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CHAPTER 3

Fatty acid and phospholipid metabolism in prokaryotes

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1. Bacterial lipid metabolism

Bacteria are a versatile tool for the study of metabolic pathways. This is especially true for the Gram-negative *Escherichia coli*. These bacteria are easy to grow, and the growth conditions can be controlled and manipulated by the investigator. Most importantly, they are suitable for genetic manipulation and their genome sequence is available [1]. *E. coli* have been studied extensively, and the genes and enzymes of phospholipid metabolism were first delineated using this organism [2]. However, *E. coli* is not typical of all Gram-negative bacteria, let alone all eubacteria. Certain key differences between this model system and other organisms will be highlighted in the relevant sections.

Phospholipids in bacteria comprise about 10% of the dry weight of the cell, and each mole of lipid requires about 32 mole of ATP for its synthesis. Thus, phospholipid synthesis requires significant investment by the cell, and the advantages of maintaining fine control over the pathway are obvious. The pathway in most bacteria is catalyzed by a series of discrete proteins: the enzymes of fatty acid synthesis are cytosolic, while those of membrane lipid synthesis are mainly integral inner membrane proteins. The differences between the bacterial and mammalian enzymes offer attractive targets for novel antimicrobial drugs, and this has been a driving force behind much of the recent research.

The study of phospholipid enzymology in *E. coli* dates back to the early 1960s, when work in the laboratory of Vagelos discovered that the intermediates in fatty acid synthesis are bound to a heat stable cofactor termed acyl carrier protein (ACP) [3]. The enzymes of fatty acid synthesis are soluble proteins whose individual activities can be assayed in crude cell extracts or purified preparations. This is markedly different from the mammalian fatty acid synthase, a large multi-functional polypeptide with intermediates covalently attached (Chapter 6). Thus, the bacterial enzymes became a focus of intensive study, especially in the laboratories of Vagelos, Bloch and Wakil. Soon, the structures of all of the intermediates were known, and the basic chemical reactions required had been described [4]. During the late 1960s, work on the enzymes of phospholipid synthesis in bacteria flourished, and, mainly through classical identification experiments in the Kennedy laboratory, the intermediates in that pathway were established [5].

A second phase of bacterial phospholipid research, during the 1970s and early 1980s,

was the identification of mutants in the pathway [6]. The genetic manipulation of *E. coli* is relatively facile. Mutants in many specific enzymes were generated by employing mutagens in combination with a battery of clever selection and screening techniques [7]. Such mutations generally fall into one of two classes. Firstly, they may confer an auxotrophy on a strain, such as a requirement for unsaturated fatty acids or glycerol phosphate. Such mutants have generally lost the ability to produce a key biosynthetic enzyme (e.g. *fabA* mutants, which require supplementation with unsaturated fatty acids), or they may be more complex (e.g. *plsB* mutants require high glycerol phosphate concentrations due to a K_m defect in the enzyme and a second site mutation). Secondly, they may be conditionally defective, usually at elevated temperatures. For example, strains with the *fabI*(Ts) mutation grow at 30°C, but are not viable at 42°C. This type of defect is usually ascribed to an amino acid change in an essential enzyme that renders it unstable at higher temperatures. Techniques using the bacteriophage P1 are available for the movement of these alleles into other host strains, thus allowing for the mapping of the genes to specific regions of the chromosome, or the generation of strains with particular combinations of mutations. Regulatory mutants were identified, affecting multiple enzymes with a single mutation, that allowed for regulatory networks to be investigated. The membrane-bound enzymes of phospholipid synthesis were not amenable to analysis using standard biochemical approaches, and the genetic approach allowed these enzymes to be identified.

Next came the cloning and detailed study of the enzymes of lipid metabolism during the late 1980s and 1990s [2]. Plasmid-based expression systems were used to examine overexpression of enzymes on pathway regulation, and purified enzymes could be more easily obtained for biochemical analysis. With the availability of the sequence data generated by these clones, more precise methods for the construction of specific mutations also became available. Specific genes, or portions of genes, could be 'knocked-out' by targeted replacement based on sequence information, as opposed to random insertion of phage DNA. In the late 1990s, the genomic sequences of a broad spectrum of bacteria started to become available [8]. These data fuel the current phase of bacterial lipid metabolic research and comparative enzymology.

With the advent of genome information, many of the genes and gene products first identified and characterized in *E. coli* (Table 1) were identified in other bacteria, thus these genes can easily be cloned and their properties compared to those of *E. coli*. The pathways, especially fatty acid biosynthesis, are generally highly conserved amongst bacteria. Coupled with recent findings about the efficacy of certain inhibitors of the pathway, considerable attention is now being focused on fatty acid synthesis as a target for novel drug design [9]. The availability of pure enzymes has also stimulated progress in structural biology, and many new 3-dimensional structures of enzymes related to lipid metabolism have been recently solved.

2. *Membrane systems of bacteria*

Phospholipids in *E. coli* and other Gram-negative bacteria are used in the construction of the inner and outer membranes (Fig. 4 in Chapter 1). The inner membrane is im-

permeable to solutes unless specific transport systems are present. The outer membrane contains pores that allow the passage of molecules having a molecular weight less than 600, and is rich in structural lipoproteins and proteins involved in the transport of high molecular weight compounds. The outer layer of the outer membrane is composed primarily of lipopolysaccharides rather than phospholipid. Between the inner and outer membranes is an osmotically active compartment called the periplasmic space. Membrane-derived oligosaccharides, peptidoglycan, and binding proteins involved with metabolite transport are found in this compartment. Gram-positive bacteria do not possess an outer membrane. Instead, they have a membrane bilayer surrounded by a thick layer of peptidoglycan decorated with proteins, carbohydrates and, often, teichoic and lipoteichoic acid.

3. *Bacterial fatty acid biosynthesis*

3.1. Acyl carrier protein

A unique feature of fatty acid synthesis in bacteria is the presence of the small (8.86 kDa), acidic and highly soluble ACP, the product of the *acpP* gene. ACP is one of the most abundant proteins in *E. coli*, constituting about 0.25% of the total soluble protein ($\sim 6 \times 10^4$ molecules/cell). The acyl intermediates of fatty acid biosynthesis are bound to the protein through a thioester linkage to the terminal sulfhydryl of the 4′-phosphopantetheine prosthetic group. The prosthetic group sulfhydryl is the only thiol group of ACP and is attached to the protein via a phosphodiester linkage to Ser-36. ACP must interact specifically and transiently with all of the enzymes of fatty acid biosynthesis (except acetyl-CoA carboxylase), and does so through interactions with exposed negative residues on ACP with a patch of positive residues on the surfaces of the *fab* enzymes.

The ACP pool in normally growing cells is approximately one-eighth the coenzyme A (CoA) pool, the other acyl group carrier in cells. The prosthetic group of ACP is produced from CoA, and a common feature of both is the pantetheine arm for thioester formation. Virtually all of the ACP is maintained in the active, holo-form in vivo indicating that the supply of prosthetic group does not limit fatty acid biosynthesis. During logarithmic growth, a significant pool of ACP is unacylated. The ACP pool must be severely depleted before an effect on fatty acid and phospholipid synthesis can be detected. Overproduction of ACP generally yields high levels of apo-ACP, which is toxic to the cell by inhibition of the glycerol phosphate acyltransferase. The 4′-phosphopantetheine prosthetic group is transferred from CoA to apo-ACP by the 14-kDa monomeric [ACP]synthase. The [ACP]synthase from *Bacillus subtilis* has been crystallized in complex with ACP to give the first detailed look at ACP–protein interactions. ACP plays other roles in cell physiology, donating acyl chains to membrane-derived oligosaccharides, lipoic acid and quorum sensors.

Table 1
Genes of lipid metabolism

Gene	Protein
aas	2-acyl-GPE acyltransferase
accA	Carboxyltransferase subunit
accB	Biotin carboxy carrier protein
accC	Biotin carboxylase
accD	Carboxyl transferase subunit
acpP	Acyl carrier protein
acpD	Azoreductase
acpS	Acyl carrier protein synthase
cdh	CDP-diacylglycerol hydrolase
cdsA	CDP-diacylglycerol synthase
cdsS	Stabilizes mutant CDP-diacylglycerol synthase
cfa	Cyclopropane fatty acid synthase
cls	Cardiolipin synthase
desA	Desaturase
dgk	Diacylglycerol kinase
fabA	β-Hydroxydecanoyl-ACP dehydrase
fabB	β-Ketoacyl-ACP synthase I
fabD	Malonyl-CoA : ACP transacylase
fabF	β-Ketoacyl-ACP synthase II
fabG	β-Ketoacyl-ACP reductase
fabH	β-Ketoacyl-ACP-synthase III
fabI	Enoyl-ACP reductase I
fabK	Enoyl-ACP reductase II
fabL	Enoyl-ACP reductase III
fabZ	β-Hydroxyacyl-ACP dehydrase
fadA	β-Ketoacyl-CoA thiolase
fadB	4-Function enzyme of β-oxidation: β-hydroxyacyl-CoA dehydrogenase and epimerase; *cis*-β-*trans*-2-enoyl-CoA isomerase and enoyl-CoA hydratase
fadD	Acyl-CoA synthetase
fadE	Electron transferring flavoprotein
fadF	Acyl-CoA dehydrogenase
fadG	Acyl-CoA dehydrogenase?
fadH	2,4-dienoyl-CoA reductase
fadL	Long-chain fatty acid transport protein precursor
fadR	Transcriptional regulator
fatA	Unknown, possible transcription factor
gpsA	Glycerol phosphate synthase
htrB	KDO_2-lipid IV_A acyloxy lauroyltransferase
kdtA	KDO transferase
lpxA	UDP-GlcNAc β-hydroxymyristoyl-ACP acyltransferase
lpxB	Disaccharide-1-P synthase
lpxC	UDP-β-*O*-hydroxymyristoyl-GlcNAc deacetylase
lpxD	UDP-β-*O*-hydroxymyristoyl-GlcN *N*-acyltransferase
lpxK	Disaccharide-1-P 4′-kinase
lpxP	KDO_2-lipid IV_A acyloxy palmitoyltransferase
mdoB	Phosphatidylglycerol : membrane-oligosaccharide glycerophosphotransferase
msbA	Lipid flippase
msbB	KDO_2-lipid IV_A acyloxy myristoyltransferase

Table 1 (continued)

Gene	Protein
pgpA	PGP phosphatase
pgpB	PGP phosphatase
pgsA	PGP synthase
pldA	Detergent-resistant phospholipase A
pldB	Inner membrane lysophospholipase
plsB	Glycerol phosphate acyltransferase
plsC	1-Acylglycerol phosphate acyltransferase
plsX	Unknown
plsD	Glycerol phosphate acyltransferase
psd	PS decarboxylase
pssA	PS synthase
tesA	Thioesterase I
tesB	Thioesterase II

3.2. Acetyl-CoA carboxylase

Acetyl-CoA carboxylase catalyzes the first committed step of fatty acid synthesis, the conversion of acetyl-CoA to malonyl-CoA. Acetyl-CoA is a key intermediate in many pathways, and forms the majority of the CoA species within the cell at concentrations of about 0.5–1.0 mM during logarithmic growth on glucose [10]. Malonyl-CoA is normally present at 0.5% of this level, and is used exclusively for fatty acid biosynthesis. The overall carboxylation reaction is composed of two distinct half reactions: the ATP-dependent carboxylation of biotin with bicarbonate to form carboxybiotin; and transfer of the carboxyl group from carboxybiotin to acetyl-CoA, forming malonyl-CoA (Fig. 1).

Each acetyl-CoA carboxylase half reaction is catalyzed by a different protein subcomplex. The vitamin biotin is covalently coupled through an amide bond to a lysine residue on biotin carboxyl carrier protein (BCCP; a homodimer of 16.7-kDa

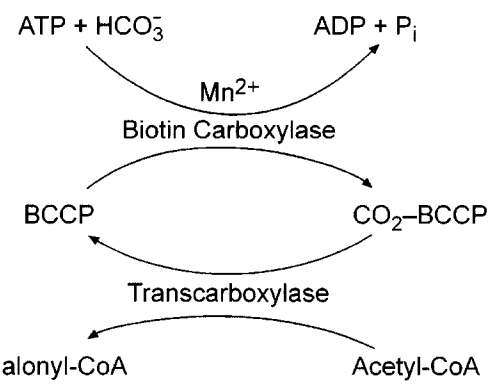

Fig. 1. The acetyl-CoA carboxylase reaction is performed in two steps. Biotin, covalently attached to BCCP (biotin carboxyl carrier protein, *accB*), is carboxylated by the carboxylase subunit (*accC*). The heterodimeric transcarboxylase (*accA* and *accD*) then transfers the CO_2 to acetyl-CoA, forming malonyl-CoA.

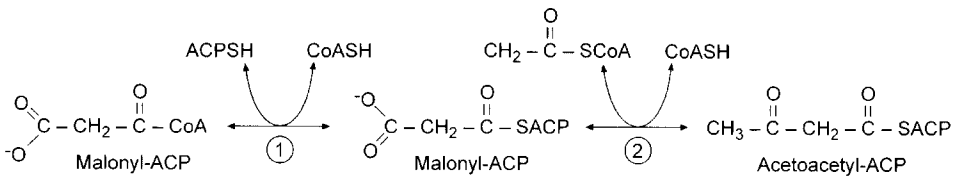

Fig. 2. Initiation of fatty acid synthesis. (1) Malonyl-CoA : ACP transacylase (FabD) transfers the malonyl group from CoA to ACP and then (2) β-ketoacyl-ACP synthase III (FabH) catalyzes the initial irreversible condensation of malonyl-ACP with acetyl-CoA to form acetoacetyl-ACP.

monomers encoded by *accB*) by a specific enzyme, biotin-apoprotein ligase (encoded by *birA*), and is essential to activity. The crystal and solution structures of the biotinyl domain of BCCP have been determined, and reveal a unique 'thumb' required for activity. Carboxylation of biotin is catalyzed by biotin carboxylase (encoded by *accC*), a homodimeric enzyme composed of 55-kDa subunits that is copurified complexed with BCCP. The *accB* and *accC* genes form an operon. The 3-dimensional structure of the biotin carboxylase subunit has been solved by X-ray diffraction revealing an 'ATP-grasp' motif for nucleotide binding. The mechanism of biotin carboxylation involves the reaction of ATP and CO_2 to form the short-lived carboxyphosphate, which then interacts with biotin on BCCP for CO_2 transfer to the 1′-nitrogen.

