH) for acetyl-CoA and *S. aureus* FabH (SaFabH) for branched-chain (C4-C5) acyl-CoAs is attributed to structural differences in the hydrophobic pocket adjacent to the active site that accommodates the acyl-enzyme intermediate. It is the organization of the residues in this pocket that determine which acyl-CoA primers can be accommodated by the enzyme. In the *E. coli* FabH structure solved by Qiu *et al.*, the binding pocket is only large enough to fit acetyl- or propionyl-CoA [21,25,26]. Although the sequence of the residues throughout the tunnel are almost identical, in *S. aureus* FabH the side chains of the residues are oriented to create space for bulkier branched-chain acyl-CoAs [21]. The shape of this pocket is thought to be a major determinant of the substrate specificity of FabHs [7,19,21,27].

Confirmation for the role that FabH plays in determining the structure of fatty acids was provided by Li *et al.* who performed an elegant study using the FabH from *Streptomyces coelicolor* (ScFabH) [28]. *S. coelicolor* phospholipids contain predominantly branched-chain fatty acids (74%), a signature of a FabH enzyme with a preference for branched-chain acyl-CoA substrates. Li *et al.* deleted the endogenous *fabH* gene in *S. coelicolor* and complemented the deletion with either *EcfabH* or *fabH* from *Streptomyces glaucescens* (*SgfabH*), another organism that synthesizes mainly branched-chain fatty acids. Complementation with SgFabH had little effect on the fatty acid composition of *S. coelicolor*, whereas complementation with EcFabH resulted in an organism with 87.5% straight-chain fatty acids compared to 25.6% with the endogenous ScFabH enzyme. The

EcFabH-complemented mutant also exhibited a major growth defect, stressing the importance of a correct fatty acid profile for bacterial membrane homeostasis.

A unique FabH initiates FASII in Mycobacteria by utilizing long-chain acyl-CoA. *Mycobacterium tuberculosis* uses a type one fatty acid synthase (FAS I) to manufacture long-chain acyl-CoA products (C14-C26) [29]. The products are utilized by MtFabH to initiate elongation by FASII enzymes to generate the very long-chain mycolic acids (C50-C56). The ability of mtFabH to utilize such long-chain primers requires some significant structural alterations compared to the *E. coli* enzyme (EcFabH). MtFabH contains an extra hydrophobic channel that permits binding of longer acyl-CoA substrates. In EcFabH, this channel is blocked by a phenylalanine whereas the corresponding threonine residue in MtFabH allows an opening into a much larger hydrophobic cavity that accommodates the fatty acid [29].

The sequence similarity of different FabH enzymes varies enormously but each is characterized by having a conserved Cys-His-Asn catalytic triad [21]. Every FabH catalyzes a Claisen-type condensation reaction between an acyl-CoA substrate and malonyl-ACP using a bi-bi ping-pong mechanism [30]. The acyl-CoA binds to the enzyme and the acyl group is inserted into the hydrophobic active site tunnel of the protein [25,26,31]. The acyl chain is transferred to the active site cysteine via nucleophilic attack of the sulfhydryl on the CoA thioester, resulting in the release of CoA [31]. Malonyl-ACP then binds to the acyl-enzyme intermediate [31], and condenses with the intermediate, releasing bicarbonate and the β -ketoacyl-ACP product. The His and Asn residues of the catalytic triad are critical for the malonyl-ACP decarboxylation half reaction and are thought to aid the process by interacting with and stabilizing the developing negative charge on the malonyl-ACP carbonyl oxygen [31].

The initiation (FabH) and elongation (FabB/F) condensing enzymes are distinguished by their active site residues. However, both FabH and FabB/F catalyze identical condensation reactions, so the distinction between FabH having a His-Asn-Cys triad versus the His-His-Cys triad of FabB/F has always been unclear. The requirement for the His-Asn-Cys catalytic triad for bacterial initiation condensing enzymes was recently challenged by Yuan *et al.* who investigated FASII initiation in *Pseudomonas aeruginosa* [32]. Using bioinformatics, four genes were identified with similarity to EcFabH and each contained at least two of the three His-Asn-Cys residues of the active site. A quadruple knockout strain of *P. aeruginosa* was constructed with all four potential FabH genes deleted. Interestingly, this mutant strain showed no obvious growth defect suggesting none of these *fabH* genes were responsible for initiating FASII. The author's identified a gene named *fabY* (PA5174) that was essential for *P. aeruginosa* viability, and restored growth in an *E. coli* FabH knockdown strain. This deviation from the FabH nomenclature was appropriate because FabY active site consists of a His-His-Cys triad that is characteristic of the elongation condensing enzyme (FabF/FabB). FabY differs from the FabF/FabB enzymes in preferring acyl-CoA substrates instead of the acyl-ACP substrates employed by the elongation condensing enzymes. A FabY structure may shed light on why this unique condensing enzyme has adopted the His-His-Cys configuration.

The question of whether *fabH* is essential for growth in *E. coli* was investigated by introducing the *Salmonella enterica fabH* gene into the *E. coli* chromosome [33]. The endogenous *fabH* gene was easily deleted from the double-*fabH* strain, but *fabH* could not be inactivated in the parent strain. This conclusion was supported by experiments in *Lactococcus lactis* where the deletion of *fabH* gave rise to exogenous fatty acid auxotrophs. These data appeared to provide convincing evidence for the essentiality of FabH in *E. coli*, except that a different *E. coli fabH* null mutant was able to grow without a fatty acid

supplement and has a cell volume that is 70% smaller [34]. The apparent discrepancy was traced to differences in the genotypes of the *E. coli* strains used in the two experiments. The strain where *fabH* essentiality was demonstrated contained both *relA1* and *spoT1* mutations that inactivate these enzymes. RelA and SpoT are involved in the regulation of the intracellular concentration of ppGpp, a small molecule that regulates a multiplicity of cellular processes [35]. However, *fabH* can be deleted in wild-type strains and a series of genetic experiments showed that a functional *spoT* gene was required in order for *fabH* to be dispensable in *E. coli* [34]. Although the accumulation of ppGpp following the inhibition of fatty acid synthesis is SpoT dependent [36] and SpoT directly interacts with ACP [37–39], the role of SpoT in FASII is a mystery and deserving for more research.

The enzyme(s) responsible for the initiation of FASII in the absence of FabH in *E. coli* remain to be determined. The fatty acid auxotrophy of the *L. lactis fabH* deletion mutant can be rescued by overproduction of the β -ketoacyl-ACP synthase II, FabF [40]. Although the catalytic mechanism of the FabF condensing enzyme is identical to FabH, FabF is unable to utilize acetyl-CoA and is instead specific for acyl-ACP primers. Importantly, as demonstrated by in vitro experiments, the FabF enzyme is able to self-prime in the absence of a suitable acyl-ACP substrate by the decarboxylation of malonyl-ACP to acetyl-ACP, which subsequently forms the acyl-enzyme intermediate. In this example, FabH is bypassed by this side-reaction of FabF [41]. The acetyl-enzyme would subsequently perform the condensation reaction with malonyl-ACP to produce the β -ketobutyryl-ACP normally generated by FabH. The efficiency of the decarboxylation reaction compared to the condensation reaction is low, explaining the need for abnormally high FabF expression to complement the FabH defect [42,43]. These studies provide insight into the phenotype in the *L. lactis fabH* mutant and suggest that either FabB or FabF may be compensating the lack of FabH in the *E. coli fabH* deletion. However, this idea remains unproven and further experimentation is required to positively identify the enzyme responsible for the initiation of FASII in the absence of FabH in *E. coli*.

2.2. The Elongation Module

The elongation module represents the central machinery of bacterial FASII (Fig. 2). These four enzymes work in concert to receive the product of the FabH reaction and elongate the acyl-ACP by two carbons with the completion of each turn of the cycle until a long-chain acyl-ACP is generated. The enzymes appear to be independent biochemical entities, and the cycle can be reconstituted in vitro by incubating the enzymes with necessary substrates and cofactors [44]. The FabH product enters the elongation cycle and is reduced by the NADPH dependent β -ketoacyl-ACP reductase (FabG) to form β -hydroxyacyl-ACP. The crystal structures of FabG from an assortment of organisms are known, and there is very little variation in the FabG structures between plant, Gram-positive and Gram-negative bacterial enzymes [45–48]. The similarities in FabG structure between organisms that produce distinctly different fatty acids suggests FabG does not play a role in determining fatty acid structure. The next step in the elongation cycle is dehydration of the β -hydroxyacyl-ACP to the *trans*-2-enoyl-ACP. In *E. coli*, FabA and FabZ are the enzymes that catalyze this reaction. The distinction between the two enzymes lies in the ability of FabA to perform the *cis-trans* isomerase reaction needed to synthesize unsaturated fatty acids (see section 2.2.3). In Gram-positive bacteria containing saturated fatty acids, only the FabZ isozyme is present, but FabZ is the only isoform in *S. pneumoniae*, which produces unsaturated fatty acids. Thus, FabZ must be capable of elongating these intermediates acyl-ACP also.

The next reaction of the elongation cycle is performed by the NAD(P)H *trans*-2-enoyl-ACP reductase, which in many bacteria is the FabI enzyme [49]. This reaction reduces the C₂-C₃ double bond on *trans*-2-enoyl-ACP to form acyl-ACP. FabI belongs to a subgroup within the short-chain alcohol reductase/dehydrogenase superfamily that is defined by the Tyr-Xaa₆-

Lys catalytic diad and a requirement for nicotinamide cofactors [50]. The lysine binds the 2'-hydroxyl group of the ribose moiety of the cofactor and the tyrosine stabilizes the enoyl-form of the thioester during catalysis. In *E. coli*, FabI has a preference for NADH over NADPH although the converse is true of *S. aureus* FabI. *Bacillus subtilis* possesses a FabL enoyl-ACP reductase in addition to a FabI [51]. This alternate reductase has an identical catalytic diad to FabI, but has a strong preference for NADPH over NADH. Neither FabI nor FabL are essential in *B. subtilis* but a double knockout cannot be obtained showing that the two enzymes function interchangeably in FASII. A structurally distinct enoyl-ACP reductase, FabV, was first discovered in *V. cholerae* [52] and subsequently in *P. aeruginosa* [53] and *Burkholderia mallei* [54] during studies on bacteria resistant to the FabI inhibitor, triclosan. The origin of FabV is indicated by the presence of an enzyme (BatG) in an operon in that produces a natural product FabI inhibitor [55]. The FabV homolog (BatG) confers resistance to bacteria toward this natural product, whereas bacteria that have only a FabI are sensitive to the natural product. FabV is relatively widespread in bacteria and two FabV structures have been solved [56,57]. FabV is 60% larger than FabI and also possesses the catalytic Tyr and Lys residues, albeit in a different configuration (Tyr-Xaa-Lys). Many *Streptococci* use an unrelated flavoenzyme, FabK, to perform the enoyl-ACP reductase reaction [58]. This enzyme lacks the Lys and Tyr catalytic residues in FabI, but uses a His and a flavin mononucleotide (FMN) for catalysis instead [59]. It is thought that this unique reductase may have an additional function as an NAD⁺ re-generating system due to its NADH oxidase activity observed in the absence of a substrate, which would promote energy metabolism via glycolysis. Despite this significant structural diversity between species, each of the different enoyl-ACP reductases can complement a temperature-sensitive *fabI* mutant in *E. coli*, indicating their common function in the FASII elongation cycle. The acyl-ACP produced by the elongation module has two fates. It can be used by the acyltransferase module (Fig. 3) or it can be used by the elongation condensing enzyme, FabB or fabF, to initiate a new round of elongation (Fig. 2).

Several of the intermediates generated in the elongation module are also required for other essential cellular processes. An example is lipoic acid, synthesized from octanoyl-ACP manufactured by the elongation cycle [60,61]. This sulfur containing coenzyme is present in almost all prokaryotic bacteria and is covalently attached to pyruvate dehydrogenase and glycine cleavage protein [60]. This modification is required for catalytic activity of these enzymes and depletion of lipoic acid in lipoic acid auxotrophic *E. coli* mutants causes growth arrest [62]. Another essential enzyme cofactor arising from FASII is biotin, which is synthesized from a pimeoyl-ACP derived from FASII [63]. The importance of biotin as a cofactor for carboxylase enzymes (including ACC) is well appreciated (section 2.1.1). In addition to enzyme cofactors, structural components not destined to the cytoplasmic membrane also require the diversion of intermediates from the elongation module. Lipid A is needed to form the hydrophobic anchor of lipopolysaccharide (LPS) in Gram-negative bacteria and usually contains at least four β -hydroxy-fatty acids derived from FASII [64]. The β -hydroxy-fatty acids are an essential structural component of the LPS and cannot be substituted for by normal fatty acids. Also, the enzymes of LPS biosynthesis are specific for ACP thioesters, thus FASII is the only source for the hydroxy-fatty acids of LPS [64], making FASII essential even in the presence of exogenous fatty acids. In Gram-positive bacteria, which lack LPS, exogenous fatty acids can be ligated to ACP and enter the elongation cycle [65]. Finally, intermediates in the elongation module are diverted to produce variety of extracellular molecules that regulate cell activity. Examples, are the quorum-sensing N-acyl-homoserine lactones that are produced by a large number of organisms [66,67]. The quinolone signals [68] and extracellular rhamnolipid surfactants [69] produced by *P. aeruginosa* are derived from FASII, as is the recently discovered diffusible signal factor, *cis*-2-decenoic acid [70].

2.2.1 The determinant role of FabI in the rate of elongation—The idea that the enoyl-ACP reductase (FabI, Fig. 2) is a rate-determining reaction in the elongation module is derived from the in vitro reconstitution of the cycle using *E. coli* enzymes. FabI catalyzes the final reaction of the elongation cycle and the resulting acyl-ACP product is either used by FabB/F to begin another round of elongation or it is incorporated into phospholipids by the acyltransferases (Fig. 2). The in vitro reactions catalyzed by the elongation module enzymes FabI, FabG, and FabB/F result in extensive product formation. However, the equilibrium of the β -hydroxyacyl-ACP dehydratase reactions performed by EcFabA or EcFabZ favor the formation of β -hydroxyacyl-ACP over enoyl-ACP [49]. The analysis of the acyl-ACP pool composition in FabI-depleted *E. coli* shows the accumulation of predominantly β -hydroxy-ACP instead of enoyl-ACP in a 9:1 ratio corroborating the in vitro results in vivo [49]. Thus, FabI pulls the elongation cycle to completion, and thus regulates of the rate at which the elongation cycle turns. Whereas this idea is consistent with all the data in *E. coli*, it would be wrong to think that the enoyl-ACP reductase plays the same role in all bacteria. We now know of the diversity in enoyl-ACP reductase structures among organisms, and it would not be prudent to conclude that these widely disparate entities have the same role in the pathway. Also, the FabZ enzymes of all bacteria may not have the same equilibrium position. Thus, understanding the fundamental regulation of the elongation module in *E. coli* may or may not bring us closer to understanding how the cycle is controlled in pathogens.

FabI also impacts the basal saturated:unsaturated fatty acid ratio in some organisms. FabA is the enzyme that catalyzes the isomerization reaction of *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP at the branch point in unsaturated fatty acid synthesis (section 2.2.3; Fig. 4). Thus, FabA and FabI compete for the *trans*-2-decenoyl-ACP. The requirement of *E. coli* for a minimum of 15% unsaturated fatty acid content for growth stresses the importance of maintaining a balance of FabA catalyzed *cis-trans* isomerization and the cycle-concluding reaction of FabI [71]. This theory is supported by studies observing growth rate attenuation in *E. coli* overexpressing *fabI* [72]. Overproduction of FabA rather surprisingly leads to an increase in saturated fatty acid synthesis due to the reversible nature of the *cis-trans* isomerase reaction [73]. This change can be counteracted by overproduction of FabB, which drags *cis*-decenoyl-ACP produced by FabA into the unsaturated fatty acid pathway [73]. Although FabI and FabA compete for the *trans*-2-C10-ACP intermediate, the absence of a change in unsaturated fatty acid composition in cells overexpressing *fabI* is consistent with FabB being the rate-limiting step in unsaturated fatty acid synthesis. The balance between the *cis-trans* isomerase reaction and the *trans*-2-enoyl-ACP reductase reaction is more important in *S. pneumoniae* which lacks a FabA and utilizes a monofunctional FabM enzyme for the isomerization of *trans*-2-decenoyl-ACP to *cis*-2-decenoyl-ACP instead [74]. FabM directly competes with FabK for the substrate, and the replacement of FabK with FabI leads to a decrease in unsaturated fatty acid synthesis due to the higher rates of enoyl-ACP utilization by FabI [74]. These data point to the importance of different enoyl-ACP reductases in different bacteria and illustrate how the unsaturated fatty acid content in *S. pneumoniae* is regulated by the expression levels of FabK and FabM.

