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***Vibrio harveyi* Acyl Carrier Protein and its Role in
Activation of Exogenous Fatty Acids**

By

Zhiwei Shen

**Submitted in partial fulfillment of the requirements for the
degree of Doctor of Philosophy at Dalhousie University**

**Department of Biochemistry and The Atlantic Research Centre
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May, 1994

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Abstract

Acyl carrier protein (ACP) is a multifunctional fatty acid carrier which interacts with several different enzymes during the synthesis of fatty acids, phospholipids, and other specialized molecules in bacteria. Luminescent marine bacteria such as *Vibrio harveyi* also require 14:0-ACP for the synthesis of the aldehyde substrate of luciferase. To characterize *V. harveyi* ACP and elucidate its role in the metabolism of exogenous fatty acid, *V. harveyi* ACP was purified to homogeneity. A degenerate oligonucleotide probe based on partial protein sequence data was used to isolate a 1.4 kb EcoRI fragment in pUC18 encoding the N-terminal two-thirds of *V. harveyi* ACP. This was subsequently used to clone an overlapping 3 kb HindIII fragment encoding the C-terminal region. The predicted amino acid sequence of *V. harveyi* ACP (76 residues; Mr = 8,734; pI = 4.0) is 86% identical to that of *E. coli* ACP, while the DNA sequence is 79% identical. Sequencing of upstream regions indicated an organization of *V. harveyi* fatty acid biosynthetic genes similar to that found in *E. coli* (*fabD-fabG-AcpP*). *V. harveyi* acyl-ACP synthetase was used to enzymatically prepare fatty acylated derivatives of *E. coli* and *V. harveyi* ACP (chain lengths C₆ to C₁₆). Both ACPs migrated anomalously as 20 kDa bands on SDS-polyacrylamide gel electrophoresis, but they exhibited different behaviour upon fatty acylation. Immunoprecipitation with anti-*V. harveyi* ACP serum was used to demonstrate that *V. harveyi* acyl-ACP can be labeled in vivo with exogenous [9,10-³H]myristic acid; chain length-dependent resolution of acyl-ACPs on urea gels revealed that most of the label was associated with chain lengths (C₈ to C₁₂) shorter than the added fatty acid. However, different labeling profiles were observed after incubation with [³H]acetate, indicating that acyl-ACP labeling with [³H]14:0 is not due to total degradation of [³H]14:0 to [³H]acetyl-coenzyme A followed by resynthesis. Additional bands which comigrated with acyl-ACP on SDS gels were identified as lipopolysaccharide. Thus, in contrast to *E. coli*, exogenous fatty acids can be activated to form acyl-ACP intermediates after partial degradation in *V. harveyi* and can effectively label products (i.e. lipid A) that require ACP as an acyl donor. The transport mechanism of exogenous long chain fatty acid in *V. harveyi* was also explored by using mutagenesis and different screening methods. The results indicated that a protein-mediated transport system similar to *E. coli* may not be required for uptake of 14:0 in *V. harveyi*. Despite overall similarities in fatty acid and phospholipid composition, therefore, fatty acid metabolism in the luminescent bacterium *V. harveyi* exhibits several key differences with that in *E. coli*.

List of Abbreviations and Symbols Used

<i>E. coli</i>	<i>Escherichia coli</i>
<i>V. harveyi</i>	<i>Vibrio harveyi</i>
<i>P. phosphoreum</i>	<i>Photobacterium phosphoreum</i>
x	multiple times concentrated
x g	relative centrifugal force
3-HO-14:0	3-hydroxymyristic acid
4:0	butyric acid
6:0	hexanoic acid
8:0	octanoic acid
10:0	decanoic acid
12:0	lauric acid
14:0	myristic acid
16:0	palmitic acid
A	adenosine
A ₆₆₀	absorbance at 660 nm
ATP	adenosine triphosphate
ACP	acyl carrier protein
BCA	bicinchoninic acid
Bis	N, N'-methylene-bis-acrylamide
bp	base pair
BSA	bovine serum albumin
C ₁₄	chain length of 14 carbons
C	cytidine
CDP	cytidine 5'-diphosphate
CL	cardiolipin
CoA	coenzyme A
d	days
Dcp	D-alanine carrier protein
Denhardt's	Denhardt's reagent (see Section II, Solutions)
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FA	fatty acid
FLP	FadL protein

FMNH ₂	flavin mononucleotide (reduced)
G	guanosine
G3P	sn-glycerol-3-phosphate
GlcNAc	N-acetyl-glucosamine
GPE	glycero-phosphoethanolamine
KAS	3-ketoacyl-ACP-synthase
kDa	kilodalton
kb	kilobase pair
K _m	Michaelis constant
LTA	lipoteichoic acid
LU	light unit
M	molar
MDO	membrane-derived oligosaccharides
MES	2-(N-morpholino)ethanesulfonic acid
mg	milligram
min	minute
ml	milliliter
mM	millimole
MW, M _r	molecular weight
NaAc	sodium acetate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
ng	nanogram
NH ₄ Ac	ammonium acetate
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NP-40	Nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDA	piperazine diacrylamide
PE	phosphatidylethanol
PEG	polyethylene glycol
PG	phosphatidylglycerol
PDA	piperazine diacrylamide
PS	phosphatidylserine
PVDF	polyvinyl-difluoride
RIPA	RIPA buffer (see section C, Solutions)

RNA	ribonucleic acid
RNase A	ribonuclease A
r, s, t	fatty acid reductase: reductase, synthetase, transferase subunits
rpm	rotations per minute
R_s	Stokes radius
SAC	fixed <i>Staphylococcus aureus</i> Cowan I
SDS	sodium lauryl sulfate (sodium dodecyl sulfate)
SSC	SSC buffer
T	thymine
TBS	Tris-buffered saline (see Section II, Solutions)
TE	Tris-EDTA buffer (see Section II, Solutions)
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	thin layer chromatography
Tris	Tris (hydroxymethyl) aminomethane
UDP	uridine 5'-diphosphate
v	volume
V_m	maximal velocity
w	weight
μg	microgram
μl	microliter
μM	micromole

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Section I. Introduction

Prokaryotic cells are important models for biochemical research because of their simple cellular organization, definable nutritional requirements, and rapid reproduction. Bacterial cells are surrounded by two lipid bilayer cell membranes that consist primarily of glycerophospholipid, lipopolysaccharide and protein. The lipid component, which accounts for more than 10% of the dry weight of the cell, is essential for membrane integrity and serves as the matrix for membrane-associated enzymes and proteins. Since fatty acid (FA) is a major constituent of glycerophospholipid and lipopolysaccharide, its metabolism is very important for maintenance of cell functions. *Vibrio harveyi*, a gram-negative marine bacterium, is a member of the luminescent bacterioplankton family and was originally isolated from sea water. Although its luminescence system has been extensively studied for decades, little is known about FA metabolism in this organism. In contrast, FA metabolism in another gram-negative bacterium, *Escherichia coli*, has been well characterized at both biochemical and genetic levels. In fact, *E. coli* is better understood than any other cellular form of life. Since FA metabolism shares similarities in most of the prokaryotic kingdom, the broadly accumulated knowledge of FA metabolism in *E. coli* provides the basic reference for research in other prokaryotic organisms. *V. harveyi* and *E. coli* are very closely related bacterial species which both belong to the γ -3 subgroup of γ -purple bacteria [244]. The work in this thesis is concerned with the structure of acyl carrier protein (ACP) and its role in FA metabolism in *V. harveyi*, and assumes that the biosynthesis and degradation of fatty acids in *E. coli* can be used as a starting point to explore this topic. For this reason our knowledge of *E. coli* FA metabolism will be one of the primary topics reviewed in this chapter.

A. Fatty acid and complex lipid biosynthesis in *E. coli*.

FA synthesis falls into two general categories. The type-I FA synthase system involves a multifunctional FA synthase complex consisting of one or two polypeptides

catalyzing all the elongation reactions in cytosol, and is found mainly in animal cells and fungi. The type-II FA synthase system, which has been found principally in plant chloroplasts and most bacteria, consists of a series of individual FA synthetic enzymes mediated by the interaction with an ACP intermediate, the common substrate for most of the enzymes in the type-II system.

1. ACP is the acyl carrier in FA synthesis.

ACP, the multi-functional fatty acyl carrier, plays a key role in lipid synthesis in bacteria and plants since it is involved at virtually each stage of FA synthesis. The fatty acyl moieties in FA synthesis are covalently linked to ACP via a thioester bond. Both ACP and FA synthetic enzymes are located primarily in cytosol [100].

The synthesis of saturated and unsaturated fatty acid begins with acetyl-CoA as the building block (Fig. 1) [46,99]. Acetyl-CoA carboxylase is composed of four individual proteins (Table I) and catalyzes the conversion of acetyl-CoA to malonyl-CoA in an irreversible reaction [2,77,215]. Both acetyl and malonyl groups can be transferred from CoA to ACP to form acetyl-ACP and malonyl-ACP, respectively (Fig. 1). The formation of malonyl-ACP is catalyzed by malonyl-CoA:ACP transacylase [84]. Temperature-sensitive mutants of acetyl-CoA carboxylase or malonyl-CoA:ACP transferase are both defective in FA synthesis at the nonpermissive temperature [83,84,215], indicating that both are essential for initiation. Malonyl-ACP is required for all subsequent condensation reactions. Acetoacetyl-ACP is formed either from condensation of malonyl-ACP and acetyl-CoA catalyzed by 3-ketoacyl-ACP synthase (KAS) III [103,109,230] or from condensation of malonyl-ACP and acetyl-ACP catalyzed by KAS-I [50]. Acetyl transfer from acetyl-CoA to ACP is catalyzed either by a thiolactomycin-insensitive acetyl-CoA:ACP transacylase [140], or by KAS-III which contains the thiolactomycin-sensitive acetyl-CoA:ACP acyl transfer activity [230]. Acetyl-ACP is also formed from decarboxylation of malonyl-ACP catalyzed by KAS-I or II (Fig. 1) [3]. It still remains unclear whether the first condensation normally involves acetyl-CoA with KAS-III or

Table IProteins or enzymes involved in FA synthesis and delivery in *E. coli**

Protein or enzyme	Gene	Reference
1. FA synthesis enzymes		
Acetyl-CoA carboxylase (complex)	(<i>fabE</i>)	
Biotin carboxylase subunit	<i>accC</i>	[126,134]
Biotin carboxyl carrier protein (BCCP)	<i>accB (fabE)</i>	[4,161]
Transcarboxylase (alpha subunit)	<i>accA</i>	[135,137]
(beta subunit)	<i>accD (eddB/usg)</i>	[135,137]
Acyl carrier protein	<i>acpP</i>	[178]
Malonyl-CoA:ACP transacylase	<i>fabD</i>	[142,236]
3-Ketoacyl-ACP synthase III	<i>fabH</i>	[230]
Acetyl-CoA:ACP transacylase (thiolactomycin-insensitive)	(not mapped)	
3-Ketoacyl-ACP reductase	(<i>fabG?</i>)	[99]
3-Hydroxyacyl-ACP dehydrase	(not mapped)	
Enoyl-ACP reductase	(<i>fabG?</i>)	[99]
3-Ketoacyl-ACP synthase I	<i>fabB</i>	[117]
3-Ketoacyl-ACP synthase II	<i>fabF</i>	[141]
3-Hydroxydecanoyl-ACP dehydrase	<i>fabA</i>	[45]
2. Glycerophospholipid synthesis		
Glycerol-3-phosphate acyltransferase	<i>plsB</i>	[138]
1-Acyl-glycerol-3-phosphate acyltransferase	<i>plsC</i>	[35]
3. Lipid A synthesis		
UDP-N-acetylglucosamine acyltransferase	<i>lpxA</i>	[106]
UDP-3-O-(R-3-hydroxymyristoyl)- glucosamine N-acyltransferase	<i>firA</i>	[54,118]

* Protein and enzymes are listed in groups according to their functions in FA metabolism.

acetyl-ACP with KAS-I or II *in vivo*. Overproduction of KAS-I may result in the formation of acetoacetyl-ACP to bypass both the acetyl transacylase and KAS-III routes (Fig. 1) [231]. However, considering the normally large acetyl-CoA pool in *E. coli* available for condensation by KAS-III [193] and the ATP consumption required for acetyl-ACP formation from acetyl-CoA via synthase I (Fig. 1), the route totally dependent on KAS-I should not dominate the first condensation.

Every cycle of fatty acyl chain elongation begins with an irreversible condensation reaction between acyl-ACP and malonyl-ACP to form 3-ketoacyl-ACP which is two carbons longer than the original acyl-ACP (Fig. 1). The 3-ketoacyl-ACP formed from condensation is subsequently reduced by an NADPH-dependent 3-ketoacyl-ACP reductase, dehydrated by 3-hydroxyacyl-ACP dehydrase and reduced again by enoyl-ACP reductase to generate a saturated acyl-ACP (Fig. 1), which in turn can serve as the substrate for another cycle of elongation. The condensation of malonyl-ACP and saturated fatty acyl-ACP is catalyzed by either KAS-I or II [50,71]. A malonyl-ACP binding site was found in all three KAS enzymes but a fatty acyl binding site only exists in KAS-I and II [50,103]. All three condensing enzymes are inhibited by the antibiotic thiolactomycin and are protected from this inhibition by malonyl-ACP, suggesting that the malonyl-ACP binding site on the enzyme is targeted by thiolactomycin [103,141,165]. Another very useful inhibitor in the study of FA synthesis is cerulenin, which specifically blocks 3-ketoacyl-ACP synthases I and II, but not III [49,109]. The cysteine residues in the active site of the condensing reaction domain were found to be covalently modified by cerulenin [65].

Upon elongation of the acyl chain to C₁₀ (ten carbons long), the biosynthetic pathways of saturated and unsaturated fatty acids diverge (Fig. 1). The 3-hydroxydecanoyl-ACP (3-HO-10:0-ACP) could be dehydrated either by 3-hydroxyacyl-ACP dehydrase or 3-hydroxydecanoyl-ACP dehydrase. The action of 3-hydroxydecanoyl-ACP dehydrase [218] not only removes a water molecule from 3-hydroxydecanoyl-ACP to form *trans*-2-decenoyl-ACP (*trans*-2-10:1-ACP) but also isomerizes a portion of this

product to *cis*-3-decenoyl-ACP (*cis*-3-10:1-ACP) which is further elongated to monounsaturated acyl-ACPs up to C₁₈. In vitro study demonstrated that 3-HO-10:0-ACP was converted into equal amounts of *trans* and *cis* products [78]. Increased intracellular levels of 3-hydroxydecanoyl-ACP dehydrase resulted in an increase in saturated rather than unsaturated FA synthesis, suggesting that the level of enzyme activity normally present does not limit the rate of unsaturated FA synthesis [32]. The condensation of malonyl-ACP with 10:1, 12:1 or 14:1-ACP is catalyzed by KAS-I (Fig. 1, enzyme 5) only, while KAS-II (Fig. 1, enzyme 6) catalyzes the conversion of *cis*-9-16:1-ACP (*cis*-9-palmitoleoyl-ACP) to *cis*-11-18:1-ACP (*cis*-11-vaccenoyl-ACP) (Fig. 1) [70,71]. Very little *cis*-11-18:1 was found in KAS-II mutants (*fabF*), indicating that KAS-II plays a critical role in synthesis of this unsaturated fatty acid. 3-Hydroxyacyl-ACP dehydrase simply catalyzes the formation of *trans*-2-decenoyl-ACP which continues the elongation of saturated fatty acids to 16 carbons [46].

The saturated fatty acid 16:0 and unsaturated fatty acids *cis*-9-16:1 and *cis*-11-18:1 are the major fatty acyl groups in glycerophospholipid (see below). This composition varies with growth temperature and growth phase. The distribution of fatty acyl groups in glycerophospholipid is 18% for *cis*-11-18:1, 35% for *cis*-9-16:1 and 45% for 16:0 in a typical *E. coli* strain grown at 37°C, compared to 45% for *cis*-11-18:1, 35% for *cis*-9-16:1 and 18% for 16:0 at 25°C [46]. A dramatic increase in *cis*-11-18:1 vs 16:0 in phospholipid was observed within 30 seconds of temperature shift from 42°C to 24°C, while synthesis of *cis*-9-16:1 was unaffected [69]. Inhibition of RNA or protein synthesis did not affect the compositional alteration of fatty acid when temperature was changed, indicating this thermal regulation is exerted at the level of enzyme activity rather than protein expression [69]. Both biochemical and genetic analyses indicated that KAS-II is the only enzyme responsible for thermal regulation since the lack of 3-ketoacyl-ACP synthase II resulted in the loss of thermal regulation of fatty acyl composition, although its function in *cis*-11-18:1 synthesis could be complemented by overexpression of KAS-I [51].

Genes encoding several enzymes of FA synthesis have been cloned and sequenced (Table I). These genes are located at several sites on the *E. coli* chromosome. It should be noted that *fabH*, *fabD*, *fabG*, *acpP* and *fabF* are organized sequentially around the 24 min position of the *E. coli* chromosome [99,178], but it is yet unclear whether all these genes are in the same operon. It was suggested from DNA sequence analysis that the *fabG* gene, cotranscribed with *acpP*, may encode one of the two reductases in FA synthesis (Table I) [99,178]. Genes for the two subunits of acetyl-CoA carboxylase, *accB* and *accC*, are also cotranscribed [134] from the 72 min position of the *E. coli* chromosome [136].

2. Acyl-ACP provides acyl groups for different biological components in *E. coli*.

When saturated or unsaturated acyl chains reach a certain length they are transferred to various biochemical components by different enzymes. The acyl-ACP pool is responsible for providing fatty acyl groups for the synthesis of glycerophospholipid, lipopolysaccharide, minor neutral lipids and for protein modification.

a) Glycerophospholipid synthesis and its relationship with FA synthesis.

Glycerophospholipids, which account for about three quarters of intracellular fatty acyl moieties, are predominant in both the inner membrane and the inner layer of the outer membrane. Their synthesis is dependent on the sequential acyl transfer from acyl-ACP to glycerol-3-phosphate (G3P) and 1-acyl-glycerol-3-phosphate (1-acyl-G3P) by membrane bound acyltransferases [35,192]. G3P acyltransferase initiates glycerophospholipid synthesis by transferring the acyl group from acyl-ACP or acyl-CoA to G3P to form 1-acyl-G3P. 1-Acyl-G3P acyltransferase transfers the second fatty acyl chain from acyl-ACP (also acyl-CoA in vitro) to form phosphatidic acid, the key intermediate in glycerophospholipid synthesis (Fig. 2). Both enzymes, as with many other enzymes of glycerophospholipid synthesis, are located on the cytoplasmic face of the inner cell membrane [35,192]. Interaction between G3P acyltransferase and ACP has been observed:

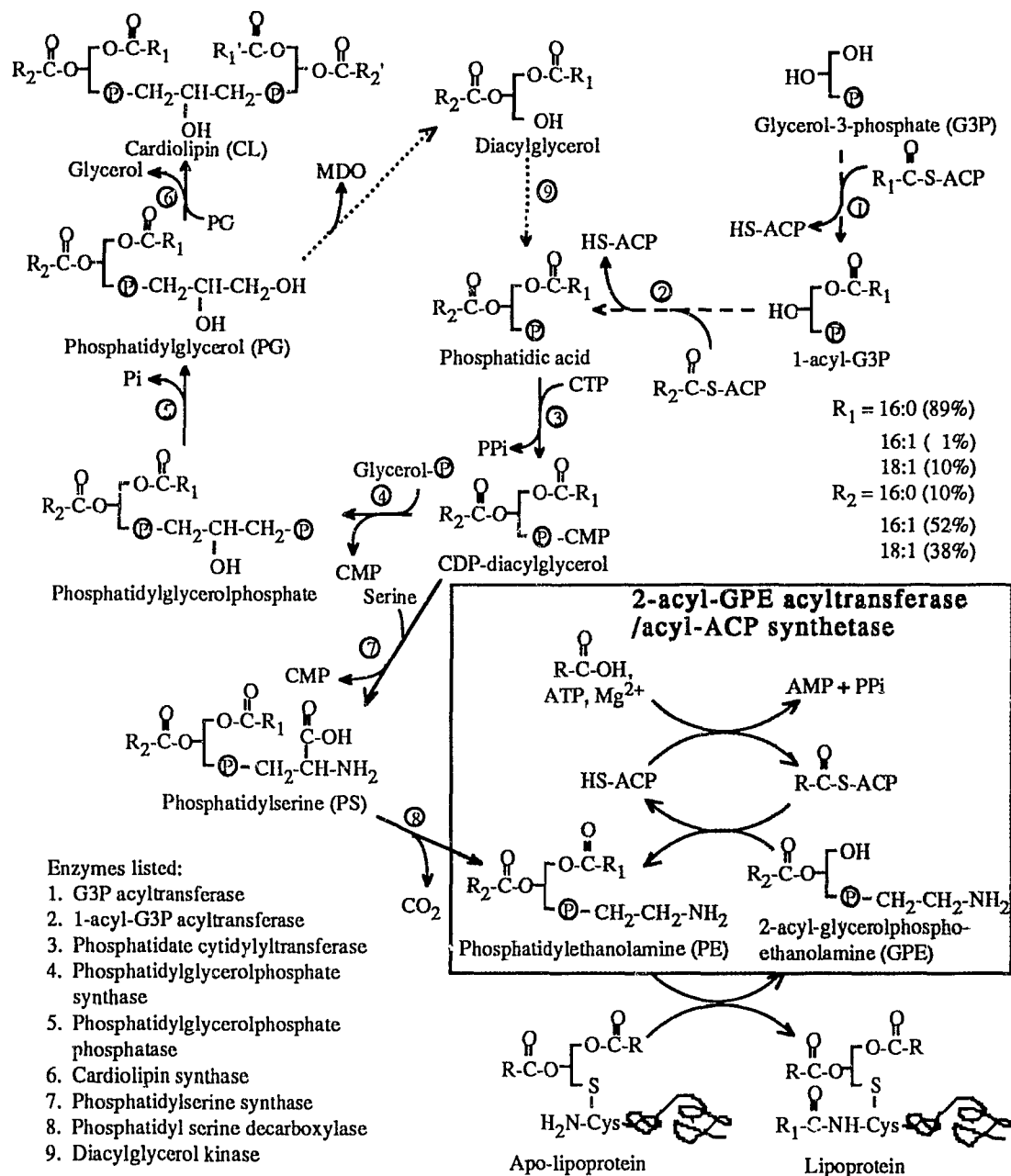


Fig. 2. Metabolic pathways of glycerophospholipid synthesis, turnover of the 1-acyl group of phosphatidylethanolamine, and the diacylglycerol cycle in *E. coli*. The delivery of acyl groups from acyl-ACP to lipid synthesis is shown with dashed lines and the acyl group specificity at two different positions of glycerol is indicated [99]. 2-acyl-GPE acyltransferase-catalyzed transacylation and fatty acyl-ACP synthesis is shown in gray. The diacylglycerol cycle is shown as dotted arrows. Enzymes catalyzing the reactions are listed on the bottom left. MDO = membrane-derived oligosaccharides $\textcircled{P}$ = phosphate.

membrane vesicles prepared from an *E. coli* strain containing amplified G3P acyltransferase have more ACP binding sites than those from wild type strain [8]. Moreover, *E. coli* ACP bearing a photoactivable aryl azide derivative can be specifically cross-linked to an inner membrane protein, probably the G3P acyltransferase [9]. A short region of homology was found among the three acyltransferases, G3P acyltransferase, 1-acyl-G3P acyltransferase and UDP-N-acetyl-glucosamine acyltransferase (see below), all of which utilize acyl-ACP as acyl donor in the acyl transfer reaction [35].