The carboxyltransferase enzyme that transfers the carboxy group from the biotin moiety of BCCP to acetyl-CoA is a heterotetramer composed of two copies of two dissimilar subunits, α (35 kDa) and β (33 kDa) (encoded by *accA* and *accD*, respectively). Sequence analysis suggests that the acetyl-CoA binding site lies within the AccA subunit. Strains with mutations in *accB* and *accD* have been obtained that are temperature sensitive for growth, indicating that this reaction is essential. It is thought that the enzyme present in vivo is composed of one copy of each subcomplex, with a combined molecular weight of 280 kDa.

3.3. Initiation of fatty acid biosynthesis

For the malonate group to be used for fatty acid synthesis, it must first be transferred from malonyl-CoA to malonyl-ACP by the 32.4-kDa monomeric malonyl-CoA : ACP transacylase, the product of the *fabD* gene (Fig. 2). A stable malonyl-serine enzyme intermediate is formed during the course of the FabD reaction, and subsequent nucleophilic attack on this ester by the sulfhydryl of ACP yields malonyl-ACP. The high reactivity of the serine in malonyl-ACP transacylase is due to the active site being composed of a nucleophilic elbow as observed in α/β hydrolases. The serine is hydrogen bonded to His-201 in a fashion similar to serine hydrolases.

The last two carbons of the fatty acid chain (i.e. those most distal from the carboxylate group) are actually the first introduced into the nascent chain, and acetyl-CoA can be thought of as the 'primer' molecule of fatty acid synthesis in *E. coli*. The initial condensation reaction, catalyzed by β-ketoacyl-ACP synthase III (FabH), utilizes acetyl-CoA and malonyl-ACP to form the four carbon acetoacetyl-ACP with concomitant loss of CO_2 (Fig. 2). FabH also possesses acetyl-CoA : ACP transacylase

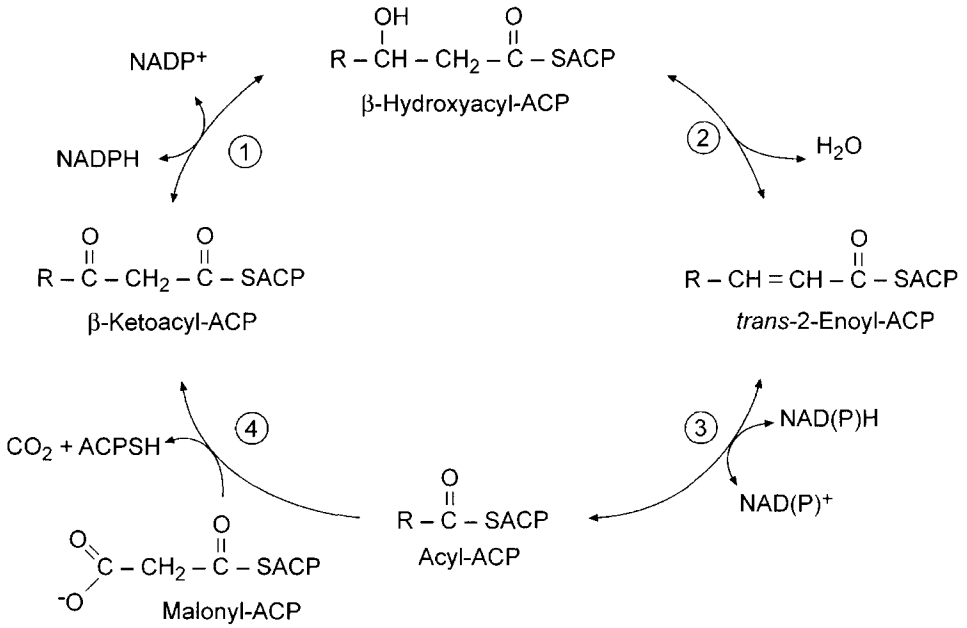

Fig. 3. Cycles of fatty acyl chain elongation. All intermediates in fatty acid synthesis are shuttled through the cytosol as thioesters of the acyl carrier protein (ACP). (1) β-Ketoacyl-ACP reductase (FabG). (2) β-Hydroxyacyl-ACP dehydrase (FabA or FabZ). (3) *trans*-2-Enoyl-ACP reductase I (FabI). (4) β-Ketoacyl-ACP synthase I or II (FabB or FabF).

activity, and for many years it was thought that acetyl-ACP was the actual primer. However, acetyl-ACP appears to be a product of a side reaction, and plays no direct role in fatty acid synthesis.

3.4. Elongation of acyl chains

Four enzymes participate in each iterative cycle of chain elongation (Fig. 3). First, β-ketoacyl-ACP synthase I or II (*fabB* or *fabF*) adds a two-carbon unit from malonyl-ACP to the growing acyl-ACP. The resulting ketoester is reduced by a NADPH-dependent β-ketoacyl-ACP reductase (*fabG*), and a water molecule is then removed by a β-hydroxyacyl-ACP dehydrase (*fabA* or *fabZ*). The last step is catalyzed by enoyl-ACP reductase (*fabI*) to form a saturated acyl-ACP, which in turn serves as the substrate for another condensation reaction.

3.4.1. The β-ketoacyl-ACP synthases

Three *E. coli* enzymes catalyze the Claisen condensation that is the β-ketoacyl-ACP synthase reaction. These enzymes are the products of the *fabB*, *fabF*, and *fabH* genes. β-Ketoacyl-ACP synthase I, or FabB, is composed of two identical 42.6-kDa subunits, and has both malonyl-ACP and fatty acyl-ACP binding sites. In the condensation reaction, the acyl group is covalently linked to the active site cysteine. The acyl-enzyme undergoes condensation with malonyl-ACP to form β-ketoacyl-ACP, CO_2, and free enzyme.

Overproduction of FabB has two effects: an increased amount of *cis*-vaccenic acid in phospholipids; and resistance to the antibiotics thiolactomycin and cerulenin (Section 12.2). β-Ketoacyl-ACP synthase II (FabF) is very similar to FabB (38% identical at the amino acid level). Like FabB, FabF has a dimeric structure of 43-kDa subunits, and is inhibited by cerulenin and thiolactomycin. FabF is not essential to growth in *E. coli*, but is essential for the regulation of fatty acid composition in response to temperature fluctuations. Mutants lacking FabF activity, unlike wild-type *E. coli*, do not produce increased amounts of the long, unsaturated fatty acid *cis*-vaccenate at lower temperatures. β-Ketoacyl-ACP synthase III (FabH) is a dimeric protein of identical 33.5-kDa subunits first detected as a condensation activity resistant to cerulenin. The FabH reaction is characterized by the preference for a CoA-linked primer, rather than acyl-ACP.

The crystal structures of all three synthases have been determined, and all share a common thiolase fold. Structures of FabB and FabF are virtually identical. Both utilize a conserved catalytic triad of Cys–His–His. FabH has a much more closed active site, reflecting its use of less hydrophobic, shorter chain, substrates than FabB and FabF. FabH also differs in that it contains a catalytic triad composed of Cys–His–Asn. This difference renders FabH more resistant to thiolactomycin and cerulenin than FabB or FabF. The exact reason for this discrepancy is not clear at this time, since the key nitrogens of the His or Asn residues of the respective synthases occupy equivalent space in the respective structures.

3.4.2. β-Ketoacyl-ACP reductase

The β-ketoacyl-ACP reductase gene (*fabG*) is located within the *fab* gene cluster between the *fabD* and *acpP* genes and is cotranscribed with *acpP*. Insertional mutants that prevent *fabG* transcription while allowing ACP to be produced were generated in Cronan's laboratory and suggest that *fabG*-encoded reductase activity is essential in *E. coli*. The *fabG*-encoded NADPH-specific β-ketoacyl-ACP reductase is a homotetrameric protein of 25.6-kDa monomers. The protein functions with all chain lengths in vitro and exhibits cooperative binding of NADPH. A dramatic conformational change occurs on cofactor binding, as evidenced by the crystal structures of the free and NADPH-bound protein.

3.4.3. β-Hydroxyacyl-ACP dehydrase

E. coli possesses two β-hydroxyacyl-ACP dehydrases (more properly termed dehydratases). One is encoded by *fabZ*, and is active on all chain lengths of saturated and unsaturated intermediates. This enzyme is distinct from the dual-function β-hydroxydecanoyl-ACP dehydrase/isomerase (encoded by *fabA*) first described by Bloch and coworkers. The FabA enzyme dehydrates saturated, but not unsaturated, fatty acid intermediates and catalyzes a key isomerization reaction at the point where the biosynthesis of unsaturated fatty acids diverges from saturated fatty acids (Fig. 4). Both enzymes share weak overall homology (28% identity and 50% similarity at the amino acid level). The mono-functional FabZ protein (17 kDa) is somewhat smaller than the *fabA*-encoded bifunctional enzyme (19 kDa). The FabA dehydrase/isomerase has been crystallized, and its structure solved. The active site His is located in a long tunnel which acts a molecular ruler to ensure that only 10-carbon intermediates are isomerized.

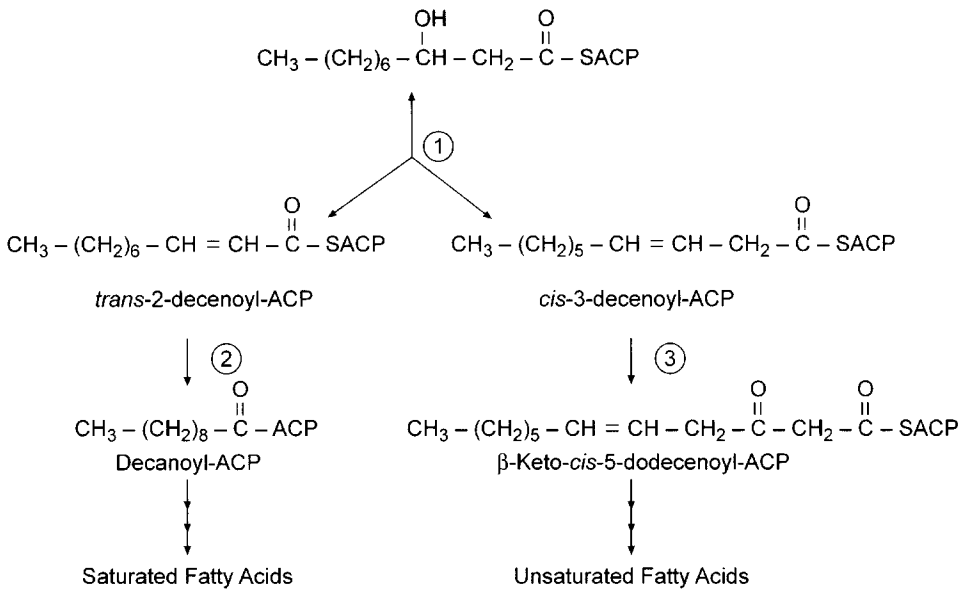

Fig. 4. Branch-point in unsaturated fatty acid synthesis. (1) FabA catalyzes the interconversion of β-hydroxydecanoyl-ACP, *trans*-2-decenoyl-ACP and *cis*-3-decenoyl-ACP. (2) *trans*-2-Decenoyl-ACP is a substrate for enoyl-ACP reductase (FabI), while (3) *cis*-3-decenoyl-ACP is elongated by β-ketoacyl-ACP synthase I (FabB). Competition between FabI and FabB is partly responsible for the ratio of unsaturated fatty acids made.

3.4.4. Enoyl-ACP reductase

The final step in each round of fatty acyl elongation in *E. coli* is the NADH-dependent reduction of the *trans* double bond, catalyzed by the homotetrameric (subunit mass of 29 kDa) NADH-dependent enoyl-ACP reductase I (encoded by *fabI*). The FabI amino acid sequence is similar (34% identical) to the product of a gene (called *inhA*) from Mycobacteria. InhA is involved in mycolic acid biosynthesis. The synthesis of these unusual 70–80 carbon mycobacterial acids requires a pathway composed of enzymes essentially identical to those of fatty acid synthesis. Missense mutations within the *inhA* gene result in resistance to the anti-tuberculosis drugs, isoniazid and ethionamide. The crystal structures of FabI and InhA have been solved, and are virtually superimposable for most of the protein. FabI has a flexible substrate binding loop that becomes ordered on binding of the specific inhibitors, diazaborine or triclosan (and presumably substrate), while InhA has two short helices in this region that move 4 Å on drug or substrate binding. Novel enoyl-ACP reductase isoforms II (FabK) and III (FabL) have been discovered recently in Gram-positive bacteria by bioinformatics (Section 11).

3.5. Synthesis of unsaturated fatty acids

The pathway described above suffices to produce the straight chain saturated fatty acids found in the membrane phospholipids, mainly palmitic acid (16 : 0) in *E. coli*.

Gram-negative bacteria also contain unsaturated fatty acids, and the ratio of saturated : unsaturated fatty acids in the membrane phospholipids is a key determinant in membrane fluidity, and changes according to temperature [11]. The *fabA*-encoded dehydrase/isomerase (Section 3.4.3) catalyzes a vital reaction at the branch point of the two pathways: the isomerization of *trans*-2-decenoyl-ACP to *cis*-β-decenoyl-ACP (Fig. 4). The *cis*-3 compound is not a substrate for the enoyl-ACP reductase, but instead is rapidly condensed in a reaction requiring FabB, but not FabF. Strains with mutations in either *fabA* or *fabB* require supplementation with unsaturated fatty acids for growth, showing the specific requirement for both enzymes. As their names suggest, these were the first two mutants in fatty acid biosynthesis identified by classical genetic techniques [12,13].