2.2.2 Condensing enzyme regulation of product structure—*E. coli* utilizes three condensing enzymes for fatty acid synthesis. Biochemical analysis of each of the three enzymes identified markedly differences in substrate specificities. β -Ketoacyl-ACP synthase III (KASIII) is the product of the *fabH* gene and is a main player during initiation of fatty acid synthesis as discussed in section 2.1.2. β -Ketoacyl-ACP synthase I (KASI), is encoded by the *fabB* gene and most efficiently catalyzes the condensation of short to medium chain acyl-ACPs with malonyl-ACP [75]. β -Ketoacyl-ACP synthase II (KASII) activity is the product of the *fabF* gene and functions in vivo to elongate palmitoleic acid (16:1 Δ 9) to *cis*-vaccinic acid (18:1 Δ 11), a reaction that FabB performs less efficiently [76]. All three

enzymes catalyze Claisen condensation reactions between an acyl donor and malonyl-ACP to generate a β -ketoacyl-ACP elongated by two carbons. Each KAS uses a ping-pong mechanism with an acyl-enzyme intermediate, but the catalytic residues vary between FabB/FabF and FabH. FabF and FabB utilize a His-His-Cys catalytic triad whereas most FabH enzymes utilize His-Asn-Cys residues. Deletion mutants of *fabB* in *E. coli* are unsaturated fatty acid auxotrophs and rely on a supply of exogenous oleic acid for growth [77]. Disruption of the *fabF* gene in *E. coli* has no discernible effect on cell growth but almost eliminates the pool of *cis*-vaccinic acid containing lipids [78]. Interestingly, the *fabF* mutant in *P. aeruginosa* deficient in all three forms of motility suggesting the *cis*-vaccinate is important to the assembly of the motility machinery [79]. The effect of *fabF* on motility in *E. coli* has not been examined. This difference in essentiality between FabB and FabF underlines the distinct but integral roles of each condensing enzyme in unsaturated fatty acid synthesis. FabB is absolutely required to feed the *cis*-decenoyl-ACP product produced by FabA into the elongation system, but has poor activity with any acyl-ACP with 16 carbons or more, including palmitoleoyl-ACP. Thus, FabF is the enzyme in *E. coli* capable of efficiently converting palmitoleic acid to *cis*-vaccinic acid [80]. The FabF reaction participates in the adaptation of *E. coli* to changes in temperature to maintain the biophysical properties of the bilayer. As the temperature decreases, the bacteria respond by increasing the percent of unsaturated fatty acid in their membranes [75]. FabF is a naturally temperature-sensitive enzyme retaining high activity at lower temperatures to increase the rate of *cis*-vaccinate production in relation to saturated fatty acids [75]. Interestingly, the lack of *cis*-vaccinate synthesis in *fabF* null *E. coli* can be recovered through the overexpression of *fabB*, offsetting the poor FabB activity associated with palmitoleoyl-ACP [81]. However, this plasmid-driven *cis*-vaccinate biosynthesis does not substitute for the temperature-dependent increase in *cis*-vaccinate in FabF-positive strains [81]. The reverse experiment of overexpression of *fabF* in a *fabB* deletion host has failed to provide useful information because the overexpression of *fabF* is cytotoxic to *E. coli* [82]. Analysis of intracellular CoA and ACP thioester pools during *fabF* overexpression suggests a block at the malonyl-ACP transacylase (FabD) reaction may be responsible because partial growth recovery is seen when *fabD* and *fabF* are co-expressed.

The length of the fatty acids produced by any bacteria is a result of competition between the condensing enzyme and phospholipid acyltransferases for the acyl-ACP intermediates in the elongation module [83]. The upper chain-length is limited to 20–22 carbons by the substrate specificity of the condensing enzymes, and the lower limit is 12–14 carbons due to the substrate specificity of the acyltransferases. This competition concept is supported by studies blocking the acyltransferases or overexpressing *fabB* in *E. coli* and observing a substantial increase in the average fatty acyl chain length [81,84]. Overexpression of the *plsB* acyltransferase leads to a decrease in the average chain length. *E. coli* has been considered the paradigm for dissociated fatty acid synthesis for decades, although only a minority of bacteria uses an elongation module with both FabB and FabF. Most bacteria accomplish acyl-chain elongation using exclusively FabF. The molecular basis for the exclusion of longer chain lengths from the elongation condensing enzymes appears to be the size of the hydrophobic pocket adjacent to the active site that accommodates the acyl-enzyme intermediate [85], but this reasonable hypothesis has received little direct experimental support. This idea highlights the need for the coordinate regulation of expression levels of the key enzymes in the elongation and acyltransferase modules.

2.2.3 Multiple solutions for the introduction of the double bond—The production of unsaturated fatty acids is an important aspect of membrane homeostasis in bacteria [5]. The physical state of a cell membrane is manipulated by the incorporation of a mixture of fatty acids with different melting temperatures into phospholipids. A decrease in temperature increases membrane rigidity and many bacteria respond by increasing the

proportion of unsaturated fatty acids incorporated into the phospholipids. When membrane fluidity increases due to rising temperature in the environment, the proportion of unsaturated fatty acids incorporated into the membrane decreases. There is considerable diversity in the mechanisms used by bacteria to generate unsaturated fatty acids. *E. coli* uses the bifunctional FabA enzyme to perform the dehydration of β -hydroxyacyl-ACP to *trans*-2-enoyl-ACP (Fig. 4), and specifically at the 10-carbon stage in the elongation module, the isomerization *trans*-2-decenoyl-ACP to *cis*-2-decenoyl-ACP [44]. The specificity of the isomerization reaction for the 10 carbon β -hydroxy-ACP is rationalized from the FabA crystal structure, which shows the active site tunnel that would perfectly fit a 10-carbon acyl-ACP [86]. But FabA cannot do the job alone. The product of the FabA isomerization reaction must be utilized by FabB to skip the reductase step and initiate the elongation of a monounsaturated fatty acid. Inactivating mutations in either the *fabA* or *fabB* genes result in unsaturated fatty acid auxotrophs. The *fabA-fabB* route to unsaturated fatty acids is common in the γ -proteobacteria, but most bacterial genomes do not contain these genes.

S. pneumoniae utilizes the FabM enzyme to introduce the double bond. This monofunctional *trans*-2, *cis*-3-decenoyl-ACP isomerase bears no similarity to FabA despite catalyzing the same reaction and having a strong substrate preference towards *trans*-2-decenoyl-ACP [74]. Interestingly, the *fabM* gene alone is not capable of complementing an *E. coli fabA* temperature-sensitive mutant. This was attributed to FabM being unable to successfully compete with FabI for the *trans*-2-enoyl-ACP substrate. This was remedied by expressing *fabM* and *fabK* on a plasmid based system and mitigating FabI activity using the potent FabI inhibitor triclosan [74]. This condition allowed the *fabA* mutant to be complemented by FabM at the non-permissive temperature. As with FabA in *E. coli*, FabM is essential for growth in *S. pneumoniae*, unless an exogenous supply of *cis*-vaccinic acid is available [87]. In contrast, a *fabM* deletion strain constructed in *Streptococcus mutans* was able to grow without unsaturated fatty acids, albeit with a doubling time of 159.9 minutes compared to 78.7 minutes in the wild type [88]. Both *S. pneumoniae* and *S. mutans fabM* deletions are unsaturated fatty acid auxotrophs, but the ability of *S. mutans* to grow without an external supply of unsaturated fatty acids identifies a difference in the requirement for unsaturated fatty acids between the two bacteria. The prototypical FabZ has a broad substrate specificity, catalyzing the dehydration of short, medium and long-chain saturated and unsaturated fatty acids [44]. The FabZ of *E. coli* cannot carry out the isomerization, and FabZ is the only isoform present in bacteria like *S. aureus* that produce only saturated fatty acids. However, this is not always the case. A FabZ-like protein in *Enterococcus faecalis*, called FabN, performs the same reaction as FabA and in this bacterium, FabF elongates the nascent unsaturated chain [77]. The reason why some dehydratases in the FabZ family can catalyze the isomerization reaction and others cannot is perplexing. They share the same catalytic residues. Domain swapping experiments suggest that perhaps the shape of the active site tunnel may be responsible for the exclusion of the kinked acyl chains with *cis* double in some enzymes [89], but this hypothesis requires further experimental validation. Recently, a gene named *ufaA* was demonstrated to be essential for unsaturated fatty acid synthesis in *Neisseria gonorrhoeae* [90]. This gene encodes a protein that is related to the FabK class of enoyl-ACP reductases, but its biochemical role in unsaturated fatty acid synthesis remains a mystery. Finally, there are many bacteria that produce unsaturated fatty acids, like *Clostridium acetobutylicum*, where the mechanism for unsaturated fatty acid synthesis remains unknown [91].

An alternate route for the generation of unsaturated fatty acids after the elongation cycle exists in some bacteria. The genome *Bacillus subtilis* does not contain a *fabA* gene but expresses a gene encoding a fatty acid desaturase, *des*, which functions to insert a double bond into phospholipid anchored acyl chains [92,93]. Unlike the anaerobic isomerization of *trans*-2-decenoyl-ACP, bacterial fatty acid desaturases are iron-containing, oxygen-

dependent enzymes. The *B. subtilis des* gene is induced during growth at low temperatures and functions to increase membrane fluidity by the generation of unsaturated phospholipids in a pre-existing saturated membrane [93]. Deletion of *des* results in a strain that has no obvious phenotype when cultured at 37°C, but is unable to grow and begins to lyse at 15°C, reinforcing the role of *des* in low-temperature adaptation in *B. subtilis*. Fatty acid compositional analysis of wild-type *B. subtilis* grown at 15°C degrees initially identified four different unsaturated fatty acid species, including 16:1Δ5, 16:1Δ9, 2-hydroxy-iso-17:1Δ7, and anteiso-17:1Δ7. All four species were absent upon deletion of the *des* gene [93]. Expression of the *des* gene in *E. coli* resulted in the production of only the 16:1Δ5 species [94]. Further mass spectroscopic investigation by Altabe *et al.* deduced that the double bond of unsaturated fatty acids in *B. subtilis* is exclusively at the Δ5 position, designating the Des protein as a specific Δ5 phospholipid desaturase [92]. The other unsaturated species without a double bond at the Δ5 position identified by Weber *et al.* were attributed to errors in the algorithm used to determine the identity of products eluted from a gas-chromatography column.

Like *E. coli*, *Pseudomonas aeruginosa* produces saturated and unsaturated fatty acids by the anaerobic FabA/B pathway, but can also generate unsaturated fatty acids by two oxygen-dependent pathways [95]. The *P. aeruginosa desA* gene introduces the double bond into acyl chains attached to phospholipids and has a similar structure to the *B. subtilis des* gene described above. The second system, *desBC*, is a 2-gene operon responsible for the introduction of a double bond into saturated acyl-CoA in a reaction analogous to the mammalian stearoyl-CoA desaturase. Both desaturase enzymes are oxygen dependent and catalyze double bond insertion specifically at the Δ9 position of the fatty acid. Single deletion mutants of *desA*, *desB/C* or *fabA* are viable, although Δ*fabA* cells exhibit a growth defect [95]. A double knockout of *fabA* and *desA* required supplementation with either saturated (16:0/18:0) or unsaturated (18:1Δ9) fatty acids for growth whereas a triple knockout of *fabA*, *desA*, and *desB* could only grow in the presence of 18:1Δ9 fatty acid. The additional desaturases in *P. aeruginosa* give the bacteria a system to modify existing phospholipids (DesA) and to produce unsaturated fatty acids from exogenous saturated fatty acids (DesBC). Unlike the *B. subtilis*, Δ5 phospholipid desaturase, the expression of *desA* does not seem to be affected by a change in temperature but instead is expressed in response to anoxic growth conditions [95]. The transcription factors controlling *desA* expression and its biological function remain elusive, but it is known that expression of *desBC* is regulated by the transcriptional repressor DesT that will be discussed in section 4.1.2.

A less well-characterized pathway for acquiring unsaturated fatty acids is through uptake of exogenous fatty acids. *S. aureus* lacks a FabA enzyme and any known desaturases but is able to activate exogenous saturated and unsaturated fatty acids through ligation to ACP and subsequent utilization by the elongation and/or acyltransfer module [65]. Considering the energy intensive process of *de novo* fatty acid synthesis, this pathway may be an energy saving mechanism. The identity and substrate specificity of the acyl-ACP synthetase is currently unknown, therefore the biological significance of this pathway remains vague. The skin pathogen *Propionibacterium acnes* incorporates polyunsaturated fatty acids into phospholipids through an unusual mechanism. The bacterium imports exogenous free fatty acids and utilizes a unique polyunsaturated fatty isomerase to convert Δ9,Δ11-linoleic acid to Δ10,Δ12-linoleic acid [96]. This isomerase is highly specific for free fatty acids as opposed to thioesters. The biological reason for this reaction remains a mystery. Fatty acid analysis of *P. acnes* indicate the bacteria only produce branched and saturated fatty acids but as the regulation of *de novo* synthesis in the bacterium is unknown, this composition could simply reflect the fatty acid composition of the Brain-Heart Infusion media [97,98].

2.3 Acyltransferases and phosphatidic acid synthesis

Glycerol-3-phosphate acyltransferases are responsible for intercepting the products of the elongation cycle and transferring the acyl-chain from acyl-ACP to either the *sn*-1 or the *sn*-2 carbons of glycerol-3-phosphate (Fig. 3). These reactions produce 1,2-diacyl-*sn*-glycerol-3-phosphate (phosphatidic acid), which is the universal phospholipid precursor in bacteria (for a recent review see [99]). These enzymes are at the interface between FASII and membrane expansion and are thus positioned to be key regulators of both fatty acid and phospholipid synthesis. Not only does the substrate specificity of these enzymes determine if an acyl-ACP undergoes additional rounds of elongation, it also defines which position and which species of fatty acid is attached to glycerol-3-phosphate. The initial reaction of phospholipid synthesis is catalyzed by glycerol-3-phosphate dehydrogenase. This reversible reaction produces glycerol-3-phosphate from dihydroxyacetone phosphate linking glycolysis and phospholipid synthesis. Deletion mutants of *gpsA* are glycerol auxotrophs and rely on a supply of exogenous glycerol-3-phosphate or GlpK, a glycerol kinase, to generate glycerol-3-phosphate from exogenous glycerol. A block at the GpsA reaction triggers an accumulation of abnormally long-chain acyl-ACPs that are not seen in cells with a functional acyltransferase system [100–102]. This illustrates the interplay between the elongation and acyltransfer modules that determines membrane fatty acid composition.

2.3.1 The PlsB/PlsC system—The first phospholipid acyltransferases to be discovered were the PlsB/PlsC acyltransferases of *E. coli* [103]. PlsB ligates a fatty acid into the 1-position of glycerol-3-phosphate and PlsC the 2-position of 1-acyl-glycerol-3-phosphate (lysophosphatidic acid). These membrane bound acyltransferases utilize acyl-ACP from the elongation cycle or acyl-CoA thioesters derived from exogenous fatty acids [104]. *E. coli* ligates exogenous fatty acids to CoA using an acyl-CoA synthetase (FadD) [105]. Although these CoA thioesters cannot be elongated by FASII, they can be placed directly into phospholipids by PlsB or PlsC or degraded by β -oxidation to generate a carbon source for growth [106]. The attachment of FASII-derived acyl chains to ACP and exogenous fatty acids to CoA serves as a biochemical tag to prevent any endogenously synthesized fatty acids from entering the degradative β -oxidation pathway. There is distinct asymmetry in the incorporation of acyl-chains into the 1- and the 2- position of glycerol-3-phosphate. In *E. coli*, the 1- position is occupied by either a 16:0 or an 18:1 fatty acid whereas the 2- position predominantly contains the unsaturated fatty acids 16:1 or 18:1 [107]. This phospholipid structure arises from the substrate specificities of the two acyltransferases [108]. However, this selectivity for different fatty acids is not absolute. In an *E. coli fabF fabA* double mutant strain that is unable to effectively synthesize unsaturated fatty acids, 16:0 can be found in both the 1 and the 2 position, suggesting a substrate preference for PlsC towards unsaturated acyl groups rather than a complete inability to utilize saturated acyl-ACPs [109]. PlsB and PlsC homologs exist in mammals, and it was long thought that the *E. coli* discoveries would also extend to all bacteria. However, with the advent of whole-genome sequencing it became readily apparent that the PlsB/PlsC system is largely limited to the γ -proteobacteria [104].

2.3.2 The PlsX/PlsY/PlsC system—In 1974, Robert Bell's group identified an *E. coli* mutant that was a G3P auxotroph [110]. They determined this phenotype was due to mutations in the *plsB* gene that resulted in a defective acyltransferase with an increased K_m for G3P, a defect that is overcome by supplying exogenous G3P in the media. The missense mutation giving rise to the *plsB26* allele was later identified [111]. Years later, the Bell group discovered that the G3P auxotroph strain they had developed also had a mutation in the enigmatic *plsX* gene, a widely-distributed gene of unknown function [112]. The second mutation was discovered after failed attempts to reproduce the G3P auxotrophy phenotype by transducing the defective *plsB* gene into a wild type strain. Transduction of the defective *plsX* gene into a wild type strain had no phenotype.