The conversion of phosphatidic acid to CDP-diglyceride is catalyzed by CDP-diglyceride synthase, an enzyme involved in initiation of glycerophospholipid synthesis (Fig. 2). Glycerophospholipid consists mainly of phosphatidylethanolamine (PE, 75% of glycerophospholipid), phosphatidylglycerol (PG, 15-20%) and diphosphatidylglycerol or cardiolipin (CL, 5-10%) (see figure 2 for biosynthesis and structures) [99].

As G3P acyltransferase is at a point that links fatty acid and glycerophospholipid synthesis (Fig. 2), this enzyme was originally considered to be the rate-controlling step in glycerophospholipid synthesis [219]. However, Rock and Jackowski [193] observed that acyl-ACPs of chain lengths suitable for G3P acyltransferase account for less than 1% of the total ACP pool and do not accumulate during cell growth unless the initial step of glycerophospholipid synthesis is arrested, strongly suggesting that this enzyme is not the rate limiting step. By examining intracellular acyl-ACP pools, they also suggested that the first condensation in acetoacetyl-ACP formation may be the rate-controlling point in fatty acid biosynthetic pathway [109]. Ninety-four percent of ATP consumed in *E. coli* glycerophospholipid synthesis is spent on FA synthesis, implying that a rate-controlling point of glycerophospholipid synthesis at an early stage of FA synthesis would be most logical to save energy consumption [99]. Li and Cronan [136] recently demonstrated that transcription of the *acc* genes for all four subunits of acetyl-CoA carboxylase (Fig. 1, enzyme 1 and Table I) is positively correlated with the cell growth rate varied by nutrition limitation, nutrition transition, or dilution of stationary-phase cultures, suggesting that this

enzyme complex may be at the control point for glycerophospholipid synthesis. Since an increased *accC* and *accB* gene copy number did not have much effect on gene expression, they suggested that a positive activator may be needed for activation of gene transcription.

Although both G3P acyltransferase and 1-acyl-G3P acyltransferase are unlikely to be rate-limiting in glycerophospholipid synthesis, they do however play a key role in the asymmetric distribution of fatty acids between the 1- and 2-positions of the glycerol backbone (Fig. 2). Saturated and unsaturated fatty acids predominate at the 1-position and 2-position, respectively, of the glycerolphosphate backbone. In vitro experiments demonstrated that G3P acyltransferase has a higher $V_{\max}$ and a lower K_m for saturated vs unsaturated fatty acyl-ACPs. Blocking acyl transfer to the 2-position in a 1-acyl-G3P acyltransferase mutant strain resulted in accumulation of 1-acyl-G3P; the ratio of 16:0 to 18:1 at the 1-position was very low at 1.5 [34], compared to 9 in normal cells (Fig. 2), and 16:1 was not detected, showing that 16:1 is a very poor substrate for G3P acyltransferase although it is the favored substrate for 1-acyl-G3P acyltransferase. The increased incorporation of 18:1 in this mutant cell is possibly due to the increased intracellular 18:1-ACP pool resulting from blocked utilization of 16:1-ACP in the 2-position. Although glycerophospholipid normally has unsaturated fatty acid at its 2-position, the deprivation of intracellular unsaturated fatty acid results in saturated fatty acid occupancy at this position. This combined evidence strongly suggests that in addition to substrate specificity of acyltransferase, the selectivity of fatty acyl transfer also depends on the available fatty acyl pool.

b) Incorporation of fatty acyl groups into lipid A.

Lipopolysaccharide is the major lipid component of the outer layer of the outer membrane and forms a protective barrier for the cell against the action of bile acids, hydrophobic compounds and lipolytic and proteolytic enzymes. It consists of three domains: lipid A, core polysaccharide and O-specific polysaccharide (Fig. 3). The highly variable O-specific polysaccharide extends outward from the membrane but is linked to

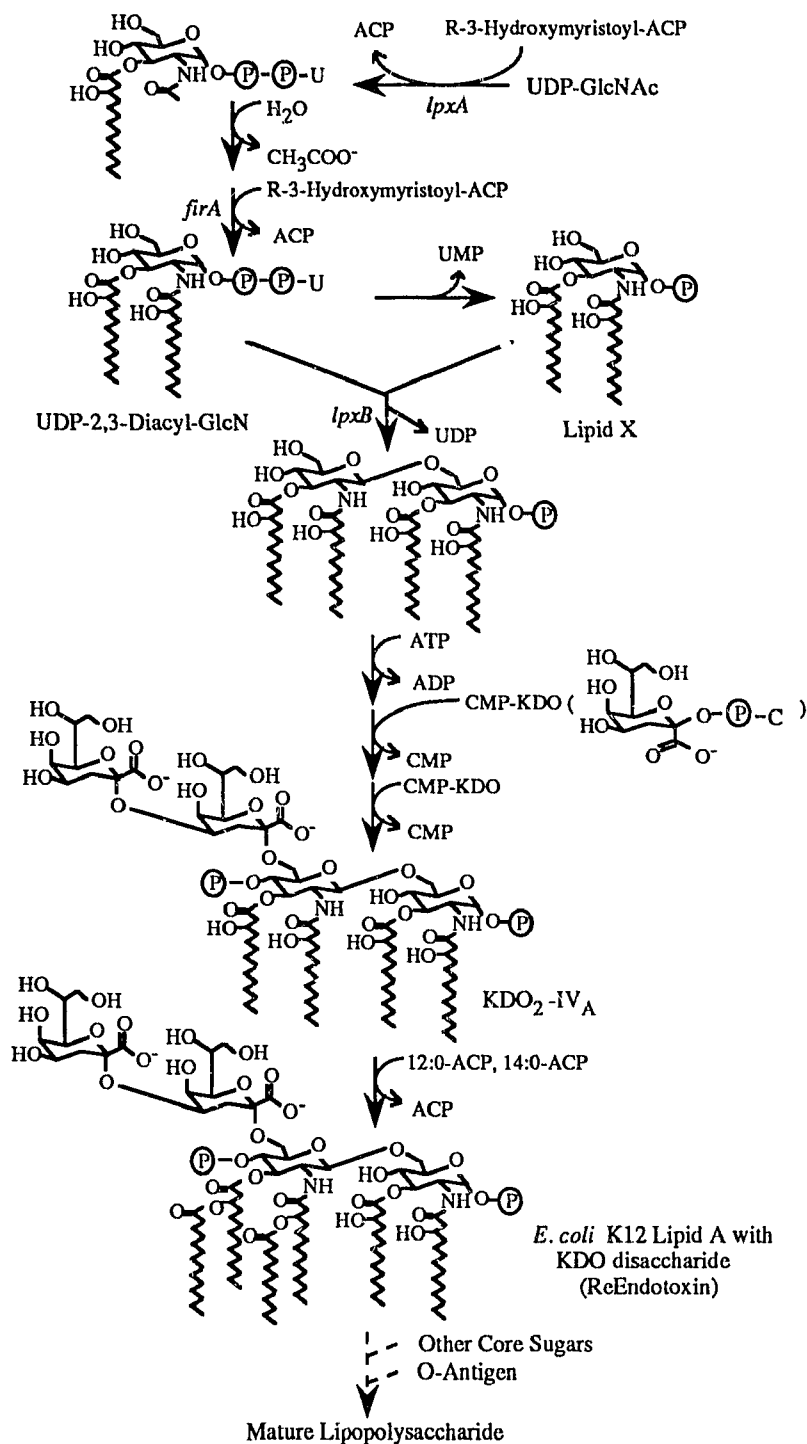


Fig. 3. Lipid A synthesis in *E. coli*. Enzymes encoded by the *lpxA* and *firA* genes catalyze the incorporation of 3-hydroxymyristoyl groups into lipid A (Table I). The incorporation of 12:0 and 14:0 is also indicated. ReEndotoxin is the minimal lipopolysaccharide required for *E. coli* growth. The picture is adapted from [177]. C, cytidine; U, uridine.

lipid A through the "bridge" of core polysaccharide. Both O-specific polysaccharide and most of the core polysaccharide can be deleted by mutation without affecting cell growth but lipid A is essential for viability.

Unlike G3P acylation, acylation during lipid A synthesis is absolutely specific for acyl-ACP rather than acyl-CoA as substrate [177]. Lipid A contains three types of acyl chains: 3-HO-14:0 (3-hydroxymyristic acid), 12:0, and 14:0. The first step of lipid A synthesis is catalyzed by UDP-N-acetylglucosamine O-acyltransferase which transfers 3-hydroxymyristate to the 3,3' (O-linked) position of the glucosamine backbone (Fig. 3). Defects in this enzyme are lethal to the cell [66]. However, this reaction is unlikely to be the committed step in lipid A synthesis as the reaction is thermodynamically unfavorable with a K_{eq} of 0.01, inconsistent with a model S→O acyl transfer reaction [5]. More likely, initiation of lipid A synthesis is committed by deacetylation, the step following this O-acyl transfer reaction [5]. UDP-3-O-(R-3-hydroxymyristoyl)-glucosamine N-acyltransferase, which is homologous to the O-acyltransferase, adds another 3-hydroxymyristoyl group to the 2,2' (N-linked) position of the glucosamine backbone (Fig. 3). Both enzymes specifically use 3-HO-14:0-ACP as their substrate [243]. The mechanism by which 3-HO-14:0-ACP is diverted into lipid A synthesis rather than continual elongation in FA synthesis remains unclear. A gene (*orf-17*) which may encode a 3-hydroxymyristoyl-ACP dehydrase (Fig. 1, enzyme 11) was recently found just upstream of UDP-N-acetylglucosamine acyltransferase gene (*lpxA*) and may be involved in controlling the flux of 3-hydroxymyristoyl-ACP to lipid A synthesis [141]. Enzymes specifically using 12:0-ACP or 14:0-ACP for the incorporation of 12:0 and 14:0 into (KDO)₂-IV_A (Fig. 3) were detected recently [20]: a cytosolic fraction was found to catalyze acyl transfer with 12:0-ACP only while a membrane fraction transferred both 12:0 and 14:0.

c) Involvement of fatty acyl-ACP in protein modification.

Acyl-ACP is also involved in protein fatty acyl modification. A novel protein fatty acylation discovered recently [96,98] specifically utilizes long chain acyl-ACP to activate

haemolysin. Haemolysin, a 110 kDa toxin protein produced and secreted by pathogenic *E. coli*, is synthesized as nontoxic prohaemolysin and then activated to toxic haemolysin by transacylation before being secreted. The transacylation is catalyzed by a 20 kDa activator enzyme HlyC, which is cotranslated with haemolysin protein, and dependent on long chain fatty acyl-ACP with 14:0-ACP being the most active. After secretion, haemolysin binds to mammalian cell membranes and lyses cells. Protein fatty acylation appears to play a key role in direct binding of haemolysin to mammalian membranes [96].

B. The metabolism of exogenous fatty acid in *E. coli*.

1. Exogenous fatty acids do not have access to biosynthetic pathways.

Although *E. coli* cells are capable of endogenous FA synthesis, they are also able to metabolize extracellular fatty acid, an adaptation to their normal environment (the intestine) rich in long chain fatty acids. When suitable exogenous long chain fatty acids such as 16:0 or 18:1 are available, intracellular FA synthesis is apparently decreased [217] and enzyme systems for FA uptake and utilization, normally expressed at very low levels, are induced. Exogenous long chain fatty acid is transported into cells and activated to form acyl-CoA. Suitable fatty acids are used not only for energy production by β -oxidation but also as substrates for G3P acyltransferase [192] (or 1-acyl-G3P acyltransferase in vitro as well) for glycerophospholipid synthesis, as discussed above.

FA synthesis and degradation may be physically separated in eukaryotic cells: synthesis occurs in cytosol while degradation occurs inside mitochondria and peroxisomes. Therefore, acyl-CoA from β -oxidation and from FA synthesis may be chemically identical but do not exchange. In contrast, the separation of different pathways in prokaryotic cells is exerted by chemical distinction or channeling of intermediates. As described above, only acyl-ACP intermediates can be used for further acyl chain elongation in *E. coli*. Fatty acids activated to acyl-CoA are primarily targeted toward degradation by β -oxidation. Exogenous

fatty acids are not elongated in *E. coli*, indicating that they do not have access to ACP-related biosynthetic pathways [216].

2. The *fad* regulon is essential for utilizing long chain fatty acid.

Only long chain fatty acids (C₁₂ to C₁₈) can induce the FA degradative system (*fad*) which is essential for the transport, activation and β -oxidation of long chain fatty acid [167]. Although the enzymes in activation and β -oxidation may also utilize medium (C₇ to C₁₁) and short (C₄ to C₆) chain fatty acyl-CoA as substrates, these fatty acids are unable to induce the *fad* system. Therefore, wild type *E. coli* can survive on long, but not medium or short, chain fatty acid as sole carbon source. The genes encoding proteins of the *fad* system are distributed over several sites on the *E. coli* chromosome and form a regulon, the *fad* regulon, controlled by FadR protein. The *fad* regulon consists of at least five members (Table II): *fadR* for the transcriptional regulator, *fadL* for a receptor protein of long chain FA transport (FLP or FadL protein), *fadD* for acyl-CoA synthetase, *fadE* for an ATP-dependent acyl-CoA dehydrogenase, and *fadA* and *fadB* for a multifunctional oxidation enzyme complex. Genes of the *fad* regulon are constitutively expressed in *fadR* mutant cells [170].

a) Transport of long chain fatty acid across cell membrane via FLP.

FLP, the long chain FA receptor and transport protein, is integrated into the outer membrane (Fig. 4) and is necessary to deliver exogenous long chain fatty acid across cell membranes. This acidic protein (pI=5.2) migrates as a 43 kDa band in SDS-polyacrylamide gel electrophoresis (PAGE) if denatured in SDS at 100°C before electrophoresis but migrates faster (apparent size 33 kDa) when solubilized in SDS at 37°C. The true size determined by a combination of DNA sequence and N-terminal amino acid sequence analysis of the protein is 46 kDa [14]. FLP is synthesised as pro-FLP with a 27 residue signal sequence which is removed when it is incorporated into the outer membrane (102). FLP contains a series of hydrophobic regions which may be important in FA transport. This protein may be a monomer within the outer membrane [15] and is associated with

Table IIProteins or enzymes involved in exogenous FA uptake in *E. coli**

Protein or enzyme	Gene	Reference
1. Long chain FA transport, activation and β-oxidation (<i>fad</i> regulon)		
<i>Fad</i> regulon negative transcriptional regulator	<i>fadR</i>	[56]
Outer membrane receptor protein	<i>fadL</i>	[14]
Acyl-CoA synthetase	<i>fadD</i>	[16]
Acyl-CoA dehydrogenase	<i>fadE</i>	gene unidentified
Multifunctional oxidation enzyme complex	<i>fadB</i> and <i>fadA</i>	[55,163]
2. Short chain FA transport, activation and degradation (<i>ato</i> system)		
Acetoacetyl-CoA transferase	<i>atoD</i> and <i>atoA</i>	[111]
Thiolase II	<i>atoB</i>	[111]
<i>Ato</i> operon positive transcriptional regulator	<i>atoC</i>	[111]
3. Acetate activation		
Acetate kinase	<i>ack</i>	[148]
Phosphotransacetylase	<i>pta</i>	[133]
Acetyl-CoA synthetase	(unidentified)	
4. Glyoxylate shunt (<i>ace</i> system)		
Isocitrate lyase	<i>aceA</i>	[41]
Malate synthase	<i>aceB</i>	[41]
Isocitrate dehydrogenase kinase-phosphatase	<i>aceK</i>	[41]
<i>AceBA</i> operon negative transcriptional regulator	<i>iclR</i>	[42,224]
5. Acyl-CoA independent long chain FA incorporation.		
2-acylglycerophosphoethanolamine acyltransferase/acyl-ACP synthetase	<i>aas</i>	[78]

* Enzymes and proteins are listed in groups according to their roles in FA metabolism but they may also be involved in the pathways of other groups.

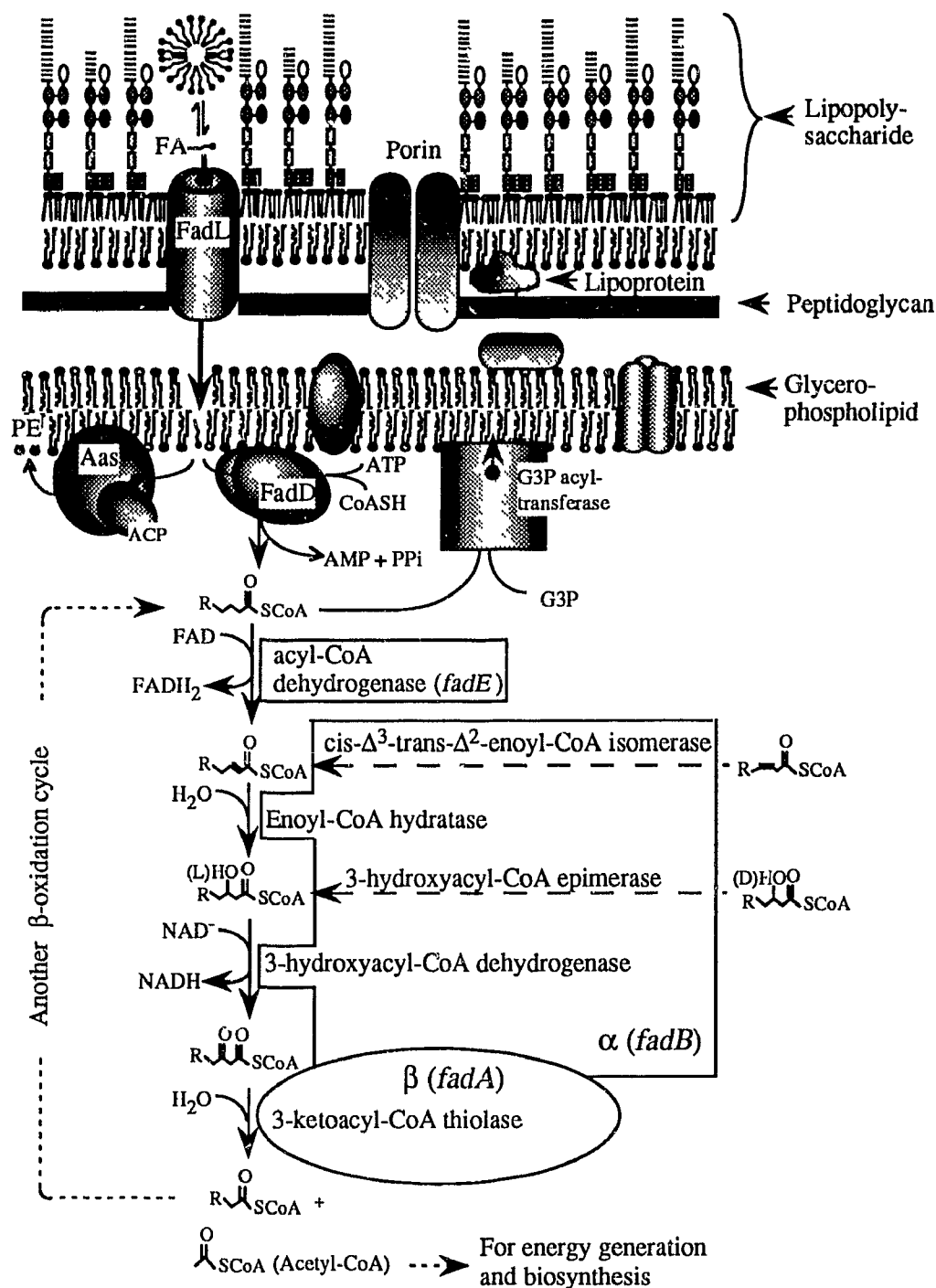


Fig. 4. Pathways of transport, activation, acyl transfer and oxidation of exogenous long chain fatty acid in *E. coli*. The abbreviations used are: Aas, 2-acyl-sn-glycero-3-phosphoethanolamine acyltransferase/acyl-ACP synthetase; ACP, acyl carrier protein; FA, fatty acid; FadD, acyl-CoA synthetase; FadL, fatty acid transport protein; G3P, glycerol-3-phosphate; PE, phosphatidyl-ethanolamine. Figure is adapted from [102] and [166].

peptidoglycan [12]. Mangroo and Gerber [146] demonstrated that FLP can be specifically labeled on isolated *E. coli* membranes with a photoreactive fatty acid analogue and that the labeling process is saturable. It appears that the cellular uptake of long chain fatty acids is tightly coupled to their activation to form acyl-CoA. When fatty acid activation is blocked by a mutation in the *fadD* gene, FA transport is inhibited allowing the FLP-specific FA binding activity to be measured. Binding studies have shown that a *fadD fadL*⁺ strain binds more long chain fatty acid than *fadD fadL* strain and that the binding of medium chain fatty acids is undetectable [13], consistent with the early observation that the *fadL* product is necessary for the transport of long but not medium chain fatty acid [168]. Long chain FA binding to *fadD fadL*⁺ strain is also saturable [13]. Some of the amino acid residues and domains of FLP responsible for FA binding or transport were identified recently [127,128]. Although FLP has been recognized as the FA receptor and transport protein on the outer membrane, it is unclear at present whether there are other FA transport proteins within the inner membrane or FA binding proteins in the periplasmic space. Labeling of *E. coli* inner membrane with a photoreactive fatty acid analogue did not reveal any FA binding proteins, suggesting that FA permeation across the inner membrane occurs by simple diffusion without protein mediation [146]. FA uptake by *E. coli* is decreased when cells are grown at high osmolarity, even in the presence of long chain fatty acid [89]. It was suggested that the depression of FA uptake by high osmolarity is due to transcriptional repression of the *fadL* gene by two other possible regulators, OmpR and EnvZ proteins.

b) Structure and function of acyl-CoA synthetase (FadD).