Both FabB and FabF are capable of participating in saturated and unsaturated fatty acid synthesis and the enzymes have been shown, in vitro, to function similarly with all long-chain acyl-ACPs except palmitoleoyl-ACP. Palmitoleoyl-ACP is an excellent substrate for FabF, but not for FabB. In vivo, the reactivity of FabF towards this substrate increases at lower temperatures, leading to increased amounts of the more fluid *cis*-vaccenate (18 : 1Δ11) in membrane phospholipids.

3.6. Afterword: dissociable or dissociated enzymes?

Historically, scientists in the field have debated whether the enzymes of bacterial fatty acid synthase system are 'dissociable' or 'dissociated'. This seemingly minor semantic distinction has larger ramifications for the in vivo physiology of the cell. The implication of the use of the word *dissociable* is that the enzymes form a complex in vivo, and only became separated (or dissociated) on cell disruption; whereas in a *dissociated* system, the enzymes do not form a complex in vivo. The concept of a large complex mimicking the multifunctional type I enzyme would support the notion of substrate channeling between the active sites, increasing the catalytic efficiency of the pathway as a whole. However, there are no data to support the existence of either a large complex or substrate channeling. A minimal fatty acid synthetic unit must consist of at least six separate activities, encoded by the *acpP*, *fabD*, *fabH*, *fabG*, *fabI*, *fabZ* and *fabB* genes. In vitro, these enzymes appear as monomers (ACP, FadD), dimers (FabH, FabZ and FabB) or tetramers (FabG and FabI). Thus, a complex of these proteins in vivo would possess 16 subunits (assuming only one of each individual complex was present) with a combined mass of over 440 kDa. With our current knowledge of the 3-dimensional structures of these proteins (only FabZ has not been solved to date), it is hard to envision how the pieces of such a puzzle would fit together. Many of the enzymes have active sites located in narrow tunnels, leaving no opportunity for the prosthetic group to swing between them. The acyl-ACP intermediate must instead completely dissociate from the enzyme to interact with the next enzyme in the pathway. Studies with the yeast two-hybrid system have also failed to detect interactions between different enzymes. Thus, type II fatty acids synthases should be considered dissociated and not dissociable.

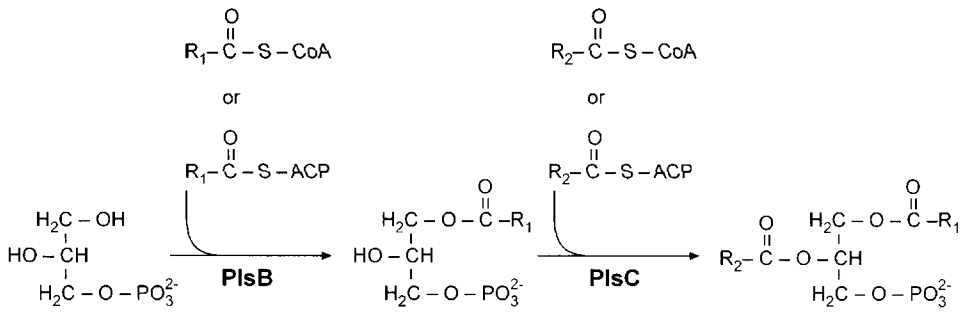

Fig. 5. Transfer of fatty acyl groups to the membrane. A (primarily saturated) fatty acid is transferred from acyl-ACP or acyl-CoA to the 1 position of *sn*-glycerol-3-phosphate by glycerol phosphate acyltransferase (PlsB). A second fatty acid is transferred to the 2-position by the 1-acylglycerol phosphate acyltransferase (PlsC).

4. Transfer to the membrane

Fatty acid biosynthesis in *E. coli* normally ends when the acyl chain is 16 or 18 carbons in length. These acyl-ACPs are now substrates for the acyltransferases that will transfer the fatty acyl chain into the membrane phospholipids (Fig. 5). Alternatively, *E. coli* can incorporate exogenous fatty acids, following esterification to CoA. The first enzyme (the *plsB* gene product) transfers fatty acids from either the soluble acyl-ACP or acyl-CoA to the 1-position of glycerol phosphate. The product of the reaction, 1-acylglycerol phosphate, partitions into the membrane. The PlsB protein is an integral inner membrane protein of 91 kDa, and has a preference for saturated fatty acids. The second acyltransferase (the *plsC* gene product), a membrane protein of 27 kDa, esterifies the 2-position of the glycerol backbone and prefers unsaturated acyl chains. Thus, bacterial phospholipids have an asymmetric distribution of fatty acids between the 1- and 2-positions of the glycerol phosphate backbone. The glycerol phosphate acyltransferase system influences the chain length of the fatty acids incorporated into the phospholipids by competition with the elongation condensing enzymes for acyl-ACP, and the rate of fatty acid biosynthesis via modulation of its activity by ppGpp (Section 10.5).

The isolation of *E. coli* mutants with defective acyltransferase activity (*plsB*) by Bell's laboratory heralded a major advance in the study of the acyltransferases. These mutants were glycerol phosphate auxotrophs and exhibited an increased Michaelis constant for glycerol phosphate in in vitro acyltransferase assays. The increased K_m was subsequently shown to arise from a single missense mutation in the open reading frame. Therefore, *plsB* mutants require an artificially high intracellular concentration of glycerol phosphate for activity. Complementation of these mutants facilitated the cloning of the glycerol phosphate acyltransferase. Plasmids that suppressed the glycerol phosphate requirement of *plsB* strains overexpressed glycerol phosphate acyltransferase activity 10-fold. The protein possesses a catalytic His–Asp dyad common to all glycerolipid acyltransferases. The glycerol phosphate acyltransferase is specifically activated by acidic phospholipids, phosphatidylglycerol and cardiolipin, as shown by

micelle assays containing detergents and phospholipid, and is active as a monomer. Glycerol phosphate acyltransferase also exhibits negative cooperativity with respect to glycerol phosphate binding, a property that may account in part for the finding that dramatic increases in the intracellular glycerol phosphate concentration do not increase the amount of phospholipid in *E. coli*.

The interpretation of the *plsB* mutants is complicated by the finding that the *plsB* growth phenotype depends on mutations in two unlinked genes. One mutation is in the *plsB* gene discussed above, and the second is in a gene called *plsX*. Both mutations are required for a strain to exhibit a requirement for glycerol phosphate since strains harboring either the *plsB* or *plsX* lesion do not have a defective growth phenotype. The *plsX* gene is located in the *fab* cluster next to *fabH* and predicted to encode a protein of 37.1 kDa. Despite extensive study, no enzymatic activity has been described for the PlsX protein. It is possible that the native PlsB protein exists in a complex with PlsX. Analysis of the genomic databases reveals many bacteria contain *plsX* homologues, but provides no further clues as to its biochemical function.

The next step in phospholipid biosynthesis is catalyzed by 1-acylglycerol phosphate acyltransferase (the *plsC* gene product) which acylates the product of the PlsB step to form phosphatidic acid (Fig. 5). Phosphatidic acid comprises only about 0.1% of the total phospholipid in *E. coli* and turns over rapidly, a property consistent with its role as an intermediate in phospholipid synthesis. The 1-acyl-glycerol phosphate acyltransferase is thought to transfer unsaturated fatty acids selectively to the 2-position.

Certain bacteria, such as Clostridia, do not possess a *plsB* homologue. Instead, the *plsD* gene was isolated from a *Clostridium difficile* genomic library by functional complementation of the *plsB* phenotype of *E. coli*. The protein encoded by *plsD* is a glycerol phosphate acyltransferase, but shares no homology with the *plsB*-encoded enzyme, except for a predicted His–Asp dyad active site. Overall, it appears more similar to the *plsC*-encoded 1-acylglycerol phosphate acyltransferase of *E. coli* in both amino acid composition (27% identity) and size (26.5 kDa). However, there are many bacterial species that lack both *plsB* and *plsD* genes, thus the enzyme(s) that acylates glycerol phosphate in these organisms remains unknown.

5. *Phospholipid biosynthesis*

E. coli possesses only three major phospholipid species in its membranes, making it one of the simplest organisms to study with regard to phospholipid biosynthesis. Phosphatidylethanolamine (PE) comprises the bulk of the phospholipids (75%), with phosphatidylglycerol (PG) and cardiolipin (CL) forming the remainder (15–20% and 5–10%, respectively). The scheme for the synthesis of membrane phospholipids follows the classic Kennedy pathway (Fig. 6).

5.1. Phosphatidate cytidylyltransferase

The key activated intermediate in bacterial phospholipid synthesis, CDP-diacylglycerol, comprises only 0.05% of the total phospholipid pool. The 27.6-kDa enzyme phosphati-

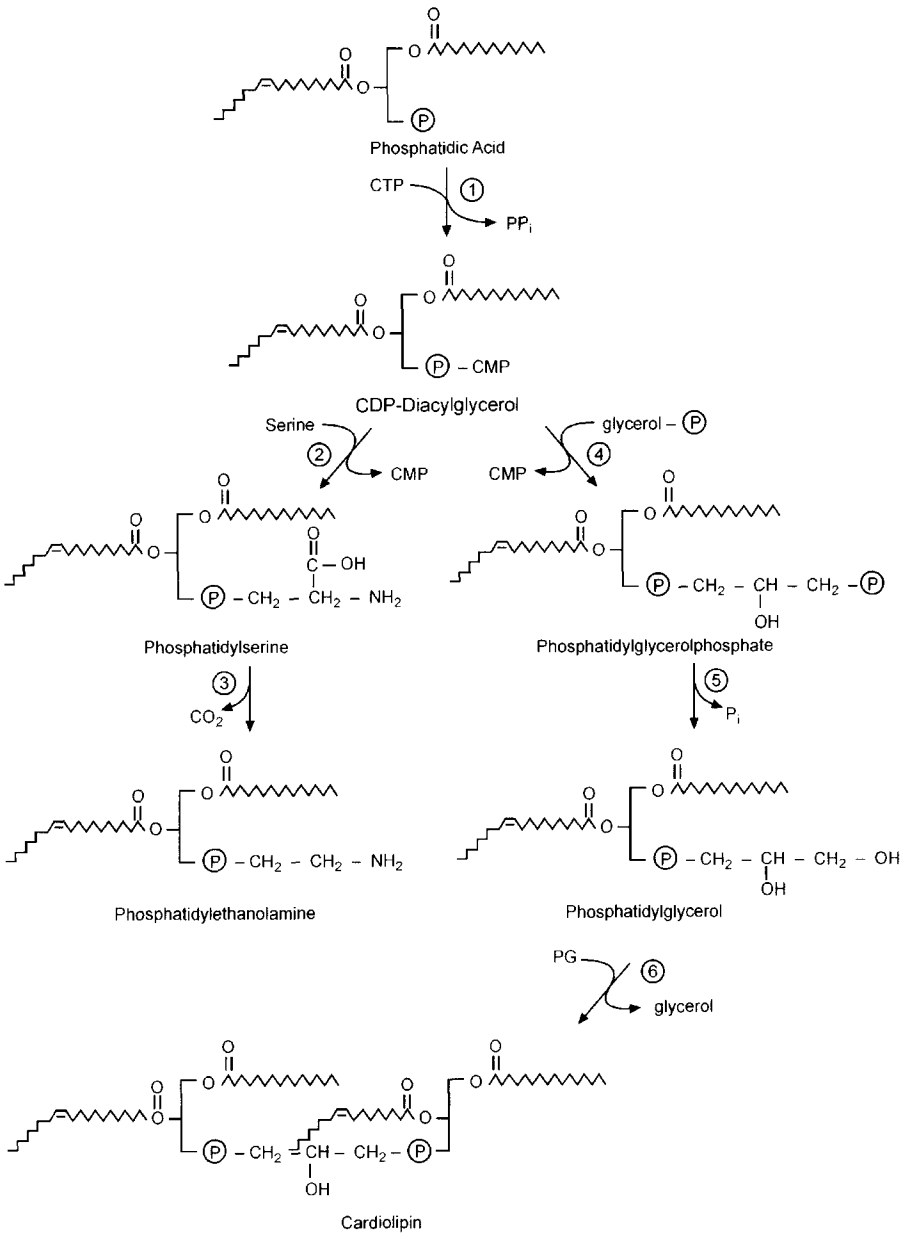

Fig. 6. Synthesis of phospholipid polar headgroups. The three major phospholipid species in *E. coli* are synthesized by a total of six different enzymatic activities: (1) phosphatidate cytidylyltransferase (Cds); (2) phosphatidylserine synthase (Pss); (3) phosphatidylserine decarboxylase (Psd); (4) phosphatidylglycerolphosphate synthase (PgsA); (5) phosphatidylglycerolphosphate phosphatase (PgpA or PgpB); and (6) cardiolipin synthase (Cls).

date cytidylyltransferase (or CDP-diacylglycerol synthase) catalyzes the conversion of phosphatidic acid to a mixture of CDP-diacylglycerol and dCDP-diacylglycerol. Strains of *E. coli* with mutations in the *cds* gene retain 5% of the normal levels of CDP-diacylglycerol synthase and grow normally under standard laboratory conditions, although are hypersensitive to erythromycin and elevated pH. Thus, CDP-diacylglycerol synthase is present in large excess of the minimum amount of enzyme required to sustain phospholipid synthesis. These mutants accumulate substantial amounts of phosphatidic acid (up to 5% of the total phospholipid). Null mutations in *cds* have not been reported, and would presumably be non-viable due to the complete lack of phospholipid synthesis from this point.

CDP-diacylglycerol stands at the branch point between PE synthesis and PG and CL synthesis (Fig. 6). It has been hypothesized that the presence of both ribo and deoxyribo forms of the liponucleotide could play a role in determining the relative amount of intermediate entering these two arms of the pathway. For this to be true, the respective synthases that utilize this compound would have to be selective toward either dCDP- or CDP-diacylglycerol. In vivo the ratio of dCDP- to CDP-diacylglycerol is 0.88. A change in this ratio to 3.1 has no effect on the relative rates of PE and PG synthesis in vivo, arguing against selectivity of the subsequent enzymes for one form of the other. Further, both ribo- and deoxyribo-liponucleotides are substrates for PS synthase in vitro, and thus, the significance, if any, of the two forms of liponucleotide remains to be determined.