The role of PlsX in phospholipid synthesis was uncovered in 2006 [104]. PlsX converts acyl-ACP to acyl-PO₄, which is subsequently utilized by the PlsY glycerol-phosphate acyltransferase. Most bacteria, including *S. aureus* and *S. pneumoniae*, use the PlsX/Y pathway for the acylation of glycerol-3-phosphate. The PlsY acyltransferase is unable to utilize acyl-ACP or acyl-CoA [104]. The essentiality of PlsX/Y and their role in coupling FASII with phospholipid synthesis was investigated in *B. subtilis*. Paoletti *et al.* [113] used a *plsX*, *plsY* and *plsC* deletion mutants complemented with inducible plasmid-based expression to investigate the consequences of blocking each of these reactions. Depletion of *plsY* apparently had no effect on the rate of fatty acid synthesis, although phospholipid synthesis was blocked and free fatty acids accumulated. The fatty acids arise from the conversion of acyl-ACP to acyl-PO₄ followed by the hydrolysis of the accumulating acyl-PO₄. Depletion of *plsC* resulted in over 200% increase in fatty acid synthesis and an accumulation of large quantities of free fatty acid and monoglyceride [113]. The free fatty acid accumulation was attributed to degradation of the monoglyceride formed by PlsY by an esterase, although the identity of this enzyme is unknown. Upon *plsX* depletion, the rate of fatty acid synthesis decreased precipitously, suggesting PlsX is a key regulatory point that synchronizes FASII and phospholipid synthesis. A block at the PlsX reaction would result in accumulation of acyl-ACP from the FASII elongation module. Acyl-ACP is a key feedback regulator of FASII in *E. coli*, and it is possible a similar regulation mechanism could be present in *Bacillus*. However, at this stage nothing is known about the biochemical regulation of the pathway in *B. subtilis*, and the biochemical analysis of FabH and ACC in *B. subtilis* is needed to validate this connection. The only instance of biochemical regulation of the acyltransferase enzymes that has been identified is *S. pneumoniae* PlsY, which is reportedly non-competitively inhibited by acyl-CoA [114]. This connection is puzzling and perhaps irrelevant because there is no known mechanism for the production of acyl-CoA in *S. pneumoniae*.

The reason for the retention of PlsX/Y in *E. coli*, which has the PlsB acyltransferase is an enigma. Yoshimura and coworkers generated targeted gene deletions of *plsB*, *plsX* and *plsY* (annotated as *ygiH*) [115]. Their studies revealed that *plsB* is an essential gene, whereas single deletion mutants of *plsX* or *plsY* have no detrimental effect on growth. However, construction of a *plsY* and *plsX* double deletion mutant was not successful, indicating an essential role for the PlsY and PlsX enzymes in *E. coli*. The expression of *B. subtilis plsX* can rescue the glycerol auxotroph *plsB26* phenotype in an *E. coli* strain expressing an inactive PlsX, and a deleted *plsY* gene [116]. This interesting observation strongly suggests an important role for acyl-PO₄ outside of lysophosphatidic acid synthesis, however, why *plsX* is retained in organisms with a PlsB/C pathway remains a puzzle that will require more research to unravel.

2.3.3 The diacylglycerol (DAG) kinases—Diacylglycerol kinases (Dgk) catalyze the phosphorylation of DAG to generate phosphatidic acid (Fig. 5). This reaction is critical for recycling DAG formed as a byproduct from the utilization of phospholipids in the biosynthesis of other macromolecular membrane components. Phosphatidic acid produced by Dgk can then feed back into the phospholipid biosynthetic pathway. The substrate for the major phospholipid breakdown pathways is phosphatidylglycerol (PtdGro), which donates its *sn*-glycerol-1-phosphate (G1P) headgroup to the either membrane-derived oligosaccharides (MDO) in Gram-negative species or lipoteichoic acid in Gram-positive bacteria. These two groups of bacteria have unique Dgks to recycle the DAG.

The first DAG kinase to be discovered was *E. coli* DgkA [117–120]. *E. coli* produces MDO to control the osmolarity in the periplasmic space. MDO are β -glucans containing 8–10 glucose units that are decorated with multiple G1P moieties to convert them into polyanionic polymers [121,122]. The glucose subunits of MDO are also substituted to a lesser extent

with succinic acid and phosphoethanolamine residues [122]. Although MDO not essential for cell survival in normal media, a role in osmotic homeostasis in *E. coli* has been established [121,122]. During osmotic stress, the periplasmic levels of MDO fall to balance the osmotic pressure and maintain a constant periplasmic volume. Strains bearing mutations in the G1P transferase gene, *mdoB*, are unable to perform the phosphoglycerol transfer reaction from PtdGro to DAG [123]. Mutants of *mdoB* and *mdoC*, the enzyme responsible for the insertion of the succinate moiety into MDO, are hypersensitive to detergent and osmotically challenging environments [124].

As expected, inactivation of the *dgkA* gene in *E. coli* results in an accumulation of DAG [120]. The *dgkA* gene is not required for growth under standard laboratory conditions where MDO synthesis is not required, but is essential during growth in osmotically challenging environments [123]. In high salt conditions, the addition of arbutin to the growth media acts as an artificial acceptor of G1P, therefore accelerating the production of DAG [123]. An accumulation of such a neutral lipid in the membrane is catastrophic for the bacteria due to disruption of the bilayer [125]. Membrane proteins were unable to assemble into liposomes containing high concentrations of diacylglycerol [126]. It was proposed that the bulky structure of DAG essentially seals the membrane in a way that the hydrophilic heads of phospholipids cannot. Thus, it is the role of DgkA to phosphorylate the accumulating DAG, allowing for new synthesis of polar lipids.

DgkA is an integral membrane protein that exists as a trimer with the active site facing the cytoplasmic side of the membrane [127]. Solving the structure of this integral membrane protein was a triumph of NMR spectroscopy and readers interested in the detailed structure and function of DgkA and its relatives are referred to the recent review by Van Horn and Sanders [128]. Because DAG is generated on the outside of the cell, it must first translocate to the inner leaflet of the cytoplasmic membrane to be acted on by DgkA. There is no known transport system for DAG and it is thought to spontaneously flip between the two sides of the bilayer. Little is known about the genetic regulation of *dgkA*, although it is clear that its transcription is controlled by multiple stress response regulators in *E. coli* [129]. Homologs of DgkA are widely distributed in bacteria, but not all of these enzymes are actually DAG kinases. It is not possible to determine from bioinformatics analysis the lipid substrates used by the DgkA homologs [128,130].

Diacylglycerol is also generated outside the cell in Gram-positive bacteria, although the G1P transferase reaction serves a different purpose. The G1P headgroup of PtdGro is used to synthesize lipoteichoic acid (LTA), a major component of the Gram-positive cell envelope. LTA is synthesized by the LtaS enzyme, which polymerizes G1P residues derived from PtdGro onto a glycolipid membrane anchor [131]. Depletion of LTA by inactivation of the *ltaS* gene in *S. aureus* results in a strain that is unable to grow at 37°C and exhibits aberrant growth at reduced temperatures [132]. Aside from the role of LTA as a major structural cell wall component, it is also thought to stabilize the cell membrane, localize the activity of cell-wall synthesis enzymes and is a major player in pathogen-host interactions during infection [133–136]. Reminiscent of the synthesis of MDO in *E. coli*, each subunit of G1P that is transferred to the LTA polymer results in a molecule of DAG formed as a byproduct. Therefore, the synthesis of a single LTA strand produces over 20–40 DAG molecules in the process [137]. Consequently, Gram-positive bacteria such as *B. subtilis* and *S. aureus* have developed a similar mechanism to *E. coli* for recycling DAG. *B. subtilis* was reported to have a *dgkA* gene but a genetic and biochemical evaluation of the enzyme identified the protein as an undecaprenol kinase [130]. The *bone fide* DAG kinase of *B. subtilis*, a soluble protein with no sequence similarity to DgkA, was discovered and named DgkB [130]. This soluble DAG kinase is highly specific for the phosphorylation of DAG over other phospholipids, and its primary sequence places it in the same protein family as the

mammalian signaling DAG kinases. Unlike the integral membrane DgkA, DgkB is an interfacial enzyme that uses positively charged lysine residues on its surface to dock on the anionic surface of the cell membrane [138]. This property is demonstrated in vitro through enzymatic assays showing a robust increase in DgkB activity when DAG is incorporated into negatively-charged PtdGro vesicles, as opposed to neutrally charged bilayers [138]. Complementation studies of a *dgkB* mutant with an inducible plasmid expressing *dgkB* revealed the gene to be essential in *B. subtilis* unless LTA synthesis is disrupted by mutations in *yfiE*, a gene homologous to *ltaS* of *S. aureus*. Cessation of LTA synthesis would prevent DAG formation through LTA turnover, preventing the need for a DAG recycling system [137]. The structure of DgkB from *S. aureus* was solved [139]. This structure illustrates how the enzyme interacts with the phospholipid bilayer, and revealed a unique structural Mg^{2+} site that explained the metal-dependent activity of this enzyme family.

2.4 Phospholipid headgroup diversity

An assortment of different phospholipid headgroups are synthesized by bacteria to create the optimum surface charge for the cell membrane. The charge of zwitterionic phospholipid headgroups must be balanced with those containing acidic headgroups such as phosphatidylglycerol. This balance in electrostatic charge is required for many integral membrane proteins to adopt the correct topology in the cell membrane [140,141]. This property highlights the need for stringent regulation of the synthesis of different headgroup moieties.

The key intermediate in bacterial phospholipid synthesis is CDP-DAG [142] synthesized by the *cds* gene product [143]. Both ribo- and deoxyribo-liponucleotides are produced by Cds [144], but the significance, if any, for the formation of these two products is unknown. Cds is an essential gene, but mutants with decreased activity have been isolated and accumulate phosphatidic acid which leads to membrane dysfunction [145–147]. A regulatory role has not been ascribed to Cds and its activity level appears to be in large excess over what is required to support phospholipid synthesis.

2.4.1 CDP-DAG-dependent reactions—In *E. coli*, the fate of CDP-DAG represents a branch point between synthesis of acidic phospholipids (PtdGro) and zwitterionic phospholipids (phosphatidylethanolamine). In the PtdGro pathway, PtdGro phosphate is synthesized from CDP-DAG by the displacement of cytidine monophosphate (CMP) by glycerol-phosphate catalyzed by the PgsA enzyme [148]. The PtdGro phosphate is dephosphorylated by a PtdGro phosphate phosphatase (Pgp) to form the end product, PtdGro. There are three *pgp* genes in the *E. coli* genome (*pgpA*, *pgpB* and *pgpC*), the last just recently discovered [149]. Perhaps not all of these are actually designed for PtdGro phosphate hydrolysis, and they may perform other functions also. The essentiality of the genes involved in PtdGro synthesis has been debated over the decades [150,151]. In *S. aureus* and *B. subtilis*, whose membranes are predominantly composed of PtdGro (50–60%), *pgsA* is an essential gene [152–155]. In *E. coli*, PtdGro is a minor component (20%) compared to phosphatidylethanolamine (PtdEtn) (70%) [150]. Weiming *et al.* were the first to examine the requirement of PtdGro for growth in *E. coli* and found that a *pgsA* and *lpp* lipoprotein deletion mutant was only able to grow when the mutation was complemented by *pgsA* expressed on a temperature sensitive plasmid [150]. However, one aspect of their experimental design was that they did not use an inducible promoter, but rather reached the conclusion by assaying growth arrest that occurred when the *pgsA* plasmid was cured by growth at 42°C. Membrane phospholipid composition is important for temperature and osmotic regulation, consequently aberration in normal membrane composition may prevent growth under strenuous conditions. This caveat was studied by Kikuchi *et al.* who observed

that a *pgsA lpp* null mutant is able to grow at 37°C, but not 42°C. Growth in low osmolarity media was also compromised presumably due to the lack of MDOs [151]. The requirement for the deletion of the outer membrane lipoprotein gene in combination with the *pgsA* gene was investigated by Suzuki *et al.* [156]. The lipoprotein requires transfer of the DAG moiety from PtdGro for maturation and removal from the inner membrane. A lack of PtdGro results in accumulation of unmodified Lpp on the inner membrane which crosslinks with peptidoglycan via the carboxyl terminus of the lipoprotein. This crosslinking causes the inner membrane to be erroneously anchored to the cell wall, resulting in lysis. These studies conclude that anionic phospholipids alone are not absolutely essential for metabolic function in *E. coli* grown under laboratory conditions that do not stress the bacterium.

The PtdGro also serves as a precursor in the synthesis of cardiolipin, a variable component of Gram-positive and Gram-negative cell envelopes [148]. Cardiolipin derived from two PtdGro molecules is synthesized in *E. coli* by the ClsA protein [157]. The *clsA* gene is dispensable in *E. coli*, although trace amounts of cardiolipin are still detected in the membranes of *clsA* deletion mutants suggesting an alternate synthetic route is present [158]. Two additional cardiolipin synthases are found in *E. coli*. The *clsB* gene encodes a cardiolipin synthase that uses PtdGro, but whether the other substrate is PtdGro or another phospholipid is unknown [159]. Recently, the *clsC* gene was discovered that encodes an enzyme that uses PtdGro plus PtdEtn instead of two PtdGro molecules [160]. *S. aureus* possesses two genes for the synthesis of cardiolipin termed *clsI* and *cls2* [161]. Transcription of *clsI* was induced under stress conditions such as osmotic stress whereas *cls2* is the constitutive cardiolipin synthase, catalyzing its formation under standard laboratory growth conditions. Neither *cls* genes were found to be essential when deleted individually or in combination although the double knockout was more susceptible to osmotic stress (25% NaCl). Thus, cardiolipin appears non-essential in most organisms under laboratory growth conditions, but the multiplicity of genes and their regulation of membrane cardiolipin content suggest that this phospholipid plays an important role(s) in the survival of bacteria in the environment.

The alternative use of CDP-DAG in *E. coli* is to synthesize the zwitterionic phospholipid PtdEtn. PtdEtn is the major component of the *E. coli* cell membrane, but surprisingly mutants completely defective in PtdEtn remain viable if the growth medium is supplemented with Ca²⁺, Mg²⁺ or Sr²⁺, despite replacing all PtdEtn with PtdGro and cardiolipin [162]. No other cations or osmolites substitute for these cations. The phosphatidylserine (PtdSer) synthase (PssA) enzyme catalyzes the first committed step in PtdEtn synthesis, which condenses serine to the phosphatidyl moiety of CDP-DAG coupled with the release of CMP. Unlike the other enzymes involved in phospholipid headgroup synthesis, *E. coli* PssA is tightly associated with ribosomes instead of the cell membrane in cell fractionation studies [163,164]. However, this is likely an artifact of the propensity of PssA to bind anionic surfaces, and purified PssA exhibits substrate-dependent association with the membrane bilayer [165]. PtdSer is a transient intermediate in *E. coli* and constitutes less than 0.1% of the detectable lipid pool [166]. The final reaction in PtdEtn biosynthesis is performed by the PtdSer decarboxylase (Psd), which decarboxylates the serine headgroup resulting in the release of CO₂. Psd uses a covalently attached pyruvate group as a cofactor that is formed by the self-cleavage of the primary transcript into two nonidentical subunits [167]. Kennedy *et al.* isolated a Psd temperature sensitive mutant strain of *E. coli* and observed an accumulation of PtdSer coupled with growth arrest [168]. However, membranes with a significant proportion of PtdSer were viable by growth of the temperature-sensitive strain at a semi-permissive temperature. In some lactic acid fermenting bacteria and nitrogen-fixing plant symbiotes, PtdSer is a major membrane lipid [169]. The specific cellular functions of PtdSer are unknown but there is a clear correlation between the presence of PtdSer in lipid membranes and the ability of the bacteria to ferment and fix nitrogen.

Two other phospholipids are less widespread in bacteria, but are nonetheless important for the physiology of the specific organisms that produce them. Phosphatidylcholine (PtdCho) is a phospholipid usually associated with eukaryotic cells, although it is estimated that 10% of bacteria produce cell membranes containing PtdCho [170]. PtdCho is present in some pathogenic bacteria, including *P. aeruginosa*, albeit only a few percent of the total lipid mass. *P. aeruginosa* uses a PtdCho synthase enzyme to condense choline with CDP-DAG. An alternate PtdCho synthesis pathway occurs in some soil-dwelling bacteria. This pathway uses choline to displace CMP similar to PssA in a reaction that is distinct from eukaryotic PtdCho biosynthesis. The diversity and regulation of these enzymes has been covered in a recent review [171]. *Agrobacterium tumefaciens* synthesizes PtdCho through successive methylations of PtdEtn via methyltransferase enzymes using S-adenosyl-methionine as a methyl donor [172]. Some species of bacteria have phosphatidylinositol (PtdIns) containing membranes [173–177]. PtdIns is another lipid normally associated with mammalian cell membranes but present in various Mycobacteria and bacteria found in some more obscure environments [173–177]. It is thought the lipid rich cell wall of Mycobacteria and some *Actinomycetes* allows them to survive in severe environmental conditions [175]. Actinobacteria (including Mycobacteria) are able to synthesize PtdIns from inositol and CDP-DAG using phosphatidylinositol synthase (PIS) and PtdIns can comprise up to 25% of the total membrane lipids [174]. The PIS is encoded by the *pgsA* gene in *M. tuberculosis* and is essential. It was not determined if the loss in viability in the *pgsA* deprived strain was due the lack of PtdIns or the lack of a metabolically deprived product of PtdIns [174].