Acyl-CoA synthetase encoded by the *fadD* gene plays a key role in transport of long chain fatty acid and its conversion to metabolically active CoA thioesters for subsequent degradation or incorporation into phospholipid (Fig. 4). This enzyme may consist of two identical subunits of 62 kDa [16,116]. Acyl-CoA synthetase activity is ATP dependent and has a broad FA chain length specificity ranging from C₁₀ to C₁₈ with 12:0 showing maximal activity in vitro [116]. *FadD* mutants are unable to accumulate exogenous fatty

acids of any length in the cytosol, indicating that acyl-CoA synthetase is required for the transport and/or activation of both long and medium chain fatty acids [167]. It is, however, unclear how acyl-CoA synthetase is involved in long chain FA transport. *E. coli* fatty acyl-CoA synthetase is a cytoplasmic enzyme since 90% of the enzyme was found in cytosol fractions [116], but it may also be loosely associated with the cytosolic face of the inner membrane [204]. Mangroo and Gerber [147] recently observed that oleoyl-CoA formation by the partially purified enzyme is increased 20-fold by adding Triton X-100 to the reaction and another 4-fold by addition of bacterial membranes. Acyl-CoA synthetase may be regulated *in vivo* by reversible association with the inner membrane, as incubation of *E. coli* with D-lactate prior to cell lysis resulted in association of all the enzyme activity with the membrane fraction, compared to 20% without preincubation.

As indicated above, *E. coli* fatty acyl-CoA synthetase (but not FLP) is also essential for uptake of medium chain fatty acid (C₇ to C₁₁). A kinetic study of medium chain FA uptake suggested that transport includes both carrier-regulated and free diffusion processes [143]. FLP may also be involved in medium chain FA transport but its mutation did not affect FA uptake [231]. Uptake of 8:0 was totally inhibited in the presence of equimolar 14:0 in a *fadL*⁺ strain, but was not depressed at any level in *fadL* mutant cells. This uptake was only 50% inhibited in the presence of medium chain fatty acid in both *fadL*⁺ and *fadL* cells, indicating that free diffusion of medium chain fatty acid may be decreased if the FLP-mediated transport system is active.

c) β -oxidation of fatty acids.

After long chain fatty acid is delivered into the cell and activated to form acyl-CoA, it is either degraded by β -oxidation or incorporated into glycerophospholipid (Fig. 4). Every cycle of fatty acyl-CoA β -oxidation creates one molecule of acetyl-CoA and an acyl-CoA shortened by two carbons. Acyl-CoA dehydrogenase is responsible for the first step of the β -oxidation cycle (Fig. 4). Since *E. coli* acyl-CoA dehydrogenase has not been purified or characterized, very little information about this enzyme has been obtained [167], although

mutants in the *fadE* gene have been isolated. More details are available about the remaining enzymes in the β -oxidation pathway. The subsequent reactions of β -oxidation are catalyzed by a multifunctional enzyme complex rather than individual enzymes (Fig. 4). The purified enzyme complex has a molecular mass of 260 kDa and has an $\alpha_2\beta_2$ subunit structure in which activities of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase are components of α -subunits while 3-ketoacyl-CoA thiolase is associated with β -subunits [175]. The degradation of unsaturated fatty acid requires two additional activities: an isomerase converting Δ^3 -cis-enoyl-CoA to Δ^2 -trans-enoyl-CoA, and an epimerase converting (D)-3-hydroxyacyl-CoA to (L)-3-hydroxyacyl-CoA. These two enzyme activities are also associated with the α -subunits. The large α -subunit encoded by the *fadB* gene has a predicted molecular mass of 80 kDa and the small β -subunit encoded by *fadA* gene is 41 kDa [55,245,246]. Both genes are in the same operon and are cotranscribed.

Mutants defective in *fadE*, *fadA* or *fadB* gene take up significantly less medium or long chain fatty acids than do wild type cells, with the *fadE* strain lacking acyl-CoA dehydrogenase in particular exhibiting low FA uptake capacity comparable to the *fadD* mutant [143]. This indicates that FA transport is tightly coupled to FA oxidation. However, exogenous long chain fatty acid retains its ability to be incorporated into glycerophospholipid even in cells >90% depleted of ACP [44], and *fadBA* or *fadE* mutations do not restrict unsaturated FA auxotrophs (*fabA*) from incorporating unsaturated fatty acids into lipids [167]. These observations suggest that acyl-CoA derived from acyl-CoA synthetase can also access lipid biosynthetic pathways through G3P acyltransferase, which normally utilizes acyl-ACP when exogenous fatty acid is not available (Fig. 2 and Fig. 4).

d) FadR protein regulates both the *fad* regulon and the *fabA* gene.

The FadR protein is a multifunctional regulator which specifically represses *fad* gene expression but activates transcription of the *fabA* gene in FA synthesis [88]. FadR protein represses gene expression by binding to the transcription beginning regions of *fadB* or

fadL [56,57]. This binding was inhibited specifically by long chain acyl-CoAs in vitro, consistent with the fact that *fad* genes are induced only by long chain fatty acid ($\geq C_{12}$) in medium. Medium chain fatty acyl-CoAs cannot bind to FadR to release transcriptional repression of *fad* genes and thus are unable to induce *fad* gene expression. Therefore, only *fadR* mutant cells in which the *fad* system is constitutively expressed are able to grow on medium chain fatty acids as a sole carbon source.

The *fabA* gene which encodes 3-hydroxydecanoyl-ACP dehydrase is positively regulated by FadR. The binding of FadR protein to the -40 position of the *fabA* gene was also antagonized by long chain acyl-CoAs in vitro [57,88], consistent with the inhibition of *fabA* gene expression *in vivo* in the presence of long chain fatty acids.

3. Other enzyme systems involved in FA catabolism in *E. coli*.

a) The *ato* system for utilization of exogenous short chain fatty acid.

The mechanism of transport of short chain fatty acid (C_4 to C_6) into *E. coli* is less understood but likely different from that for long or medium chain fatty acids. The transport and activation of short chain fatty acid requires acetoacetyl-CoA transferase, a tetrameric protein composed of two α -subunits (encoded by the *atoA* gene) and two β -subunits (encoded by the *atoD* gene) [111]. This enzyme replaces the acetyl group of acetyl-CoA with a short chain fatty acid to form short chain fatty acyl-CoA that is further β -oxidized by the same enzymes used in long chain FA degradation. Thiolase II (encoded by the *atoB* gene) is also needed for the cleavage of acetoacetyl-CoA into two molecules of acetyl-CoA. These genes are contained within an operon that is regulated by a positive regulatory element (encoded by *atoC* gene). The *atoDBA* operon is not constitutively expressed but can be induced by acetoacetate. Both 4:0 and 6:0 are unable to induce the expression of either *ato* genes or the *fad* system in wild type *E. coli* cells and, therefore, cannot be used as sole carbon source for *E. coli* growth except by a double mutant where both *ato* and *fad* systems are expressed constitutively [112]. The transport of short chain fatty acids is independent of *fadL* and *fadD* products and they do not competitively inhibit

the uptake of medium or long chain fatty acid [201]. However, this uptake is also contingent upon subsequent FA metabolism [167].

b) Enzymes involved in acetate activation.

Acetate must be activated to acetyl-CoA for use as sole carbon and energy source by *E. coli*. Two pathways may be involved in acetate activation. One is catalyzed by two constitutively expressed enzymes, acetate kinase (encoded by *ack* gene) and phosphotransacetylase (encoded by *pta*) (Fig. 5) [133]. Mutants defective in either gene cannot utilize acetate as sole carbon source, indicating both enzymes are essential for *E. coli* to grow optimally on acetate as sole carbon source. The fact that *ack* or *pta* mutant cells grown on glycerol, but not glucose, are able to incorporate labeled acetate led to the discovery of another activation pathway (Fig. 5), involving acetate activation by an inducible acetyl-CoA synthetase which is regulated by catabolite repression [19].

c) The glyoxylate shunt.

It should be noted that the induced *fad* or *ato* systems alone cannot fully support growth of *E. coli* on fatty acid as sole carbon source since both systems ultimately produce acetyl-CoA. Every molecule of acetyl-CoA is completely degraded by the citric acid cycle (Fig. 5) to generate 24 molecules of ATP and 2 molecules of CO₂. Thus, no net carbon accumulation from acetyl-CoA can occur by this means. Acetyl-CoA can be utilized for FA biosynthesis, as indicated before, but cannot be converted to other key intermediates necessary for synthesis of such biological components as carbohydrates, protein and nucleic acid. To grow on fatty acid or acetate as sole carbon source, a metabolic pathway called the glyoxylate shunt is induced when *E. coli* is cultured with these nutrients. The induced isocitrate lyase (encoded by *aceA*) and malate synthase (encoded by *aceB*) plus other enzymes in the citric acid cycle divert isocitrate from this pathway, bypassing the CO₂-creating steps and converting two molecules of acetyl-CoA to one molecule of oxaloacetate which is further used for cellular biosynthesis (Fig. 5). The *aceB*, *aceA* and *aceK* (see below) genes are in same operon which is controlled by a repressor protein

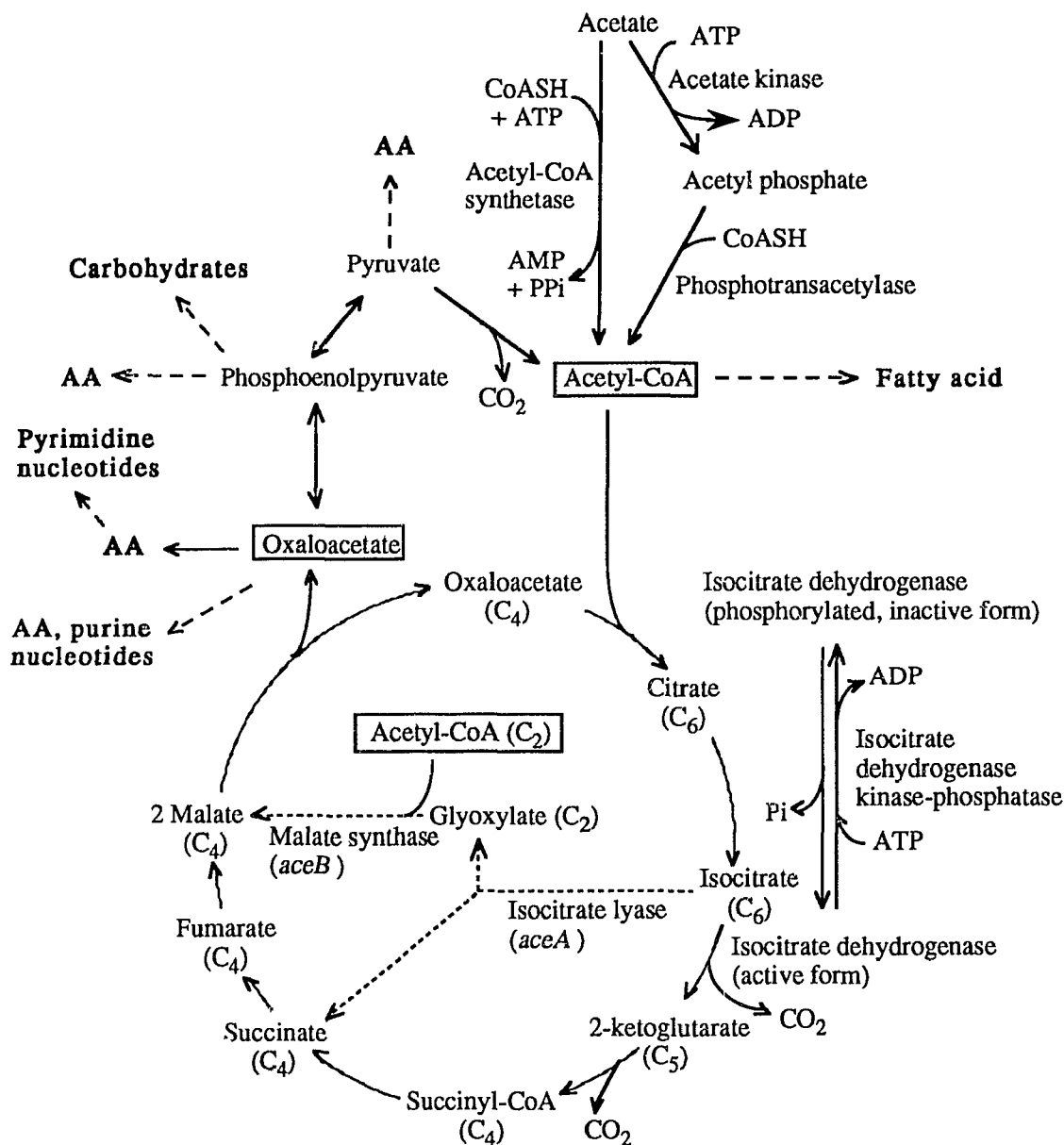


Fig. 5. Pathways of acetate activation, glyoxylate shunt and citric acid cycle in *E. coli*. Shown is the net conversion of two acetyl-CoA into one oxaloacetate molecule through the glyoxylate shunt which is indicated with dotted lines. The solid line indicates single step reactions while dashed lines indicate several metabolic steps. The basic biochemical components are highlighted. The abbreviation used is: AA, amino acid. Part of the figure is adapted from [167].

encoded by the *iclR* gene adjacent to the *ace* operon [144]. Transcription of the *ace* operon is also inhibited by FadR protein, and the combined action of FadR and IclR results in full repression of the *ace* operon. Besides transcriptional regulation, the glyoxylate shunt is also regulated by isocitrate dehydrogenase, the citric cycle pathway enzyme which competes with isocitrate lyase (Fig. 5). The phosphorylation of isocitrate dehydrogenase catalyzed by isocitrate dehydrogenase kinase-phosphatase (encoded by the *aceK* gene) inactivates isocitrate dehydrogenase and thus forces isocitrate through the glyoxylate bypass [68,131]. Biochemical and genetic evidence suggests that this enzyme also catalyzes the dephosphorylation of isocitrate dehydrogenase to restore enzyme activity (Fig. 5) [130].

4. Other pathways for exogenous long chain FA incorporation in *E. coli*.

a) 2-acylglycero-phosphoethanolamine acyltransferase/acyl-ACP synthetase.

Although acyl-CoA synthetase (FadD) is generally required for transport and activation of exogenous long chain fatty acid, some incorporation of long chain fatty acid transported by FLP into phospholipid persists in the absence of FadD [179]. This additional CoA-independent pathway is dependent on an inner membrane enzyme (Aas) (Figs. 2 and 4), which contains both 2-acylglycerophosphoethanolamine (2-acyl-GPE) acyltransferase and fatty acyl-ACP synthetase activities [95]. ACP is tightly but not covalently bound to this enzyme with a K_m of 64 nM under standard assay conditions for 2-acyl-GPE acyltransferase activity. The affinity for acyl-ACP is much less with an apparent K_m of 13 μ M [38]. The *aas* gene sequence predicts an 81 kDa protein which has been overexpressed in *E. coli* [102]. This enzyme has been widely used in preparations of long chain acyl-ACP with chain lengths C_{10} to C_{18} . Triton X-100 is required for acyl-ACP synthetase activity and high concentrations of LiCl are essential for release of acyl-ACP product.

As discussed previously, glycerophospholipid and lipopolysaccharide account for the major portion of fatty acyl moieties found in *E. coli*, but fatty acyl groups are also found in lipoproteins, a group of bacterial membrane proteins with covalently attached lipids (Fig. 4). Although lipoproteins were found to be the major proteins in the outer membrane, some lipoproteins were located in the inner membrane as well [247]. The lipoproteins may function differently from each other, but all are synthesized as protein precursors with a consensus signal sequence which is identified and processed by a lipid modification system. Lipid modification is important for lipoprotein anchoring on the membrane surface. As indicated in Fig. 2, the mature lipoprotein has an amino terminal cysteine that is modified with a sulfhydryl-linked diacylglycerol and an amide-linked fatty acid. The latter is mainly provided by transferring the 1-position fatty acyl moiety of phosphatidylethanolamine [108]. The resulting 2-acyl-GPE stays as a membrane component and can be re-esterified at the 1-position by 2-acyl-GPE acyltransferase (Aas) [185]. This acyl transfer reaction can either utilize acyl-ACP as an acyl donor or fatty acids in the presence of ATP and Mg^{2+} [185]. In *E. coli fadD* mutants, exogenous long chain FA incorporation into lipid was decreased to 2.5% of wild type levels and was exclusively in the 1-position of phosphatidylethanolamine, indicating that incorporation through acyl-CoA synthesis is normally the major pathway [194]. Mutation of the *aas* gene (Table II) blocked acyl-CoA-independent long chain FA incorporation [95], whereas an *fadD-aas* double mutant completely lost the ability to incorporate long chain fatty acid into phospholipid [102]. The *aas* mutants also accumulated 2-acylglycerophosphoethanolamine in the cell membrane, suggesting that 2-acyl-GPE acyltransferase/acyl-ACP synthetase is very active under normal growth conditions [95]. Actually, the 1-position FA turnover of phosphatidylethanolamine accounts for about 3-5% of fatty acyl moieties in this position from FA synthesis in *E. coli* [185].

Although 2-acyl-GPE acyltransferase exhibits a lower K_m for long chain fatty acid than for acyl-ACP *in vitro*, it remains unclear which substrate the enzyme utilizes *in vivo*

during cell growth in the absence of exogenous fatty acid. Acyl-ACPs produced from FA synthesis are relatively abundant, but are unlikely to be the natural substrates for the acyltransferase since the enzyme binds product (ACP) more tightly than acyl-ACP when the latter is used as substrate in vitro. However, another question arises if fatty acid is the natural substrate for the acyltransferase. In contrast to acyl-ACP, very little free fatty acid is found during *E. coli* cell growth even in mutants deficient in FA degradation [124], unless phospholipid synthesis is also inhibited resulting in accumulation of abnormally long and metabolically active free fatty acids inside the cell [47]. *E. coli* contains two thioesterases that could theoretically cleave the thioester bonds of acyl-CoA or acyl-ACP to provide free fatty acid, although acyl-ACPs are much poorer substrates than acyl-CoA in vitro [222]. However, defects in thioesterase I (*tesA*⁻) or II (*tesB*⁻) or both (*tesA*⁻*tesB*⁻) did not display any growth phenotype [30,162]. A possible way to examine whether acyl-ACP or free fatty acid is utilized by 2-acyl-GPE acyltransferase under normal growth conditions is to analyze whether the 1-position FA turnover of phosphatidylethanolamine or glycerophospholipid composition is differentially affected by mutation of the two thioesterases.

b) Uptake of exogenous long chain fatty acid in *fadDfadL* mutant cells.

Early studies to discern the role of FLP in long chain FA transport concluded that FLP is absolutely required [167], since the *fadL* strain failed to deliver long chain fatty acids ($\geq C_{12}$) into the cell [143]. However, this conclusion was challenged recently in a study of protein myristoylation in recombinant *E. coli* [125]. The *Saccharomyces cerevisiae* *NMT1* gene encodes myristoyl-CoA:protein N-myristoyltransferase (Nmt1p) which catalyzes the cotranslational transfer of 14:0 from 14:0-CoA to *S. cerevisiae* ADP-ribosylation factor 1 (Arf1p). Both Nmt1p and Arf1p are cytosolic proteins. Expression of Nmt1p and Arf1p simultaneously in *E. coli* resulted in labeled Arf1p when cells were incubated with exogenous [³H]14:0. Acyl-CoA synthetase was necessary for utilization of exogenous myristate by Nmt1p since labeled Arf1p was undetectable in *fadD* mutants.

However, defective FLP did not block the labeling of Arf1p by [^3H]14:0, indicating that FLP is not essential for import of 14:0 for this purpose. More interestingly, comparison of radioactive products in *fadRfadD*⁺ and *fadRfadD* mutants derived from [^3H]14:0 or [^3H]16:0 labeling indicated that a defect in the *fadD* gene causes accumulation of free [^3H]14:0 or [^3H]16:0 inside the cells. Based on their observations, Knoll and Gordon suggested that FadD is not necessary for import of 14:0 and 16:0 [125]. It should be noted, however, that the cell labeling method in Knoll and Gordon's experiment was different from that by which Nunn et al [143] earlier concluded that both FLP and FadD are necessary for long chain FA uptake. The latter method, which has been used frequently for long chain FA uptake analysis, routinely starves *E. coli* cells in minimal medium to deplete carbon sources prior to assaying FA uptake, to avoid dilution of the radioactive fatty acid in the assay [127,143]. The method of Knoll and Gordon involved direct labeling of *E. coli* cells ($A_{660} = 0.6$) in TB medium without prior starvation and medium change [125]. Moreover, the differing sensitivities of detection of radiolabeled protein (Knoll and Gordon) vs. radiolabeled lipids or cells (earlier method) may have also led to different conclusions.