5.2. Phosphatidylethanolamine production

5.2.1. Phosphatidylserine synthase

The first step in the synthesis of PE is the condensation of CDP-diacylglycerol with serine catalyzed by PS synthase to form PS. During cell disruption, the 58-kDa PS synthase appears associated with ribosomes, but reattaches to the membrane vesicles once substrate is added. PS is a minor membrane constituent of *E. coli* since it is rapidly converted to PE by PS decarboxylase. Mutants in the *pss* gene encoding PS synthase are viable only when supplemented with divalent metal ions. PE is capable of forming the hexagonal (non-bilayer) H_{II} lipid phase, and Dowhan has demonstrated that the divalent cations interact with CL to replace the function of PE in the formation of an H_{II} phase (Chapter 1). The cells lack PS synthase activity, and thus contain no PS or PE in their membranes. There are also perturbations in the function of permeases, electron transport, motility and chemotaxis.

5.2.2. Phosphatidylserine decarboxylase

PS is decarboxylated by PS decarboxylase to yield the zwitterionic PE. This inner membrane enzyme has a subunit molecular mass of 36 kDa. PS decarboxylase has a pyruvate prosthetic group that participates in the reaction by forming a Schiff base with PS. Overproduction of the enzyme 30–50-fold by plasmid-borne copies of the *psd* gene has no effect on membrane phospholipid composition indicating that the level of this enzyme does not regulate the amount of PE in the membrane. The majority of the PE is found in the periplasmic leaflet of the inner membrane, and there is a rapid flipping from the inner to outer leaflet by the MsbA lipid flippase (Section 7).

Mutants with a temperature-sensitive decarboxylase accumulate PS at the non-permissive temperature. The mutants continue to grow for several hours after the shift to the non-permissive temperature, despite the reduced levels of PE and the concomitant increase in PS. Complete inactivation of *psd* by insertional mutagenesis has the same divalent cation-requiring phenotype as the *pss* mutants described above. The requirement for CL is consistent with the inability to introduce a null *cls* allele into *pss* strains. Thus, PE is essential for the polymorphic regulation of lipid structure. Evidence from the Dowhan laboratory demonstrates that PE is a molecular chaperone that is essential for the proper folding of integral membrane proteins (Chapter 1). All of the physiological processes dependent on the formation of local regions of non-bilayer structure or that specifically require PE remain to be elucidated, but the process of cell division, the formation of contacts between inner and outer membranes, and the translocation of molecules across the membrane are viable candidates.

5.3. Phosphatidylglycerol synthesis

5.3.1. Phosphatidylglycerolphosphate synthase

CDP-diacylglycerol is condensed with glycerol phosphate to form phosphatidylglycerolphosphate (PGP), an intermediate in the production of the acidic phospholipids PG and CL (Fig. 6). The reaction is analogous to the synthesis of PS, with the product CMP being released. Mutants (*pgsA*) defective in PGP synthesis contain less than 5% of normal PGP synthase activity in vitro, however, there is no growth phenotype associated with these mutants. The PgsA protein is predicted to be a 20.7-kDa integral membrane protein. It has long been thought that PGP synthase is essential, and that cells cannot survive without acidic phospholipids. There are many important cellular functions that are affected by reduced PG and/or CL content of the membrane. PG is required for protein translocation across the membrane, and acidic phospholipids are required for channel activity of bacterial colicins and the interaction of antibiotics with the membrane. Cell division proteins such as FtsY also apparently require acidic phospholipids for activity, as does the DnaA protein involved in chromosome segregation. However, Matsumoto has recently inactivated the *pgsA* gene with a kanamycin cassette. This *pgsA*::*kan* strain has no detectable PG or CL, is not viable above 40°C, and contains increased concentrations of PA, which may at least partially compensate for the absence of PG and CL.

5.3.2. PGP phosphatases

The second step in the synthesis of PG is the dephosphorylation of PGP (Fig. 6). Two independent genes have been identified, *pgpA* and *pgpB*, that encode PGP phosphatases based on an in vitro assay. Both proteins are small (19.4 and 29 kDa, respectively) but share no sequence homology. In vitro, the *pgpA*-encoded phosphatase specifically hydrolyzes PGP, whereas the PgpB phosphatase also hydrolyzes phosphatidic acid. However, disruption of both of these genes in a single strain did not impair PG synthesis, although the respective phosphatase activities were reduced. Thus, neither of these phosphatases is required for PG synthesis suggesting that another phosphatase capable of operating in the PG biosynthetic pathway remains to be discovered.

5.4. Cardiolipin biosynthesis

Unlike in mammalian mitochondria, where CL is synthesized by the reaction of CDP-diacylglycerol with PG (Chapter 8), CL is produced in bacteria by the condensation of two PG molecules (Fig. 6). CL accumulates as the cells enter the stationary phase of growth, and is required for prolonged survival of the bacteria. CL synthase is post-translationally processed from a 55-kDa precursor to a 45–46-kDa form. Mutants deficient in CL synthase (*cls*) possess very low levels of CL and lose viability in stationary phase. The mutants also grow at a slower rate and to a lower density than the corresponding wild-type cells. Low, residual concentrations of CL are present in the *cls* null mutants, hampering efforts to isolate the role of this lipid in cell physiology. A second gene has recently been described that can catalyze the formation of CL in vitro, but does not appear to do so in vivo. Amplification of CL synthase leads to the overproduction of CL, a decrease in membrane potential, and loss of viability. Therefore, *E. coli* can tolerate changes in the overall CL content but the elimination or significant overproduction of CL leads to significant physiological imbalance.

5.5. Cyclopropane fatty acids

Fatty acids attached to membrane phospholipids can be post-synthetically converted to their cyclopropane derivatives during the stationary phase of bacterial growth. Their biosynthesis and function have been elucidated by the Cronan laboratory. *E. coli* mutants that completely lack cyclopropane fatty acid synthase activity (owing to null mutations in the *cfa* gene) grow and survive normally under virtually all conditions, except that *cfa* mutant strains are more sensitive to freeze–thaw treatment and acid shock than are isogenic cfa^+ strains. Thus, the stable cyclopropane derivative protects the reactive double bond from adverse reactions during stationary phase. Cyclopropanation involves a significant energy commitment by the cell: the reaction uses *S*-adenosylmethionine, which requires three molecules of ATP are required for regeneration. It is not known how the soluble enzyme and substrate gain access to the phospholipids of the inner and outer membranes.

The 44-kDa cylcopropane synthase protein is metabolically unstable, but protein levels peak sharply due to increased *cfa* transcription as cultures enter the stationary phase. Cfa levels drop in late stationary phase cultures as the enzyme is destroyed by proteolysis, probably by a protease of the heat shock response. The *cfa* gene possesses two promoters of approximately equal strengths, with the more distal promoter functioning throughout the growth cycle. The proximal promoter requires the specialized sigma factor, σ^S (encoded by *rpoS*), for transcription, and is thus active only as cultures enter stationary phase. Indeed, the cyclopropane fatty acid content of *rpoS* strains is low and transcription from the proximal promoter is absent in these strains. As cells remain in stationary phase and phospholipid biosynthesis ceases, the low levels of Cfa that do persist no longer encounters an expanding substrate pool. Thus, an increasing amount of the fatty acyl chains are converted to their cyclopropane derivatives over time. The instability of Cfa results in little carry-over of synthetic capacity when exponential growth resumes, and the existing cyclopropane fatty acids are quickly diluted by de novo phospholipid synthesis.

6. *Lipid A biosynthesis*

Lipopolysaccharides (LPS) form the majority of the outermost leaflet of the membrane in most Gram-negative bacteria (Fig. 4 in Chapter 1), and display a tremendous amount of structural variability [14]. LPS is essential to the growth of Gram-negative bacteria, and provides an effective hydrophobic barrier to toxic compounds. LPS are comprised of three components: the O-antigen, a core polysaccharide and lipid A. The O-antigen is a polysaccharide that extends from the cell surface. O-antigens are constructed from 10 to 30 repeats of specific β-6 sugar oligosaccharide units, and each is essentially unique to a given serotype of bacteria. The O-antigen is linked to the core polysaccharide region, which is common to groups of bacteria. The membrane associated portion of LPS is lipid A. The core polysaccharide is attached to lipid A by a 2-keto-β-deoxyoctonate (KDO) disaccharide. Lipid A anchors the LPS to the outer membrane and functions as an endotoxin and a mitogen during bacterial infections. The lipid A is synthesized and ligated to the oligosaccharide core on the cytoplasmic face of the inner membrane, while the O-antigen is added in the periplasm. O-antigen is not essential for the viability of *E. coli*, and is in fact missing from *E. coli* K12, making it safe for laboratory use. Details of the synthesis of the O-antigen and core region are outside the scope of this discussion and can be found in a review by Raetz [14].

The pathway of lipid A synthesis has been determined mainly in the laboratory of Raetz (Fig. 7). The first step in lipid A synthesis is the reversible transfer of the β-hydroxymyristoyl group from ACP to UDP-*N*-acetylglucosamine (UDP-GlcNAc) by the UDP-GlcNAc acyltransferase (*lpxA*). Competition between LpxA and the FabZ dehydratase of fatty acid synthesis help determine the rate of lipid A synthesis. The second step in the pathway is the deacetylization of UDP-3-Acyl-GlcNAc by the zinc-dependent 34-kDa UDP-β-*O*-acyl-GlcNAc deacetylase (*lpxC*). The *lpxC* gene was first described as *envA*, an essential gene involved in envelope production. Mutations in *lpxC* cause a plethora of effects, including increased sensitivity to antibiotics, increased dye permeability and defects in cell division. Null mutations in *lpxC* are lethal. The LpxA-catalyzed acyltransfer step is thermodynamically unfavorable, thus the irreversible deacetylase reaction is the first committed step in the pathway. The essential nature of LpxC has driven the development of a novel group of antimicrobial compounds active against this step (Section 12). These compounds are effective against a wide range of Gram-negative bacteria in vivo.

The third step is a second β-hydroxymyristoyl-ACP acyltransferase, catalyzed by UDP-β-*O*-[β-hydroxymyristoyl]GlcN acyltransferase (*lpxD*). Like the LpxA acyltransferase, the 36-kDa LpxD possesses repeating hexapeptide units and will presumably have a fold and trimeric structure similar to LpxA. The product of the LpxD reaction is UDP-2,3-diacyl-GlcN. UMP is removed from this compound to form 2,3-diacyl-GlcN-1-phosphate (lipid X), but the enzyme responsible for this reaction is not known. There is an approximately 10-fold excess of lipid X over UDP-2,3-diacyl-GlcN in wild-type cells. Mutations in *lpxB*, cause a 500-fold increase in the amount of lipid X in the membrane, although LpxB is not a pyrophosphatase. LpxB, a dimer of 42-kDa monomers, catalyzes the condensation of lipid X with UDP-2,3-diacyl-GlcN to form the lipid A disaccharide-1-phosphate. *lpxB* is present in a complex cluster with *lpxA*, *lpxD*

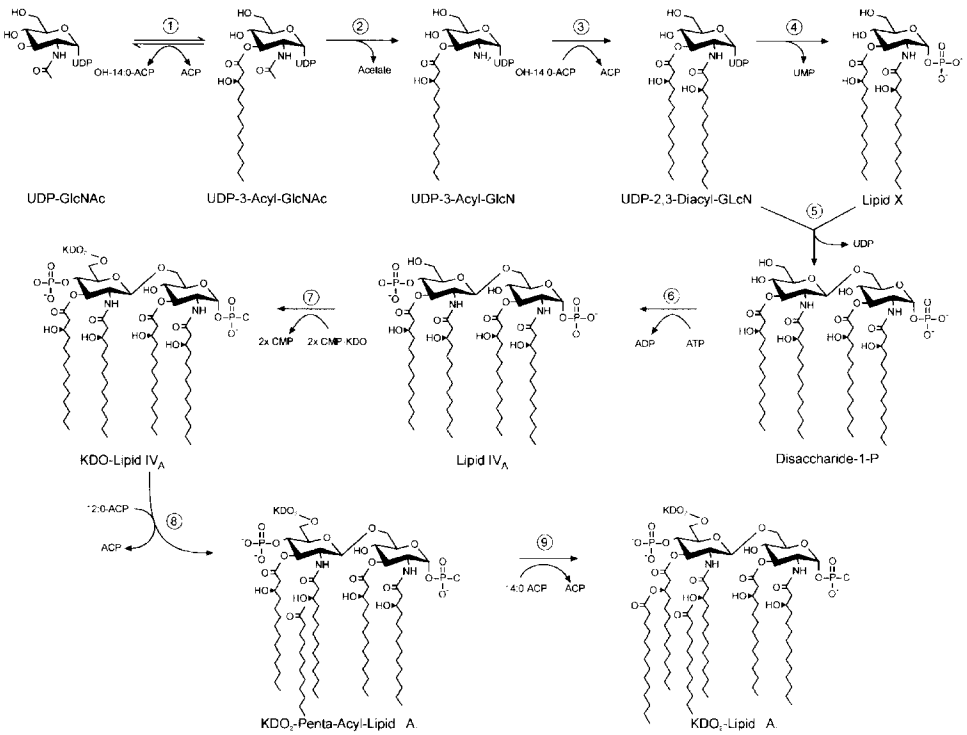

Fig. 7. Biosynthesis of endotoxin in *E. coli*. The first step (1) in the pathway is catalyzed by UDP-N-acetylglucosamine (UDP-GlcNAc) acyltransferase (LpxA). (2) The committed step is catalyzed by the LpxC deacetylase, followed by (3) a second acyltransferase (LpxD). (4) Lipid X is generated by the removal of UMP from UDP-2,3-diacyl-GlcNac by an unknown enzyme. (5) Lipid X and UDP-2,3-diacyl-GlcNac are then condensed together by LpxD to form lipid IV$_A$. (6) A 4′-kinase phosphorylates the disaccharide to produce lipid IV$_A$. (7) Two consecutive additions of KDO by KdtA, and two O-acylations by (8) HtrB and (9) MsbB yield KDO$_2$-lipid A. Subsequent addition of core sugars and O-antigen chains (not shown) yield the mature lipopolysaccharide.

and *fabZ*, and other genes encoding enzymes of phospholipid synthesis, outer membrane proteins, DNA synthesis and fatty acid synthesis.