2.4.2 DAG-dependent reactions—DAG is predominantly a byproduct of either MDO synthesis in *E. coli* or LTA biosynthesis in Gram-positive organisms. However, there are some lipids that require DAG as a precursor as opposed to CDP-DAG. In Gram-positive bacteria and some cyanobacteria, DAG is used to synthesize glycolipids [178]. In cyanobacteria, the glycolipids form part of the energy-generating photosystem I [179]. In Gram-positive *B. subtilis* and *S. aureus*, the glycolipids function as a scaffold for the LTA polymer to be synthesized and to physically anchor the LTA to the cell membrane. LTA is constructed from three components: (i) the glycolipid anchor that attaches the LTA to the membrane, (ii) the poly(glycerophosphate) residues derived from successive GIP transfer reactions from PtdGro and (iii) the D-alanyl moieties linked to the poly(glycerophosphate) [178]. The species of glycolipid found in *B. subtilis* and *S. aureus* is diglucosyldiacylglycerol (Glu₂DAG). The enzyme that catalyzes the glucotransferase reaction (YpfP) was initially discovered in *B. subtilis* [180]. YpfP utilizes uridine diphosphate-glucose (UDP-glucose) as a carbohydrate donor. *B. subtilis ypfP* (BsYpfP) was cloned and overexpressed in *E. coli*, an organism that does not produce glycolipids [180]. They found that YpfP enzyme is capable of transferring up to four glucose residues to a DAG molecule, although in *B. subtilis* only the product of one or two glucotransferase reactions are observed. In addition to its function as the glycolipid anchor, Glu₂DAG also exists as a membrane lipid, constitution approximately 8% of the molar proportion of glycolipid in *S. aureus* [181]. Other bacterial glycolipids appear to be synthesized by closely-related glycosyltransferases with different specificities for UDP-sugars [182].

The role of glycolipids in membrane homeostasis is highlighted by research using the *Acholeplasma laidlawii* model system. Alterations in membrane fatty acid composition, cholesterol content and growth temperature induce significant changes in the ratio of monoglycosyl-DAG (MGDG) to diglycosyl-DAG (DGDG) [183–186]. The MGDG:DGDG ratio increases with increasing unsaturated fatty acids or temperature suggesting that the ratio is an adaptive response to increasing membrane disorder. This body of work is interpreted to describe a compensatory mechanism that maintains the biophysical properties of the membrane close to the lamellar to hexagonal phase transition. MGDG forms inverted nonlamellar structures as opposed to the bilayer conformation of DGDG. It has been

suggested that the bacterial cell membrane is always kept at the limit of stability by the introduction of non-bilayer lipids into membrane to allow a flexible response to extracellular stimuli that perturb the membrane biophysical properties [180,187]. How this is accomplished is mechanistically unclear, but perhaps the activities of the interfacial glycosyltransferases [188] may be regulated by the physical properties of the membrane containing their substrates, but this idea needs to be put to the test.

It is difficult to examine the effect of selectively removing glycolipids from most bacteria by inactivation of the glycosyltransferases because elimination of glycolipids also results in a dramatic reduction in the amount of LTA produced [189]. Deletion of the *ltaS* gene is lethal in *S. aureus* unless cells are grown with osmoprotectants or in combination with a cyclic diadenosine monophosphate phosphatase (*gdpP*) deletion [131,132,178,190]. Interestingly, a 90% reduction in LTA biosynthesis through deletion of *ypfP* in *S. aureus* SA113 is viable with minimal impact on growth in standard laboratory media or osmotically stressful conditions (2.5 M NaCl) [189]. There appears to be a significant difference in the regulation of LTA biosynthesis between *S. aureus* strains RN4220 and SA113 as the rate of cell-associated LTA biosynthesis remains unchanged in an RN4220 *ypfP* knockout, despite the lack of a Glu₂DAG anchor. There is a two-fold increase in the amount of LTA secreted from RN4220 *ypfP* mutant, perhaps representing the lack of a stable connection to the cell membrane [189]. SA113 *ypfP* deletion mutant attaches the G1P polymer of LTA directly to DAG instead of Glu₂DAG. Perhaps this is the normal reaction that is followed by the transfer of the G1P polymer to the Glu₂DAG. Synthesis of Glu₂DAG also requires a α -phosphoglucomutase (*PgcA*) and UTP: α -glucose-1-phosphate uridylyltransferase (*GtaB*) to produce the UDP-glucose for *YpfP* [137]. The carbohydrate moiety of the glycolipid is exclusively glucose in *S. aureus* and *B. subtilis* but this is not true throughout the bacterial kingdom. A comprehensive review by Norman Shaw describes the different carbohydrate subunits present in bacterial glycolipids that includes galactose, mannose, rhamnose and glucaronic acid [191]. Unfortunately in many cases the composition of the diglyceride remains a mystery although bacteria containing plant-like digalactosyldiacylglycerol glycolipids have been identified [192,193]. Some *Rhodococcus* species and *Mycobacterium tuberculosis* synthesize triacylglycerol from DAG to use as an energy store in the form of lipid droplets [194–196]. These storage lipids are normally only found in eukaryotes but have been hypothesized to aid the TB bacterium during the unique dormant phase in latent tuberculosis infections [195]. Most bacteria use polyhydroxyalkanoates as storage lipids [197], and in some cases these precursors are derived from the β -hydroxy-intermediates of the FASII elongation module [69].

2.5 Non-phosphorus lipids

In addition to glycolipids, some bacteria are able to form phosphorus-free lipids in response to phosphate-limited environments. These bacteria ligate fatty acids onto an alternative backbone to glycerol-3-phosphate. The most common substitutes are amino acids [198,199]. Although lipids synthesized using lysine, glycine, glutamine and serineglycine have been reported, most research has focused on ornithine lipids [199]. Ornithine lipids in *S. meliloti*, *R. capsulatus*, *B. abortus* and *P. aeruginosa* are synthesized by ligation of a fatty acid from acyl-ACP onto the α -amino group and the 3-hydroxyl group of ornithine resulting in an amide linked and an ester linked fatty acid. The *PlsB/C* acyltransferases are not capable of performing the aminoacyltransferase reactions, but rather related acyltransferases, *OlsB* and *OlsA*, catalyze the stepwise transfer the fatty acids from acyl-ACP to the amino and hydroxyl group, respectively. Ornithine lipid production is an adaptation to low phosphate environments (for reviews, see [198,199]). During growth in phosphorus-limiting conditions, some α -proteobacteria such as *Rhodobacter sphaeroides* and *S. meliloti* replace the majority of their lipids with ornithine lipids plus two additional classes of phosphorus-

free lipids: betaine lipids and sulfolipids [170,200–202]. In *S. meliloti*, the switch to non-phosphorus lipids is transcriptionally activated by the PhoB- and PhoU-dependent phosphate sensor. Deletion mutants of either PhoB or PhoU are unable to induce non-phosphorus lipid synthesis [201]. The most abundant lipid formed under phosphorus-limitation is the betaine lipid diacylglycerol-*N,N,N*-trimethylhomoserine (DGTS). Sulfoquinovosyl diacylglycerol (SL) is a less abundant sulfur lipid that is also formed under phosphorus-limitation. The enzymes involved in DGTS and SL syntheses both utilize DAG as a precursor. In an effort to liberate phosphorus contained within preexisting phospholipids, *S. meliloti* uses a phospholipase C (PlcP) to remove the phosphocholine from PtdCho and CgmB to remove phosphoglycerol from PtdGro [200]. This turnover of phospholipids releases phosphorus to other metabolic processes that are also suffering from the phosphorus limiting growth conditions. Despite the ability of several bacteria to exclude phosphorus from membrane lipids, no organisms have been isolated that are completely devoid of glycerophospholipids. This observation begs the question whether it is possible to form biological membranes in the absence of these phospholipids.

2.6 Bacterial Sphingolipids

Sphingolipids are a class of lipids containing an aliphatic amino alcohol connected to a sphingoid backbone and a fatty acid *N*-linked via an amide. Sphingolipids are structural and signaling components of mammalian and yeast cells but in rare cases have been observed in bacteria. Bacterial sphingolipids are structurally distinct from eukaryotic sphingolipids. Eukaryotic sphingolipids characteristically have an unsaturated amino alcohol and an unbranched acyl-chain of 18–20 carbons. In contrast, bacterial sphingolipids are typically saturated and often branched with no more than 19 carbons [203]. Eukaryotic sphingolipids usually possess a phosphocholine headgroup, whereas most bacteria utilize an array of different headgroups not including phosphocholine [203]. Sphingolipids have been identified in a handful of bacterial genera but specifically in the *Sphingomonas* and *Sphingobacterium* classes [204–207]. *Sphingomonas paucimobilis* lacks LPS in their outer membrane and alternatively utilizes two glycosphingolipids [205]. Similarly to LPS, glycolipids of *Sphingomonas* are potent activators of natural killer T-cells during infection [208,209]. The functions of the membrane sphingolipids in bacteria must be deduced from their structure and localization because there are no genetic studies that directly demonstrate their role in membrane homeostasis. One interesting study by An *et al.*, examined the effect of inhibiting sphingolipid biosynthesis in the sphingolipid producing bacterium *Bacteroides fragilis* [203]. *B. fragilis* forms part of the normal flora in the human small intestine but is also associated with urinary tract infections [210]. The investigators utilized myriocin, a potent, specific inhibitor of eukaryotic sphingolipid synthesis to prevent sphingolipid formation in *B. fragilis*. Myriocin inhibits the serine palmitoyltransferase enzyme that catalyzes the first committed step in sphingolipid biosynthesis (condensation of palmitoyl-CoA with serine to form 3-ketosphingosine) [203]. When sphingolipid synthesis was inhibited through myriocin treatment, *B. fragilis* is less tolerant to oxidative and heat shock stress. The authors showed that exogenous cholesterol and sphingolipids are also required to manage DNA damage stress. It is unknown if this decrease in resilience is due to the requirement of sphingolipids as a structural component, or the role sphingolipids could play in signal transduction by formation of sphingolipid microdomains in the cell envelope – a characteristic of eukaryotic sphingolipids [203].

3. Biochemical regulation of phospholipid synthesis

Biochemical regulation exerts a powerful influence in the control of membrane lipid homeostasis. Biochemical regulation is normally in the form of negative feedback, by which the product or intermediate of a metabolic pathway feedback inhibits an enzyme upstream. Not only does this allow for an extremely fast and effective response, it can overcome

increases in gene expression by adjusting the net activity regardless of the overall enzyme amount. Most of the energy that it takes to construct a phospholipid is used in the biosynthesis of fatty acids. In almost all bacteria, fatty acids have no alternate fate to membrane formation. Therefore, tight biochemical regulation of FASII is an important aspect of membrane lipid homeostasis and bacterial fitness.

3.1 Regulation in the initiation module

The initiation module is an ideal point of regulation in membrane lipid biosynthesis. The two most significant enzymes in the initiation module are ACC and FabH. The ACC performs the first committed step in FASII and every turnover of FabH results in the formation of a fatty acid. In *E. coli*, the ACC is feedback inhibited by acyl-ACP [11]. Davis *et al.* synthesized acyl-ACPs of different chain lengths (C6-C20) and measured ACC activity in the presence of the different ACP species. They observed robust inhibition of ACC activity (70–75%) in the presence of 40 μ M acyl-ACP, regardless of the chain length or the saturation of the acyl-chain. Although 40 μ M may appear to be a high concentration for physiologically relevant inhibition, ACP is the most abundant soluble protein in *E. coli*. The inhibitory effect was specific for bacterial ACP, as ACP from spinach had no effect. Similarly, unacylated ACP showed no inhibition. Assays of the biotin carboxylase and carboxyltransferase reactions individually did not show any inhibition in the presence of acyl-ACP. The lack of inhibition in the ATP dependent biotin carboxylase reaction suggests that acyl-ACP does not interfere with ATP binding. Since the authors used the reverse reaction (decarboxylation of malonyl-CoA) to assay the biotin carboxylase, no information regarding the mode of inhibition with respect to acetyl-CoA can be discerned. Assay of the complete reaction showed a mixed type inhibition with respect to acetyl-CoA: a combination of competitive and non-competitive inhibition. This feedback loop was investigated *in vivo*, by Heath *et al.* who monitored the quantities of malonyl-CoA in *E. coli* after a block at either the elongation cycle or the acyltransfer module. The block at the elongation cycle was achieved by treatment of the cells with cerulenin, a specific FabB/FabF inhibitor, whereas the block at the acyltransfer module was formed by depriving using an *E. coli plsB* mutant of the glycerol it requires for growth [211]. Treatment with cerulenin resulted in the accumulation of malonyl-CoA after 10 minutes of treatment. Similarly, treatment of *E. coli* with the FabB/F inhibitor thiolactomycin also resulted in the sustained production of malonyl-CoA [212]. Although these data illustrate that ACC is not regulated under these conditions, the nature of the ACP thioesters that accumulate following the block in elongation remain to be precisely determined. The molecular basis for the inhibition of the ACC by acyl-ACP is unclear, but it may be that similar residues that are involved in acetyl-CoA binding are involved due to both molecules sharing a common 4'-phosphopantetheine moiety. However, the acetyl-CoA binding pocket is not large enough for ACP and an ACP binding surface on ACC has not been identified [213]. This regulatory system has been also been identified in rapeseed (*Brassica napus*) [214] suggesting that it is widespread. Unlike in *E. coli*, *S. pneumoniae* can ligate exogenous fatty acids directly to ACP, allowing them to feed exogenous fatty acids into either the elongation or acyltransfer modules as ACP thioesters [65]. Upon treatment with exogenous oleate for 20 minutes, the intracellular malonyl-CoA pool fell to <5% of the untreated. The effect was similar to treatment of a *S. aureus* culture with andrimid, an ACC inhibitor. Although the role of acyl-ACP in this physiological response has not been confirmed, it seems like a reasonable intermediate to repress ACC activity. Inhibition of the acetyl-CoA carboxylase by acyl-ACP is a logical feedback regulatory system linking the end product of the pathway with the initial reaction, and is likely to be widespread in bacteria.

Overproduction of the ACC in *E. coli* does not result in a significant increase in the rate of fatty acid synthesis, unless a soluble acyl-ACP thioesterase (TesA) is co-expressed to

uncouple acyl-ACP utilization from phospholipid synthesis [18]. These data confirm the extremely tight regulation involving acyl-ACP in *E. coli* and also point to another regulatory control point in FASII outside of the ACC. This additional control point was identified as the FabH enzyme based on biochemical analysis [7]. Unlike the acyl-chain length independent effect of acyl-ACP on the ACC, the potency of the acyl-ACP increases with the length of the fatty acid (20% inhibition for 12:0-ACP and 70–80% inhibition with 20:0-ACP). This graded response helps to tune the activity of the initiation module to the elongation and acyltransfer modules, ensuring fatty acids of the desired length are synthesized. The authors determined the mode of inhibition was competitive with respect to malonyl-ACP and mixed inhibition with acetyl-CoA, indicating the acyl-ACP can bind to the apo-enzyme and the acyl-enzyme intermediate. The acyl-ACPs appear less potent to FabH compared to ACC with the authors performing in vitro inhibition assays with 100 μ M of acyl-ACP to achieve similar inhibition to the ACC at 40 μ M [7]. Further evidence of the regulatory effect in vivo was provided by employing an *E. coli plsB* mutant glycerol auxotroph. Using this strain, starving the cells of glycerol prevents utilization of acyl-ACP by the acyltransfer module, resulting in long-chain acyl-ACP accumulation. Examination of the short chain acyl-ACP pool showed depletion of the products of the FabH reaction, malonyl-ACP and acetyl-ACP (potentially arising from decarboxylation of malonyl-ACP by FabF), due to accumulation of long-chain acyl-ACP [33]. The combinatory regulation of the ACC and FabH by the product of the elongation cycle allows for some delicate synergistic feedback regulation of the initiation cycle to control the quantity of fatty acid produced in *E. coli* during a reduction in the rate of acyltransferase activity or consequently an increase in the rate of fatty acid elongation. The inhibition of FabH by acyl-ACP has only been characterized in *E. coli* but is likely to be present in other organisms also. *S. aureus* treated with oleic acid reduces lipid synthesis by 50%, although malonyl-CoA levels remain unchanged [65]. The lack of repression of ACC activity combined with the reduction of lipid synthesis points to FabH as the regulatory point and potentially acyl-ACP as the regulatory ligand, but further studies are needed to confirm this.