C. Structure and function of acyl carrier protein.

1. *E. coli* ACP.

a) Primary structure and genomic organization.

ACP is a small acidic protein with a highly conserved amino acid sequence. *E. coli* ACP is by far the best characterized among ACPs of different species. Its amino acid sequence was first determined by Vanaman *et al* in 1968 [235], and its DNA sequence was also determined recently [178], predicting one residue different from Vanaman's protein sequence. *E. coli* ACP contains 77 amino acid residues (Fig. 6), among which 20 are acidic residues (26%) and 6 are basic (8%) [178]. The calculated molecular weight of *E. coli* ACP is 8,860 and the isoelectric point is pH 4.1. Pantetheine 4'-phosphate is

ACP source:

- Gram-negative bacteria: 1. *Escherichia coli* [178]
2. *Rhizobium meliloti* (nodulation bacterium) [172]
3. *Rhodobacter sphaeroides* (phototrophic bacterium) [172]
4. *Anabaena variabilis* (Cyanobacteria) [64]
- Plant: 5. maize [96]
6. spinach-I [96]
7. spinach-II [96]
8. barley-I [96]
9. barley-III [96]
10. Rape 28F10 [200]
- Chloroplast genome: 11. *Cryptomonas* [240]
12. *Cylindrotheca* [97]
- Mitochondria: 13. *Neurospora crassa* [199]
- Gram-positive bacteria: 14. *Saccharopolyspora erythraea* [182]
15. *Streptomyces glaucescens* [210]
- Component of polyketide synthase (PKS) 16. Unknown PKS, *Saccharopolyspora hirsuta* [74]
17. Actinorhodin PKS, *Streptomyces coelicolor* [120]
18. Granaticin PKS, *Streptomyces violaceoruber* [214]
19. Oxytetracycline PKS, *Streptomyces rimosus* [120]
20. Tetracenomycin PKS, *Streptomyces glaucescens* [11]
- NodF protein: 21. *Rhizobium meliloti* [52]
22. *Rhizobium leguminosarum* [52]
23. *Rhizobium trifolii* [52]

Fig. 6. Sequence comparison of different ACPs and ACP-like proteins. The amino acid sequences shown are either determined from protein sequencing or deduced from the DNA sequence and aligned around the highlighted serine which is the phosphopantetheine attachment site. Deleted residues are shown by dots to facilitate better alignment among ACPs. ACPs are arranged in groups and the source of the ACPs are listed. Amino acid sequences surrounding the phosphopantetheine attachment site are underlined. *, identical residues among the group; ≡, highly conserved residues among the group.

A. ACP from Gram-negative bacteria.

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      10      20      30      40      50      60      70
1.  STIEERVKKIIGEQLGVKQ.EEVTNNASFVEDLIGADSLDTVELVMALEEEFDTEIPDEEA EKITTVQAAIDYINGHQA
2.  SDIAERVKKIVIDHLGV.DAEKVSEGA SFIDDLGADSLDTVELVMALEEEFGVEIPDDAADSILTVGDAVKFIEKAQAA
3.  SDIADRVKKIVVEHLGVEE.EKVTETTSFIDDLGADSLDTVELVMALEEEFGIEIPDDAAETIQTFGDAP
4.  SQSETFEKVKKIVIEQLSVENPDTVTPEASFANDLOADSLDTVELVMALEEEFDIEIPDEAAEKITTVQAAVD?IN
      *   ~~~~~~ ~ * *   ~ *   ** ~** ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~

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B. Plant ACP.

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5.  AAKKDTTDKVCEIVKKQLALPDHTEVCGESKFS.ELGADSLDTVEIVMSLEEHFDISVEESSAQTIATVEDAADLIDKLVAGKA
6.  AAKKETIDKVS DIVKEKLALGADVVTADSEFSK.LGADSLDTVEIVMNLEEEFGINVEDDKAQDISTIQQAADVIESLL.EKK
7.  AAKPEMVTKVSDIVKSQLALAEAKVTGETKFS.ELGADSLDTVEIVMKLEEEFGVTVEEENAQTITTIQEAADMIEALQONK
8.  AAMGEAQAKKETVDKVMIVKKQLAVPDGTPVTAESKFS.ELGADSLDTVEIVMGLEEEFNITVDETSAQDIATVQDAANLIEKLVTEKTA
9.  AKQDTVEKVCEIVKKQLAVPEGTEVCGTTKFS.DLGADSLDTVEIVMGLEEEFOITWQETSQAQIATVEDAATLIDKLVS AKSS
10. AAKQETVEKVSEIVKKQLSLKDDQQVVAETKFV.DLGADSLDTVEIVMGLEEEFGIQMAEKAQKIATVEQAELIEELMQAKK
      ** ~ ~ ~** ~~~~ ~~~~ ~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~

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C. ACP sequence encoded by chloroplast genome.

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11. NEQEIFEKVQTIISEQLGVDKSQ.VTKDANFANDLGADSLDTVELVMALEEA FNIEIPDDAAEQISNLQQA VDFISQKVAA
12. MSDTLKRLQKIVSE.QLSVDPEK.VTSTADFGKQLGADSLDTIELTMTIYEENIDIEDHYASKITTVQDALNYIENKIKQK

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D. Mitochondrial ACP

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13. SAGGHLKKDQVFSRIAQVLSGFDKVNDPKNITETAHFANDLGLDSLDTVEVVMALEEEFSIEIPDKDADQIHSVDKAVEYILSQPDAN

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E. ACP from gram-positive bacteria

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14. MAATQEEIVAGLADIVNEIAGIPV.EDVQLDKSFTDDLDVDSLMEVVVAAEERFDVKIPDEDVKNLKTVGDATEYILKHQA
15. MDRKEIFERIEQVLAELQLGIPA.EQITEEADLREDLSLGMDLDLVELVSALEDEVGMRVEQSQLEGIETVGHVMELTDLIDVARLATASAADKPEAAS

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F. ACP-like protein of polyketide synthases.

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16. MSKLTIDDLRRILVDCAGEDEAAELGADALDTEFD.DLGYSLALMETAARIHOELGVLIPEDQVAELRTPRQVLDLVNGAAVETA
17. MATLLTTDDLRRALVE CAGETDGTDLSGDFDLRFE.DIGYDSLALMETAARLESRYGV SIPDDVAGRVDTPRELLDLINGALAEAA
18. MARLTL DGLRTILVACAGEDDGV DLSGDILDTFE.ELGYDSLALMESASRIERELGVALADGDINEELTPRVLLDLVNGAQAEAA
19. MTLTTLSDLLTLLRECAGEEESIDLGGDVEDVAFDA.LGYDSLALINTVGRIERDYGVQLGDDAVEKATTPRALIEMTNASLTGASPSPGAARD
20. MVPQIGLPRLVEIIRECAGDPDERDLGDILDVITYQ.DLGYSIALLEISAKLEODLGVSIPGE...ELKTPRHTLHLVNTETAGEVA
      ~ ~   *   ~   ~~~~~ ~ ~* ~* ~ ~ ~~~~~~ ~ ~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~

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G. NodF proteins from nodulation bacteria.

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21. MVDQLESEIIGIKNRVESEGGDGETALIVGDLTAATELTALGVDSLGLADIWDVFOAYGIRIEMNTAEAWSDLQNVGDIVGAIRGLLTGKA
22. MADGLTVEIIIAIKNLAQSENGGRIPAAI.GDITADRQLTSLGIDSLALADVLWDLEOAYGIRIEMNTADAWSNLKNIGDVVEAVRGLIAKEA
23. MADQLTLEIISAINKLKVAENGERTSVAL.GEITDTFELTSLGIDSLGLADYLVWDLEOLYGIKIEMNTADAWSNLNNIGDVVEAVRGLLTKEV
      * * * ~~~~ *   *   ~ ~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~

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Fig. 6.

covalently linked through a phosphodiester bond to a serine residue at position 36 of the protein, and fatty acyl moieties are attached as thioesters to the only sulfhydryl group on the protein at the terminus of 4'-phosphopantetheine (Figs. 6 and 7). *E. coli* apo-ACP (ACP without the 4'-phosphopantetheine prosthetic group) can be prepared by chemical synthesis [82] or by cleavage of ACP with hydrofluoric acid [176]. Enzymatic attachment of the prosthetic group to chemically synthesized apo-ACP by [ACP]synthase results in biologically active ACP (or holo-ACP).

Although the primary structure of *E. coli* ACP was determined long ago, the search for the *E. coli* ACP gene (*acpP*) took much longer probably due to the toxic effect of over-expression of ACP when the gene is cloned into multicopy plasmids [17]. Using a synthetic ACP gene as probe, the chromosomal *acpP* gene was cloned and sequenced in 1992 [178], 24 years after its amino acid sequence was reported [235]. The *acpP* gene is located between *fabG* (a 3-ketoacyl-ACP reductase gene) and *fabF* (3-ketoacyl-ACP synthase II gene); a noncoding region of 210 bp separates *fabG* and *acpP*. Although no obvious sequences were found to match promoter and ribosome binding sites within the noncoding region between *fabG* and *acpP*, results from maxicell experiments indicated that *acpP* is transcribed from two promoters: one is located in the noncoding region while the other is located upstream of *fabG* [178].

b) Secondary and tertiary structure of ACP and acyl-ACP.

Although *E. coli* ACP has a calculated molecular weight of 8,860, it behaves as a 20 kDa protein in SDS-PAGE and as a 5.5 kDa protein in SDS-urea-PAGE [186]. It is believed that anomalous migration of ACP in SDS-PAGE is due to decreased SDS binding to the acidic protein and a paucity of hydrophobic amino acid residues [186]. *E. coli* ACP and acyl-ACP also exhibit properties of a 20 kDa globular protein in gel filtration chromatography at neutral pH [57]. Since the amino acid composition does not suggest abnormal protein hydration, it was concluded that this anomalous behaviour of *E. coli* ACP in gel filtration is due to molecular asymmetry [186].

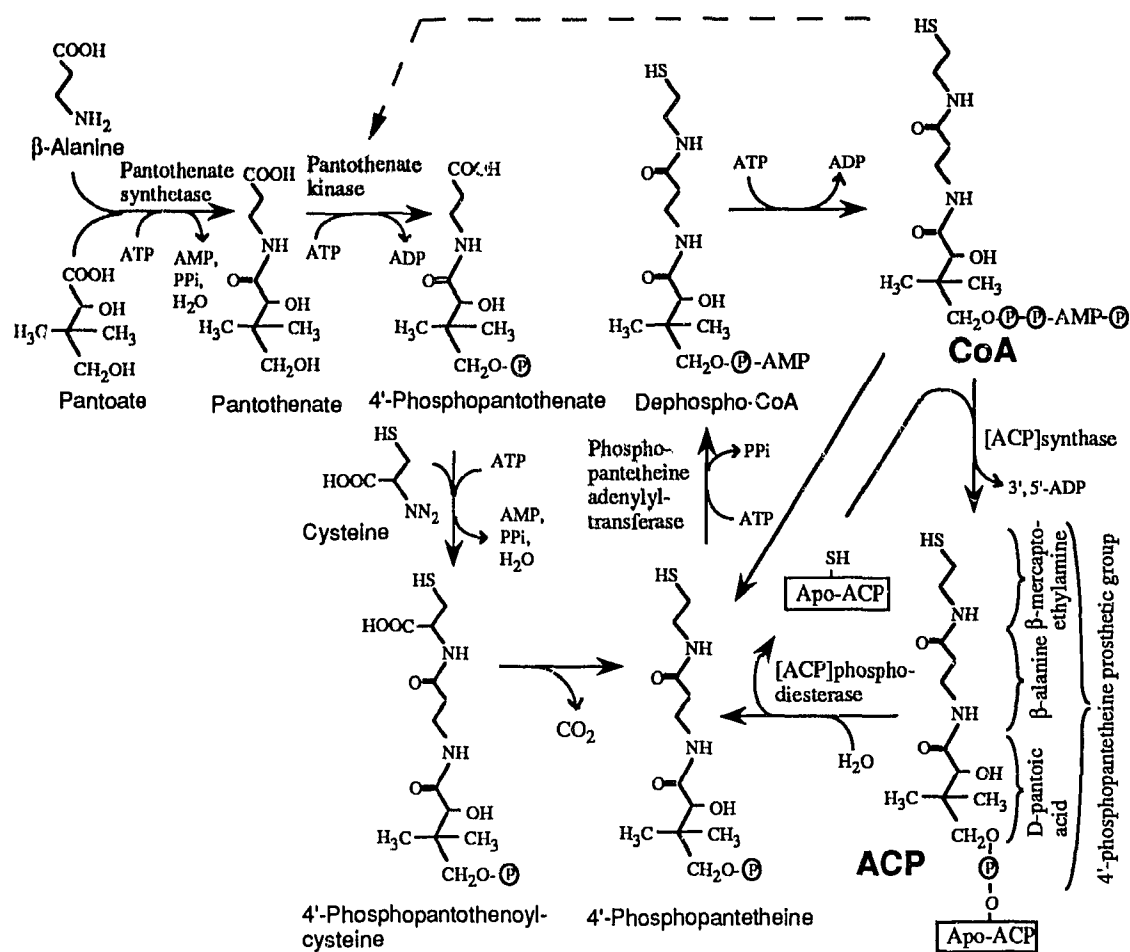


Fig. 7. The phosphopantetheine structure of ACP and CoA and its metabolic pathways in *E. coli*. Phosphopantetheine prosthetic group is linked to 3', 5'-ADP in CoA or linked to Ser-36 residue of ACP. Two regulated steps in the pathway are highlighted in gray. Dashed line indicates the feedback inhibition of pantothenate kinase by CoA levels. The excess phosphopantetheine from biosynthesis or ACP turnover is excreted from the cell. PPi, pyrophosphate; ACP, acyl carrier protein; CoA, coenzyme A. The figure is adapted from [141].

E.coli ACP was crystallized and X-ray diffraction data reported in 1985, but no crystal structure has been published [152]. Analysis of *E. coli* ACP structure has been primarily based on two-dimensional nuclear magnetic resonance (2D-NMR) data. Rock & Cronan [186] first proposed a detailed secondary structure model in 1979 on the basis of the Chou-Fasman predictive algorithm. Later, on the basis of this model and nuclear Overhauser effect (NOE) cross-relaxation rates from one-dimensional NMR spectra, Prestegard et. al [151] suggested a hypothetical tertiary structure model which places most of the hydrophobic residues in the interior and most of hydrophilic residues on the surface of the protein. More accurate tertiary models of *E.coli* ACP have subsequently been established. The principal structural motif is believed to consist of three roughly parallel amphipathic α -helices: I (residues 3-14), II (residues 37-51) and III (residues 65-75), as well as a hydrophobic cleft running along the surfaces of helices II and III [92,122]. Overall, there are two distinct conformers of *E. coli* ACP in dynamic equilibrium in solution [122,123]. Due to the dynamic equilibrium, individual conformers of *E. coli* ACP cannot be resolved. However, recognition of two sets of NMR resonances for spinach ACP strongly suggests that these states could be characterized if the conversion between two alternate conformers was slow enough [121].

The α -helix content of *E. coli* ACP, which accounts for more than 50% of total amino acid residues, was reversibly decreased at high pH as judged by optical activity [206], resulting in a large increase in the hydrodynamic radius of ACP [186]. The Stokes radius (R_s) of ACP doubled from 19.6 Å at neutral pH to 37 Å when the pH was increased to 9.4. The expansion of ACP can be analyzed either by gel filtration [186] or, more conveniently, by conformationally-sensitive PAGE (native PAGE) since proteins with smaller hydrodynamic radii migrate faster in native PAGE [187]. In the absence of SDS, acyl-ACPs with chain lengths longer than C₆ migrated 15% faster than ACP in native PAGE, while acetyl-ACP was not resolved from ACP and 4:0-ACP migrated about half way between acetyl-ACP and other acyl-ACPs, indicating that acyl-ACPs with chain

lengths longer than C₄ have more compact molecular shapes or smaller R_s than ACP [43]. Moreover, acyl groups render ACP more resistant to pH-induced hydrodynamic expansion since an R_s of 30.5 Å was observed for acyl-ACP (C₈ to C₁₈), compared to 37 Å for ACP [186]. The degree of stabilization provided by shorter acyl chains was less than that with long-chain acyl-ACPs and was a direct function of acyl chain length (C₈ > C₆ > C₄ > C₂ > ACP) [43]. The fact that apo-ACP has a larger R_s than ACP suggested the prosthetic group helps stabilize the protein [187]. Divalent cations, such as Ca²⁺, also stabilize ACP conformation since thermal stability was greatly increased in the presence of divalent cations [207].

Although tertiary structure models of ACP have been described, little detail is known about orientation of the prosthetic group and acyl chain of acyl-ACP. The secondary structure of ACP is largely conserved upon acylation [115]. Holak et. al [93] suggested that most of the prosthetic group is not in direct contact with ACP since no NOEs could be detected between the prosthetic group and protein residues. Hydrophobic binding studies with octyl-Sepharose showed that saturated long chain acyl-ACPs (C₁₀ - C₁₆) were bound much more tightly than 6:0-ACP and 8:0-ACP, while ACP, 2:0-ACP and 4:0-ACP did not bind to octyl-Sepharose. Overall, the strength of binding was a direct function of acyl chain length [43,190]. Based on these results, Cronan [43] suggested that there is an acyl-chain binding site on ACP which prevents the first 6 to 8 carbon atoms of the acyl chain from interaction with octyl-Sepharose. Mayo and Prestegard examined ACP and saturated fatty acyl-ACPs (C₂ to C₁₀) by NMR and observed that acylation with chain lengths of eight carbon atoms or less induced a conformational change which caused small resonance chemical shifts of several aromatic amino acid residues all over the molecule. Besides these minor conformational changes, they also observed a major NOE change at Phe-50 between 4:0-ACP and 6:0-ACP and assumed this chemical shift was largely due to the direct interaction between the acyl chain and Phe-50 [150]. Later, they reported that the fifth methylene group of the acyl chain interacts with Ile-54 and Ala-59 [114]. Replacement of

Ile-54 or Ala-59 with Val resulted in structural destabilization of acyl-ACP as judged by native-PAGE [17].

c) Metabolism and turnover of ACP.

ACP, one of the most abundant proteins in *E. coli* (about 6×10^4 molecules per cell) [17], is synthesized as a functionally inactive apoprotein which is activated by the attachment of phosphopantetheine prosthetic group to the Ser-36 residue. The prosthetic group of ACP undergoes metabolic turnover *in vivo* [106] controlled by two enzymes: [ACP]synthase transfers the prosthetic group from CoA to apo-ACP and [ACP]phosphodiesterase cleaves the prosthetic group from ACP [62] (Fig. 7). Since phosphopantetheine is the functional group in both ACP and CoA, its metabolism may be important for the regulation of intracellular activity of these cofactors. As indicated in Fig. 7, *E. coli* is able to utilize pantoate and β -alanine to synthesize pantothenate that is further phosphorylated by pantothenate kinase. Although both β -alanine and pantothenate are metabolically available intermediates in *E. coli*, they can also be obtained by uptake from the medium if exogenous sources are available. Therefore, *E. coli* ACP and CoA can be specifically labeled *in vivo* with radiolabeled β -alanine or pantothenate, providing a very useful tool to analyze the intracellular pool of ACP and CoA. Pantothenate kinase is the primary rate-controlling step of CoA and ACP synthesis and is regulated by feedback of CoA levels, while 4'-phosphopantetheine adenylyltransferase catalyzes another rate-limiting step [104,107]. *E. coli* cells do not maintain a large phosphopantetheine pool and the excess phosphopantetheine from biosynthesis or ACP turnover is excreted from the cell [107]. Apo-ACP is not detectable in either logarithmic or stationary phase *E. coli* cells unless [ACP]synthase is defective, indicating that phosphopantetheine modification is tightly coupled to protein synthesis [105]. The turnover of ACP phosphopantetheine amounted to about 25% of the ACP pool per generation during logarithmic growth [106]. Although ACP levels remained constant and apo-ACP remained undetectable when the intracellular CoA pool was severely depressed to 10% of normal levels [105], the turnover

rate of ACP phosphopantetheine was indeed dependent on the intracellular CoA concentration [106]. Since the 4'-phosphopantetheine from ACP turnover can also re-enter CoA synthesis (Fig. 7), and ACP contains about 10% of the total intracellular 4'-phosphopantetheine, the high turnover rate of ACP phosphopantetheine may be important for CoA synthesis [106].

Long chain acyl-ACP amounts to less than 10% of the total ACP pool in *E. coli* cells during normal exponential growth and the main chain length of acyl-ACP is between C₁₂ to C₁₅ [109,193]. Inhibition of acyl transfer to phospholipid synthesis caused dramatic accumulation (>5 fold) of acyl-ACP within 5 min, with 16:0-ACP the major acyl-ACP component while ACPSH (holo-ACP) content almost vanished [193]. Overexpression of ACP is lethal to cells: much of the overexpressed ACP is the apo-protein [141], while short chain fatty acyl-ACP is also observed [17].

2. The common features of ACPs of different organisms.

As described above, ACP plays a key role in FA synthesis (type-II system) and as a donor of fatty acyl moieties for glycerophospholipid and lipopolysaccharide synthesis and protein modification in *E. coli*. Besides *E. coli*, ACP and ACP-like proteins have been identified and characterized from other organisms, and their functions have been extensively studied.