Strains mutated in *lpxK* accumulate lipid A disaccharide-1-P, which led to the cloning and characterization of lipid A disaccharide kinase, a Mg^{2+}-dependent 4′-kinase activity stimulated by CL, that makes the key intermediate, lipid IV$_A$. The presence of the 4′-phosphate is essential for the recognition of lipid A by mammalian cells during endotoxin stimulation. Two subsequent additions of KDO are catalyzed by the KDO transferase, the 47-kDa KdtA protein. A single protein catalyzes both additions, since overexpression of *kdtA* causes a large increase in both transfers. KDO transferase activity is essential for growth, and conditional mutants accumulate massive amounts of lipid IV$_A$ prior to cessation of growth at the non-permissive temperature.

In the final steps of lipid A synthesis, two fatty acids are transferred to the hydroxyl groups of the β-hydroxymyristate on the distal unit. The first of these is usually laurate (12 carbons) added from lauroyl-ACP by the enzyme HtrB, and then MsbB adds

myristate from myristoyl-ACP. During cold shock (on shifting *E. coli* from 30 to 12°C), palmitoleate appears in the lipid A at the expense of laurate. A novel gene, *lpxP*, is induced for this reaction, and null mutants in HtrB are not defective in this adaptation. Double null *htrB msbB* mutants can also be generated. Thus, the extensive acylation of the lipid A is not absolutely required for its proper insertion into the outer membrane or the formation of a hydrophobic barrier.

7. Phospholipid flippase

All of the enzymes of phospholipid and lipid A biosynthesis are either cytosolic or located on the inner aspect of the inner membrane. How then do the lipids get to the outer face of the inner membrane, or to the outer membrane? Recent work from Raetz's laboratory has shown that the product of the *msbA* gene is a lipid 'flippase' required for the trafficking of lipids across the inner membrane. A temperature-sensitive mutant, *msbA*(Ts), with a A270T substitution in the MsbA protein, shows a rapid and dramatic reduction in the export of all major lipid classes, including PE and core-lipid A, to the outer membrane. Lipid export is inhibited by >90% after 30 min at the non-permissive temperature, while protein transport is not affected. *E. coli* harboring this mutation possess duplicated inner membranes at the elevated temperature. Null mutations in *msbA* are lethal, and this is the only bacterial transporter that has been shown to be essential. The MsbA protein is a member of the ATP-binding cassette (ABC) family of transporters, and has most similarity to mammalian P-glycoprotein multiple-drug resistance ABC transporters (>30% identity). MsbA exists as a homodimer of 64.6-kDa monomers, each of which has a single membrane spanning region (comprised of six transmembrane helices) and a nucleotide binding domain. This arrangement is distinct from the mammalian multiple-drug resistance pumps, which have two membrane spanning regions and two nucleotide binding domains fused into a single polypeptide. Thus, MsbA is a 'half-transporter', and is actually homologous to both amino- and carboxy-terminal halves of the mammalian multiple-drug resistance protein.

The crystal structure of the integral membrane MsbA has been determined by Chang and Roth to 4.5 Å [15]. The protein traverses the entire inner membrane and is cone-shaped, with an opening of about 25 Å on the cytoplasmic side, leading into a chamber of sufficient size for core-lipid A. The nucleotide binding domains are on the cytoplasmic face, and share no contacts. Dimer contacts are made in the half of the protein in the outer membrane. A model for transport has been suggested in which the lipid A or phospholipid enters the chamber through the cytoplasmic membrane, the chamber then closes allowing for flip-flop of the molecule, and then the chamber re-opens, and the lipid is ejected. Presumably, the lipid is released into the outer leaflet of the inner membrane, since MsbA does not extend across the periplasm. Thus, lipids that are required in the outer membrane must also traverse the periplasm. LPS molecules must also be flipped across the outer membrane to be displayed on the surface of the bacteria. Currently, nothing is known about these processes.

8. *Degradation of fatty acids and phospholipids*

8.1. β-Oxidation of fatty acids

8.1.1. Transport of fatty acids across the membrane

Exogenous long chain fatty acids are utilized by *E. coli* in two ways. Firstly, they can be incorporated into the membrane phospholipids by the acyltransferase system (PlsB and PlsC; Section 4). Secondly, they can be used as the sole carbon source for growth, and are in fact an important source of energy for *E. coli* in their normal habitat, the intestine [16]. The CoA thioester of the fatty acid is the substrate for both of these pathways. Fatty acids greater than 10 carbons in length require the *fadL* gene product to be taken up from the growth medium in sufficient quantities to support growth. FadL is a 46-kDa outer membrane protein produced following the cleavage of a 28-residue signal peptide from the propeptide. Fatty acid uptake is closely coupled to acyl-CoA formation, since very low levels of free fatty acid are found in the cells. The acyl-CoA synthetase, encoded by *fadD*, is a homodimer of 62-kDa subunits, and associates with the cytoplasmic leaflet of the inner membrane. Strains mutated in *fadD* cannot produce acyl-CoA and thus cannot grow on exogenous fatty acids, nor incorporate them into their membrane phospholipids. The esterification of the free fatty acid to CoA traps the fatty acid inside the cell, driving its transport across the inner membrane, and the net accumulation of fatty acid from the medium. Medium chain fatty acids do not require FadL to enter the cells, and may traverse the outer membrane by passive diffusion.

8.1.2. Degradation of fatty acids

Degradation of fatty acids proceeds via an inducible set of enzymes that catalyze the pathway of β-oxidation [16]. β-Oxidation occurs via repeated cycles of reactions that are essentially the reverse of the reactions of fatty acid synthesis (Fig. 8). However, three major differences distinguish the two pathways. Firstly, β-oxidation utilizes acyl-CoA thioesters, and not acyl-ACPs. Secondly, the β-hydroxy intermediates have the opposite stereochemistry (L in β-oxidation and D in synthesis). Finally, the enzymes of β-oxidation share no homology with those of synthesis.

The first step in the pathway is the dehydrogenation of acyl-CoA by the enzyme acyl-CoA dehydrogenase. While other organisms have several dehydrogenases with different chain length specificities (i.e. for short, medium or long acyl chains), it has been reported that *E. coli* has one enzyme active on all chain lengths. The dehydrogenase has been linked to two mutations in the 5 min region of the *E. coli* chromosome, *fadF* and *fadG*. However, the genome sequence suggests that a single gene in this region, *yahF*, encodes for a 92-kDa dehydrogenase. Thus, *fadF* and *fadG* probably represent different mutations in the same YahF dehydrogenase, and do not encode two distinct proteins. The acyl-CoA dehydrogenase is a flavoprotein, and is linked to a electron transferring flavoprotein (*fadE*). Further confusion exists in the literature as many acyl-CoA dehydrogenase (YafH) homologues are annotated as FadE homologues. Mutant strains of *E. coli* blocked in β-oxidation with *fadE* or *fadF* (*yahF*) mutations can accumulate acyl-CoA species, but cannot degrade them.

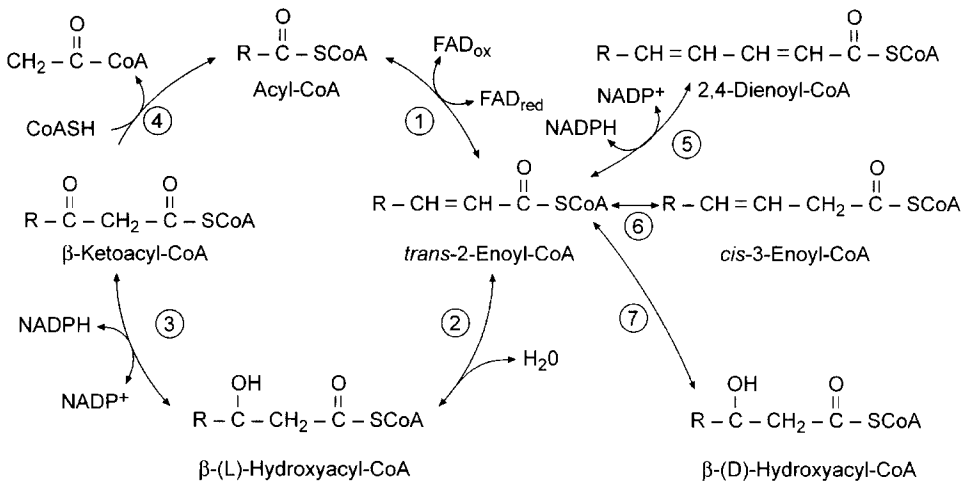

Fig. 8. β-Oxidation of fatty acids in *E. coli*. Long chain fatty acids are transported into the cell by FadL and converted to their CoA thioesters by FadD (not shown). The acyl-CoAs are substrates for the (1) acyl-CoA dehydrogenase (YafH) to form a *trans*-2-enoyl-CoA. The double bond is reduced by (2) trans-2-enoyl-hydratase (crotonase) activity of FadB. The β-hydroxyacyl-CoA is then a substrate for the $NADP^+$-dependent dehydrogenase activity of FadB (3). A thiolase, FadA (4), releases acetyl-CoA from the β-ketoacyl-CoA to form an acyl-CoA for subsequent cycles. (5) Polyunsaturated fatty acids are reduced by the 2,4-dienoyl-CoA reductase (FadH). (6) FadB also catalyzes the isomerization of *cis*-unsaturated fatty acids to *trans*. (7) The epimerase activity of FadB converts D-β-hydroxy thioesters to their L-enantiomers via the *trans*-2-enoyl-CoA.

The second step in the cycle is enoyl-CoA hydratase, an activity commonly referred to as 'crotonase'. Traditionally, in vitro measurements of this activity utilize crotonoyl-CoA (*trans*-2-butenoyl-CoA) as the substrate. Crotonase activity in *E. coli* is one function present in a multifunctional protein encoded by *fadB*. The next step in the cycle is β-hydroxyacyl-CoA dehydrogenase, another function of the FadB enzyme. The β-ketoacyl-CoA produced in this reaction is a substrate for the monofunctional *fadA*-encoded β-ketoacyl thiolase, which cleaves acetyl-CoA from the acyl-CoA to produce an acyl-chain two carbons shorter than when it entered the cycle. The cycle is then repeated until the fatty acid is metabolized. The FadB protein is a homodimer of 78-kDa subunits, and is purified in complex with the homodimeric 42-kDa *fadA* gene product. The total complex is thus an $\alpha_2\beta_2$ heterotetramer with an apparent mass of about 260 kDa. The *fadA* gene encodes the β-subunit, while the *fadB* gene gives the α-subunit, the confusing nomenclature a remnant of the days of classical genetics.

Unsaturated fatty acids can also be degraded by the β-oxidation pathway. The FadB protein possesses *cis*-β-enoyl-CoA isomerase activity, which converts *cis*-3 double bonds to *trans*-2 (Fig. 8). A 2,4-dienoyl-CoA reductase encoded by *fadH* is also required for the metabolism of polyunsaturated fatty acids (Fig. 8). This protein is a 73-kDa monomeric, $NADP^+$-dependent, 4Fe–4S flavoprotein. The FadH protein can utilize compounds with either *cis* or *trans* double bonds at the 4-position. An epimerase activity of FadB allows for the utilization of D-hydroxy fatty acids. The epimerase is actually a combination of a D-β-hydroxyacyl-CoA dehydratase and the

Table 2
Phospholipid degradative activities in *E. coli*

Enzyme	Gene	Location	Substrates
Phospholipase A1	*pldA*	Outer membrane	Phosphatidylethanolamine, phosphatidylglycerol, cardiolipin and lyso derivatives
Phospholipase A		Cytoplasm	Phosphatidylglycerol
Lysophospholipase L2	*pldB*	Inner membrane	Lyso-phosphatidylethanolamine
Lysophospholipase		Cytoplasm	Lyso-phosphatidylethanolamine, lyso-phosphatidylglycerol
Phospholipase C		Unknown	Phosphatidylethanolamine
Phospholipase D		Cytoplasm	Cardiolipin
Phospholipase D		Cytoplasm	Phosphatidylserine
Lipase		Membrane	Triacylglycerol
CDP-diacylglycerol hydrolase		Inner membrane	CDP-diacylglycerol
Phosphatidic acid phosphatase		Membrane	Phosphatidic acid
Thioesterase I	*tesA*	Periplasm	Acyl-CoA
Thioesterase II	*tesB*	Cytoplasm	Acyl-CoA

crotonase (hydratase) activities, resulting in the conversion of the D to the L enantiomer (Fig. 8).

The substrate specificities of the enzyme complex in vitro suggests that all of the enzymes can utilize all chain lengths of substrates, with the possible exception of the crotonase activity. This function of FadB appears somewhat limited to short chain substrates, and it has been suggested that a separate long chain enoyl-CoA hydratase may exist in *E. coli*. Two open reading frames in the *E. coli* genome, discovered by bioinformatics, are predicted to encode homologs of FadA and FadB. Thus, two complexes may be present with preferences for long or short chain acyl-CoAs.

8.2. Phospholipases

Based mainly on cell free assays, 10 enzymatic activities that degrade phospholipids, intermediates in the phospholipid biosynthetic pathway, or triacylglycerol have been reported (Table 2). The detergent-resistant phospholipase A_1 (encoded by *pldA*) of the outer membrane, characterized by Nojima and colleagues, is the most studied of these enzymes. This enzyme is unusually resistant to inactivation by heat and ionic detergents and requires calcium for maximal activity. The mature phospholipase has a subunit molecular mass of 31 kDa. Hydrolysis of fatty acids from the 1-position of phospholipids is the most rapid reaction, but the enzyme will also hydrolyze 2-position fatty acids, as well as both isomeric forms of lysophosphatides and mono- and di-acylglycerols. A detergent-sensitive phospholipase A_1 has also been described, although this activity has not been designated to a gene. This enzyme differs from the detergent-resistant protein in that it is located in the soluble fraction of the cell, is inactivated by heat and ionic detergents, and has a high degree of specificity for PG. The cytoplasmic phospholipase A also requires calcium for activity. There are also inner membrane and

cytoplasmic lysophospholipases. The best characterized of these is the inner membrane lysophospholipase L2 (*pldB*) which hydrolyzes 2-acyl-glycerophosphoethanolamine efficiently, but is barely active on the 1-acyl isomer. This lysophospholipase also catalyzes the transfer of fatty acids from 2-acyl-glycerophosphoethanolamine to PG to form acyl-PG.