3.2 Control of fatty acid elongation

The determinant role of the elongation enzymes in the structure of fatty acids is discussed in section 2.2.2. The activity and substrate specificity of the elongation enzymes are finely balanced to produce: 1) fatty acids of the desired length; and 2) to produce the correct ratio of unsaturated:saturated fatty acids. The roles of FabZ/FabA from *E. coli* and FabM from *S. pneumoniae* in regulating the unsaturated fatty acid content are also relevant (section 2.2.3). The acyl chain length of the fatty acids generated by the cycle is a product of competition for the acyl-ACP between the G3P acyltransferases and FabB/F (or FabF in most species with a single elongation condensing enzyme). The longer chain acyl-ACPs are poor substrates for FabB and ideal substrates for the acyltransferases. A shift in the activity ratio in *E. coli* is accomplished by overexpressing FabB or depleting the cells of PlsB to alter the kinetic balance between the elongation and acyltransfer modules. Both perturbations result in abnormally long fatty acids [215]. The acyl-chain length specificity of FabB is explained by the size of substrate binding tunnel adjacent to the active site. Many crystal structures illustrate that the pocket accommodates acyl-chains up to 16 carbons and limit the enzyme to producing 18-carbon fatty acids [46,216–219]. The *trans*-2-enoyl-ACP reductase (FabI) is also inhibited by acyl-ACP. This effect is attributed to product inhibition, a property of all enzymes, but nonetheless may be very relevant to slowing the rate of the elongation module when long-chain acyl-ACP are abundant [220]. In summary, biochemical regulation at multiple steps in the pathway work in concert to provide a tight control over the amounts of fatty acid produced and their structures.

3.3 Coordination of lipid and macromolecular synthesis

The synchronization of lipid synthesis with DNA, RNA and protein synthesis must occur to maintain the correct proportion lipid to protein in the membrane. How the cell correctly balances lipid and macromolecular synthesis such that the membrane protein:lipid ratio remains constant at different growth rates is a problem in bacterial physiology that requires additional experimentation. There have been only a few studies on this topic over the past four decades. The first reports of the cross-talk between lipid and protein synthesis were published by Glaser *et al.*, who apparently used a temperature-sensitive glycerol-phosphate acyltransferase *E. coli* mutant to examine the effect of diminished phospholipid synthesis on the production of DNA, RNA and protein syntheses [221]. Growth of the mutant at the non-permissive temperature resulted in an abrupt halt to DNA, RNA and protein synthesis, but it was later found that mutation in the *plsA* mutation, originally thought to be a acyltransferase, was actually a defective adenylate kinase [222]. Inhibition of phospholipid synthesis in mutants expressing a defective *plsB* gene did not cause other branches of macromolecular syntheses to slow until the cells have divided 1–2 times, despite the nucleotide pool remaining largely unchanged [223–225]. This lack of coordination was confirmed when the membranes of glycerol-starved *E. coli gpsA* mutants were discovered to contain significantly more protein than their glycerol-supplemented counterparts [224]. Nonetheless, protein synthesis does eventually stop under these conditions, demonstrating there is a factor which senses a reduction in growth rate or perturbation of the membrane and feeds back to other arms of metabolism. After glycerol is made available to the growth-arrested cells, lipid synthesis and growth rapidly resume, but there is a delay in the initiation of DNA, RNA and protein synthesis. This observation argues for a sensor which detects the ratio of protein to lipid in the membrane and does not restart other macromolecular syntheses until the incorrect lipid to protein ratio of the membrane has been remedied [225]. A similar pattern was observed in *E. coli* cells treated with the FabB inhibitor cerulenin. Despite a 90% reduction in lipid synthesis after 40 minutes of treatment, only a moderate (25%) reduction in nucleotide synthesis was observed with no change in protein synthesis [226]. Reminiscent of the *E. coli gpsA* mutant during glycerol starvation, after 1–2 hours complete termination of all macromolecular syntheses was observed and when the cerulenin was removed from the growth medium, lipid synthesis restarted significantly faster than protein synthesis. These studies hint at a connection between lipids and other branches of metabolism, but these studies all used strains of *E. coli* that also possess lesions in the synthesis of the global regulatory signalling molecule, ppGpp. Most were defective in both *relA1* and *spoT1* alleles that result in absence of ppGpp regulation [227]. The RelA protein catalyzes the synthesis of ppGpp from GTP and GDP whereas SpoT catalyzes its synthesis and degradation [228]. The importance of ppGpp in linking protein synthesis and lipid synthesis was investigated by one group that compared incorporation of [¹⁴C]acetate into phospholipids during starvation of leucine [229]. They compared wild-type and *relA* strains and found that upon leucine deprivation, the wild-type strain responded by reducing lipid synthesis by approximately 50%, whereas the *relA* strain did not. This effect was attributed to biochemical inhibition of the carboxyltransferase subunit by ppGpp in the wild-type strain that was absent in the *relA* strain. Multiple groups also demonstrated that ppGpp can inhibit the PlsB activity in vitro when palmitoyl-CoA is used as the acyldonor [230,231]. Cerulenin treatment triggers a SpoT-dependent increase in ppGpp [36], suggesting that SpoT activity is responsible for the coordination of fatty acid and protein synthesis. The discovery of a ppGpp-mediated regulatory link between amino acid availability and lipid synthesis calls to question the relevance of earlier studies to normal physiology and stresses the importance of knowing the status of the *relA* and *spoT* genes used in the experiments. More recently, Yao *et al.* were reminded of the connection between ppGpp and regulation of lipid synthesis when selecting for *E. coli* mutants that were able to grow in the presence of a defective LPS synthesis pathway [34]. Their selection conditions yielded mutations in *fabH* and *accD*,

allowing the cells to grow during limited LPS biosynthesis. Based on this result, the researchers generated a targeted *fabH* deletion in the parent strain which they observed was viable. The debate about the essentiality of *fabH* was discussed in Section 2.1.2, but it was uncovered that a *fabH* null mutation was not viable in an *E. coli* *recA1 spoT1* strain. The explanation for this is the lack of pathway regulation by ppGpp, which accumulates during fatty acid starvation/inhibition, but to date a conclusive mechanism has not been provided [36,232].

3.4 Phospholipid headgroup homeostasis

3.4.1 Regulation of PtdSer Synthesis—Synthesis of the PtdEtn and PtdGro in *E. coli* was covered in Section 2.4.1. The balance of PtdEtn to PtdGro is maintained by a series of strict biochemical regulatory controls. The branch point between zwitterionic phospholipids (PtdEtn) and anionic phospholipids (PtdGro and cardiolipin) arises after phosphatidic acid has been converted to CDP-DAG. The PtdSer synthase (Pss) and the PtdGro-phosphate synthase (PgsA) enzymes compete for the CDP-DAG. Plasmid driven overexpression (800 fold) of Pss or PgsA individually in *E. coli* has minimal effect on the composition of PtdEtn and PtdGro in the membrane due both enzymes being tightly controlled by independent feedback regulatory loops [233–235]. Evidence for independent regulation of Pss and PgsA comes from experiments with *E. coli* treated with the glycerol-1-phosphate acceptor arbutin, that stimulates the transfer of G1P from PtdGro to form arbutin-phosphoglycerol and DAG [234]. The arbutin-mediated removal of G1P from PtdGro did not significantly reduce the PtdGro content of the cell membrane. This demonstrated a huge increase (7 fold) in PgsA activity to maintain the PtdGro content of the membrane despite constant conversion of PtdGro to DAG by arbutin. No additional PgsA activity was detected in cell extracts from cells treated with arbutin, indicating this increase in *in vivo* activity was due to biochemical feedback regulation as opposed to increased gene expression. The synthesis of PtdEtn was not increased, proving PgsA and Pss are regulated independently.

During the purification of Pss, it was noted that it was primarily associated with the ribosomal fraction of the cell instead of the membrane fraction as expected [165]. However, if membranes supplemented with CDP-DAG are added to the ribosomal fraction, Pss switches location and associates with the membrane. Thus, CDP-DAG essentially activates Pss and promotes association of the enzyme with the membrane rather than ribosomes [165]. Pss is an interfacial enzyme which needs to associate with anionic lipids on the surface of the membrane to be catalytically active [165,236]. The requirement for anionic phospholipids for Pss activity results in a clever detection mechanism that can respond to an increase in anionic membrane phospholipids by stimulating PtdSer (and consequently PtdEtn) synthesis. This also provides an explanation for the lack of increased PtdEtn synthesis following a 800-fold overexpression of Pss because only the membrane-bound form is catalytically active. Pss from *B. subtilis* is an integral membrane protein with no association with the ribosomal fraction of the cell [237]. Sara *et al.* introduced the Pss from *B. subtilis* into an *E. coli* Pss null strain under the control of an IPTG inducible promoter [237]. In contrast to overproduction of *E. coli* Pss, increasing amounts of IPTG (and therefore *B. subtilis* Pss) caused a steady-state increase in the PtdEtn content up to a critical level, where expression of the gene became lethal [1,237]. The membrane bound *B. subtilis* Pss does not have the regulated activity of the *E. coli* enzyme and therefore must be controlled differently than in *E. coli*. This multilayered regulation of *E. coli* Pss is a combination of strict feedback regulation and a sensing mechanism for acidic phospholipids that drives the membrane association and activation of Pss.

Phosphatidylserine decarboxylase (Psd) is an intrinsic membrane protein that catalyzes the formation of PtdEtn from PtdSer. The fact that only trace amounts of PtdSer are detected in

E. coli cells suggests that Psd is extremely efficient at performing the reaction. Psd is an interesting enzyme that utilizes a covalently bound pyruvate prosthetic group that is essential for catalytic activity [238]. Unlike most of the enzymes involved in membrane lipid synthesis in *E. coli*, Psd requires a post translational modification for activity. Li *et al.* reported Psd is translated as a 36 kDa proenzyme that is rapidly cleaved by reductive amination into two non-identical subunits of 7 kDa and 29 kDa [239]. This cleavage results in Ser254 being converted to the N-terminal pyruvate of the 7 kDa subunit [239]. Mutagenesis of Ser254 to a cysteine or threonine reduces the cleavage reaction whereas substitution to an alanine completely inhibits the reaction [167]. The mechanism for the cleavage is unclear but is thought to follow an autocatalytic serinolysis reaction comparable to that of the histidine decarboxylase enzyme [240]. The requirement for posttranslational processing of Psd seems to be widespread as the enzyme of *Vibrio cholerae* also undergoes autocatalytic cleavage into a 27.9 kDa and a 3.6 kDa subunit and the *B. subtilis* Psd contains a conserved cleavage segment, although the cleavage mechanism has not yet been confirmed [241–243].

3.4.2 The MprF system—Some firmicutes, including *S. aureus* and *B. subtilis* synthesize the unique lipid, lysyl- phosphatidylglycerol (Lys-PtdGro). This positively charged phospholipid is synthesized by the aminoacylation of PtdGro by the transmembrane MprF enzyme utilizing Lys-tRNA as a lysine donor (for review see ref [244]). The lysinylation of the anionic PtdGro changes the net charge of the phospholipid from -1 to +1 [245]. The enzyme was named MprF as a notation for multiple peptide resistance factor, in reference to the increased susceptibility of a *S. aureus mprF* null mutant towards cationic antimicrobial peptides (CAMPs) and lipopeptide antibiotics such as defensins and daptomycin [246,247]. The cationic nature of CAMPs result in high-affinity to anionic bacterial membranes. The positive net charge introduced due to the production of Lys-PtdGro perturbs this electrostatic attractions and mitigates the antimicrobial effects of the CAMPs. Although Lys-PtdGro does not have a defined structural role, it clearly has an integral role in pathogenesis in *S. aureus* because *mprF* deletion mutants have attenuated virulence [248]. Why a non-pathogenic soil-dwelling organism like *B. subtilis* would produce Lys-PtdGro seems less obvious, but it has been hypothesized that many soil dwelling organisms do so to mitigate the effect of antimicrobial peptides secreted by themselves and other soil bacteria [249]. MprF from *S. aureus* produces strictly Lys-PtdGro, whereas an MprF-homologue from *P. aeruginosa* produces only Ala-PtdGro [250].

The MprF enzyme from *Enterococcus faecium* and *B. subtilis* have a less strict substrate specificities and are able to utilize both Lys and Ala to modify PtdGro [251]. The MprF enzyme houses two different catalytic domains. The C-terminal domain is the Lys-PtdGro synthase domain that catalyzes the transfer of the lysine from the aminoacyl-tRNA to the hydroxyl group of terminal glycerol of PtdGro. The amino-terminal domain is the hydrophobic flippase components that catalyzes the translocation of Lys-PtdGro from the inner leaflet of the cell membrane where it is produced, to the outer leaflet where it can function to repel CAMPs. Ernst *et al.* [246] demonstrated that production of Lys-PtdGro alone on the inner leaflet is not sufficient to induce resistance to CAMPs. This was accomplished by expressing a truncated form of MprF that only contains the Lys-PtdGro synthase domain that actively produced Lys-PtdGro. Expression of the synthase domain in combination with the flippase domain restored resistance to CAMPs, signifying the requirement for presenting the Lys-PtdGro on the exterior of the membrane to exert its CAMP repulsing effect. The regulation of MprF activity and gene expression remains elusive. Nothing is known about the biochemical control of MprF activity in any organism.

In *P. aeruginosa*, Ala-PtdGro synthesis is induced by acidic growth conditions and Lys-PtdGro production in *S. aureus* varies throughout the stage of growth [246,250]. The *mprF*

gene and other resistance mechanisms are upregulated in response to CAMPs by the ApsRSX three component regulator [252–255]. ApsS is the membrane bound sensor kinase that interacts with CAMPs and activates ApsR by phosphorylation, inducing transcription of *mprF* and genes involved in lysine biosynthesis [252,255].

3.5 Acyl-chain turnover

The turnover of PtdGro in *E. coli* to generate MDO and in *S. aureus* during LTA synthesis was discussed in Section 2.3.3, but in *E. coli* production of lipoproteins results in the removal of acyl chains from established phospholipids. Lipoproteins serve a plethora of different functions in the bacterial kingdom, from hemolysins that are essential for pathogenesis to peptidoglycan associated lipoproteins that have a role in cell wall maintenance [256,257]. Lipoproteins are abundant in *E. coli* with 1% to 3% of genes predicted to encode fatty acid or diacylglycerol modified proteins. The most thoroughly studied is the outer membrane lipoprotein Lpp [258]. There are two generalized structures of lipoproteins in bacteria: diacylated lipoproteins and triacylated lipoproteins. In *E. coli*, the synthesis pathway of the triacylated form is well-established [257]. The lipoprotein is translated as a preprolipoprotein containing a 20 amino acid amino-terminal signal peptide. The lipoprotein DAG transferase (Lgt) enzyme attaches a DAG molecule from PtdGro to the sulfhydryl group of a conserved cysteine. The lipoprotein signal peptidase enzyme (Lsp) specifically cleaves the prelipoprotein upstream of the lipidated Cys residue resulting in the Cys becoming the amino-terminal residue of the lipoprotein. The final stage to generate a triacylated lipoprotein by the lipoprotein N-acyl transferase (Lnt) catalyzed transfer of a fatty acid to the terminal amine residue of the cysteine. The source of the fatty acid involved in the α -aminoacylation of the lipoprotein is derived from the 1-position of PtdEtn in *E. coli*, creating the lysophospholipid 2-acyl-glycerophosphoethanolamine (2-acyl-GPE) in the process [259,260]. Gupta and coworkers demonstrated that the Lnt reaction isn't absolutely specific for PtdEtn, as in the absence of PtdEtn, the fatty acid from the 1-position of PtdGro or cardiolipin can be used [261]. It is not desirable for the cell membrane to contain lysophospholipids, therefore a lysophospholipid repair or degradation pathway must be implemented to dispose of these lipids. 2-acyl-GPE can be degraded into free fatty acid and GPE by the lysophospholipase L2 enzyme (PldB). The alternative is conversion of 2-acyl-GPE to PtdEtn through the acyl-ACP synthetase:2-acyl-GPE acyltransferase enzyme (Aas) [262–265]. This membrane-bound enzyme contains two activity domains: the first catalyzes the ATP dependent formation of acyl-ACP from free fatty acid and unesterified ACP that remains tightly associated with the enzyme. The second activity transfers the fatty acid from the acyl-ACP to the 1-position of 2-acyl-GPE, in essence recycling the lysophospholipid into a bilayer forming membrane lipid. Unlike the acyl-ACP synthetase produced by *V. harveyi* that generates free acyl-ACP from free fatty acid and ATP, the acyl-ACP from the *E. coli* Aas enzyme does not dissociate from the enzyme unless non-physiological high ionic strength is applied (0.4 M LiCl) [263,266]. Other methods of removing 2-acyl-GPE include a membrane bound transacylase activity that combines two 2-acyl-GPEs to produce PtdEtn and GPE and another phospholipase that degrades 2-acyl-GPE [267,268]. In *E. coli*, lysophospholipids can also be generated through transfer of an acyl chain from membrane bound phospholipids to lipid A [269,270]. The recycling system is thought to be the same mechanism that is used to repair lysophospholipids generated during Lpp maturation. It is important to note that the reactions of lipoprotein acylation occur outside the cytoplasmic membrane justifying the use of phospholipids, rather than acyl-ACP, for these reactions. However, this does create a topological problem in that the phospholipid products must be transferred inside the cell to re-enter the biosynthetic pathway. DAG is thought to spontaneously cross the plasma membrane, but there is a transporter (flippase) required to move 2-acyl-GPE from the periplasm to the cell interior where it can be used by the Aas

enzyme. This is accomplished that the LplT transporter that is found in the same operon as Aas [271]. In some bacteria, the proteins are fused as is found in the MprF system.