Although the structure of ACP varies among species, all share a few common features. All the ACPs characterized so far from different organisms are small acidic polypeptides which use a serine residue located in the middle of the peptide chain as the site for attachment of the phosphopantetheine prosthetic group (Figs. 6 and 7). The amino acid sequence in the region surrounding the serine site is highly conserved among different ACPs (Fig. 6). Moreover, the Ala-59 residue which has been implicated as a fatty acyl chain binding site in *E. coli* ACP is definitely conserved among all ACPs and even some ACP-like proteins, whereas different amino acid residues are found in position 54, another site potentially related to FA binding. The high degree of homology may have functional

consequences. Expression of spinach ACP-I in *E. coli* resulted in its acylation, indicating that it can be recognized by *E. coli* FA synthesis enzymes [79]. On the other hand, the homology between ACPs and ACP-like proteins is considerably less, suggesting the latter may have tertiary structures different from ACP which would be specifically recognized by enzyme systems other than type II FA synthase. Expression in *E. coli* of an ACP-like protein from *Streptomyces glaucescens* tetracenomycin C polyketide synthase in *E. coli* resulted mainly in apo-protein, indicating it was a poor substrate for *E. coli* [ACP]synthase [210].

In animal cells and fungi, the multifunctional type-I FA synthase contains an ACP-like domain which has the acyl carrier function and thus a separate ACP is not required. The ACP domain on type-I synthase also shows a high degree of homology with *E. coli* ACP. It has been suggested that Gram-positive bacteria *Streptomyces* and *Saccharopolyspora* have the type-I FA synthase. However, ACP genes were cloned recently from these two species (Fig. 6). The deduced amino sequence surrounding serine-36 showed 55-70% identity with the corresponding *E. coli* sequence. However, whether these ACPs are involved in FA synthesis remains unclear.

3. Other ACPs involved in FA synthesis.

a) Plant chloroplast ACPs.

De novo FA synthesis in plant cells takes place primarily in chloroplast stroma by a type II FA synthase system. The fatty acyl moiety of long chain acyl-ACP synthesized in chloroplasts may either be transacylated by a chloroplast acyltransferase during glycerophospholipid synthesis or be cleaved by thioesterases for export from the chloroplast for phospholipid synthesis in endoplasmic reticulum [139,197]. Like bacterial ACP, plant ACPs are also low molecular weight acidic proteins (Fig. 6) and play an important role in FA synthesis. It is believed that plant ACP is a nuclear-coded protein synthesized on cytoplasmic ribosomes as a precursor with an N-terminal transit peptide. The ACP precursor is post-translationally imported into the chloroplast, involving cleavage

of the signal sequence [195]. The phosphopantetheine modification of ACP may occur before or after translocation since [ACP]synthase activity was found both inside and outside of the chloroplast [58,59]. Mature plant ACPs have 83-90 residues with over 50% identity to *E. coli* ACP and do not contain arginine [221]. The amino acid sequence surrounding the prosthetic group attachment site is highly conserved among different plant ACPs with overall a 95% similarity to the corresponding *E. coli* region (Fig. 6, underlined region).

Plant nuclear genomes usually contain more than two ACP genes which encode different ACP isoforms [7]. The expression of ACP isoforms are tissue-dependent. Isolation and characterization of cDNA clones in spinach revealed two ACP isoforms: the major isoform ACP-I is expressed only in leaves whereas the minor isoform ACP-II is expressed in leaves, roots and developing seeds. In vitro studies showed that the two isoforms possess differential reactivity in thioesterase and G3P acyltransferase reactions [80], suggesting that tissue-dependent isoform expression may be important for targeting synthesized fatty acyl groups to fulfill specific requirements of the various cell types. In barley, one major leaf-specific isoform (ACP-I) and two minor isoforms (ACP-II and ACP-III) were also characterized in seedling leaves, and genes encoding ACP-I and ACP-III were located on different chromosomes. Even more isoforms have been reported in rape seed (*Brassica napus*), which contains six distinct nuclear ACP genes encoding five different mature ACP isoforms [200], and in *Arabidopsis* which may have more than four nuclear genes. Although most studies indicate that nuclear genomic DNA encodes cytosolic ACP, recent evidence has revealed that some chloroplast or plastid genomes also contain ACP genes (Fig. 6) [97,240]. However, it is unclear whether these chloroplast genome-encoded ACP genes are expressed inside the chloroplast.

b) Mitochondrial ACP.

It was believed that all organisms contain either a type I or a type II FA synthase, so it was surprising to discover that *Neurospora crassa*, a fungus with a type I cytoplasmic

synthase contains a discrete ACP in its mitochondria [18]. Although mitochondria have been characterized as sites of FA metabolism, such as β -oxidation or acyl chain elongation, only CoA derivatives were previously identified in these processes. Subsequently, it was proven that the presence of ACP in mitochondria is not unique to fungi and that antibody against *Neurospora* ACP could recognize similar mitochondrial proteins in pea leaves, potato and yeast [31]. The mitochondrial ACP of *N. crassa*, which is post-translationally transported into mitochondria, contains 87 amino acid residues with overall 42% identity to *E. coli* ACP but 85% identity around the prosthetic group binding site (Fig. 6, underlined region) [199]. ACPs from both *N. crassa* and bovine heart mitochondria were found to be subunits of NADH:ubiquinone reductase, a respiratory chain complex with more than 30 subunits [198,199]. Further evidence showed that mitochondrial ACP was involved in a *de novo* FA synthesis pathway and saturated or unsaturated fatty acyl-ACPs up to C₁₈ were identified in *N. crassa* mitochondria [157,248]. It remains unknown why ACP is bound to NADH:ubiquinone reductase complex if its function is only in FA synthesis. Mitochondria are not autonomous in membrane lipid synthesis and most mitochondrial phospholipid (except cardiolipin) is synthesized in endoplasmic reticulum and transported into the organelle. Therefore, the unsolved question is why mitochondria need an additional FA synthesis system. To define the function of ACP in mitochondria, the destination of its acyl moieties must be determined. It should be very interesting to find out whether mitochondria contain acyltransferases which transfer these endogenous fatty acyl moieties to other biochemical components.

4. ACP-like proteins.

a) Polyketide biosynthesis.

Bacteria, fungi and plants synthesize polyketides, a group of secondary metabolites, in a manner similar to FA synthesis [94]. Polyketide synthesis begins with an activated carboxylic acid, such as acetyl-CoA for actinorhodin synthesis in *Streptomyces coelicolor* [213], followed by successive condensations with different building blocks of acetate (C₂),

propionate (C₃) or butyrate (C₄) during chain elongation. The type II polyketide synthase consists of multiple dissociable enzymes. These enzymes are encoded by adjacently located genes, such as the *act* genes (actinorhodin synthase) in *S. coelicolor* which consist of contiguous organized genes encoding two ketoacyl synthases, one keto reductase, one ACP-like protein and one cyclase/dehydrase [94]. Similar gene clusters were also observed in other *Streptomyces* strains [74]. The deduced sequences of five ACP-like proteins from *Streptomyces* aromatic polyketide synthases and *Saccharopolyspora* (Fig. 6, sequences 16-20) are 83-93 residues long, among which 19 residues are identical and 17 are highly conserved [120]. The phosphopantetheine prosthetic group is attached to serine-41 of these ACP-like proteins. Although considerable homology is observed among these proteins, their overall homology to *E. coli* ACP is less than 25%. As shown in the underlined regions of Fig. 6, the amino acid sequence surrounding the prosthetic group attachment site is highly similar ($\geq 90\%$ identical) among chloroplast, mitochondrial and Gram-negative bacterial ACPs. However, the identity of this region between *E. coli* and *Streptomyces* ACP-like protein is less than 35%, suggesting this protein should be called an "ACP-like protein" rather than ACP. Mutagenesis experiments in *S. coelicolor* indicated that the ACP-like protein was essential for actinorhodin synthesis [119], but its role remains ambiguous. Gene replacement of this protein with other heterologous *Streptomyces* ACP-like proteins resulted in functional synthase, suggesting most or all of the heterologous ACP-like proteins are competent to interact with the other components of the actinorhodin polyketide synthase [120].

b) Lipo-oligosaccharide biosynthesis.

Another ACP-like protein, NodF protein, was identified in nodulation bacteria. In the process of plant infection, the soil bacterium *Rhizobium* is capable of synthesizing lipo-oligosaccharide extracellular signals (Nod factors) in response to host plant signals, and the Nod factors are able to elicit early nodulation events in the plant host [234]. The common basic structure of the Nod factors consists of a β -1,4-linked tetramer or pentamer of D-

glucosamine which is N-acylated on the non-reducing terminal residue; the other N-acetylated residues are modified variously in different rhizobial strains [205,208]. The fatty acyl moiety of lipo-oligosaccharides, diversified in chain length or in the position and number of double bonds among different strains, is very important in bacteria-host specificity [53]. Two *nod* gene products, NodF and NodE are required for the synthesis of specific fatty acyl moieties since mutations in *nodE* or *nodF* resulted in alterations in the lipo-oligosaccharide N-acyl composition [53,72] as well as in host specificity. Sequence data suggested that the *nodE* gene encodes a cytoplasmic membrane-bound condensing enzyme by its homology with 3-ketoacyl synthases, and the *nodF* gene encodes an ACP-like protein [11,209]. A phosphopantetheine prosthetic group was found in purified NodF protein of *R. meliloti* [72]. Amino acid sequence analysis demonstrated that NodF products of three *Rhizobium* strains shared 75% identity among the group, but less than 25% identity with *E. coli* ACP [52] (Fig. 6). Interestingly, the similarity between NodF protein and ACP-like protein of *Streptomyces* aromatic polyketide synthase is much higher than of either to *E. coli* ACP, suggesting they may belong to a group of specialized ACP-like proteins whose function is mediating the synthesis of specialized lipids separately from normal cellular lipid synthesis.

c) Polysaccharide biosynthesis.

Besides the Nod factor, *R. meliloti* synthesizes a special polysaccharide that is also involved in symbiotic nodule development. DNA sequence analysis of a gene cluster which is essential for this synthesis revealed six genes similar to those of individual FA synthase enzymes, among them an ACP-like protein [171]. The overall amino acid sequence identity between this ACP-like protein and constitutively expressed *R. meliloti* ACP is 32%. The amino acid sequence surrounding the phosphopantetheine attachment site in this ACP-like protein showed 61% similarity to the corresponding region of *R. meliloti* ACP and 50% to that of NodF [171]. Therefore, *R. meliloti* provides an interesting model in which one constitutively expressed ACP and two ACP-like proteins have been identified so far.

d) Lipoteichoic acid biosynthesis.

A small, heat-stable and acidic protein has been recently identified in the Gram-positive bacterium *Lactobacillus casei*, and is an essential cofactor in synthesis of D-alanyl-lipoteichoic acid (LTA), a macroamphiphile which plays a key role in the assembly of the cell wall [87]. The N-terminal sequence of this protein is similar to *E. coli* ACP and a 4-phosphopantetheine prosthetic moiety is also observed. This protein was named D-alanine carrier protein (Dcp) since D-alanine is activated to form a D-alanyl-Dcp intermediate before it is transferred to membrane-bound LTA. The alanine acylation is catalyzed by a D-alanine-Dcp ligase. A *Bacillus subtilis* gene which may encode a Dcp was also identified recently [73].

5. Other functions of ACP.

Although ACP has proven to be an essential cofactor in different metabolic pathways, all functions mentioned so far are related to the phosphopantetheine prosthetic group of ACP or ACP-like proteins and its role as a carrier. Recently, it has been surprising to find that some functions of ACP, such as synthesis of membrane-derived oligosaccharides (MDO), are independent of its phosphopantetheine prosthetic group. *E. coli* MDO, a group of periplasmic glucans, are important in osmotic adaptation and their synthesis is regulated by the osmolarity of the medium. MDO consists of 6 to 12 glucose units joined by β -1 $\rightarrow$ 2 and β -1 $\rightarrow$ 6 linkages and substituted with phosphoglycerol or phosphoethanolamine residues via phosphodiester bonds at the 6-position of the sugar residues. The phosphoglycerol and phosphoethanolamine residues are derived from the head groups of membrane glycerophospholipids. Therisod and Kennedy [228] discovered a membrane-bound glucosyltransferase which catalyzed the elongation reaction that condenses a UDP-glucose unit to the polyglucose chains via a β -1 $\rightarrow$ 2 linkage. They also reported that the reaction requires a heat-stable cytoplasmic protein which turned out to be ACP. Interestingly, however, they found that *E. coli* apo-ACP, ACPSH and 16:0-ACP all are functional in the activation of the transglucosylase [227], and no glucan intermediates

bound to ACP have yet been detected [242]. The role of ACP in the activation of glucosyltransferase is so far unclear. In *Rhizobium*, periplasmic cyclic β -1,2-glucans were found to have similar structure to *E. coli* MDO, yet while *Rhizobium* ACP is 60% identical to *E. coli* ACP it failed to stimulate the activity of *E. coli* transglucosylase [172]. Indeed, only *E. coli* ACP has been so far reported to activate *E. coli* transglucosylase.

Clearly, ACP is a multifunctional protein. In addition to the functions listed above, ACP has been found to be involved in providing substrates for bacterial luminescence (see below) and in the formation of monogalactosyldiacylglycerol in cyanobacteria [29]. Recently, Niki et al [164] demonstrated that ACP is firmly attached to the *E. coli* MukB protein during its purification; MukB is a filamentous protein involved in chromosome partitioning during cell division. Therefore, it appears that the list of ACP functions is still expanding.

D. FA metabolism in the luminescent bacterium *Vibrio harveyi*.

Research in the past few years has demonstrated that *E. coli* is not a completely suitable model for FA metabolism in luminescent bacteria, possibly because of an additional requirement for 14:0 in bioluminescence. Luminescent bacteria are the most widely distributed and abundant luminescent organisms and are very common in marine environments, where they exist as plankton or as parasites and light-organ symbionts. The mechanism of luminescence has been studied mainly in five species: *Vibrio harveyi*, *Vibrio fischeri*, *Photobacterium phosphoreum*, *Photobacterium leiognathi* and *Xenorhabdus luminescens* [143]. As light emission of luminescent marine bacteria is highly dependent on cell density, single bacteria living freely in the ocean would not be expected to emit light. Functional benefits of luminescence to bacteria remain unknown but a few hypotheses have been suggested [49]. Light produced by luminescent bacteria associated with faecal debris may attract coprophagous animals to ingest faecal debris and the bacteria, and these animals would, in turn, provide a nutrient-rich environment for gut symbiotic luminescent bacteria [42].

V. harveyi is widely distributed in marine coastal waters. Although this bacterium is frequently isolated from the warmer surface water, it also occurs as a gut symbiont or as a parasite on crustacea, but has not been isolated from luminous organs of fish [233]. *V. harveyi* wild type cells carry naturally occurring plasmids and are ampicillin resistant. The nutrient composition of culture medium and temperature are very important for maximal growth and light emission of luminescent bacteria. Although *V. harveyi* grows well in a minimal medium with glycerol as carbon source, addition of tryptone and yeast extract (complex medium) is necessary for faster growth and optimal luminescence.

1. Mechanism and regulation of bacterial luminescence.

a) The luciferase reaction.

Light emission in *V. harveyi* and other luminescent organisms is catalyzed by enzymes generically known as luciferases. In luminescent bacteria, the light-emitting reaction involves oxidation of a long-chain fatty aldehyde and reduced flavin mononucleotide (FMNH₂) in the presence of oxygen and results in the emission of a photon of blue-green light at 490 nm (Fig. 8) [132]. The energy source for luminescence (60 kcal Einstein⁻¹) is supplied by the conversion of aldehyde to fatty acid and the oxidation of FMNH₂.

Luciferase catalyzes the ordered formation of a stable luciferase-flavin-oxygen-aldehyde intermediate, the decay of which yields the emission of light and the oxidation of substrates. Bacterial luciferase is a heterodimer of approx. 80 kDa consisting of two subunits: α -subunit (40-44 kDa) encoded by the *luxA* gene and β -subunit (30-40 kDa) encoded by the *luxB* gene. The two polypeptides share about 30% identity in amino acid sequence and the β -subunit may have evolved from the α -subunit after gene duplication [169]. Comparisons of the amino acid sequence have also demonstrated close homologies (45 to 88% identity) among the luciferases of different bacteria, suggesting they share similar reaction mechanisms. From mutant analysis, chemical modification, and subunit hybridization studies it has been shown that the catalytic activity is mainly associated with

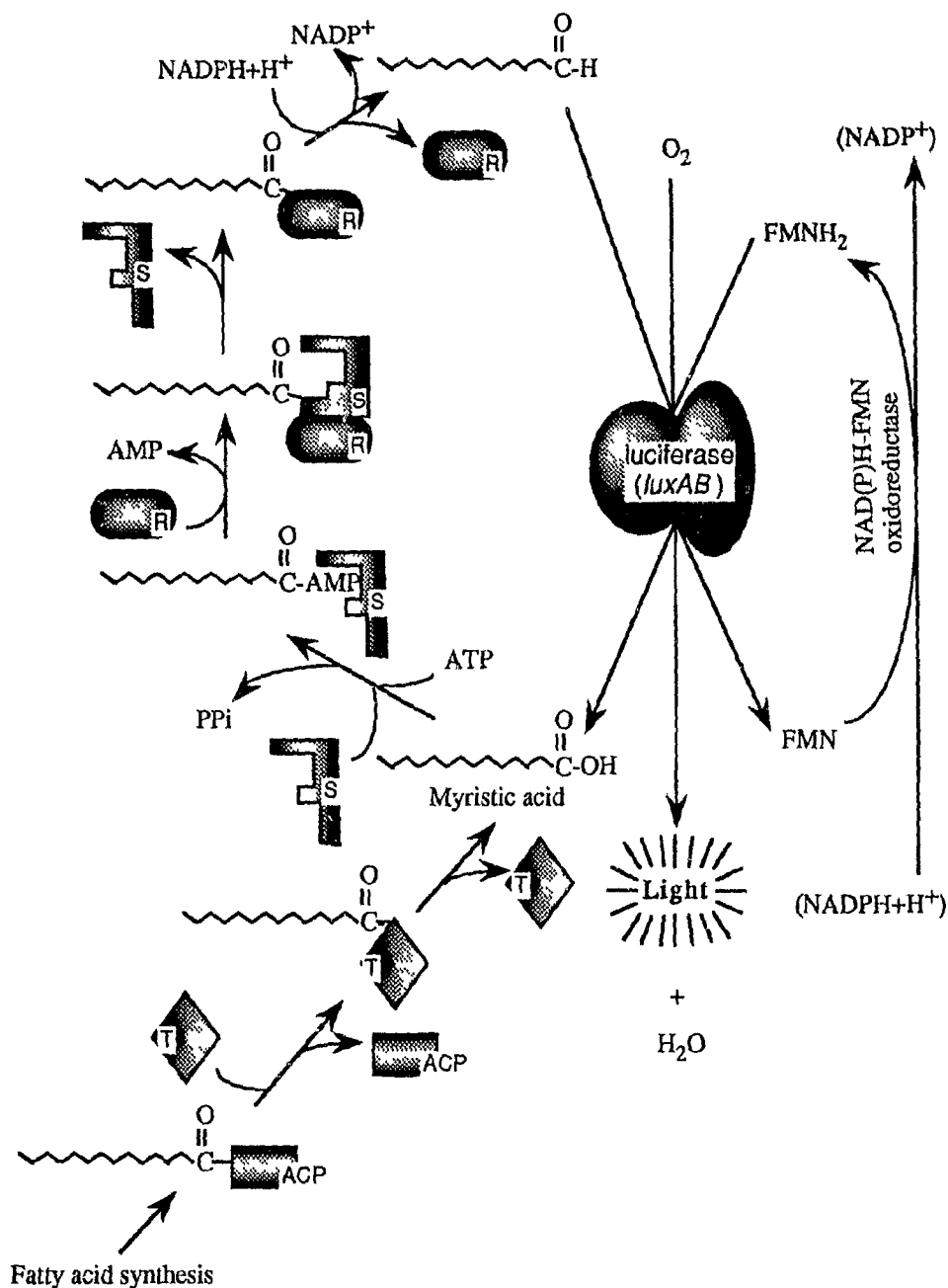


Fig. 8. Luciferase reaction and aldehyde synthesis in luminescent bacteria. 14:0-ACP from FA synthesis is converted to aldehyde catalyzed by fatty acid reductase which includes three enzymes: acyltransferase (T), synthetase (S) and reductase (R). All three enzymes form a covalently linked fatty acyl-enzyme intermediate during the reactions. The figure is adapted from [156].

the α -subunit [169]. C-terminal deletions of the β -subunit generated a truncated luciferase which showed less thermostability, indicating that the β -subunit is important for temperature stability of luciferase.

FMNH₂ is obtained from FMN in the presence of NAD(P)H, catalyzed by FMN reductases. The luminescent system does not appear to be tightly coupled with FMN reductases since the latter enzymes are not co-induced with luciferase [86], and are also found in non-luminescent bacteria such as *E. coli*.

b) Biosynthesis of fatty aldehyde.

The conversion of long chain fatty acid to aldehyde for the luminescent reaction is catalyzed by a fatty acid reductase consisting of synthetase (s) and reductase (r) activities, while the generation of fatty acid from acyl-ACP is catalyzed by a transferase (t) (Fig. 8). The synthetase, the reductase and the transferase are encoded by *luxE*, *luxC* and *luxD*, respectively [156]. These enzymes were found to form a large multienzyme FA reductase complex (r₄s₄t₂₋₄) in *P. phosphoreum*, where r, s, and t are 54 kDa, 42 kDa, and 33 kDa, respectively [156]. Fatty acid reductase activity has not been detected in *V. harveyi* cell extracts, but corresponding polypeptides of the enzyme can be identified by acylation with radioactively-labeled long chain fatty acid [239].