The physiological role of these degradative enzymes remains unknown. Mutants lacking the detergent-resistant phospholipase (*pldA*), lysophospholipase L2 (*pldB*), or both enzymes do not have any obvious defects in growth, phospholipid composition, or turnover. Moreover, strains that overproduce the detergent-resistant enzyme also grow normally. It has been established that the detergent-resistant phospholipase is responsible for the release of fatty acids from phospholipids that occurs during infection with T4 and λ phages. However, phospholipid hydrolysis is not essential for the life cycle of these bacteriophages. One possible function for the hydrolytic activities with unassigned genes is that they are actually biosynthetic proteins (acyltransferases) that act as lipases in the absence of suitable acceptor molecules in the assay systems employed. An example of such an enzyme is PS synthase, which catalyzes both phospholipase D and CDP-diacylglycerol hydrolase reactions. PS synthase appears to function via a phosphatidyl–enzyme intermediate, and in the absence of a suitable acceptor such as serine or CMP, the phosphatidyl–enzyme complex is hydrolyzed by water; thus, the enzyme acts as a phospholipase D. Also, some of these enzyme activities may reflect a broad substrate specificity of a single enzyme rather than the presence of several distinct protein species. For example, the observed lipase activity that cleaves the 1-position fatty acids from triacylglycerols (a lipid usually not found in *E. coli*) may arise from the detergent-resistant phospholipase A_1 acting on triacylglycerol as an alternate substrate. Further, a lysophospholipase L(1) activity has been attributed to thioesterase I. Three open reading frames (*ybaC*, *yhjY*, and *yiaL*) present in the *E. coli* genome potentially encode proteins with lipase activity, but have not been studied to date.

8.3. Thioesterases

Thioesterases preferentially cleave the thioester bond of acyl-CoA molecules to produce CoA and free fatty acid. *E. coli* contains two well characterized thioesterases. Thioesterase I (encoded by *tesA*) is a periplasmic enzyme of 20.5 kDa, with a substrate specificity for acyl chains >12 carbon atoms. Thioesterase I hydrolyzes synthetic substrates used in the assay of chymotrypsin, which led to the initial conclusion that TesA was a protease ('protease I'). However, the purified protein does not cleave peptide bonds. Thioesterase I also appears to possess lysophospholipase L(1) activity. Thioesterase II is a cytosolic tetrameric protein composed of 32-kDa subunits encoded by the *tesB* gene. Thioesterase II cleaves acyl-CoAs of >6 carbons and β-hydroxyacyl-CoAs, but is unable to cleave acyl-pantetheine thioesters. The physiological function of thioesterases I and II is unknown. Null mutants have been constructed in both *tesA* and *tesB*, and the double mutant strain generated. None of these strains has an observable growth phenotype indicating that neither protein is essential. However, the *tesAB* double-null mutant still retains about 10% of the total wild-type thioesterase activity indicating the existence of a third unidentified thioesterase in *E. coli*.

9. *Phospholipid turnover*

9.1. The diacylglycerol cycle

The polar head group of PG is rapidly lost in a pulse-chase experiment, whereas that of PE is stable. The conversion of PG to CL, catalyzed by CL synthase, does not account for all the loss of ^{32}P-labeled PG observed in the pulse-chase experiments. A phosphate-containing non-lipid compound derived from the head group of PG was sought, which led to the discovery of the membrane-derived oligosaccharides (MDO) by Kennedy's group [17]. These compounds are composed of *sn*-glycerol-1-phosphate (derived from PG), glucose and (usually) succinate moieties, and have molecular weights in the range of 4000–5000. They are found in the periplasm of Gram-negative bacteria, an osmotically sensitive compartment. The synthesis of the MDO compounds is regulated by the osmotic pressure of the growth medium and decreased osmotic pressure gives an increased rate of MDO synthesis. Thus, MDO compounds seem to be involved in osmotic regulation.

In the synthesis of MDO, the *sn*-glycerol-1-phosphate polar headgroup of PG is transferred to the oligosaccharide, with 1,2-diacylglycerol as the other product (Fig. 9). The 85-kDa transmembrane protein that catalyzes this reaction is encoded by *mdoB*. Diacylglycerol kinase phosphorylates the diacylglycerol to phosphatidic acid, which reenters the phospholipid biosynthetic pathway (Fig. 6) to complete the diacylglycerol cycle (Fig. 9). In the overall reaction only the *sn*-glycerol-1-phosphate portion of the PG molecule is consumed; the lipid portion of the molecule is recycled back into phospholipid. MDO synthesis is responsible for most of the metabolic instability of the polar group of PG, since blocking MDO synthesis at the level of oligosaccharide synthesis by lack of UDP-glucose greatly reduces PG turnover. Moreover, the rate of accumulation of diacylglycerol in strains lacking diacylglycerol kinase (*dgk*) correlates with the presence of both the oligosaccharide acceptor and the osmolarity of the growth medium. *dgk* is immediately upstream of *plsB* in *E. coli*. The protein is a trimer of identical 13-kDa subunits, each with three predicted trans-membrane helices. Activity appears to be limited by diffusion of substrate across the membrane to the cytoplasmic active site. It should be noted that some species of MDO contain phosphoethanolamine. Although direct proof is lacking, it is likely that the ethanolamine moiety is derived from PE, as this is the only known source of ethanolamine.

9.2. The 2-acylglycerolphosphoethanolamine cycle

2-Acylglycerolphosphoethanolamine is a minor membrane lipid in *E. coli* generated from fatty acid transfer of the acyl moiety at the 1-position of PE to the outer membrane lipoprotein. 2-Acylglycerolphosphoethanolamine acyltransferase is an inner membrane enzyme that esterifies the 1-position of 2-acylglycerolphosphoethanolamine utilizing acyl-ACP (and not acyl-CoA) as the acyl donor. The acyltransferase was first recognized as a protein called acyl-ACP synthetase that catalyzes the ligation of fatty acids to ACP, hence the gene designation *aas*. ACP acts as a bound subunit for accepting the acyl intermediate in the normal acyltransferase reaction and high salt

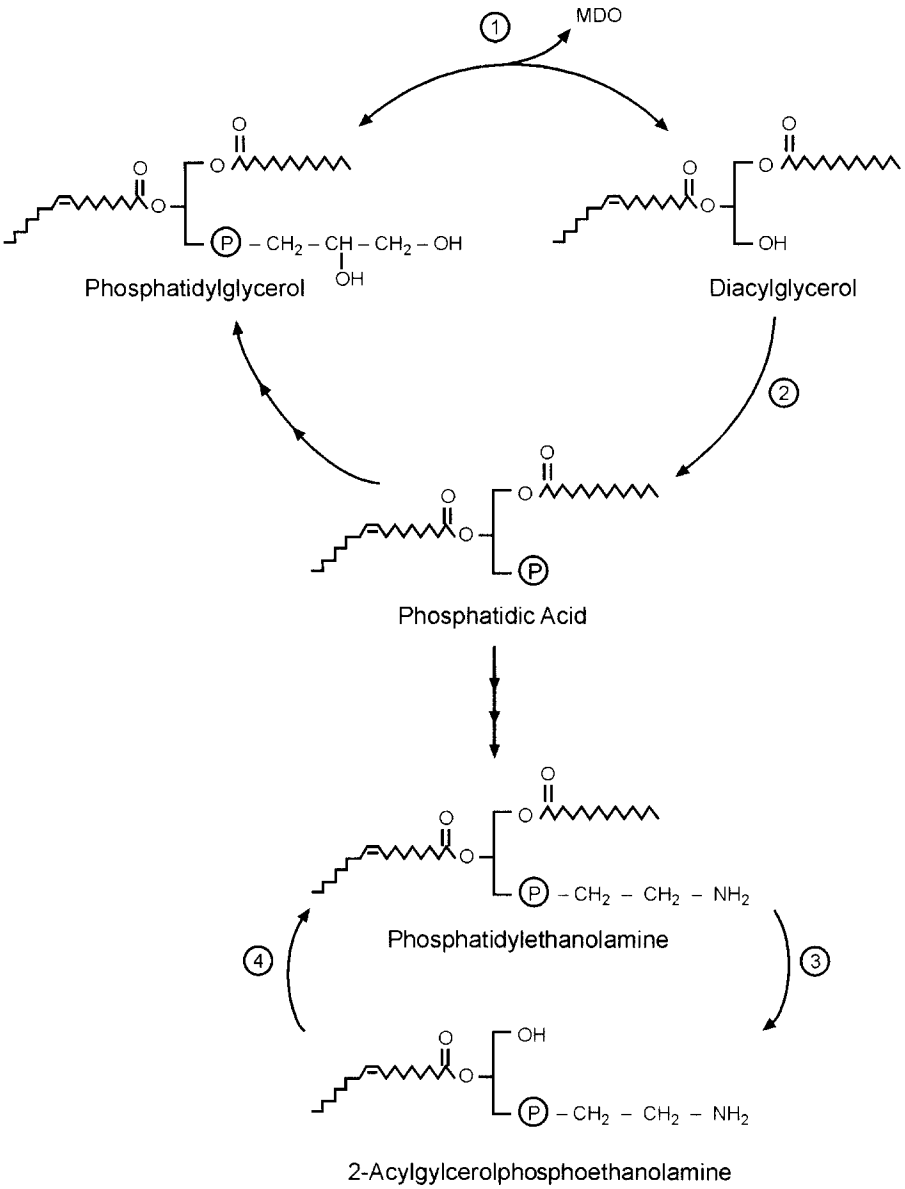

Fig. 9. Phospholipid turnover. The 1,2-diacylglycerol kinase cycle involves (1) the transfer of the *sn*-1-glycerol-phosphate moiety from phosphatidylglycerol to membrane derived oligosaccharides (MDO) by the enzyme MdoB. (2) Diacylglycerol kinase converts the diacylglycerol to phosphatidic acid, which can regenerate the phosphatidylglycerol (see Fig. 6). Phosphatidylethanolamine cycling involves (3) the transfer of an acyl chain to membrane lipoprotein and (4) re-esterification of the 1-position by 2-acylglycerophosphoethanolamine (Aas).

concentrations are required to dissociate the acyl-ACP intermediate from the enzyme in vitro. However, 2-acylglycerolphosphoethanolamine acyltransferase is the only reaction catalyzed by Aas in vivo. *aas* mutants are defective in both acyl-ACP synthetase and 2-acylglycerolphosphoethanolamine acyltransferase activities in vitro. They do not accumulate 2-acylglycerolphosphoethanolamine in vivo unless they are also defective in the *pldB* gene which encodes a lysophospholipase that represents a second pathway for 2-acylglycerolphosphoethanolamine metabolism. The acyl-ACP synthetase reaction has proven extremely valuable in the preparation of acyl-ACPs for use as substrates and inhibitors for the enzymes of fatty acid synthase.

10. Regulation of lipid metabolism

10.1. Regulation of fatty acid chain length

Fatty acyl chains in the membrane phospholipids of *E. coli* are normally 16 or 18 carbons in length. This specificity is a result of a combination of two factors: the poor reactivity of the β-ketoacyl-ACP synthases for longer chains; and the high specificity of the acyltransferases for 16- and 18-carbon products. Overexpression of FabB leads to the overproduction of *cis*-vaccenate, which is incorporated into the membranes. Overexpression of FabH causes a decrease in the average fatty acid chain length and the appearance of significant amounts of myristic acid (14 : 0) in the phospholipids. This effect is attributed to an increased rate of fatty acid initiation, which leads to a deficiency in malonyl-ACP for the terminal elongation reactions. The fatty acid biosynthetic machinery has the capacity to produce longer chains fatty acids. Under normal conditions, the 16 or 18 carbon chains are removed from the cytoplasm by the action of the acyltransferases. However, when phospholipid synthesis is blocked at the acyltransferase step, the fatty acids that accumulate have abnormally long chain lengths (e.g. 20 and 22 carbons). Conversely, overproduction of the acyltransferase results in a somewhat decreased average chain length, represented mainly by an increase in myristic acid. Thus, competition among the elongation synthases, the supply of malonyl-ACP, and the utilization of acyl-ACPs by the acyltransferase are the most significant determinants of fatty acid chain length.

10.2. Temperature modulation of fatty acid composition

All organisms regulate the fluidity of their membranes to maintain a membrane bilayer in a largely fluid state. As temperatures are lowered, membranes undergo a reversible change from a fluid (disordered) to a non-fluid (ordered) state. In *E. coli*, the temperature of the transition point depends on the fatty acid composition of the membrane phospholipids [11]. At lower temperatures, the amount of *cis*-vaccenic acid is rapidly (within 30 s) increased due to the increased activity of FabF. Synthesis of mRNA and protein are not required. Overexpression of FabB also increases the amount of this fatty acid in the membrane, although in a temperature-independent manner. Mutants that lack FabF are unable to modulate their fatty acid composition in a temperature-

dependent manner. Thus FabF, and not FabB, is involved in the thermal regulation of the fatty acid composition of the membranes.

As a result of the specificity of the acyltransferases, palmitic acid occupies position 1 of the phospholipid backbone at 37°C, whereas palmitoleic and *cis*-vaccenic acids are found at position 2. As the growth temperature is lowered, *cis*-vaccenic acid competes with palmitic acid for position 1 of the newly synthesized phospholipids. This indicates that the specificity of the acyltransferases changes during this temperature shift also, although how this happens is not yet understood.