It was thought for some time that Gram-positive organisms could only generate diacylated lipoproteins due to the lack of a Lnt homologue. This view was challenged when a triacylated protein, SitC, was discovered through a mass spectrometry study in *S. aureus* [272,273]. This finding was not without controversy as another group almost simultaneously reported the same protein was only diacylated [274]. The discrepancy was resolved by Kurokawa *et al.* who determined that the acylation state of SitC was dependent on the growth conditions [275]. Growth of *S. aureus* in a low pH, high temperature or high salt environment or post-exponential growth phase accumulated the diacyl SitC, whereas growth under more favorable conditions resulted in accumulation of the triacylated isoform. The identity of the enzyme that creates triacylated lipoproteins in Gram-positive bacteria remains to be identified.

A additional mechanism for acyl-chain turnover in *E. coli* was proposed by Kol and coworkers. They examined the fate of exogenous short chain (6:0) PtdEtn and PtdSer in a Pss mutant that is unable to synthesize PtdEtn [276]. Their results showed that exogenous PtdEtn was remodeled and the short-chain fatty acids replaced with palmitate acyl chains. Exogenous PtdSer was rapidly decarboxylated to form PtdEtn, prior to remodeling. The authors think this process involved two successive deacylation and reacylation steps, but the enzymes involved remain unknown. The steady state level of phospholipid remodeling in *E. coli* has been reported to increase during stationary phase. Pech-Canul *et al.* [277] were examining the role of a *fadD* null mutant in *E. coli* and *S. meliloti* and observed that during stationary phase, cells lacking the FadD acyl-CoA synthetase accumulated free fatty acids. They determined that the fatty acids did not arise from an exogenous source by observing the same phenomenon after deleting FadL, the fatty acid transporter preventing utilization of fatty acids in the medium. This observation highlights the potential role of FadD in activating endogenous fatty acids released from membrane lipids and the increased rate of deacylation of membrane phospholipids during stationary phase in *E. coli* and *S. meliloti*.

4. Transcriptional regulation of bacterial lipid metabolism

4.1 Genetic regulation of lipid metabolism in Gram-negative bacteria

There is a sophisticated network of diverse transcriptional regulators of lipid metabolism in bacteria that work in concert with biochemical regulation to control the pathway (Table 1) [278]. The expression levels of different fatty acid synthesis biosynthetic genes are coordinated with growth rate, nutrient availability and environmental stimuli. In organisms possessing a fatty acid β oxidation pathway in addition to biosynthesis, the expression of the degradation machinery is balanced with fatty acid synthesis enzymes.

4.1.1 Coordination of fatty acid synthesis and degradation by FadR—The FadR transcription factor has been most thoroughly studied in *E. coli* (EcFadR), but homologs are present in Gram-positive and Gram-negative bacteria. *E. coli* FadR is a member of the GntR family of transcription factors and functions as a classical repressor of genes involved in fatty acid degradation and an activator of some genes involved in fatty acid synthesis [279,280]. The genes encoding enzymes involved in β -oxidation are spread throughout the chromosome and are responsible for the transport (*fadL*), activation (*fadD*) and degradation (*fadABEFHIJ*) of exogenous fatty acids [281]. β -Oxidation liberates one molecule of acetyl-CoA with each turn of the cycle, that can be further catabolized by the citric acid cycle and enzymes involved in glyoxylate bypass [282]. FadR is not the sole transcriptional regulator of genes involved in fatty acid degradation in *E. coli* as the ArcAB system also negative

regulates the pathway whereas the global cyclic AMP receptor protein-cAMP complex activates the *fad* genes [281,283,284].

The binding of the FadR repressor to DNA is antagonized by the binding of long-chain acyl-CoA thioesters, resulting in a conformational change of FadR and a reduced affinity for its DNA binding site [279,285,286]. The FadL enzyme transports a fatty acid from the growth medium across the outer membrane and into the periplasmic space [287,288]. The fatty acids are moved from the periplasm to the cytoplasm through an unknown mechanism (probably spontaneous flipping) where acyl-CoAs are generated through the activation of exogenous or endogenous free fatty acids by the interfacial enzyme acyl-CoA synthetase (FadD) [105,277,289]. The acyl-CoA products of the FadD reaction can either be utilized as acyl-donors for PlsB and PlsC during phosphatidic acid biosynthesis or used by enzymes encoded by the *fad* regulon, that are able to disassemble the fatty acid completely to acetyl-CoA by β -oxidation. Therefore, FadR recognizes the availability of acyl-CoAs as an energy source and de-represses the genes involved in consuming the fatty acid as an energy source. An additional member of the *fad* regulon was recently found that encodes an acyl-CoA thioesterase, FadM [290]. The physiological role of FadM (formerly YbaW) has been postulated to be hydrolysis of 3,5-tetradecadienoyl-CoA and 9-*cis*,11-*trans*-octadecadienoyl-CoA that are intermediates in β -oxidation degradation of oleic acid and conjugated linoleic acid respectively [291,292]. The free acids are released from the cell and secreted into the growth medium. The proposed role of the thioesterase is to release the intermediates that are resistant to further degradation that would otherwise accumulate and inhibit the flow of metabolites through the pathway. Similarly to FadL and FadD, FadM is only weakly derepressed in a Δ *fadR* strain (2–3 fold) whereas the enzymes involved in degradation, FadH and FadBA, increase expression 5–10 fold [290,293]. The *fadL* and *fadD* genes are the only members of the *fad* regulon containing two FadR binding sites, despite a lesser influence of FadR on repression of *fadL* and *fadD* [293]. The less stringent regulation of FadL and FadD is logical as these enzymes are required to generate the regulatory acyl-CoA.

FadR also functions as an activator of two genes involved in unsaturated fatty acid synthesis, *fabA* and *fabB*, in addition to *iclR* [294–297]. The IclR protein is a repressor of the *aceAB* genes that encode enzymes involved in the glyoxylate shunt pathway [294]. The activation of *fabA* and *fabB* by FadR is abolished upon binding of FadR to acyl-CoA. The simultaneous activation of fatty acid degradation and repression of unsaturated fatty acid synthesis in response to exogenous fatty acids coordinates the two pathways. A possible explanation put forward by John Cronan to explain the selective deactivation of the unsaturated arm of FASII by FadR is based on the essentiality of saturated fatty acids in *E. coli* [295]. As described in Section 2, *E. coli* requires 3-hydroxymyristoyl-ACP to synthesize the lipid A component of LPS. Although exogenous fatty acids can fulfill the unsaturated fatty acid requirement of the cell, 3-hydroxymyristate is a product of saturated fatty acid biosynthesis and can only be utilized from the endogenous pathway as an ACP thioester. Biochemical studies have concluded FadR has the highest affinity for long-chain acyl-CoAs (C16–C18), and little affinity for <C10-CoA or free fatty acids [279]. The dissociation constants for palmitoyl and oleoyl-CoA are in the nanomolar range, underlining the high sensitivity of FadR to acyl-CoAs [279].

Structural data has been obtained for three forms of FadR: the free protein, FadR-DNA complex and the FadR-acyl-CoA complex [298–301,301]. The structures illustrate that FadR contains a typical DNA binding winged-helix motif at the N-terminus and a collection of α -helices as the C-terminal domain. The free protein and DNA-bound structures are almost identical whereas the acyl-CoA bound structure shows the interaction of the CoA thioester with the C-terminal domain. In order to accommodate the acyl-CoA, several

residues from the DNA-binding region change conformation causing the helices in the DNA binding domain to separate. This conformational change reduces the affinity of FadR to its recognition sequence and dissociates the repressor from the DNA.

Although *E. coli* is considered the paradigm for bacterial FASII, the advent of genome sequencing has revealed variations in the machinery and the regulation of the pathway. FadR is not present in bacteria without a β -oxidation pathway and is not present in all bacteria which have the pathway. Not only does the affinity for acyl-CoAs vary between species, the genes within the *fad* regulon are also different [302]. For example, a FadR homologue was discovered in *B. subtilis* that also represses the genes of β -oxidation but shows no evidence of the activator function of observed in *E. coli*, most likely due to the lack of a FabB/FabA mediated unsaturated fatty acid synthesis pathway [303]. Although *B. subtilis* and *E. coli* FadR proteins are functionally similar, they are structurally distinct with the *B. subtilis* protein belonging to the TetR family while the *E. coli* enzyme is a GntR like repressor. An additional member of the FadR regulon was identified in *Vibrio cholerae* where FadR binds to the promoter region and represses transcription of *plsB* [304]. The potential explanation for the absence of this transcriptional regulation in *E. coli* arises from the different acyltransferase machinery. *E. coli* uses solely PlsB and PlsC to transfer fatty acids from acyl-CoA or acyl-ACP to G3P, whereas *V. cholerae* appears to use different acyltransferases for exogenous and endogenous fatty acids. Although *E. coli* possesses *plsX* and *plsY* genes, the functions remain unknown. *V. cholera* utilizes the PlsX/PlsY system for the acylation of glycerol-phosphate from *de novo* synthesized fatty acids and PlsB for exogenous fatty acids. Based on the poor affinity of *B. subtilis* PlsX for acyl-CoAs compared to acyl-ACPs, it is possible *V. cholerae* PlsX could behave the same, thereby utilizing one acyltransferase for exogenous fatty acids (PlsB) and another set for endogenously synthesized acyl chains (PlsX/PlsY) [104]. This idea is supported by data indicating a transposon insertion into *plsB* of *V. cholerae* is not lethal while *plsB* is essential in *E. coli* [115,305,306]. In contrast, PlsX is a dispensable gene in *E. coli* but not in *V. cholera* [115,305,306]. If PlsB functions purely as an exogenous fatty acid acyltransferase in *V. cholerae*, it seems logical that it is derepressed in response to exogenous fatty acid.

The widespread nature of the FadR enzyme indicates an important role in metabolism in several different bacteria. Deletion of *fadR* appears to have no effect in *E. coli* and *S. enterica* unless fatty acids are the sole carbon source, where deletion of *fadR* causes accelerated growth as the fatty acid degradation enzymes are overexpressed [307]. In contrast, disruption of *fadR* in *V. vulnificus* had a severe effect on cell growth in rich media and resulted in attenuated virulence in a murine subcutaneous infection model [308]. The virulence attenuation could be attributed to the lack of expression of the *fabA* gene in the *fadD* mutant, which causes a significant (13%) decrease in the unsaturated fatty acid content of the bacteria.

4.1.2 FabR/DesT control of unsaturated fatty acid synthesis—In addition to the influence of *fabA* and *fabB* in controlling unsaturated fatty acid biosynthesis, *P. aeruginosa* also possesses Δ^9 -desaturases (discussed in Section 2.2.3) that insert a double bond into the acyl-chains of existing phospholipids (DesA) or saturated acyl-CoAs (DesBC) [95]. The expression of *fabAB* and *desBC* is controlled by the DesT repressor [309,310]. DesT is a TetR family transcription factor and functions to sense the overall fatty acid composition of the acyl-CoA pools. DesT binds saturated and unsaturated acyl-CoAs with equal affinity but binding to DNA is enhanced when DesT is bound to an unsaturated acyl-CoA and perturbed when DesT is bound to a saturated acyl-CoA [309,310]. This allows *P. aeruginosa* to respond to the availability of exogenous saturated and unsaturated fatty acids and adjust gene expression to maintain a constant membrane fluidity. DesT only detects exogenous fatty acids in the form of long-chain acyl-CoA thioesters as acyl-ACP, short chain acyl-

CoAs and free fatty acids did not alter DNA binding [309,310]. Growth of *P. aeruginosa* on oleate repressed expression of *desC* and *fabAB* 2- and 12-fold, respectively. In contrast, growth on stearic acid stimulated transcription of *desC* by approximately 8-fold [309,310]. The differential influence of DesT binding between the *fabAB* and *desCB* genes was attributed to the slightly different recognition palindromes in the promoters with the *desCB* recognition sequence binding DesT with the highest affinity and exerting more stringent regulation [309,310]. X-Ray crystallography of the different forms of DesT provided a molecular insight into the conformational changes that govern the binding/dissociation of DesT from its cognate DNA upon acyl-CoA binding. Miller and coworkers [311] solved crystal structures of the DesT-18:1 Δ 9-CoA-DNA complex and the DesT-16:0-CoA complexes. They determined that DesT adopts two conformations: a “relaxed” conformation when bound to an unsaturated acyl-CoA that facilitates DNA binding and a “tense” conformation when bound to a saturated acyl-CoA species that exhibits a lower affinity for the DNA target sequence. The L-shaped oleoyl-CoA ligand slots into the hydrophobic core of the DesT ligand binding domain and becomes an integral component of the hydrophobic domain allowing it to adopt its relaxed state. As DesT binds 16:0-CoA, the acyl chain is inserted into the protein core forcing the phenylalanine rich cluster in the hydrophobic pocket to adjust its position to accommodate the straight acyl chain locking DesT into the tense conformation. The conformational changes that occur to accommodate the saturated acyl-CoA result in a shift of some of the helices in the DNA binding domain away from their optimal DNA binding orientations present in the relaxed form. A key residue in the phenylalanine cluster is Phe166, which is perfectly positioned where the *cis*-double bond of oleate sits and is thought to detect the unsaturation of the acyl-chain. Despite retaining affinity for acyl-CoA, mutagenesis of Phe166 to an alanine prevents detection of the kink in oleate and locks the protein in the tense state, preventing DNA binding. Phe166 is positioned below one of the helices in the DNA binding domain and functions to stabilize its orientation when oleoyl-CoA is bound.

There is a second transcription factor, designated FabR, which contributes to fine tuning the biophysical properties of the membrane. FabR binds to regions of the *fabA* and *fabB* promoter downstream of the FadR recognition sequence to repress transcription [280,312–314]. FabR was discovered in 2001 after McCue *et al.* examined a sequence upstream of the *fabA* and *fabB* genes that is conserved in *E. coli* and several other γ -proteobacteria [315]. Using an oligonucleotide affinity column, they isolated proteins from an *E. coli* crude extract that bound to the *fabA* and *fabB* promoter sequence and used proteomic techniques to identify interacting proteins. This strategy led to the identification of FabR, which was subsequently followed by genetic studies examining the transcriptional effects of deleting the *fabR* gene. Deletion of *fabR* in *E. coli* causes a 2–4 fold increase in *fabA* and *fabB* mRNA, confirming the role of FabR as a repressor [312]. The effect of deleting *fabR* was most pronounced on the expression of *fabB*, whereas FadR is the most prominent regulator of *fabA*. Deletion of FabR causes an increase in the unsaturated to saturated fatty acid ratio from approximately 1 to 2, consistent with its role in repressing transcription of *fabA* and *fabB*, the primary determinants of unsaturated fatty acid synthesis in *E. coli* [314]. Analysis of the an *fabR* insertional inactivation strain from the Keio collection did not show an increase in unsaturated fatty acid content when compared to the parent strain [278,306]. However, sequencing of the *fabR* gene from the parent strain used in the Keio collection identified a missense mutation in the DNA binding domain of the gene encoding the FabR that likely antagonizes the binding of FabR to its cognate sequence, presenting the phenotype of a FabR deletion mutant [278]. Zhu *et al.* [278] identified the regulatory ligands that increase the affinity of FabR for DNA as unsaturated acyl-CoA and acyl-ACP thioesters. FabR demonstrated high affinity for long-chain unsaturated acyl-CoA/acyl-ACP thioesters that induce binding of FabR to DNA, suppressing new unsaturated fatty acid synthesis. Both FadR and FabR can simultaneously bind to the *fabB* promoter sequence,

demonstrating combinatorial transcriptional regulation. Binding of the FabR-18:1 Δ 9 CoA complex to DNA was antagonized by the presence of saturated 16:0-CoA/16:0-ACP to increase the unsaturated fatty acid content when an abundance of saturated fatty acids are available. This observation is consistent with the theory that unsaturated fatty acid ligands induce a conformational change in FabR that increases its affinity to DNA, whereas binding of saturated ligands does not induce the change in conformation. FabR essentially measures the unsaturated: saturated fatty acid ratio in the cell, as opposed to absolute amounts of either. This property is crucial as during cell growth, the concentration of intracellular acyl-ACP does not vary whereas the unsaturated content can change. This allows FabR to tune the expression of *fabA* and *fabB* according to the unsaturated fatty acid content of the ACP pool. The ability of FabR to bind both CoA and ACP thioesters indicates FabR is a sensor of both de novo and exogenously obtained fatty acids, in contrast to FadR that only senses the exogenous acyl-CoA pool.