As indicated in Fig. 8, the acyl group of acyl-ACP is transferred to a serine residue in the active domain of the transferase, which in turn transfers the acyl group to a water molecule to form fatty acid [60]. This serine is located in the middle of a Gly-X-Ser-X-Gly motif which is essential for transferase function and is present in both *V. harveyi* and *P. phosphoreum* transferases as well as in other serine esterases [60]. A point mutation replacing the first glycine with glutamic acid in the motif of *V. harveyi* transferase results in a dark mutant strain of *V. harveyi* (M17) which lacks functional transferase [159]. Transformation of the M17 mutant with cloned wild type *luxD* gene complemented the defective transferase and fully restored luminescence [160]. Myristoyl-ACP is believed to be the natural substrate for bacterial transferases based on several lines of evidence: (i)

bioluminescence can be significantly restored in M17 by adding exogenous 14:0 but not other fatty acids [233]; (ii) acyl-ACP is cleaved *in vivo* by *V. harveyi* transferase [27]; (iii) purified *V. harveyi* and *P. phosphoreum* transferases both cleave 14:0-ACP more efficiently than other acyl-ACPs *in vitro* [60]. The purified *V. harveyi* transferase is a 32 kDa protein and, in addition to water, can transfer acyl groups from either acyl-ACP or acyl-CoA to other acceptors, such as glycerol [22].

The synthetase subunit catalyzes ATP-dependent FA activation to form an acyl-AMP intermediate, and the acyl group is then transferred to a cysteine residue near the synthetase C-terminus in the presence of the reductase subunit (Fig. 8). Site-specific substitution of this cysteine with serine in the *P. phosphoreum luxE* gene prevented acylation of the mutant enzyme but did not affect its ability to catalyze FA activation to acyl-AMP [220]. Interaction with the reductase subunit mediates a conformational change in the synthetase which is necessary for its activity [220]. Subsequently, the acyl group is transferred and covalently attached to the reductase subunit, where it is finally reduced to aldehyde by NADPH (Fig. 8) [237]. *In vitro* experiments showed that the reductase can also covalently accept acyl groups from acyl-CoA, followed by reduction to aldehyde or transfer in reverse to form acylated synthetase [238]. However, it seems unlikely that acyl-CoA can be used by reductase *in vivo* based on observations with a *V. harveyi* luminescent mutant strain (A16) which is defective in the synthetase. If acyl-CoA is the natural substrate for the reductase, the restoration of A16 luminescence by addition of 14:0 might be expected since 14:0 can be activated to 14:0-CoA (see below) upon uptake into the cell. However, luminescence of A16 is stimulated by exogenous aldehyde but not 14:0 [239]. Another *V. harveyi* luminescent mutant strain (M42), which contains a defective reductase, also cannot use 14:0 for luminescence but emits light in the presence of aldehyde. Luminescence is inhibited by cerulenin, an inhibitor of 3-ketoacyl-ACP synthase. This inhibition is not due to the effect of cerulenin on FA synthesis [76], but rather due to covalent acylation by cerulenin of the reductase subunit [28].

V. harveyi also contains an aldehyde dehydrogenase that possesses both acyl-CoA reductase and thioesterase activities [21]. This enzyme is co-induced with luminescence *in vivo* but has not been found in other luminescent bacteria and its function remains unknown.

c) Regulation of the *lux* operon.

Genes encoding bacterial luciferase (*luxAB*) and the fatty reductase complex (*luxCDE*) from *X. luminescens*, *V. harveyi*, *V. fischeri*, *P. leiognathi* and *P. phosphoreum* have been cloned. The five genes are clustered to form a *lux* operon in the order *luxCDABE* in all species. Other genes, such as *luxG* and *luxH* in *V. harveyi*, have also been identified within the *lux* operon in some species, but none are present in all five species [156].

Light emission by luminescent bacteria is highly dependent on cell density. The genes of the *lux* operon are not expressed following subculture into fresh liquid medium until middle to late logarithmic growth phase, and the dramatic expression of luminescence during this period is due to "autoinduction" of the *lux* operon. Autoinducers, such as β -ketocaproyl-N-homoserine lactone in *V. fischeri* and β -hydroxybutyryl-N-homoserine lactone in *V. harveyi*, are synthesized and secreted by luminescent bacteria [155]. Accumulation of the autoinducer to a critical level in the medium is necessary for induction. Regulation of the *lux* operon has been most extensively characterized in *V. fischeri*, where two regulatory genes (*luxI* and *luxR*) are located immediately upstream of the *luxCDABE* genes. The *luxI* gene is cotranscribed with the other *lux* structural genes and is the structural gene for autoinducer synthesis. The *V. fischeri luxR* gene, which is located immediately upstream of the *lux* operon but is transcribed in the opposite direction, may encode a receptor for autoinducer since the autoinducer can specifically bind to this protein [155]. Although the mechanism is unclear, a simple model suggests that autoinducer binds to the LuxR receptor, positively stimulating transcription of *luxICDABE*, and increased synthesis of LuxI causes synthesis of more autoinducer, resulting in a positive-feedback

loop which increases bioluminescence several orders of magnitude within a relatively short time [155].

A *V. harveyi* gene which is also named *luxR* and is not within the *lux* operon, has also been cloned, but sequence analysis indicated that the gene product is not related to the *V. fischeri* LuxR protein. It was demonstrated recently that the *V. harveyi* LuxR protein is a transcriptional activator that stimulates transcription initiated 26 bp upstream of the *lux* operon [226], and the purified protein specifically binds to the AT-rich region upstream of the *V. harveyi lux* operon [225] independent of autoinducer. This regulatory region upstream of the *V. harveyi lux* operon is necessary for expression of luminescence both in *V. harveyi* and in recombinant *E. coli*. However, wild type *E. coli* transformed with the *V. harveyi lux* operon and this upstream region emits only very low levels of light unless the *V. harveyi luxR* gene is co-transformed as well. Although the *V. harveyi* autoinducer has a structure similar to the *V. fischeri* autoinducer, genes similar to *V. fischeri luxR* and *luxI* have not yet been identified in *V. harveyi*.

V. harveyi luminescence is inhibited by growth in the presence of glucose. This catabolite repression is due to decreased expression of FA reductase and luciferase, and is partially alleviated in the presence of cyclic AMP [25,155]. Luminescence is repressed irrespective of cell density in *V. harveyi* cells cultured in minimal medium. However, addition of arginine to minimal medium will stimulate luminescence induction. It was suggested that the arginine stimulation occurs at the transcriptional level of *lux* operon expression [86].

2. The effect of luminescence development on FA metabolism.

The FA reductase complex has provided an excellent model for studying FA channeling and metabolism. The transferase subunit plays a key role in diverting fatty acids from FA synthesis to luminescence. One milliliter of bright *P. phosphoreum* cells emitting light at a rate of 1×10^{13} quanta/sec would require a minimum aldehyde synthesis rate of 0.9 nmol/min. In vitro experiments showed that the FA reductase complex in 1 ml of this

cell extract can catalyze reduction of 14:0 at a maximum rate of 1 nmol/min while the transferase subunit can cleave 14:0-ACP at a maximum rate of 0.2 nmol/min. This suggests that 14:0 produced in the luciferase reaction may be recycled by the FA reductase complex for aldehyde synthesis, since 14:0 generated by the transferase is not sufficient to maintain luminescence [154]. Moreover, luminescence of *V. harveyi* M17 incubated with a limiting amount of 14:0 or tetradecanal was increased 60-fold by cyanide and other agents that block respiration and increase NADPH levels, while incubation with the M42 mutant (reductase deficient) only resulted in a 2-fold increase in luminescence [233]. This observation also indicated that *V. harveyi* FA reductase can recycle 14:0 created in the luciferase reaction.

V. harveyi has been the main species for study of the effect of luminescence development on FA and phospholipid metabolism. Labeling experiments with [1-¹⁴C]acetate showed that radioactivity was incorporated mainly into three phospholipids: phosphatidylethanolamine (63%), phosphatidylglycerol (11%), and cardiolipin (5%), which is comparable to the phospholipid composition of *E. coli* [23]. The fatty acid composition of wild type *V. harveyi* grown at 27°C prior to luminescence induction consists of 14:0 (10%), 16:0 (19%), 16:1 (56%) and 18:1 (10%) [23]. Analysis of fatty acid mass composition of phospholipid indicated that diversion of 14:0-ACP from FA biosynthesis to the aldehyde synthesis did not substantially alter the overall FA composition during development of luminescence in *V. harveyi* [23]. However, pulse-label studies with [1-¹⁴C]acetate revealed a small but significant increase in the ratio of labeled 14:0 to 16:0 in the FA composition with the induction of luminescence in wild type strains. Wild type cells did not accumulate free fatty acid or aldehyde under normal growth conditions, but a small transient free 14:0 pool was detectable inside the cells by [1-¹⁴C]acetate labeling when fatty acid and lipid synthesis were perturbed by medium change [23]. In contrast to the wild type strain, no such accumulation was observed in preinduced wild type cells, or

in M17 and A16 mutants, indicating that the activities of acyltransferase and synthetase are coupled in *V. harveyi*.

3. Exogenous fatty acids can be elongated in *V. harveyi*.

Our laboratory demonstrated a few years ago that the metabolism of exogenous fatty acid in *V. harveyi* differs from that in *E. coli*. *V. harveyi* normally grows well in a medium containing glycerol as carbon source. However, unlike *E. coli*, the growth of *V. harveyi* in minimal media containing 14:0 (1 mM) or sodium acetate (0.4%) as sole carbon source was insignificant, with A_{660} doubling every 2 to 3 days after a growth lag of several days [24]. Since exogenous [1- ^{14}C]14:0 was β -oxidized in *V. harveyi*, releasing $^{14}\text{CO}_2$ at a rate comparable to that in *E. coli*, this observation suggested a difference in the glyoxylate shunt between the two species. When bacterial cells were incubated with [^{14}C]14:0 in a medium containing glycerol, both *V. harveyi* and *E. coli* utilized the [^{14}C]14:0 for phospholipid synthesis [24]. However, *P. phosphoreum* did not accumulate radioactivity in either phospholipid or CO_2 , suggesting that this species lacks acyl-CoA synthetase or other enzymes necessary for utilization of exogenous fatty acids. In contrast to *E. coli*, FA uptake does not appear to be inducible in *V. harveyi*.

Also in contrast to *E. coli*, elongation of radiolabelled exogenous fatty acid was observed, indicating that exogenous fatty acids have access to FA biosynthetic pathways [24]. Incubation of *V. harveyi* with [^{14}C]8:0 resulted in the formation of phospholipid containing all the major saturated and unsaturated labelled fatty acids, while incubation with ^{14}C -labelled 12:0, 14:0 or 16:0 resulted in labelled saturated fatty acid only ($\leq \text{C}_{16}$), suggesting that unsaturated FA synthesis is diverted at the C_{10} stage in *V. harveyi*, as in *E. coli*. Elongation of exogenous fatty acid was not affected by the induction of luminescence. Incubation with cerulenin prior to ^{14}C -fatty acid (8:0, 12:0 and 14:0) labelling inhibited elongation, but the direct incorporation of 12:0 and 14:0 into phospholipid was enhanced,

indicating that activation of fatty acid per se is not affected by cerulenin in *V. harveyi* and that exogenous and endogenous fatty acid compete for phospholipid synthesis.

4. *V. harveyi* fatty acyl-ACP synthetase.

A fatty acyl-ACP synthetase, which was first identified in soluble extracts of *V. harveyi* a few years ago [26], was purified recently in our laboratory [61]. After *E. coli* acyl-ACP synthetase, this is the only other acyl-ACP synthetase so far described in bacteria. Both enzymes are dependent on ATP and MgCl_2 for acylation activity and involve an acyl-AMP intermediate. However, while the *E. coli* enzyme requires Triton X-100 (0.07%) and high concentrations of LiCl (0.4 M) in the reaction mixture for acyl-ACP synthetase activity [101], *V. harveyi* acyl-ACP synthetase is inhibited under these conditions [61]. Purified *V. harveyi* acyl-ACP synthetase migrates as a 62 kDa band in SDS-PAGE and elutes as an 80 kDa protein by gel filtration under reducing conditions. This enzyme has a K_m of 7 μM for 14:0 and a K_m of 20 μM for *E. coli* or *V. harveyi* ACP. Active with fatty acids from C_8 to C_{18} , *V. harveyi* acyl-ACP synthetase prefers fatty acids ≤ 14 carbons in length [25]. The isolated *V. harveyi* enzyme is very unstable in the absence of glycerol and low concentrations of Triton X-100.

The function of *V. harveyi* acyl-ACP synthetase remains unclear but it may be part of an enzyme complex *in vivo* since acyl-ACP synthetase activity is eluted from Sephacryl S-300 HR column with an apparent mass of 300 to 500 kDa in the early stages of purification [61]. Acyl-ACP synthetase is constitutively expressed in *V. harveyi* and *V. fischeri* but is not detected in *P. phosphoreum*, indicating that the enzyme is not essential for luminescence [26].

E. Research objectives.

Fatty acid metabolism in *E. coli* has been well characterized. However, previous studies from our laboratory have shown that the metabolism of exogenous fatty acids in the luminescence bacterium *V. harveyi* exhibits a number of significant differences from that

in *E. coli*, despite overall similarities in fatty acid and phospholipid compositions of these Gram-negative organisms. Exogenous fatty acid can be directly elongated in *V. harveyi*, probably by an ACP-mediated pathway, and a novel *V. harveyi* acyl-ACP synthetase has been described which could play a key role in this process. This is clearly in contrast to *E. coli*, where FA degradation pathways for exogenous fatty acid and FA biosynthetic pathways are maintained by chemically distinct intermediates (CoA vs ACP).

Due to the importance of FA activation in determining the fate of fatty acids in metabolic pathways and the central role of ACP in this process, further knowledge of the structure and function of ACP is essential to a complete understanding of exogenous FA transport and activation in *V. harveyi*. Unique features of ACP from this organism, perhaps related to its additional function in bioluminescence, might also be expected. The primary goal of this research project was therefore directed towards characterization of *V. harveyi* ACP at the molecular level and elucidation of its role in the metabolism of exogenous fatty acids. The work has been broadly divided into four categories, each interrelated on theoretical and practical levels:

- (a) purification of *V. harveyi* ACP and determine *V. harveyi* ACP sequence, to provide important structural information and material for further research;
- (b) characterization of *V. harveyi* ACP, including development of analytical methods for immunodetection and acylation of ACP, to study its role in FA metabolism;
- (c) analysis of intracellular ACP and acyl-ACP intermediates derived from exogenous FA, to determine the mechanism of activation and metabolic pathways;
- (d) investigation of long chain FA transport in *V. harveyi*, to establish whether a protein-mediated system(s) might target exogenous fatty acids to different metabolic fates.

Section II. Materials.

A. General chemicals, kits and other materials.

<u>Name</u>	<u>Company</u>	<u>Address</u>
Alkaline phosphatase colour reagent	BioRad (Canada) Ltd.	Mississauga, ON
Amberlite Resin CG-4B	Haltech Scientific Ltd.	Dartmouth, NS
Bacto-tryptone	Difco laboratories	Detroit, MI (U.S.A.)
DEAE-Sepharose (Fast Flow)	Pharmacia (Canada) Inc.	Baie d'Urfe, PQ
DNA sequencer	Life Technologies (Canada) Inc.	Burlington, ON
Decanoic acid	Sigma Chemical Co.	St. Louis, MO (USA)
<i>E. coli</i> ACP	Sigma Chemical Co.	St. Louis, MO (USA)
En ³ Hance	Du Pont-NEN	Boston, MA (USA)
Exonuclease III	Life Technologies (Canada) Inc.	Burlington, ON
Extract of yeast powder	BDH, Inc.	Darmstadt (Germany)
Freund's complete adjuvant	Sigma Chemical Co.	St. Louis, MO (USA)
Freund's incomplete adjuvant	Sigma Chemical Co.	St. Louis, MO (USA)
Fixed <i>Staphylococcus aureus</i> Cowan I (SAC)	Terochem Laboratories Ltd.	Markham, ON
GeneClean II Kit	Bio 101 Inc.	La Jolla, CA (USA)
Goat anti-rabbit IgG alkaline phosphatase conjugate	BioRad (Canada) Ltd.	Mississauga, ON
Kodak XAR 5 film	Eastman Kodak Company	Rochester, NY (USA)
Immobilon PVDF Transfer Membrane	BioRad (Canada) Ltd.	Mississauga, ON
Low molecular weight SDS-PAGE standards	Pharmacia (Canada) Inc.	Baie d'Urfe, PQ
Lauric acid	Sigma Chemical Co.	St. Louis, MO (USA)
Lysozyme	Boehringer-Mannheim (Canada)	Laval, PQ

Micro-BCA protein assay reagent kit	Pierce Chemical Co.	Rockford, Ill. (USA)
Myristic acid	Sigma Chemical Co.	St. Louis, MO (USA)
Nitrocellulose membrane	BioRad (Canada) Ltd.	Mississauga, ON
Nylon membrane	Boehringer-Mannheim (Canada)	Laval, PQ
Octanoic acid	Sigma Chemical Co.	St. Louis, MO (USA)
Octyl-Sepharose CL-4B	Pharmacia (Canada) Inc.	Baie d'Urfe, PQ
Palmitic acid	Sigma Chemical Co.	St. Louis, MO (USA)
Peptone (casein trypsin-digested)	BDH, Inc.	Darmstadt (Germany)
Piperazine diacrylamide (PDA)	BioRad (Canada) Ltd.	Mississauga, ON
PAGE and protein transblot apparatus	BioRad (Canada) Ltd.	Mississauga, ON
Prestained SDS-PAGE standards (low range)	BioRad (Canada) Ltd.	Mississauga, ON
Restriction endonucleases	Life Technologies (Canada) Inc. BioRad (Canada) Ltd.	Burlington, ON Mississauga, ON
RNase A	Sigma Chemical Co.	St. Louis, MO (USA)
S1 nuclease	Pharmacia (Canada) Inc.	Baie d'Urfe, PQ
Sephacryl S-200 (High Resolution)	Pharmacia (Canada) Inc.	Baie d'Urfe, PQ
Sequenase enzyme system Version 2.0 kit	US Biochemical Co.	Cleveland, OH (USA)
T ₄ -polynucleotide kinase	Life Technologies (Canada) Inc.	Burlington, ON
Trans-Blot immobilon PVDF membrane (0.2 micron)	BioRad (Canada) Ltd.	Mississauga, ON
Yeast extract	Difco laboratories	Detroit, MI (U.S.A.)

B. Radiosotopes.

<u>Compound</u> (specific radioactivity)	<u>Company</u>	<u>Address</u>
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[α - ^{35}S]dATP (600 Ci/mmol)	ICN Biomedicals Canada Ltd	Mississauga, ON
[γ - ^{32}P]ATP (3000 Ci/mmol)	Du Pont-NEN	Boston, MA
[1- ^{14}C]Butyric acid (10.6 Ci/mol)	Sigma Chemical Co.	St. Louis, MO
[1- ^{14}C]Decanoic acid (10.6 Ci/mol)	Sigma Chemical Co.	St. Louis, MO
[1- ^{14}C]Hexanoic acid (8 Ci/mol)	Sigma Chemical Co.	St. Louis, MO
[1- ^{14}C]Lauric acid (57 Ci/mol)	Amersham Canada Ltd.	Oakville, ON
[1- ^{14}C]Myristic acid (54 Ci/mol)	Amersham Canada Ltd.	Oakville, ON
[1- ^{14}C]Octanoic acid (53.5 Ci/mol)	Du Pont Canada Inc.	Mississauga, ON
[1- ^{14}C]Palmitic acid (58 Ci/mol)	Du Pont Canada Inc.	Mississauga, ON
[9,10- ^3H]Myristic acid (20 Ci/mmol)*	Amersham Canada Ltd.	Oakville, ON
[^3H] β -alanine (1.6 Ci/mol)	Du Pont Canada Inc.	Mississauga, ON
Sodium [^3H]acetate (22 Ci/mmol)	ICN Biomedicals Canada Ltd	Mississauga, ON

*[9,10- ^3H]Myristic acid was obtained by thin layer chromatographic purification of the products of tritiation of myristoleic acid prepared by Amersham.

C. Solutions.

<u>Denhardt's (50 x) [203]</u>	1% Ficoll (w/v), 1% polyvinylpyrrolidone (w/v), 1% BSA (w/v).
<u>Fatty acid solutions</u>	All ^{14}C -labeled, ^3H -labeled, and unlabeled fatty acids were dissolved in ethanol except short chain fatty acids, 4:0 and 6:0, which were dissolved in H_2O . If [^{14}C]fatty acids were supplied in hexane, they were dried under N_2 and redissolved in ethanol.
<u>Phosphate buffer (1 M)</u>	1 M NaH_2PO_4 and 1 M K_2HPO_4 mixed in a ratio of 1:2.4 (v/v) ; pH adjusted to 7.5.
<u>RIPA buffer [85]</u>	150 mM NaCl, 1% Nonidet P-40 (v/v), 0.5% deoxycholate (w/v), 0.1% SDS (w/v), 50 mM Tris-HCl, pH 7.5.
<u>SDS sample buffer (2 x)</u>	60 mM Tris-HCl, pH 6.8, 10% v/v glycerol, 2% SDS, 0.001% bromphenol blue.
<u>SSC (20 x) [203]</u>	3 M NaCl, 3 M sodium citrate, pH 7.0.

<u>TBS buffer</u> [229]	0.9% NaCl (w/v), 10 mM Tris-Cl, pH 7.5, 0.02% sodium azide (w/v).
<u>TBS-Tween buffer</u>	TBS containing 0.2% Tween-20 (v/v).
<u>TE buffer</u> [203]	0.1 M Tris-Cl, pH8.0, 10 mM EDTA.

D. Bacterial growth media.

1% NaCl Complex medium [184]	Per liter: 10 g NaCl, 3.7 g Na ₂ HPO ₄ , 0.5 g (NH ₄)H ₂ PO ₄ , 1 g KH ₂ PO ₄ , 0.2 g MgSO ₄ ·7H ₂ O, 5 g Bacto-tryptone (or Peptone), 0.5 g yeast extract (or extract of yeast powder), 2 ml glycerol.
1% NaCl Minimal medium [184]	Same as complex medium except without Bacto-tryptone (or Peptone) and yeast extract (or extract of yeast powder).
LB medium [203]	Per liter: 10 g NaCl, 10 g Bacto-tryptone (or extract Peptone), 5 g yeast (or extract of yeast powder).