10.3. Transcriptional regulation of the genes of fatty acid synthesis and degradation

The known genes of fatty acid synthesis are scattered along the genome with only two clusters, the minimal *accBC* operon and the *fab* cluster. The *fab* cluster contains the *fabH*, *fabD*, *fabG*, *acpP*, and *fabF* genes and may have functional significance, since the work of Cronan has demonstrated that several genes are cotranscribed. However, most genes also appear to have a unique promoter, and the full relevance of this cluster is not yet fully understood.

For balanced production of each member of the acetyl-CoA carboxylase complex, one might expect each gene to be regulated in the same manner. However, while transcription of all four *acc* genes is under growth rate control, with the rate of transcription decreasing with decreased growth rate, the *accBC* operon seems to be regulated by a mechanism that differs from the regulation of the *accA* and *accD* genes. The *accBC* operon is transcribed from a promoter located unusually far upstream of the *accB* gene. The major *accA* promoter lies within the coding sequence of the upstream *polC* (*dnaE*) gene, although transcription through *polC* and perhaps other upstream genes also reads through the *accA* sequence. The *accD* gene is transcribed from a promoter located within the upstream *dedA* gene.

Transcriptional regulation of the other genes of fatty acid synthesis is no less complicated. The FadR protein, which was first identified as a repressor of transcription of genes in the *fad* regulon of β-oxidation and fatty acid transport, also positively regulates *fabA* transcription. FadR binds to DNA in the absence of acyl-CoA, to repress the β-oxidation regulon and activate *fabA*. Acyl-CoA (formed from exogenous fatty acids transported into the cell), binds FadR and the protein is released from the DNA. The molecular details of these interactions have been examined by the crystallization of FadR and the FadR–acyl-CoA and FadR–DNA complexes. Whether FadR activates or represses transcription depends on the location of its binding site within the promoter region. For repression, FadR binds in the -30 to $+10$ region of the promoter and prevents binding of DNA polymerase. For activation, the FadR operator site is located in a 17-bp region at -40 of the *fabA* promoter, and FadR binding promotes DNA polymerase binding. In *fadR* null mutants, the *fabA* gene is transcribed from two weak promoters of about equal strength whereas in wild-type strains, a 20-fold increase in transcription from the proximal promoter is seen in the absence of acyl-CoA. Thus, FadR monitors the intracellular concentration of acyl-CoA and coordinately regulates fatty acid synthesis and β-oxidation in response to these compounds.

The *fabB* gene, encoding β-ketoacyl-ACP synthase I, possesses a nucleotide sequence in its −40 region that matches perfectly with the highest affinity FadR operator sites. Accordingly, *fabB* is also positively regulated by FadR; however, the changes in *fabB* mRNA levels due to FadR regulation are much lower than for *fabA*.

10.4. Regulation of phospholipid headgroup composition

Within a given strain of *E. coli*, the phospholipid ratio (PE : PG : CL) is maintained under a variety of growth rates and conditions. The exception to this is the increased conversion of PG to CL during the stationary phase (Section 5.4). Thus, a mechanism must exist to maintain phospholipid homeostasis. Regulatory mutants resulting in the overexpression of PS synthase (*pssR*) and diacylglycerol kinase (*dgkR*) have been identified, suggesting the existence of *trans* acting factors that control the expression of these key enzymes, but their significance is unclear. The hypothetical 32-kDa PssR protein is similar to the LysR family of transcriptional regulators. Overexpression of PS synthase, PGP synthase or CL synthase in plasmid-based systems do not lead to dramatic changes in the membrane phospholipid composition. Thus, modulation of protein level is unlikely to have a role in the regulatory scheme.

So how is phospholipid homeostasis maintained? Control of the individual enzymes at the level of activity by feedback-regulation appears a more probable mechanism. Perturbations in the ratio of phospholipids were attempted experimentally by the activation of phosphoglycerol transferase I (*mdoB*). This enzyme, involved in MDO synthesis, catalyzes the transfer of glycerol phosphate from PG to the extracellular arbutin (4-hydroxyphenyl-*O*-β-D-glucoside). Treatment with arbutin (a MDO substrate analogue) causes a 7-fold increase in the rate of PG synthesis without a concomitant increase in PGP synthase proteins levels or significant changes in membrane phospholipid composition. Thus, PS synthase and PGP synthase are independently regulated by phospholipid composition. Similarly, purified CL synthase is strongly feedback inhibited by CL, and this inhibition is partially relieved by PE. Thus, the regulation of phospholipid content in *E. coli* appears to be an intrinsic property of the enzymes.

10.5. Coordinate regulation of fatty acid and phospholipid synthesis with macromolecular biosynthesis

Fatty acid biosynthesis is coordinately regulated with phospholipid synthesis since, in growing cultures of *E. coli*, there is no significant accumulation of any of the intermediates in fatty acid synthesis. Following inhibition of phospholipid biosynthesis, fatty acid biosynthesis carries on at about 10–20% of the uninhibited rate. Measurements of this rate were severely hampered by the requirement to first prevent β-oxidation of newly synthesized fatty acids, and second to use a strain in which acetate was solely channeled into fatty acid synthesis, so that incorporation of ^{14}C-label from the acetate could be measured.

Labeling the ACP moiety of the fatty acid intermediates by growth of a *panD* strain on medium containing tritiated β-alanine, a precursor of 4′-phosphopantetheine, also shows that long chain acyl-ACPs accumulate for a short period following the cessation

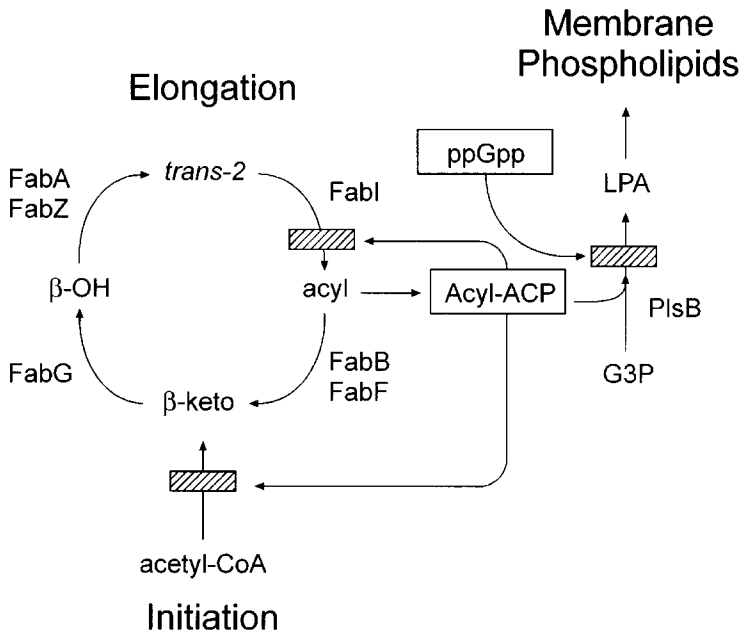

Fig. 10. Coordinate regulation of fatty acid and phospholipid metabolism. The pleiotropic regulator ppGpp regulates transfer of fatty acids to the membrane via inhibition of the PlsB acyltransferase step, coordinating phospholipid synthesis with macromolecular synthesis. Long chain acyl-ACPs accumulate for a period, then feedback inhibit their own synthesis at the point of initiation (inhibition of acetyl-CoA carboxylase and/or FabH) and elongation, by inhibition of FabI.

of phospholipid synthesis. This accumulation does not continue indefinitely, however, and reaches a plateau after about 20 min following inhibition of phospholipid synthesis. Thus, de novo fatty acid synthesis ceases, probably by a feedback inhibition mechanism involving long chain acyl-ACPs inhibiting early steps in the fatty acid biosynthesis pathway (Fig. 10). A significant finding in support of this idea is that overexpression of a thioesterase (which prevent the accumulation of acyl-ACP by cleavage of the thioester linkage and release of the acyl chain), allows continued fatty acid synthesis following cessation of phospholipid synthesis. This also further suggests that acyl-ACP and not free fatty acids mediate the inhibition. A reduction in total ACP is not responsible for the inhibition of fatty acid synthesis, since the free ACP pools of the glycerol-starved *plsB* mutants are not significantly depleted, and overproduction of ACP fails to relieve inhibition of fatty acid synthesis. A *fadD* mutant strain, which cannot produce acyl-CoA, overexpressing a thioesterase gave the same results as strains blocked elsewhere in β-oxidation or wild-type strains, thus ruling out a role for acyl-CoA.

As *E. coli* enter the stationary phase, levels of an unusual nucleotide, guanosine 5′-diphosphate-3′-diphosphate (ppGpp), rise [18]. Wild-type strains of *E. coli* undergo the so-called 'stringent response' following starvation for a required amino acid, an effect also mediated by increased intracellular ppGpp. Increased levels of ppGpp cause a strong inhibition of stable RNA synthesis, and inhibition of protein and

phospholipid synthesis. Mutant strains (*relA*) do not undergo the stringent response, due to the lack of ppGpp synthase I, a ribosomal protein that produces ppGpp in response to uncharged tRNA. The interaction of ppGpp with RNA polymerase mediates the inhibitory effects on stable RNA synthesis. ppGpp directly inhibits phospholipid biosynthesis by inhibition of the glycerol phosphate acyltransferase (PlsB) and causes an accumulation of long chain acyl-ACPs, which in turn lead to the inhibition of fatty acid biosynthesis. Overexpression of the acyltransferase relieves the inhibition on both fatty acid and phospholipid synthesis.

The target enzyme of the acyl-ACP feedback inhibition is not clear, and indeed several enzymes are still under consideration (Fig. 10). An obvious target is acetyl-CoA carboxylase, the first enzyme of the pathway. Inhibition of malonyl-CoA production would prevent fatty acid synthesis and elongation. Cronan has shown that acetyl-CoA carboxylase appears to be a rate-limiting step for fatty acid synthesis and that the enzyme is inhibited by acyl-ACP in vitro. However, β-ketoacyl-ACP synthases I and II (FabB and FabF) are potential regulators due to their ability to degrade malonyl-ACP to acetyl-ACP, an activity stimulated by the presence of long chain acyl-ACP, and hence attenuate cycles of fatty acid elongation. FabH catalyzes the first step in the pathway, and inhibition of this enzyme would halt initiation of new acyl chains, but would allow the elongation of existing fatty acid intermediates. Inhibition of FabH by physiologically relevant concentrations of long chain acyl-ACPs has been demonstrated in vitro. Finally, enoyl-ACP reductase (FabI) is a potential target since the activity of this enzyme is a determining factor in completing rounds of fatty acid elongation and acyl-ACP could act as product inhibitors. Accumulation of the precursors of the enoyl-ACP step can be seen in vivo following inhibition of the acyltransferase. It remains possible that multiple of steps may be targeted to different degrees. The identification of the inhibited enzyme(s) and their relative contribution to the regulation will require an in vitro system including acetyl-CoA carboxylase that accurately reflects in vivo metabolism, and the isolation of mutants refractory to inhibition.

The inhibition of fatty acid biosynthesis triggers the stringent response. The accumulation of ppGpp is in this case dependent on the activity of the *spoT* gene product (ppGpp synthase II). How this regulatory system operates is unknown and it will be important to determine whether intermediates, such as acyl-ACP or malonyl-CoA, are the intracellular metabolites that mediate ppGpp synthase II activity.

11. Lipid metabolism in other bacteria

11.1. Analysis of lipid metabolism by genomic inference

The availability of genomic sequences for a variety of bacteria [8] allows for the rapid assessment of the lipid metabolic pathways present. Open reading frames encoding species specific isoforms of known genes can be amplified by the polymerase chain reaction, the protein expressed in *E. coli*, and the properties of the enzyme compared to the *E. coli* or other known proteins. This approach has been used extensively in Rock's laboratory for the genes of fatty acid synthesis, with isoforms from pathogenic

bacteria being isolated to assess their unique biochemical characteristics and for use in drug-screening programs. For example, the enoyl-ACP reductase isoforms from *E. coli*, *Staphylococcus aureus* and *B. subtilis* have been compared. Subtle differences in cofactor specificity could be detected in this way that are not obvious from the primary sequences.

Novel proteins have been identified using genomic information [19]. The *fabI*-encoded enzyme is the sole reductase present in *E. coli*, and it was assumed that this was probably true for all bacteria. However, analysis of the genome of *Streptococcus pneumoniae* did not reveal a *fabI* homologue, while genes for all of the other enzymes required for saturated fatty acid biosynthesis could be identified in a gene cluster. Thus, a novel enoyl-ACP reductase isoform was sought, and the *fabK*-encoded enoyl-ACP reductase II identified. The *fabK*-encoded protein possesses no homology to the FabI protein, and utilizes flavin mononucleotide as a cofactor, and is resistant to inhibition by the antibiotic triclosan (Section 12.3).

A third enoyl-ACP reductase isoform was discovered in *B. subtilis*, which also possesses *fabI*. A gene, *fabL*, was identified with low overall homology to *fabI*, although it contained lysine and tyrosine residues in the distinct motif of the catalytic residues in the enoyl-ACP reductase I. Heath and coworkers cloned the gene and demonstrated that the product possesses enoyl-ACP reductase activity in vitro. Insertional inactivation of either *fabI* or *fabL* in *B. subtilis* results in no obvious growth phenotype, but the double null strain could not be constructed. Thus, *fabL* encodes enoyl-ACP reductase III.

11.2. Branched chain fatty acid biosynthesis

Not all bacteria regulate membrane fluidity through the production of straight chain unsaturated fatty acids. In fact, Gram-positive bacteria often use branched chain fatty acids to modulate membrane fluidity [20]. The branch is a methyl group in the iso- or anteiso-position in the chain (i.e. the second or third carbon from the distal end of the chain). Based on the concept of acetyl-CoA as a primer for straight chain fatty acid synthesis in *E. coli*, it can be seen that the methyl group could be introduced using a 'branched-chain' primer. Indeed, isotope labeling and biochemical analysis identifies precursors of the branched chain amino acids, valine and isoleucine (isobutyryl-CoA or 2-methylvaleryl-CoA, respectively), as the primers for branched chain fatty acid synthesis. As in straight chain synthesis, the primer is condensed with malonyl-ACP by the action of FabH. The substrate specificities of the FabH enzyme(s) present in the bacteria determine the relative amounts of the respective fatty acids produced. *B. subtilis* contains a high proportion of branched chain fatty acids in its membranes, and has two FabH enzymes, each of which prefer the branched chain substrates over acetyl-CoA. *E. coli* FabH cannot use branched chain primers. Why *B. subtilis* possesses two FabH enzymes, with only minor differences in substrate specificity, is not understood.