The FabR model described by Zhu *et al.* [314] was questioned by Feng and Cronan [313], who observed binding of FabR to DNA in the absence of a ligand. They observed upregulation of *fabA* and *fabB* upon deletion of *fabR* and repression of *fabA* and *fabB* after treatment of growing cells with oleate. However, depletion of the unsaturated acyl-ACP pool through specific inhibition of FabA with 3-decynoyl-*N*-acetylcysteamine did not result in depression of *fabAB*. Under their assay conditions, Feng and Cronan showed through electrophoretic mobility shift assays that FabR is able to bind to a *fabB* and *fabA* promoter sequence without addition of a ligand. On the surface, this finding seems to argue against the model of Zhu *et al.*, but the equilibrium between the two conformations of FabR or DesT can be shifted by either altering the ligand or DNA concentrations in the assay [278,311]. More research is needed to clarify the details of FabR-DNA, FabR-ligand and FabR-ligand-DNA interactions, but it is clear that the properties of FabR and DesT monitor the ratio of the saturated to unsaturated acyl-CoA pools to allow *P. aeruginosa* and *E. coli* to tune its gene expression to modify fatty acid synthesis to provide the acyltransferases with an optimal selection of saturated and unsaturated substrates. The ability of these transcription factors to examine the composition of the acyl-CoA (and acyl-ACP in *E. coli*) pools provides an elegant mode of regulation when compared to classical repressors that respond to ligand concentration rather than ligand composition.

4.2 Genetic regulation of lipogenic genes in Gram-positive Bacteria

Firmicutes, including *S. aureus*, *B. subtilis* and *S. pneumoniae*, lack the FabB and FabA enzymes for synthesizing unsaturated fatty acids. *S. aureus* and *B. subtilis* produce primarily branched-chain saturated fatty acids, whereas *S. pneumoniae* utilizes the FabM enzyme for the *cis-trans* isomerization reaction to initiate unsaturated fatty acid synthesis. This difference in the fatty acid biosynthetic machinery mitigates the requirement for a Gram-negative type of transcriptional regulation identified in *E. coli* and *P. aeruginosa* to control the expression of the *fabAB*. In addition to the biochemical regulatory control of the key fatty acid synthesis pathway enzymes, at least two different systems for the transcriptional control of FASII gene expression exist in Gram-positive bacteria.

4.2.1 The FapR system—The first transcriptional regulator of FASII to be discovered in Gram-positive bacteria was the FapR repressor of *B. subtilis*. Bioinformatic homology searches suggested FapR exists in several different species of *Bacilli* in addition to *Staphylococci* and *Clostridia* [316]. Far from the specific gene regulators described in Section 4.1, FapR is a global regulator of phospholipid biosynthesis genes. The *fap* regulon of *B. subtilis* consists of the genes encoding the condensing enzymes, *fabI*, *fabD*, *fabG*, *plsX* and two unknown proteins (*yhfC* and *ylpC*) [316]. FapR is a classical repressor that detects malonyl-CoA, which fuels the elongation cycle [316,317]. Structural studies have revealed

that FapR exists as a homodimer and contains a “hot dog” fold, which is commonly seen in thioesterases that are involved in acyl-CoA and acyl-ACP hydrolysis [318,319]. The ligand binding domain of the ligand-free FapR exists as an open cleft that surrounds the malonyl-CoA upon ligand binding. The malonyl-CoA induced structural rearrangements pull the DNA binding domains of the homodimer apart, reducing the cooperative association of the two monomers and disrupting the competent operator binding arrangement. The response of FapR to increases in malonyl-CoA levels essentially allows FapR to respond to defects in the elongation cycle by monitoring the levels of fatty acid precursor (malonyl-CoA) and increase transcription of the pathway genes accordingly [320]. Inducing a defect in the pathway by treatment of *B. subtilis* or *S. aureus* with a FabF inhibitor results in malonyl-CoA accumulation and consequently an increase in expression of genes in the *fap* regulon [65,316]. Transcription of FASII genes is constitutively high in cells lacking FapR and they are unaffected by pathway inhibition [316]. Unlike FadR that has a nanomolar dissociation constant for its acyl-CoA ligands, FapR is relatively insensitive to malonyl-CoA with a concentration of 25 μ M required to disrupt DNA binding [317]. Similar concentrations of malonyl-ACP also prevent DNA binding by FapR but slightly less effectively. Considering the concentration of ACP in *E. coli* is approximately 40 μ M and assumed to be similar in *B. subtilis*, it seems unrealistic to assume that the majority of the intracellular ACP must be converted to malonyl-ACP to exert its physiological effect [321,322]. Similarly, the steady state levels of malonyl-CoA in *E. coli* were calculated to be approximately 24 μ M, which is slightly lower than the calculated K_d for DNA bound FapR for malonyl-CoA (25 μ M) [323]. This would in theory give the bacterium enough dynamic range to modulate expression of the *fap* genes. An increase in malonyl-CoA utilization through overexpression of the condensing enzymes would be expected to have the opposite effect and decrease expression of the *fap* regulon, as steady-state malonyl-CoA levels are decreased. However, this possibility has not been investigated. The significance of malonyl-CoA as a regulatory molecule in addition to its role as a precursor of fatty acids suggests that the malonyl-CoA pool in FapR-containing organisms must be tightly regulated by a currently unknown mechanism, potentially product inhibition or feedback regulation by acyl-ACPs as seen in *E. coli*. The importance of maintaining optimum expression of the genes in the *fap* regulon became evident after it was discovered that a *B. subtilis* *fapR* null mutant has a defective growth phenotype when grown in hypothermic conditions (15°C) [316]. Compositional analysis of the mutant strain compared to the wild type indicated longer fatty acids in the *fapR* strain, which would be expected to increase membrane rigidity during growth at low temperatures. The compositional defect arising from an excess of pathway enzymes could contribute to the cold intolerance of *B. subtilis* *fapR* mutants.

4.2.2 The FabT system—The fatty acid composition of membrane lipids in *S. pneumoniae* closely resembles the unsaturated/saturated fatty acid profile of *E. coli*. Despite this similarity, both organisms have evolved distinctly different mechanisms for transcriptional control. Instead of the FadR/FabR system, *S. pneumoniae* employs FabT as a transcriptional repressor to govern gene expression of FASII genes [17,324]. The genetic organization of the genes encoding FASII machinery in *S. pneumoniae* reveals they all reside in a single locus on the chromosome, including the *fabT* gene. The genes are divided into two operons, the first containing *fabT-fabH-acpP*, and the second includes *fabK-fabD-fabG-fabF-accD-fabZ-accC-accD-accA* with a FabT operator sequence included in the promoter region of *fabT* and *fabK* [17]. Disruption of the *fabT* gene distinctly altered the fatty acid composition of membrane phospholipids, sharply reducing the unsaturated:saturated fatty acid ratio from 2.6 to 0.9 and increasing the abundance of longer chain C18 species as compared to C16. The increase in acyl chain length is reminiscent of the chain lengthening effect of disrupting *fapR* in *B. subtilis* [17,316]. The ligand that enhances FabT binding to DNA is long-chain acyl-ACP. Upon binding to acyl-ACP, the

affinity of FabT for the DNA binding site is significantly increased and the FabT-acyl-ACP complex docks on the DNA and represses transcription of the FAS cluster [324]. FabT binds acyl-ACPs of all chain lengths tested but only the long-chain acyl-ACPs induce the conformational change that stimulates DNA binding. *cis*-Vaccinate (18:1 Δ 11) is the most abundant fatty acid in *S. pneumoniae* and is the most effective of the acyl-ACP derivatives tested at inducing DNA binding [324]. The transcriptional feedback regulation involving long-chain acyl-ACPs links accumulation of the end product of the pathway with expression of the genes encoding the pathway enzymes. This regulatory circuit allows *S. pneumoniae* to adjust gene expression of all the FASII genes in response to a lack or an accumulation of the end product of the pathway. This effect is complemented by biochemical regulation of the initiation enzymes in response to exogenous fatty acids, presumably acyl-ACP [65]. However, the biochemical regulatory mechanism remains poorly understood.

B. subtilis lacking FapR and *S. pneumoniae* deficient in FabT do not produce increased quantities of lipid, despite overexpression of the pathway enzymes [17,325]. This observation strongly argues for the presence of biochemical regulatory mechanisms in both organisms that override transcriptional control to prevent excess production of phospholipids and maintain the appropriate lipid:protein ratio in the membrane.

4.3 Regulation of lipid metabolism by stress response regulators

4.3.1 The DesR thermometer—In addition to the controlling the rate of fatty acid synthesis in response to a shift in the composition of pathway intermediates, *B. subtilis* has developed regulatory mechanisms to detect environmental changes in temperature and adjust the expression of desaturating enzymes accordingly (for review see [326]). The ability to adjust the unsaturation of membrane phospholipids is crucial to counteract the increase in rigidity that arises when the temperature of biological membranes is reduced. Membrane lipids are fluid in the liquid crystalline state but become rigidified when the temperature drops below the melting temperature (T_m) of the membrane. The fluidity of the membrane is required for movement and activity of membrane proteins, therefore it is paramount to bacterial survival that the T_m is adjusted during growth at lower temperatures [327]. Section 2.2.3 discussed various desaturase enzymes from *P. aeruginosa* and the Des protein from *B. subtilis*, which inserts a *cis*-double bond into the $\Delta 5$ position of existing phospholipids [93]. Experiments demonstrated that *des* mRNA is virtually undetectable in *B. subtilis* cultures grown at 37°C, but is dramatically induced upon cold shock at 20°C [328]. The temperature-dependent expression of *des* suggested there is a temperature responsive transcription factor regulating the expression of the desaturase in *B. subtilis*. The discovery of the DesRK two-component regulator identified such a system. Two component regulators typically consist of a sensor histidine kinase/phosphatase component, which phosphorylates/dephosphorylates a response regulator that exerts its effect, usually through activation of a specific target gene. The *B. subtilis* system consists of the membrane-associated kinase, DesK, which phosphorylates DesR and drives transcription of the *des* gene by recruiting RNA polymerase to the promoter [326,329–333]. It has been postulated DesK acts as a thermometer, detecting the ambient temperature by adopting different signaling states depending on the membrane fluidity and activating the response regulator, DesR by phosphorylation [331]. This regulation could occur through the phosphatase activity dominating at high temperatures and the kinase activity dominating at low temperatures. Evidence that DesK detects perturbations in the membrane fluidity are supported by experiments that show no *des* induction during cold shock when *B. subtilis* is treated with unsaturated fatty acids. The unsaturated fatty acids increase membrane fluidity when incorporated into phospholipids, reducing the need for further desaturation of existing lipids by the Des enzyme [331]. An increased proportion of branched chain *anteiso* fatty acids in the *B. subtilis* membrane also increases membrane fluidity in the absence of unsaturated

fatty acids. The quantity of branched chain fatty acids produced in *B. subtilis* can be controlled by the availability of isoleucine in the growth medium, which is utilized by the branched-chain α -ketoacid dehydrogenase to generate the branched chain *anteiso* precursors utilized by FabH [334]. Starvation of *B. subtilis* for isoleucine at 37°C also triggers induction of *des*, in an attempt to increase membrane fluidity to counteract reduced branched-chain fatty acid synthesis [330]. This effect is alleviated through the addition of unsaturated fatty acids to the isoleucine free media.

The mechanism underlying the phosphorylation of DesR begins with the autophosphorylation of a histidine residue within DesK [333]. Phosphotransfer then occurs between DesK and an aspartate residue on DesR. As with the previous transcription factors discussed in this review, DesR exists in solution as a dimer. Phosphorylation by DesK promotes the association of two dimers to form a tetramer on the *des* promoter region where each dimer binds an adjacent, nonidentical DesR-P binding site on the *des* promoter DNA. The first palindrome, termed “RA”, is a high-affinity binding site and low concentrations of DesR-P bind only this site. The lower affinity site, termed “RB”, consists of only a partial repeat. Binding of DesR-P to both sites is necessary for recruitment of the RNA polymerase, as point mutations in RA or RB abolish *des* activation [333]. The working hypothesis for the mechanism of RNA polymerase recruitment requires binding of a dimer to the RA site initially, followed by binding to the RB site. It is the interaction of the second dimer of the tetramer at the RB site that is thought to physically interact with the RNA polymerase [332]. The requirement for binding of DesR-P to the low affinity and high affinity sites in the *des* promoter ensures that Des activation only occurs when a threshold amount of DesR phosphorylation occurs. The unusual dual binding site mechanism is responsible for the fine tuning of *des* expression and ensuring that the biophysical properties of the membrane phospholipids remain within an optimum fluidity range.

4.3.2 Alternate sigma factors—In addition to the DesRK system to sense changes in the membrane biophysical properties, *B. subtilis* expresses seven extracytoplasmic function (ECF) σ factors [335]. Several of the σ factors detect changes in membrane integrity and elicit a response to counteract the problem. A prime example is σ^w , which in contrast to DesKR, responds to an increase in membrane fluidity. σ^w is activated under conditions of membrane perturbation, such as high concentrations of detergent or when the lipid:protein ratio is disrupted [336–338]. σ^w reacts to an increase in membrane fluidity by elevating straight-chain fatty acid synthesis by the down-regulation of *fabHa* and increasing the average chain length by increased *fabF* expression [339]. There is a σ^w binding site within the *fabHa* coding sequence in the *B. subtilis fabHa-fabF* operon, which when occupied upregulates transcription of *fabF* and downregulates *fabHa*. Unlike most bacteria discussed in this review, the *B. subtilis* chromosome encodes two initiation condensing enzymes termed FabHa and FabHb, which differ in their substrate specificities [20]. FabHa has a preference for the branched chain precursors isobutyryl, isovaleryl and 2-methylbutyryl-CoA, whereas FabHb shows maximum activity with the straight-chain precursor acetyl-CoA. The authors confirmed the enzymological analysis by examining the fatty acid composition of *B. subtilis* strains deficient in either FabHa or FabHb, which showed a decrease in either branched-chain and straight-chain fatty acids, respectively. Overexpression of σ^w by plasmid based expression increased transcription of *fabF* and decreased *fabHa* transcripts. The opposite changes in expression of the two condensing enzymes generates a fatty acid profile containing longer fatty acids due to the overexpression of *fabF* and a decreased quantity of branched-chain fatty acids produced as a consequence of *fabHa* transcript suppression. The decrease in branching and increase in chain length allows tighter packing of the acyl chains within the membrane and reduces the fluidity of the membrane. Deletion of σ^w is associated with increased susceptibility to environmental hazards such as detergents and membrane active compounds; however many

of these physiological effects are likely due to the plethora of efflux pumps and detoxification enzymes that are concurrently induced as opposed to specific modification of membrane structure [340]. Whether σ^w is induced under other conditions which would increase membrane fluidity, such as growth under high temperature or excessive unsaturated fatty acid production remains undetermined. Therefore, the direct role of σ^w in membrane homeostasis may be a more general stress response as opposed to a specific reaction to changes in the biophysical properties of the membrane. *Streptococcus pneumoniae* uses the two component regulator WalRK (previously designated YycFG or VicRK) to increase the acyl chain length by upregulating *fabF* and downregulating *fabH* [341,342]. However, the WalRK mediated change in gene expression is modest (1.5–2x) and is not specific to *fabH* and *fabF* as all the pathway enzymes except *fabH*, *fabM* and *acpP* are upregulated. Regardless, activation of the WalRK two-component regulator causes an increase in 18-carbon fatty acid species compared to 16-carbon. It is not known whether WalRK is activated in response to increased membrane fluidity, but it does participate in a cell wall damage response pathway [342]. *Listeria monocytogenes* only expresses a single FabH isoform and lacks transcriptional control characterized in *B. subtilis* to regulate membrane fluidity. Instead, *L. monocytogenes* utilizes a FabH that displays an increased preference for *anteiso* precursors at low temperatures, increasing acyl chain branching and membrane fluidity. Other ECFs also regulate the membrane lipid composition in *B. subtilis*. σ^x senses membrane damage from antimicrobial peptides and responds by inducing the synthesis of the zwitterionic lipid PtdEtn, thereby decreasing the net anionic charge of the membrane [343]. Promoter regions of the *pss* and *psd* genes in *B. subtilis* also contain σ^x binding sites. The principal behind σ^x mediated resistance to antimicrobial peptides is similar to that of MprF present in several Gram-positive bacteria that synthesizes the positively charged Lys-PtdGro to electrostatically repel the peptides [244].