E. Bacterial strains.

<u>Name</u>	<u>Type</u>	<u>Reference</u>
<i>Escherichia coli</i> DH5α'	<i>RecA</i> 1	[81,203]
<i>Escherichia coli</i> MR24	JM103 harboring pMR24	[178,203]
<i>Escherichia coli</i> RR1	<i>RecA</i> ⁺	[145,203]
<i>Vibrio harveyi</i> B392	Wild type	[181]
<i>Vibrio harveyi</i> M17	Dark mutant	[232]
<i>Vibrio harveyi</i> MBM1	Double dark mutant	This work
<i>Vibrio harveyi</i> MBM4	Double dark mutant	This work
<i>Vibrio harveyi</i> MBM6	Double dark mutant	This work
<i>Photobacterium phosphoreum</i> NCMB 844	Wild type	[153]

Section III. Methods.

A. General methods.

1. Growth conditions for different bacterial strains.

For liquid culture, *V. harveyi* cells were grown at 27°C in a rotary shaker (200 rpm) in complex or minimal medium. *Photobacterium phosphoreum* cells were grown at 18°C in complex medium containing 3% NaCl. Cell growth was monitored by measuring absorbance of the cell culture at 660 nm (A_{660}), and a red filter was inserted between the cuvette and photocell to block blue light emission of luminescent bacteria. For *V. harveyi*, one A_{660} unit corresponds to 5×10^8 cells per ml of cell culture [24]. Unless specified, *E. coli* was grown in LB medium at 37°C.

Solid plates containing 1.2% agar (w/v) and 3% NaCl (w/v) in complex or minimal medium were used for *V. harveyi*, while 1.5% agar (w/v) in LB medium was used for *E. coli* cell culture. *V. harveyi* and *P. phosphoreum* strains were maintained on agar plates at room temperature and subcultured every two weeks. *E. coli* strains stored at -70°C in LB medium containing 20% glycerol were used directly for inoculation of media.

2. Measurement of bioluminescence.

The bioluminescence of *V. harveyi* was measured with a photomultiplier photometer (manufactured by Brian Millier, Dalhousie University) [158] using 1 ml of cell culture in a 20 ml glass scintillation vial. One light unit (LU) is defined as 5×10^9 quanta s^{-1} by comparison to a light standard [23].

The light emission of bioluminescence mutants of *V. harveyi* in the presence of myristic acid or decanal was measured immediately (within 5 sec) after mixing 1 ml of cell culture and 10 μ l of either 10 mM 14:0 or 10 mM decanal, in a glass scintillation vial. Myristic acid-stimulated light emission of the M17 mutant usually reaches a maximum within 30 seconds and then decreases; the maximum value was recorded.

3. Incubation of bacteria with radioactive precursors.

For labeling of ACP with [^3H] β -alanine, 4 μl of *V. harveyi* cells in exponential growth were subcultured into 1 ml of minimal medium containing 0.3 μM [^3H] β -alanine (87 Ci/mmol) and grown to an A_{660} of 2 - 3. For [^3H]14:0 or [^3H]acetate labeling, 1 ml of cells in minimal or complex medium were incubated with 20 μl of 0.2 mM [^3H]14:0 (20 Ci/mmol) or 20 μl of 3.3 mM sodium [^3H]acetate (22 Ci/mmol) for 10 min at 27°C.

For "chasing" with unlabeled fatty acid, cells were labeled with [^3H]14:0 and washed as described below (Method A4), then resuspended in 1 ml of minimal medium and incubated with 5 μM 14:0 for a further 15 min. After chase labeling, cells were washed and extracts prepared in the same way as for radiolabeled cells.

4. Preparation of cell extracts.

Unless specified, radiolabeled or unlabeled cell pellets (from 1 ml culture) were collected in a microcentrifuge (7,000 x g for 1 min), washed once with 1 ml of minimal medium, and resuspended in 50 μl of RIPA buffer. Cells were lysed by sonication (two 20 s bursts at 60% of total energy output) with an ultrasonic processor (Ultrasonic Dismembrator, Artek System Co., Farmingdale, NY) using a 5/32" diameter microtip. After sonication, the lysate was centrifuged at 13,000 x g for 5 min to remove unbroken cells, then heated at 80°C for 5 min and centrifuged again to remove denatured proteins. The supernatant was frozen in liquid nitrogen or used immediately for electrophoresis or immunoprecipitation. The above procedure was done quickly to prevent possible metabolism or degradation of acyl-ACPs. In the case of multiple sample preparation, cell pellets were frozen in liquid nitrogen before being sonicated and only one sample was handled at a time.

5. Analysis of incorporation of [^3H]14:0 into phospholipid and lipid A.

Extraction and analysis of phospholipid and lipid A were carried out essentially as described by Galloway and Raetz [67]. *V. harveyi* M17 cells (1 ml) labeled with 10 μl of 0.2 mM [^3H]14:0 (20 Ci/mmol) in complex medium (Method A3) were centrifuged and

washed once with 1 ml of complex or minimal medium containing 0.1% BSA. The cell pellet was resuspended in 1 ml of complex medium and mixed with 3.75 ml of methanol/chloroform (2:1, v/v) for 1 hour. After centrifugation, the supernatant containing phospholipid was removed from the insoluble residue containing lipopolysaccharide. To prepare monophosphoryl lipid A degradation products from lipopolysaccharide, the insoluble residue was resuspended in 0.25 ml of 0.2 N HCl by sonication and heated at 100°C for 90 min [67]. After neutralizing the hydrolysate with 2N NaOH, thin layer chromatographic (TLC) analysis was performed using silica gel G plates developed in chloroform/methanol/water/ammonia (40:25:4:2, v/v). The supernatant containing phospholipid was mixed thoroughly with 1.25 ml of water and 1.25 ml of chloroform and then centrifuged at low speed. The chloroform phase (lipid extract) was separated by TLC under the above conditions or developed in chloroform/methanol/acetic acid/water (25:15:4:2, v/v). Radiolabeled bands on the plate were either visualized by spraying the plate with En^3Hance prior to fluorography or quantitated by scanning the plate using a BioScan System 200 Radioimaging Detector. Different fractions of the preparation were also counted in a liquid scintillation spectrometer or examined by SDS-PAGE.

6. Polyacrylamide gel electrophoresis and fluorography.

SDS-PAGE was performed according to Laemmli [129] with a few modifications. The resolving and stacking gels consisted of 15% and 4% (w/v) acrylamide, respectively. Unless specified, the running buffer consisted of 50 mM Tris, 384 mM glycine and 0.1% (w/v) SDS per liter. Polyacrylamide gels containing 2M urea were prepared as described by Post-Beittenmiller *et al* [174] with 15% and 5% (w/v) acrylamide in resolving and stacking gels. No urea was added to the running buffer (25 mM Tris and 192 mM glycine).

SDS-PAGE was carried out using either a PROTEAN II or Mini-PROTEAN II dual slab cell (BioRad) while urea-PAGE was performed only with the PROTEAN II dual slab cell. SDS-PAGE using the mini-PROTEAN II system was carried out at 200 volts for 45

min (at 4°C) with gels of 0.75 mm thickness. Inconsistent results were obtained with gel thickness of 1 mm or greater due to overheating.

Cell extracts (Method A4) or enzymatically synthesized acyl-ACP standards (Method C5, below) were diluted with equal volumes of 2 x SDS sample buffer without boiling before loading onto either SDS- or urea-polyacrylamide gels. After electrophoresis, protein bands in SDS gels were visualized by staining with Coomassie Blue and the labeled proteins were visualized by fluorography using En³Hance [239]. Urea-gels were soaked in at least two changes of fixing solution (10% acetic acid and 30% methanol) overnight to remove urea before soaking in En³Hance for fluorography. After fluorography, some ³H-labeled protein bands were cut from the dried gel and reswollen in 200 µl of water in a scintillation vial. Paper and cellophane backing were removed and 0.5 ml of Protosol was added, and the sample was incubated overnight at 37°C. The sample was mixed with 10 ml of cocktail and incubated overnight again at 37°C before counting in a Beckman LS7800 liquid scintillation spectrometer (29% efficiency) [238]. The relative incorporation of label into acyl-ACP intermediates was determined by densitometric scanning of fluorograms with an Apple OneScanner and National Institutes of Health Image 1.45 software. Variation in the labeling of individual acyl-ACP species from samples incubated with [³H]14:0 in duplicate was less than ± 2% of the total acyl-ACP label.

B. Enzymatic analyses.

1. Measurement of ACP concentration with *V. harveyi* acyl-ACP synthetase.

The relative *V. harveyi* ACP concentration during purification was measured by conversion to ³H-acyl-ACP. The acylation mixture (20 µl) containing ACP sample, 0.01 units of *V. harveyi* acyl-ACP synthetase (one unit = 1 nmol acyl-ACP formed per min in the standard assay) [212], 0.1 M Tris-HCl (pH 7.8), 10 mM MgSO₄, 10 mM ATP, 1 mM DTT and 65 µM [³H]14:0 (0.8 Ci/mmol) was incubated at 37°C. Ten-microliter samples

were removed at 10 and 20 min and applied to Whatman 3 MM filter paper (2 cm²) which was washed three times with methanol/chloroform/acetic acid (6:3:1, v/v) to remove unbound fatty acid. The [³H]14:0-ACP product was counted in a liquid scintillation spectrometer. Quantitative conversion was achieved with incubation times longer than 4 hours. Counting efficiency of ³H- or ¹⁴C-labeled acyl-ACP in the filter disk assay was obtained by comparing cpm of purified radiolabeled acyl-ACP products in solution vs filter disks (see below, Method C5).

2. Analysis of 14:0-ACP using *V. harveyi* transferase.

The biological activity of [³H]14:0-ACP synthesized with *V. harveyi* acyl-ACP synthetase was examined by incubating the product with *V. harveyi* luminescence-induced transferase. This enzyme hydrolyses the thioester bond of 14:0-ACP between the acyl group and ACP *in vivo* and is functionally absent in the dark M17 mutant (see Introduction D1). A reaction mixture (total 30 µl) containing 50 mM phosphate, pH 7.0, 1 mM DTT, 70 pmol of [³H]14:0-ACP substrate and 3 µl of transferase (see below, Method C3) which was partially purified from wild-type cells or M17 mutant (control) were incubated at 37°C for different times. The reaction was terminated by adding 30 µl of 2 x SDS sample buffer and the cleavage of [³H]14:0-ACP was monitored by 15% SDS-PAGE and fluorography.

C. Protein purification and characterization.

1. ACP purification.

All purification procedures were carried out at 0-4°C except for operation of the Waters 650 Advanced Protein Purification System (Millipore Canada Ltée., Ville St-Laurent, Quebec) which was performed at room temperature.

Wild type or mutant type M17 of *Vibrio harveyi* B392 was grown as described above in 5 liters of complex medium to an A₆₆₀ of approximately 2. Cells were harvested by centrifugation at 9,000 x g for 20 min and stored at -20°C or used immediately for ACP purification.

V. harveyi ACP was purified by a modification of the procedure described by Rock and Cronan for *E. coli* ACP [189] and ACP activity during purification was monitored by conversion to ^3H -acyl-ACP (Method B1). The cell pellet (35 g) was thawed and suspended in 35 ml of 10 mM MES (pH 6.0) containing 10 mM DTT and 1 mM EDTA. Cells were lysed by sonication (10 bursts of 15 s each at 60% of total energy output) and the lysate was centrifuged at 27,000 x g for 30 min. The pellet was resuspended in the same volume of the buffer, sonicated and centrifuged again. An equal volume of 2-propanol was slowly added to the combined supernatants with continual stirring. The resulting mixture was stirred for an additional 30 min and centrifuged at 19,000 x g for 15 min. The supernatant was kept overnight at 4°C and centrifuged again to remove new precipitate.

The supernatant from 2-propanol precipitation was applied to a DEAE-Sepharose column (2.5 x 15 cm) that had been equilibrated with 80 ml column buffer (10 mM MES, pH 6.0, and 2 mM DTT). The column was eluted sequentially with 120 ml of column buffer, 100 ml of column buffer containing 0.25 M LiCl, and 100 ml of column buffer containing 0.5 M LiCl. The 0.5 M LiCl eluate containing ACP (5-10 ml) was loaded onto a Sephacryl S-200 column (2.5 x 25 cm) which was eluted with column buffer. Fractions containing ACP were pooled and applied to a DEAE-5PW column on a Waters 650 Advanced Protein Purification System. Fractions containing ACP were pooled and 1.6 μl of acetic acid was added per ml of pooled sample to obtain a pH of 4. After overnight precipitation at 4°C, the ACP pellet was collected by centrifugation at 12,000 x g for 10 min and dissolved in 1 ml of 100 mM MES (pH 6.0) and 5 mM DTT. Protein concentrations were determined by the micro-BCA protein assay according to manufacturer's instructions with bovine serum albumin as a standard. *P. phosphoreum* ACP was also purified using the above procedure.

2. Partial purification of *V. harveyi* acyl-ACP synthetase.

V. harveyi acyl-ACP synthetase, which was used for assay of ACP concentration and for preparation of acyl-ACPs, was partially purified using a simple two-step procedure

[212]. Cell extract was prepared in the same way as for ACP purification (Method C1) except that lysis buffer was 20 mM Tris-HCl (pH 7.5) containing 10% glycerol, 1 mM EDTA and 10 μ M DTT. In the first step of purification, the enzyme in cell extract was bound to Cibacron blue F3GA agarose with lysis buffer containing 10 mM MgSO_4 and eluted with lysis buffer containing 0.5 M NaCl. The pooled acyl-ACP synthetase was precipitated with 75% saturated ammonium sulfate, dissolved in lysis buffer, and further purified by Sephacryl S-300 gel filtration [212].

3. Partial purification of *V. harveyi* transferase.

Cell free extracts from either *V. harveyi* wild type or mutant M17 were made as in Method C1 except that cell pellets (0.25 g) were lysed by sonication in 1.5 ml of 20 mM phosphate (pH 7.0), 10 mM β -mercaptoethanol, and the pellet was discarded after centrifugation at 13,000 x g for 5 min in microcentrifuge. Solid ammonium sulfate was added to the cell free supernatant to obtain 30% saturation, stirred for 30 min, and centrifuged again at 13,000 x g for 30 min to remove precipitate. The supernatant was made 50% saturated with ammonium sulfate, stirred for another 30 min, and supernatant was removed by centrifugation at 13,000 x g for 30 min [22]. The precipitate fraction of 30-50% saturated ammonium sulfate was dissolved in 0.25 ml of 20 mM phosphate, pH 7.0 and 2 mM DTT.

4. Protein sequencing of ACP.

To transfer ACP to PVDF membranes for protein sequencing, purified *V. harveyi* ACP was fractionated (10 μ g/well) by 15% SDS-PAGE using a Mini-PROTEAN II dual slab cell (Method A6), with the following modifications introduced to minimize chemical modification. The cross-linker N,N'-methylene-bis-acrylamide (Bis) was replaced with piperazine diacrylamide (PDA). The acrylamide stock solution [29.2% acrylamide (w/v) and 0.8% PDA (w/v)] was mixed with 1% Amberlite (w/v) for 15 min at room temperature and filtered prior to polymerization, and the polymerized gel was aged for 24 hours before use. Immobilon PVDF Transfer Membrane, prepared as suggested by the manufacturer,

was used for protein transblotting (Method E2). After transfer, ACP was visualized by staining the membrane with 0.025% Coomassie brilliant blue in 40% methanol for 10 min and destaining in 50% methanol for 10 hours. The ACP bands were cut from the membrane and stored in distilled water at -70°C, and the protein sequence was determined by Edman degradation using an Applied Biosystems 473A Sequencer (Department of Biochemistry, Queen's University, Kingston, Ont.).

5. Acyl-ACP preparation and purification.

For large scale preparation of acyl-ACP derivatives, purified ACP from *E. coli* or *V. harveyi* (50-70 μ M, about 0.44-0.62 mg/ml) was incubated as described in Method B1 with *V. harveyi* acyl-ACP synthetase (0.5-1.5 units/ml) and ^3H - or ^{14}C -labeled fatty acids of varying chain length in a total volume of 0.3 ml at 37°C. The mixtures were incubated for more than 4 hours or overnight for maximal conversion of ACP to acyl-ACP. Conversion of ACP to labeled acyl-ACP was monitored by SDS-PAGE or by filter disk assay (above). For SDS-PAGE, the reaction was terminated by adding an equal volume of 2 x SDS sample buffer and applied to 15% polyacrylamide slab gels in the absence of reducing agents.

In some experiments, the labeled acyl-ACP product was purified further by modifications of published methods [191]. The reaction mixture was applied to a small (1 ml) DEAE-Sepharose column equilibrated in 10 mM MES (pH 6.0). After washing with the same buffer to remove unbound proteins, the column was washed with this buffer containing 80% 2-propanol to remove free fatty acid, followed by 10 mM MES (pH 6.0) again to remove 2-propanol. ACP and acyl-ACP were subsequently eluted with 10 mM MES containing 0.6 M LiCl [191]. This fraction was further applied to an octyl-Sepharose column to separate acyl-ACP from ACP. Only acyl-ACP, but not ACP, was bound to octyl-Sepharose in 10 mM MES (pH 6.0) and was eluted from the column with this buffer containing 45% 2-propanol (v/v). The fractions containing acyl-ACP were reapplied to

DEAE-Sepharose to remove 2-propanol and eluted with 10 mM MES containing 0.5 M LiCl [191].

D. Molecular biology methods.

1. Synthesis of radiolabeled oligonucleotide probes.

Pooled sense oligonucleotides (AP-1, 26 bases) based on a portion of the amino acid sequence of *V. harveyi* ACP, Asp¹⁸-Glu-Ala-Glu-Val-Lys-Asn-Glu-Ala²⁶, were synthesized in the laboratory of Dr. Paul Liu, Department of Biochemistry, Dalhousie University. Among four possible codons for Ala²⁰ and Val²², only one GCA and GTT were chosen, respectively, while GC was used for Ala²⁶. For other amino acid residues, which are specified by two codons only, both possible codons (at a 1:1 ratio) were chosen in the synthesis. Another oligonucleotide probe (AP-2, 18 base, 5' AAGCTTCTTTTCGTTGACG 3') was synthesized using a Biosearch 8750 synthesizer (Department of Biochemistry, Queen's University, Kingston, Ont.)

The oligonucleotides were labeled by phosphorylation of 5' termini with [γ -³²P]ATP. The reaction mixture (20 μ l) contained oligonucleotides (17 pmol), 2 μ l of 10 x kinase buffer provided by the manufacturer, 50 μ Ci of [γ -³²P]ATP (3000 Ci/mmol) and 8 units of T4 polynucleotide kinase. The labeled probes were purified by precipitation with ethanol and dissolved in 100 μ l TE buffer [203].

2. Preparation of DNA.

The genomic DNA from both *V. harveyi* and *E. coli* was extracted. Bacterial cell pellets collected from 40 ml of cell culture ($A_{660} \approx 2$) were resuspended in 1 ml lysozyme solution (10 mM Tris-Cl, pH 8.0, 0.15 M NaCl, 0.1 M EDTA and 2 mg/ml lysozyme) in a glass tube, incubated at 37°C for 30 min, and then frozen in liquid nitrogen. The frozen sample was quickly thawed by mixing with eight volumes of lysis buffer (0.1 M Tris-Cl, pH 8.0, 0.1 M NaCl and 1% SDS) and repeatedly inverting the tube. The thawed sample was extracted once with an equal volume of phenol (saturated with 0.1 M Tris-Cl, pH 8.0)

to clear unbroken cells and proteins and then transferred to a clean beaker. Two volumes of ethanol were added to the beaker followed by slowly stirring with a glass rod to mix the two separate phases, and the genomic DNA was gradually precipitated around the rod. The DNA pellet was washed with 70% ethanol, dried in air until ethanol had evaporated, and then solubilized in 3 ml of TE buffer. Once the nucleic acids were dissolved, RNase A was added to a final concentration of 60 $\mu\text{g/ml}$. Following incubation at 37°C for 30 min, the sample was extracted three times with an equal volume of phenol/chloroform (1:1, v/v), once with an equal volume of chloroform, and DNA was precipitated by addition of 1/10 volume of 3 M NaAc and two volumes of ethanol. The genomic DNA was washed once with 70% ethanol, air dried again, and finally dissolved in 1.5 ml TE buffer by incubation overnight at 4°C.

Preparation of plasmid DNA was carried out by the alkaline lysis method [203] with some modification. Briefly, bacterial cells centrifuged from 1.5 ml culture were resuspended in 100 μl of 25 mM Tris-Cl (pH 8.0) containing 50 mM glucose and 10 mM EDTA, and then mixed quickly with 200 μl of 0.2 N NaOH, 1% SDS before addition of 150 μl ice-cold 5 M KAc. The mixture was vortexed gently, incubated on ice for 5 min, and extracted directly with an equal volume of phenol/chloroform (1:1, v/v) without prior precipitation. Plasmid DNA was precipitated by addition of 2 volumes of ethanol, washed with 70% ethanol and dissolved in TE buffer containing RNase at 20 $\mu\text{g/ml}$.

For DNA sequencing, the recombinant plasmid pUSS11 and nested deleted plasmids generated from pUSS11 were purified by a modified alkaline lysis method [203]. Briefly, bacterial cells precipitated from 150 ml of cell culture were resuspended in 10 ml of 25 mM Tris-Cl (pH 8.0) containing 50 mM glucose and 10 mM EDTA, then mixed quickly with 20 ml of 0.2 N NaOH, 1% SDS and incubated at 0°C for 5 min. After addition of 15 ml of ice-cold 5 M KAc, the mixture was vortexed gently, incubated for another 5 min on ice, and centrifuged to remove precipitate. Two volumes of ethanol were added to the supernatant and DNA was precipitated after centrifugation and washed with 70% ethanol.