11.3. Other ways to make unsaturated fatty acids

The *fabA* gene encodes the dehydratase/isomerase specifically required for the production of unsaturated fatty acids in Gram-negative bacteria. Gram-positive bacteria do not

contain an identifiable *fabA* homologue in their genome, but do possess unsaturated fatty acids. In *Bacillus*, it has been shown that a cold-shock induced gene (*desA*) encodes a desaturase that is active on the existing fatty acids present in membrane phospholipids. Desaturase activities are dependent on oxygen, and thus an aerobic lifestyle. This mechanism is analogous to that observed in plants (Chapter 4). *Streptococcus pneumoniae*, on the other hand, has a fatty acid profile similar to *E. coli*, but possesses neither an identifiable isomerase nor a desaturase. Thus, these facultatively anaerobic bacteria must contain a novel gene that encodes an isomerase with no homology to *fabA*. Gram-positive bacteria possess a *fabZ* homologue that performs all of the dehydration steps.

11.4. Bacteria with other phospholipid headgroups

E. coli have a very simple phospholipid composition with just three major forms, PE, PG and CL. However, the prokaryotic kingdom possesses a wide array of headgroups that defy adequate description in this short space; hence the reader is referred to Goldfine's review [21] for a comprehensive treatment of bacterial phospholipid structures. The phosphocholine headgroup stands worthy of mention for its uniqueness and distinct mechanisms of synthesis. Phosphatidylcholine (PC) had long been considered a eukaryotic phospholipid, where it is synthesized by transfer of the choline from CDP-choline to diacylglycerol, or by methylation of PE (Chapter 8).

Rhodospeudomonas spheroides, *Bradyrhizobium japonicum* and a few other specialized photosynthetic or nitrogen-fixing bacteria synthesize PC by three subsequent methylations of PE. The first methylase, encoded by *pmtA*, has been disrupted in *Bradyrhizobium japonicum*, and the mutants, which contain significantly reduced PC content, are less able to fix nitrogen in colonization assays. Thus, PC seems to be involved in host : bacteria interactions to establish symbiosis. The prokaryotic PE methyltransferases share weak homology to other bacterial methyltransferases, but no homology with their eukaryotic counterparts. *Rhodospeudomonas spheroides* are also somewhat unique amongst bacteria in that they contain intracellular membranes that hold the photosynthetic machinery. The amount of the intracellular membrane correlates to the amount of incident light, indicating a light-specific regulation of phospholipid synthesis in these organisms.

Sinorhizobium meliloti has been shown to synthesize PC by direct condensation of choline with CDP-diacylglycerol, as well as by the methyltransferase pathway. The *pcs* gene was identified and expression in *E. coli* demonstrates that it does code for a PC synthase [22]. The genomes of *Pseudomonas aeruginosa* and *Borrelia burgdorferi* contain similar genes, and have been reported to possess PC in their membranes. The PC synthase protein shares weak homology with PS synthase (a CDP-diacylglycerol : serine *O*-phosphatidyltransferase) from other bacteria, but not to any eukaryotic proteins.

A genus of bacteria, termed the Sphingobacterium, produce sphingolipids by a pathway similar to that in mammals. However, little is known about the enzymes involved at this time. Clostridia produce plasmalogens (1-alk-1′-enyl lipids) by an anaerobic pathway clearly different to the O_2-dependent pathway in mammals (Chapter 9). Branched chains fatty acids are also found in which the methyl group is inserted

post-synthetically into the middle of the chain, in a manner analogous to cyclopropane fatty acid synthesis. *S*-Adenosylmethionine is also the methyl donor for these reactions.

11.5. Bacteria with a type I fatty acid synthase

A general distinction between prokaryotic and eukaryotic fatty acids synthases is that bacteria possess the dissociated enzymes described above (type II), while higher organisms have a single, multifunctional, protein (type I) that catalyses all of the reactions. There are exceptions to this rule, however. Mycobacteria, for example, possess a type I fatty acid synthase for the production of their membrane fatty acids. This enzyme is a homohexamer of 290-kDa subunits. Each subunit possesses the six different active sites required to generate a fatty acid. Unlike the type II system, the products of a type I enzyme are acyl-CoAs. For the mycobacterial enzyme, the saturated acyl chains produced are between 16 and 24 carbons in length. Unsaturations must be added post-synthetically by a desaturase. Even more unusual is that the Mycobacterium possesses a type II synthase system for the further elongation of the fatty acyl products of the type I system into the 70–80 carbon mycolic acids. *Brevibacterium ammoniagenes*, a highly developed bacteria thought to be a progenitor of the fungi, possesses a type I fatty acids synthase that is capable of producing both saturated and unsaturated fatty acids anaerobically.

11.6. Lipid synthesis in Archea

Archea are a group of organisms, previously classified as bacteria, from which eubacteria and other life may have evolved. A melavonic acid (6 carbon) building block is used for synthesis instead of acetic acid. The generated phytanyl chains are attached to glycerol moieties of complex lipids by ether linkages. Thus, these lipids are unlike anything found in eubacteria or eukaryotes today.

11.7. Other organisms with a bacterial-like fatty acid synthase system

The dissociated enzymes that form the fatty acid synthesis system of most bacteria are not limited to the prokaryotic kingdom [23]. Plants utilize a homologous series of enzymes for synthesis of their fatty acids (Chapter 4). Although the genes are present on the nuclear chromosomes, fatty acid biosynthesis occurs in the chloroplasts of plants. It is thus hypothesized that the pathway evolved from the endosymbiont bacteria that became the chloroplast in plants. Recently, is has also been shown that the apicomplexans, a group of intracellular parasites including *Plasmodium falciparum*, the causative organism of malaria, possess a bacterial-like fatty acid synthase system [24]. These eukaryotic organisms possess an organelle described as a vestigial chloroplast (the apiplast), and thus are presumably evolved from chloroplast-containing algae. Plasmodium have been shown to be sensitive to the antibiotics thiolactomycin and triclosan, indicating that this pathway could be exploited for the development of novel antimalaria drugs.

12. Inhibitors of lipid metabolism

Much of the work on prokaryotic membrane biosynthesis in recent years has been directed towards analysis of the pathway enzymes as potential targets for the discovery on new antimicrobial agents [9]. It should be remembered that bacterial fatty acid biosynthesis is catalyzed on a series of small, discrete proteins and utilizes the soluble ACP to shuttle the soluble intermediates within the cytosol. This setup is quite different from mammalian fatty acid synthesis, and thus should allow for specific inhibitors to be designed with minimal mammalian toxicity.

12.1. β-Decynoyl-N-acetylcysteamine

The β-hydroxydecanoyl-ACP dehydrase (the *fabA* gene product) is specifically and irreversibly inhibited by the synthetic acetylenic substrate analog β-decynoyl-*N*-acetylcysteamine. A covalent adduct is formed with the active site histidine and results in the loss of both dehydrase and isomerase reactions. The description of this mode of inhibition was the first report of a suicide or mechanism-based inhibitor [25]. β-Decynoyl-*N*-acetylcysteamine concentrations between 10 and 50 μM are sufficient to completely inhibit unsaturated fatty acid synthesis and bacterial growth. Growth inhibition is relieved by the addition of unsaturated fatty acids to the medium. Saturated fatty acid synthesis continues normally in the presence of β-decenoyl-*N*-acetylcysteamine due to the activity of the *fabZ*-encoded dehydrase. Since most Gram-positive bacteria do not contain unsaturated fatty acids, inhibitors of this step are not being intensively developed.

12.2. Cerulenin and thiolactomycin

Cerulenin, (2*R*)(3*S*)-2,β-epoxy-4-oxo-7,10-dodecadienolyamide, is a fungal product that irreversibly inhibits β-ketoacyl-ACP synthases I and II. It is extremely effective in blocking the growth of a large spectrum of bacteria. Cerulenin forms a covalent bond with the active site cysteine of the synthases. Cerulenin can be seen in the hydrophobic acyl binding tunnel in the crystal structures of both FabB and FabF complexed with this drug. Although cerulenin is a versatile biochemical tool, it is not a suitable antibiotic for clinical use because it is also a potent inhibitor of the multifunctional mammalian fatty acid synthase, which contains similar active site residues and catalyzes the same chemical reaction.

Thiolactomycin, (4*S*)(2*E*,5*E*)-2,4,6-trimethyl-β-hydroxy-2,5,7-octatriene-4-thiolide, inhibits all three β-ketoacyl-ACP synthases of bacterial fatty acid synthesis in vivo and in vitro, but not the multifunctional fatty acid synthases. Malonyl-ACP protects the synthases from thiolactomycin inhibition, indicating that this antibiotic targets a different site on the condensing enzyme from that targeted by cerulenin. The crystal structure of FabB in complex with thiolactomycin reveals that the drug does in fact bind in the malonyl-ACP binding pocket [26], which is presumably distinct from the malonyl-pantotheine site of mammalian type I fatty acid synthase. The structure of the mammalian enzyme has not been solved to date, due in part at least to the large size of the enzyme. Overproduction of FabB but not FabH imparts thiolactomycin resistance.

Since FabF is not essential, these data suggest that FabB is the relevant thiolactomycin target in vivo. Thiolactomycin resistance phenotype maps to the *emrR* locus of the *E. coli* chromosome and results in the inactivation of a repressor that governs the expression of the *emrAB* multidrug resistance pump. The subtly different active site architecture of FabH compared to FabB and FabF makes it resistant to both cerulenin and thiolactomycin [26].

12.3. Diazaborines, isoniazid and triclosan

Diazaborines, a group of boron-containing antibacterial heterocyclic compounds, inhibit fatty acid synthesis in *E. coli* by inhibition of the enoyl-ACP reductase I. Inhibition by diazaborines requires the presence of NAD or NADH, and the drug mimics the binding of the substrate. The discovery that the FabI analog in *M. tuberculosis* (InhA) is the target for isoniazid and ethionamide, drugs used to treat tuberculosis, illustrates the potential importance of enoyl-ACP reductase I as an antibiotic target. Isoniazid is first activated by the KatG catalase/peroxidase, and then forms a covalent adduct with the nictinamide cofactor [27]. It was recently discovered that the enoyl-ACP reductase was also targeted by another antimicrobial compound in widespread use. Triclosan, the active component in a plethora of household items including toothpaste, hand soaps, cutting boards, mattress pads and undergarments, is a very effective inhibitor of the enoyl-ACP reductase I. Its ubiquitous use has been justified by the claim, based on improperly analyzed data, that it caused non-specific membrane disruption, a delayed leakage of cytoplasmic material and finally cell death. This phenotype is essentially identical to that observed by Egan and Russell in 1973 [28] for the original temperature-sensitive *fabI* mutant at the non-permissive temperature. Mutants of *E. coli* can be generated that are resistant to triclosan, and contain a Gly to Val substitution in the active site of enoyl-ACP reductase I. The mutant protein is resistant to triclosan in vivo and in vitro. Definitive proof that triclosan does not disrupt the membranes of Gram-negative bacteria in a non-specific manner comes from the overexpression of the triclosan-resistant enoyl reductase II (from a plasmid-borne copy of the *fabK* gene) in *E. coli*, which increases resistance by over 10,000-fold.

A key feature of triclosan's mode of action is the slow but complete inactivation of the enoyl reductase. The drug binds to the active site of the enzyme and forms very strong, non-covalent interactions with active site residues and the nicotinamide ring of the cofactor. The half life of the ternary enzyme–NAD^+–triclosan complex is over 1 h. The mutation that leads to triclosan resistance is the same as that for diazaborine resistance, and adjacent to the mutation required for isoniazid resistance in *M. tuberculosis*. There is some concern that the unregulated use of triclosan in the household setting, where it has not been proven to be effective in promoting public health, will lead to the generation of bacteria resistant to all enoyl-ACP reductase I-directed inhibitors.

12.4. Lipid A biosynthesis inhibitors

A new class of antibiotics was recently discovered that inhibit lipid A biosynthesis [29]. These compounds are still in their infancy, but could prove useful additions to

our antibacterial arsenal. The first generation of compounds are effective inhibitors of bacterial growth in vivo, and potent inhibitors of the LpxC deacetylase, the second step in the pathway, in vitro. Optimization of this family of compounds may lead to effective agents against Gram-negative bacteria.

13. Future directions

Much progress has been made over the last several years in elucidating the details of the enzymes of fatty acid biosynthesis. This pathway has become a major target for therapeutic intervention in bacterial-mediated disease. Significant inroads have also been made into the molecular mechanisms that regulate fatty acid synthesis. The availability of genomic data has also allowed for the facile translation of many of the findings made in *E. coli* to other bacteria facilitating the discovery of novel genes involved in lipid biosynthesis.

Many of the details of the regulation of fatty acid biosynthesis are still to be worked out, including the probable discovery of new transcription factors and new effector molecules. The fine details of the enzymatic mechanisms, and the comparison of the biochemical properties and functions of different isoforms, will continue in an effort, in part, to elucidate probable activity spectrums of next generation antibiotics that will surely be generated against this pathway. The rush to study fatty acid synthesis has somewhat overshadowed bacterial lipid synthesis in recent years, and discoveries in this area are more difficult since the enzymes and substrates involved are membrane-associated. However, techniques to study the structure and function of membrane proteins are rapidly evolving and will certainly be applied to resolving outstanding issues in bacterial lipid biogenesis.

Abbreviations

ACP	acyl carrier protein
CoA	coenzyme A
fabA, FabA	lowercase italics indicates gene, while uppercase Roman type indicates the protein product of the gene
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PS	phosphatidylserine
PGP	phosphatidylglycerolphosphate
CL	cardiolipin
LPS	lipopolysaccharides
ABC	ATP-binding cassette
MDO	membrane-derived oligosaccharides

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