Transcriptional control by the ECF sigma factors is highly complex with many being allosterically regulated by anti-sigma factors, which in turn can be controlled by anti-anti sigma factors [344]. In addition to the autoregulation that occurs, cross regulation is also evident. An example is the σ^w promoter region that also contains a σ^a binding site. This paints a rather complex picture of the mechanisms that control each particular ECF sigma factor; however their roles in the transcription of several genes controlling phospholipid biosynthesis are evident through the genetic and physiologic studies.

5. The impact of bacterial lipid metabolism on translational medicine

5.1 Development and utility of FASII inhibitors

The emergence of multiple drug resistant bacteria has fuelled the development of drugs that target alternate macromolecular processes. The absolute growth requirement for membrane phospholipid synthesis has highlighted FASII as a potential target for development of new antimicrobial compounds. The role of the enoyl-ACP reductase, FabI, as the rate-limiting reaction of the elongation cycle has marked this enzyme as the first candidate for contemporary antimicrobial drug discovery. The anti-mycobacterial compound isoniazid is a front line treatment for latent tuberculosis, but it was almost four decades after its discovery that it was determined to be a specific inhibitor of the enoyl-ACP reductase (InhA) of *M. tuberculosis* [345]. Isoniazid is a prodrug and requires activation by the mycobacterial enzyme KatG for activity [346]. The dependence on KatG is basis for why isoniazid is a selective inhibitor of *M. tuberculosis* and has little inhibitory activity against many other pathogens. Another fatty acid synthesis inhibitor in wide use is the topical antibacterial agent, triclosan. Triclosan is an inhibitor of the enoyl-ACP reductase (FabI) enzyme in several Gram-positive and Gram-negative bacteria [347,348]. It is so effective that it has been included in an assortment of different household items and sanitizing products as a broad spectrum antimicrobial agent [349]. Despite the criticized overuse of triclosan, no

triclosan resistant bacteria have been reported in the environment [347,350,351]. Triclosan shows excellent topical efficacy against *E. coli* and *S. aureus*, but very few studies have been performed examining the efficacy of systemic delivery, likely due to the poor pharmacological properties of the hydrophobic molecule. Triclosan has a low LD₅₀ in rats when delivered intravenously (29 mg/kg), although when administered orally or subcutaneously it is much more tolerable (LD₅₀ of 4000 mg/kg and 14,700 mg/kg respectively). Triclosan administered to mice subcutaneously can reduce the severity of a pathogenic *E. coli* infection, although very few groups have investigated further the concept of using triclosan systemically due to its extremely high serum binding capacity (>99.5%) [350,352]. With the exception of triclosan as a topical antibacterial agent, no small molecules are in clinical use that target fatty acid synthesis in the dangerous pathogen methicillin-resistant *S. aureus* (MRSA). The increased prevalence of MRSA in healthcare institutions and more recently in individuals with no connection to healthcare facilities, has stressed the need for the development of new therapeutic compounds to counteract the increase in resistance [353]. The viability of FASII as an effective antimicrobial target has been validated in several different animal infection studies examining the efficacy of inhibitors of FabI, FabF and ACC against *S. aureus* infections [352,354–359]. Many of the compounds tested were natural products, which are hindered in their therapeutic value by poor pharmacokinetics and/or complex and laborious total syntheses. Unlike cell wall active antibiotics that result in cell lysis, like β -lactams and glycopeptides, FASII inhibitors are initially bacteriostatic [360,361]. Bactericidal antibiotics were historically considered superior to bacteriostatic antibiotics for treatment of many different bacterial infections, but more recent clinical data suggest this may not always be true [361]. Based on the success of triclosan and isoniazid, there are numerous drug discovery efforts to produce inhibitors of the enoyl-ACP reductase [359,362–366] (for review see [366]). Affinium Pharmaceuticals has developed AFN-1252, a small molecule inhibitor optimized against *S. aureus* FabI that is currently in clinical trials [359,362,363]. The targeted approach of developing the compounds to selectively target *Staphylococcal* FabI has resulted in an extremely potent compound with minimum inhibitory concentrations (MICs) of less than 8 ng/ml against a collection of multidrug-resistant *S. aureus* strains [65,362]. The successful use of AFN-1252 in the treatment of septicemia in a mouse infection model and the imminent clinical deployment of AFN-1252 represents an exciting frontier for the future treatment of MRSA infections by utilizing inhibitors of FASII.

5.2 Is FASII a viable target for therapeutics discovery?—The development of FASII inhibitors has not been without controversy. One group questioned the viability of FASII inhibitors to treat Gram-positive bacterial infections, on the basis that in vivo fatty acids for growth can be salvaged from the host serum [367]. In Gram-negative bacteria, the *de novo* FASII pathway is essential for generating 2-hydroxymyristic acid necessary for the synthesis of the Lipid A component of LPS [64]. Pathogenic *E. coli* and *P. aeruginosa* activate exogenous fatty acids through ligation to CoA, however they cannot enter the elongation cycle to introduce the hydroxyl group and they are not recognized by the ACP specific machinery of Lipid A biosynthesis. Gram-positive bacterial cell walls do not contain LPS, and require only small amounts of intermediates to synthesize cofactors such as lipoic acid and biotin, both which can potentially be obtained from the growth medium. Some bacteria, including *S. pneumoniae* lack enzymes for synthesizing lipoic acid and consequently completely rely on scavenging lipoic acid for growth from the host/growth medium [368,369]. Brinster *et al.* [367] disputed the suitability of inhibiting FASII in Gram-positive bacteria through studies using the Gram-positive opportunistic pathogen *Streptococcus agalactiae*. They reported that it is possible to delete each of the pathway enzymes during growth in media containing fatty acids or serum. In addition, treatment with the FabF inhibitor cerulenin had no effect if *S. agalactiae* is grown with fatty acids.

Balemans *et al.* [352] countered by reporting that FASII inhibitors are effective against *S. aureus* even in the presence of fatty acids or serum, which was criticized by Brinster *et al.* for not allowing *S. aureus* to adapt to utilizing the fatty acids [352,370]. However, Balemans *et al.* did confirm FASII inhibitors are ineffective at preventing growth of *S. agalactiae* in the presence of human serum. This vigorous debate identified a crucial lack of understanding in the dispensability of the FASII machinery in *Staphylococci* and *Streptococci*. The key to understanding the phenomenon was uncovered by Parsons *et al.* [65] who defined a crucial difference in the biochemical regulation of FASII by exogenous fatty acids between the two pathogens. During growth in media supplemented with oleate, *S. aureus* reduces the rate of fatty acid synthesis by approximately 50% as it incorporates oleate into its phospholipids. In contrast, during growth of *S. pneumoniae* in media containing fatty acids, complete repression of *de novo* fatty acid synthesis occurs as the over 95% of acyl chains in pneumococcal phospholipids were derived from the growth medium. It was revealed that exogenous fatty acids inhibit the activity of the ACC in *S. pneumoniae* but not *S. aureus*. A block at the FabI enzyme in *S. aureus* by AFN-1252 results in depletion of the ACP pool and an accumulation of short-chain acyl-ACP intermediates. The consequence of ACP depletion is an inability to activate exogenous fatty acids and synthesize new acyl chains. In *S. pneumoniae*, the repression of ACC activity prevents the fatal accumulation of acyl-ACPs in the elongation cycle and allows the unrestricted utilization of exogenous fatty acids for phospholipid biosynthesis. These data strongly suggest that FASII is an effective target in *S. aureus* and agree with the numerous reports demonstrating *in vivo* efficacy of several FASII inhibitors [352,354–359]. The reluctance of *S. aureus* to incorporate host fatty acids could reflect the differences in the fatty acid composition of *S. aureus* membrane phospholipids compared to human serum. *S. aureus* synthesizes primarily branched-chain fatty acids, unique to bacteria [65]. The fatty acid profile of *S. pneumoniae* is similar to the unsaturated/saturated fatty acid profile of human serum, implicating that it could conserve energy by salvaging fatty acids from the host, avoiding the energy intensive process of *de novo* synthesis [65,371]. In *S. aureus*, a membrane composed of solely host derived saturated and unsaturated fatty acids will likely drastically alter the biophysical properties of the membrane, resulting in potentially deleterious consequences for cell growth.

The critical differences in the regulation of FASII by exogenous fatty acids between *S. aureus* and *S. pneumoniae* highlight the dangers of extrapolating data with one species of bacteria to all others. Some basic experiments on the bench must be performed as to test the efficacy of a FASII inhibitor against a particular bacterium in the presence and absence of serum or fatty acids to answer the question. There is no dispute in the field regarding the essentiality of FASII in many Gram-negative bacteria and with the discovery of the metabolic basis of FASII inhibitor resistance in Gram-positive bacteria, there has been no additional debate interrogating the suitability of FASII as an antibiotic target in *S. aureus*. The importance of understanding the regulation of phospholipid synthesis in pathogens and the potential impact on human health must not be underestimated.

6. Perspectives

There is an incredible diversity in the structure, regulation and biosynthetic machinery of fatty acid synthesis in bacteria. The ability to alter the fatty acid composition depending on its environment is crucial for survival in the wide range of environmental conditions where bacteria thrive. *E. coli* was considered the paradigm for fatty acid synthesis for decades until key differences in the biosynthetic apparatus were discovered by genome sequencing. The more research that is performed on different bacteria, even within the Gram-positive and Gram-negative grouping, emphasizes how the regulation of each bacterium must be assessed on a species-specific basis. Fatty acid synthesis is an extremely energy intensive pathway and it is absolutely critical that the biophysical properties of the phospholipid membrane

maintain a constant fluidity based on the quantity and the fatty acid composition of the phospholipids produced. Different bacteria possess distinctive biochemical and transcriptional regulatory checkpoints to control the rate of fatty acid synthesis. The initiation, elongation and acyltransferase modules of FASII all work in harmony to ensure the correct quantity and structure of fatty acids are produced. The biochemical regulation that occurs is extremely finely balanced and aberrant gene expression of the pathway enzymes in *E. coli* can have a significant influence on the structure of the fatty acids produced. This emphasizes the need for stringent biochemical and transcriptional control, which sense not only the accumulation intermediates, but in the case of FabR and DesT, the composition of a pathway intermediate pool. An understanding of the biochemical regulation of FASII in Gram-positive pathogens has influenced and will continue to guide the progression of therapeutic compounds targeting FASII. There are still huge gaps in our understanding of FASII in many bacteria, even *E. coli*. How are fatty acids ligated to ACP in Gram-positive bacteria? It is known that *S. aureus* ligates exogenous fatty acids to ACP, but how? What further undiscovered biochemical regulatory mechanisms are there in *E. coli*, *S. aureus*, *B. subtilis*, etc? There are currently no X-ray crystal structures of any of the acyltransferase enzymes, therefore much is not known about the catalytic mechanisms. As more discoveries are made which help fill these gaps each year, the more we can appreciate just how perfectly evolution has designed bacterial fatty acid synthesis.

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Figure 1. The initiation module of type II fatty acid synthesis

The priming reaction for the elongation cycle is performed by the by the initiating condensing enzyme, FabH, which condenses an acyl-CoA with malonyl-ACP. In bacteria that produce straight chain fatty acids (SCFA), acetyl-CoA is employed. Bacteria producing branched-chain fatty acids (BCFA) utilize an amino acid derived branched chain acyl-CoA precursor. Malonyl-CoA is formed by acetyl-CoA carboxylase, which is composed of four different protein subunits encoded by separate genes (AccABCD). Malonyl-ACP is formed by the transacylase, FadD.

Figure 2. The elongation module

The enzymes of the elongation module work in concert to receive the β-ketoacyl-ACP and malonyl-ACP from initiation module and extend the fatty acid by two carbons with the completion of each cycle. There is a β-ketoacyl-ACP reductase (FabG) that appears to be present in all bacteria. FabZ is a universally-expressed dehydratase. FabA is always present with FabB and is also involved in unsaturated fatty acid synthesis (Fig. 3). There are multiple isoforms of enoyl-ACP reductase (FabI/K/L/V), and some organisms express more than one of these isoforms. FabF is the most-uniformly expressed elongation condensing enzyme. FabB is always found with FabA and has a special role in unsaturated fatty acid synthesis (Fig. 3). When the fatty acyl chain becomes long enough, the acyl-ACP can be utilized by the acyltransferase module (Fig. 4).

Figure 3. Branch points in unsaturated fatty acid synthesis

Two established mechanisms exist to produce unsaturated fatty acids. The *E. coli* system utilizes a bifunctional dehydratase/isomerase enzyme (FabA), which acts specifically on β -C10:0-ACP. The *trans*-2-C10:1-ACP product of the dehydratase reaction can either be reduced by FabI to continue through the saturated fatty acid pathway or undergo the FabA-dependent isomerization reaction to generate *cis*-3-C10:1-ACP. FabB is required to elongate this intermediate leading to an unsaturated fatty acid. In *S. pneumoniae*, there is a specific isomerase (FabM) that competes for substrate with the FabK enoyl-ACP reductase. A FabF isoform elongates unsaturated fatty acids in this organism. Other mechanisms exist, but the mechanistic understanding is limited (see section 2.2.3).

Figure 4. The acyltransferase module of bacterial lipid biosynthesis

Transfer of fatty acids to glycerol-3-phosphate is accomplished by two different systems in bacteria. (A) The PlsX/Y/C system is a widely-distributed pathway and predominates in Gram-positive organisms. PlsX produces acyl-PO₄ from the acyl-ACP end-products of FASII. PlsY is an acyl-PO₄-specific glycerol-phosphate acyltransferase that transfers the acyl chain to the 1-position. PlsC is an acyl-ACP specific 1-acyl-glycerol-phosphate acyltransferase. (B) The PlsB/C system is primarily associated with γ -proteobacteria. PlsB transfers an acyl chain from either acyl-ACP or acyl-CoA to glycerol-3-phosphate and PlsC transfers an acyl-chain from acyl-ACP/CoA to 1-acyl-glycerol-3-phosphate to generate phosphatidic acid.

Figure 5. Phospholipid headgroup synthesis

Phosphatidic acid is the precursor for glycerophospholipids in bacteria. Diacylglycerol is formed as a byproduct of membrane-derived oligosaccharides (MDO) synthesis (MdoB) in *E. coli* and lipoteichoic acid (LTA) synthesis (LtaS) in Gram-positive organisms. This intermediate is recycled to phosphatidic acid by an integral membrane kinase DgkA in Gram-negative bacteria or by a soluble, interfacial kinase, DgkB in Gram-positive bacteria. Glycolipids are produced from UDP-sugars and diacylglycerol (YfpP). Phosphatidic acid is converted to the key intermediate by CdsA, and the enzymes and pathways to the major bacterial phospholipids are illustrated. MprF is restricted to Gram-positive bacteria.

Table 1

Transcriptional regulation of bacterial lipid synthesis.

Transcription Factor	Organism	Activation	Repression	Regulator	Reference
FadR	<i>Escherichia</i> , <i>Salmonella</i> , <i>Vibrio</i> , <i>Shigella</i> , <i>Haemophilus</i> , <i>Klebsiella</i> , <i>Yersinia</i>	<i>fabA</i> , <i>fabB</i> , <i>iclR</i>	<i>fadI</i> , <i>fadD</i> , <i>fadBA</i> , <i>fadE</i> , <i>fadF</i> , <i>fadH</i> , <i>plsB</i>	Acyl-CoA	[280] 288,[304] [295]
FabR	<i>Escherichia</i> , <i>Salmonella</i> , <i>Vibrio</i> , <i>Shigella</i> , <i>Haemophilus</i>	None	<i>fabA</i> , <i>fabB</i>	Acyl-CoA and acyl-ACP	[312,372]
FapR	<i>Bacillus</i> , <i>Staphylococcus</i> , <i>Clostridium</i> , <i>Desulfotobacterium</i> , <i>Carboxidotherrmus</i> , <i>Clostridium</i>	None	<i>fabH</i> , <i>fabF</i> , <i>ydH</i> , <i>fapR</i> , <i>fabI</i> , <i>fabD</i> , <i>fabG</i> , <i>plsX</i>	Malonyl-CoA, Malonyl-ACP	[317,325]
FabT	<i>Streptococcus</i> , <i>Enterococcus</i> , <i>Lactococcus</i>	None	<i>fabT</i> , <i>fabH</i> , <i>fabK</i> , <i>fabD</i> , <i>fabZ</i> , <i>fabG</i> , <i>fabF</i> , <i>accABCD</i>	Acyl-ACP	[17,324]
DesT	<i>Pseudomonas</i>	None	<i>desCB</i> , <i>fabAB</i>	Acyl-CoA	[309,310]
DesR	<i>Bacillus</i>	None	<i>desA</i>		[328]