The pellet was dissolved in 1 ml of TE buffer containing RNase A (50 µg/ml) and incubated at 37°C for 30 min. The sample was extracted twice with an equal volume of phenol/chloroform (1:1, v/v) and twice with an equal volume of chloroform, and then mixed with 0.6 ml of 20% PEG in 2.5 M NaCl. The mixture was incubated at 0°C for at least 1 hour and centrifuged in a microcentrifuge (300 rpm for 7 min) if still turbid after incubation. The supernatant was further centrifuged at 14,000 rpm for 7 min, and the plasmid DNA precipitate was washed with 70% ethanol and dissolved in TE buffer. The plasmid DNA from 150 ml cell culture was enough for 5 double-stranded DNA sequencing reactions.

3. Southern hybridization.

V. harveyi or *E. coli* genomic DNA was digested by overnight incubation of 1 µg DNA with 10-20 units of restriction endonucleases in 30 µl reaction mixture while purified plasmid DNA was hydrolyzed in 10 µl containing less than 50 ng DNA and 2 units of restriction enzymes for 2 hours in the appropriate buffer supplied by the manufacturer. The resulting DNA fragments were separated in a 0.8% agarose gel and transferred to positively charged nylon membrane using 20 x SSC as transfer buffer [203]. DNA was crosslinked to the membrane in a UV crosslinker (Stratagene Cloning System, La Jolla, CA), and the membrane was treated with prehybridization solution (6 x SSC, 5 x Denhardts, 20 mM NaH₂PO₄ and 0.5 mg/ml denatured sheared salmon sperm DNA) for 2 hours at 42°C and hybridized by overnight incubation with hybridization solution (6 x SSC, 20 mM NaH₂PO₄, 0.4 % SDS, 0.5 mg/ml denatured sheared salmon sperm DNA and 1 x 10⁷ dpm [³²P]oligonucleotide probe) at 42°C in a hybridization oven (Hybaid Ltd.). After washing with 6 x SSC and 0.1 % SDS for 15 min once at 42°C, twice at 46°C and three times at 51°C, the membrane was covered with Saran Wrap and exposed to Kodak XAR 5 film at -70°C for 1 to 5 days.

4. Cloning and screening of the *V. harveyi* ACP gene.

V. harveyi DNA was hydrolyzed by single or double digestion with restriction enzymes and separated in a 0.8% agarose gel as described above. DNA of appropriate molecular weight, determined by positive hybridization of the [³²P]oligonucleotide probe, was extracted from the agarose gel with a GeneClean II Kit. The extracted fragments were ligated to pUC18 or pUC118 vector DNA which was digested with the same restriction enzyme(s). DH5 α ' competent cells were transformed with recombinant plasmid DNA, plated on LB-agar containing 50 μ g ampicillin per ml, and incubated overnight at 37°C. The screening of *E. coli* colonies that contain part of the ACP gene on the recombinant plasmid was performed as described by Forster et al [63]. Briefly, bacterial colonies were transferred from agar plate to Whatman 42 filter paper by pressing the filter against the plate and then peeling it from the plate after it was wet. After floating the filter on 2 x SSC and 5% SDS for 2 min, the clones were fixed by treating the filter in a microwave oven with rotating turntable for 2.5 min at full setting. Thirty ml of cold water in a small beaker was microwaved simultaneously to prevent overheating of the filter. The agar plate was incubated at 37°C until small colonies appeared and then stored at 4°C. The fixed filter was used for hybridization with [³²P]oligonucleotide probe, using the same procedures for prehybridization, hybridization and washing as described for Southern hybridization above. Clones giving positive hybridization were picked for further analysis which included mapping the insert fragment with different restriction enzymes and subcloning the insert fragment into pUC118 vector.

5. Sequencing of the *V. harveyi* ACP gene.

The recombinant plasmid pUSS11 hydrolysed with PstI and BamHI was used to generate a set of nested deletions from the BamHI site with exonuclease III and S1 nuclease [203]. DNA fragments of different length from nested deletion were ligated, transformed into DH5 α ' competent cells, and purified as described above (Method C2). For the double-stranded DNA sequencing reaction, the plasmid DNA was partially denatured by adding

1/10 volume of 2 N NaOH, incubated at room temperature for 5 min, and precipitated again by adding 0.4 volume of 5 M NH₄Ac and 3 volumes of ethanol. After 10 minutes at -70°C the precipitated DNA pellet was collected by centrifugation at 14,000 rpm for 20 min in a microcentrifuge, washed once with 70% ethanol and dried. The sequencing of denatured double-stranded DNA was performed using the dideoxy chain-termination method, with the Sequenase enzyme system and α -[³⁵S]dATP according to instructions from the manufacturer. The amino acid sequence was deduced from the gene sequence, and the MW and pI were calculated using DNAid+1.6 software.

E. Immunochemical methods.

1. Preparation of anti-*V. harveyi* ACP immune serum.

A rabbit anti-ACP immune serum was prepared against purified *V. harveyi* ACP by intramuscular injection of 250 µg ACP in 0.4 ml of Freund's complete adjuvant. Three booster injections, each with 200 µg ACP in incomplete adjuvant, were given at 2-3 week intervals. Serum was collected two weeks after the last injection. The relative level of anti-ACP antibody in serum was monitored by antibody capture assay in a microtiter plate [85]. Purified *V. harveyi* ACP (50 µl/well, 20 µg/ml) was added to the plate and incubated for 2 h. The plate was washed twice with PBS and then incubated with blocking solution (PBS-Tween) for 2 h. After washing the plate with PBS, antiserum (1:500-1:5000 diluted in PBS) was added to the plate (50 µl/well) for another 2 h incubation. The plate was washed with PBS-Tween and then with 50 mM Tris-HCl (pH 7.5) containing 0.9% NaCl (w/v) and 0.05% Tween (v/v), followed by 2 h incubation with 1:500 diluted secondary antibody, goat anti-rabbit IgG alkaline phosphatase conjugate (50 µl/well). Unbound secondary antibody was removed by washing twice with 50 mM Tris-HCl (pH 7.5) containing 0.9% NaCl (w/v) and 0.05% Tween (v/v), then twice with 10 mM diethanolamine containing 0.5 mM MgCl₂. Ten grams of p-nitrophenyl phosphate, a substrate for alkaline phosphatase, was dissolved in 10 ml of 10 mM diethanolamine

containing 0.5 mM MgCl₂, and added to the plate (50 µl/well) for color reaction. Color development was terminated by adding 0.2 volume of 0.5 mM EDTA and quantitated spectrophotometrically at 405 nm in a microplate reader.

2. Detection of ACP by western blot.

Cell pellet from 1.5 ml culture of *V. harveyi* or *E. coli* in complex medium ($A_{660} \approx 2$) was suspended in 100 µl of 10 mM MES (pH 6.0) containing 10 mM DTT and 1 mM EDTA and lysed by sonication (2 bursts of 15 s each). The supernatant was collected in a microcentrifuge at 13,000 x g for 10 min. Protein (5 µg per lane) was separated by mini-SDS-PAGE and electrophoretically transferred to nitrocellulose membrane in 25 mM Tris and 192 mM glycine at 300 mA for 2 hours according to the procedures provided by the manufacturer (Mini Trans-Blot Cell, Bio-Rad). After transblotting, the gel was stained with Coomassie Blue to check the transfer efficiency. The blot was rinsed twice with TBS-Tween (0.9% NaCl, 10 mM Tris-Cl, pH 7.5, 0.02% sodium azide and 0.2% Tween-20), incubated with blocking solution (TBS-Tween containing 5% instant nonfat dry milk) for 2 hours, and washed twice with TBS-Tween again for 10 min each. The antiserum was diluted 1:5000 in blocking solution containing 1% NP-40 (v/v) before use; the secondary antibody, goat anti-rabbit IgG alkaline phosphatase conjugate, was diluted in the same solution at 1:3000. After incubation with diluted antiserum by gentle agitation at room temperature for 1.5 hours, the blot was washed once with TBS-Tween, once with RIPA buffer (pH 7.5), and twice with TBS-Tween for 20 min each, respectively. The blot was incubated with secondary antibody for 1.5 hours, followed by the above wash steps and two additional washes with TBS (without Tween-20) for 10 min each. ACP and its derivatives were observed by developing the membrane using alkaline phosphatase colour reagents provided by Bio-Rad. To specifically detect ACP from *V. harveyi*, but not *E. coli*, the antiserum was preincubated with *E. coli* ACP (12 µg ACP per µl immune serum) overnight at 4°C, and the mixture was diluted in blocking solution containing 1% NP-40 and used as described above.

3. Immunoprecipitation of radiolabeled ACP and acyl-ACP.

V. harveyi cells from 1 ml cell culture were labeled with [^3H] β -alanine or [^3H]14:0 and lysed as described previously (Method A3 and A4). For immunoprecipitation [85], 30 μl of labeled cell extract was incubated with 20 μl of antiserum or preimmune serum and 5 μl of 5 x RIPA buffer at 0°C for 90 min. One hundred μl of 20% (w/v) fixed *Staphylococcus aureus* cells (SAC), which had been washed with and resuspended in RIPA buffer, was added for a further 2 h at 0°C. The pellets were washed three times with RIPA buffer and collected by centrifugation, resuspended in 200 μl of SDS sample buffer and heated to 85°C for 5 min to release antigen. After centrifugation, the supernatant was applied to SDS- or urea-acrylamide gel.

4. Co-precipitation of acyl-ACP synthetase by anti-ACP immunoserum.

The following procedures were carried out on ice except for sonication and centrifugation which were at 4°C. *V. harveyi* cell pellet (0.7 g) was suspended and sonicated in 1.4 ml of 20 mM Tris-Cl (pH 7.5) containing 50 μM DTT, 10 % glycerol and 1 mM EDTA. After centrifugation at 13,000 x g for 5 min to remove unbroken cells, the cell extract was pre-incubated for 1 hour with 0.2 volumes of preimmune serum and then incubated with 20 μg of SAC pellet, which had been washed twice with 10 mM Tris-Cl (pH 7.5), for another hour. The pellet was removed by centrifugation and the supernatant was treated again in the same way with washed SAC to ensure that all the antibody was cleared from the cell extract. Then, the preimmune serum-treated cell extract was divided into two equal portions (about 0.8 ml) and incubated with 10 μl anti-ACP antiserum or preimmune serum for 1.5 hour, followed by incubation with 10 μg washed SAC pellet for 3 hours. The pellet was precipitated by centrifugation, washed three times with lysis buffer, and incubated with 0.5 M phosphate buffer (pH 7.5) containing 2 mM DTT and 10% glycerol (v/v) for 10 min at 0°C. After centrifugation, the pellet was removed and the activities of ACP and acyl-ACP synthetase in the supernatant were analyzed [212].

F. Isolation and analysis of *V. harveyi* FA mutants.

1. Mutagenesis of *V. harveyi* cells.

V. harveyi wild type or M17 mutant cells in exponential growth were subcultured 1:1000 into 50 ml of minimal medium and grown to $A_{660} = 0.4$. Cells were then centrifuged and resuspended in minimal medium to give an A_{660} of 2. One ml aliquots of cell suspension were mixed with different concentrations of nitrosoguanidine (0-100 $\mu\text{g/ml}$). After incubation at 27°C for 30 min with gentle shaking, the cells were centrifuged, washed once with minimal medium, resuspended in 1 ml of complex medium, mixed with 0.25 ml of glycerol and stored at -70°C. The nitrosoguanidine-treated cell samples were diluted at 10^3 or 10^4 with complex medium and spread on agar plates to determine cell survival. Preparations with cell survival rates of 5% to 10% (from 50-100 $\mu\text{g/ml}$ nitrosoguanidine treatment) relative to control were used for further experiments [33].

2. Screening of double bioluminescent mutations in *V. harveyi* M17.

Nitrosoguanidine-treated *V. harveyi* M17 cells were grown in complex medium to $A_{660} \approx 0.1$ and spread on agar plates to give about 400 colonies per plate. Single colonies on the plates were picked up and resuspended in 0.28 ml of complex medium in a microtiter plate (0.4 ml/well, 12 x 8 wells) and incubated in a rotary shaker (27°C, 200 rpm) until $A_{660} \approx 1$. Ten microliters of 20 mM 14:0 was added to each well using a multi-channel micropipettor. After adding 14:0, bioluminescence of cells was observed visually in a dark room and dim mutants were chosen for further investigation.

3. Selection of fatty acid transport mutants by ^3H suicide.

Nitrosoguanidine-treated *V. harveyi* M17 cells were cultured to $A_{660} \approx 0.3$, and then 3.5 ml of cells were incubated with 50 μl of 0.2 mM [^3H]14:0 (20 Ci/mmol) or 0.2 mM unlabeled 14:0 (control) for 30 minutes [90]. The cells were washed once with minimal medium, twice with minimal medium plus 4 μM myristic acid and twice with complex

medium. Following the washes, cells were resuspended in complex medium, mixed with 2/3 volume of 50% glycerol and dispensed into aliquots for storage at -70°C. The incorporation of [^3H]14:0 into cells was measured by counting washed cell samples in a liquid scintillation spectrometer. Periodically, these cells were spread on agar plates and survival determined by colony counting. The incorporation of [^3H]myristic acid into phospholipids of surviving cells was measured as described in Method A5.

Section IV. Results.

A. *V. harveyi* ACP purification and sequence determination.

1. Purification of *V. harveyi* ACP.

To investigate the role of ACP in activation of exogenous fatty acid and other processes in *V. harveyi*, ACP was purified by modification of procedures used for the isolation of *E. coli* ACP. The initial steps included isopropanol extraction and batch chromatography on DEAE-Sepharose as described by Rock and Cronan [189], and most (99%) of the contaminating protein was removed from the sample at this stage. Further purification involved successive chromatography on Sephacryl S-200 and DEAE-5PW columns. Although ACP was not completely separated from the major protein peak in gel filtration (Fig. 9), this step was found necessary to remove high molecular weight contaminants that comigrated with ACP upon subsequent DEAE-5PW chromatography (Fig. 10). The ACP fractions from the DEAE-5PW column were concentrated and further purified by acid precipitation at pH 4 [189]. A pH of 6 was maintained during all column purification steps and in storage to reduce the formation of disulfide-linked ACP dimers [188,189]. MES buffer was found to give better results than phosphate buffer since a sharper ACP peak was observed in chromatography on DEAE-5PW. The purified ACP was very stable during storage in MES buffer (pH 6) at -20°C for several months.

The amount of *V. harveyi* ACP at different purification stages was analyzed by acylation with [³H]14:0 in the presence of partially purified *V. harveyi* acyl-ACP synthetase. Under these conditions, ACP is the limiting substrate and can be quantitatively measured. Previous experiments showed that *E. coli* ACP at concentrations from 1 to 400 pmol can be measured using the filter disk assay after acylation with acyl-ACP synthetase (0.5 units/ml) in a 4 h incubation [212]. Table III summarizes the recovery of ACP at various stages during the purification. As indicated in Table III, ACP was purified 1700-fold with 62% recovery. Higher amounts of ACP were observed at the DEAE-Sepharose

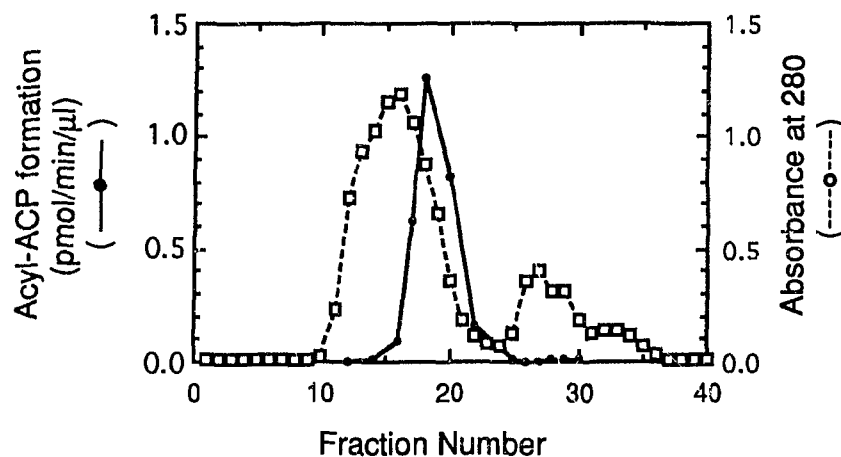


Fig. 9. Gel filtration of *V. harveyi* ACP. Active fractions from DEAE-Sephacryl chromatography were combined and applied to a Sephacryl S-200 HR column (19 x 2.5 cm) in 10 mM MES (pH 6.0) and 2 mM DTT. The sample contained 16 mg of protein in 6 ml. The flow rate was 1.2 ml/min and 3 ml fractions were collected. Elution was monitored by absorbance at 280 nm (----) and ACP activity (—) was measured as pmol [^3H]14:0-ACP formed per min per μl of fraction as described in Method B1.

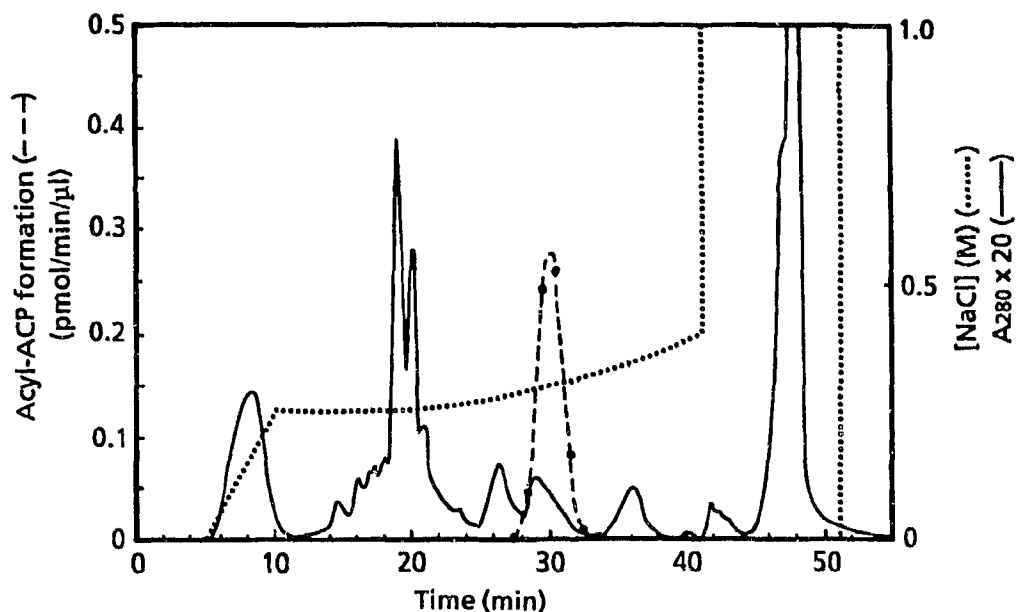


Fig. 10. Purification of *V. harveyi* ACP by anion-exchange chromatography. Fractions containing ACP from Sephacryl S-200 gel filtration were pooled and applied (about 1 mg protein in 2.5 ml) to a DEAE-5PW column (0.8 x 7.5 cm) operated on a Waters 650 Advanced Protein Purification System. The column was equilibrated in 10 mM MES (pH 6.0) and 2 mM dithiothreitol, and eluted with a nonlinear gradient of NaCl (····) in this buffer. The flow rate was 1 ml/min and elution was monitored by absorbance at 280 nm (—). One ml fractions were collected and ACP concentration (—•—) was measured as described in Method B1.

Table IIIPurification of acyl carrier protein from *V. harveyi*

Step	Total protein ^a (mg)	Total activity ^b (nmol formed/min)	Specific activity (nmol/min/mg)	Purification (n-fold)
Cell extract	3203	10.2	0.0032	---
2-Propanol	273	5.2	0.0191	6
DEAE-sepharose	31	13.2	0.438	137
Sephacryl-S200	12	16.4	1.369	428
HPLC & Acid precipitation	1.2	6.7	5.550	1734

a. Protein concentration was measured by the micro-BCA method.

b. ACP activity was measured using *V. harveyi* acyl-ACP synthetase as described in Method B1. ACP activity is defined as nmole [³H]14:0-ACP formed per minute.

and Sephacryl-S200 stages than in the lysate. This may be due to inhibition of acyl-ACP synthetase by some factors in the latter. Both 2-propanol and LiCl strongly inhibit acyl-ACP synthetase, making an accurate assessment of ACP concentration in the stages of 2-propanol precipitation and DEAE-Sepharose separation difficult. In a typical preparation, the yield of ACP was 60 mg per kilogram wet weight of *V. harveyi* cells.

Purified *V. harveyi* ACP migrated as a single 20 kDa band on SDS-PAGE (Fig. 11), similar to *E. coli* ACP ($M_r = 8,860$) which is known to exhibit anomalous migration in this gel system [186].

2. ACP protein sequencing.

After gel electrophoresis and transfer to Immobilon membrane, purified *V. harveyi* ACP was used for protein sequencing to obtain information for further molecular characterization. The sequence of the first 55 amino acids from the N-terminus was determined on an Applied Biosystems 473A Sequencer and is shown in Fig. 12. The corresponding *E. coli* ACP sequence [178] is shown for comparison. The two ACPs are highly similar: both have an N-terminal serine residue and a serine for phosphopantetheine attachment at the 36 position. An overall 85% identity was observed between amino acid residues 1 and 55 while the sequence surrounding serine-36 (residues 31 to 55) is identical. Less identity (38%) was observed between amino acid residues 18 and 25 of the two ACPs, which was important for the design of a specific oligonucleotide probe for cloning of the *V. harveyi* ACP gene (see below).

3. Cloning of the *V. harveyi* ACP gene.

To further characterize *V. harveyi* ACP, its gene was cloned in *E. coli*. The strategy for cloning the *V. harveyi* ACP gene (*acpP*) was to design an oligonucleotide probe, based on the amino acid sequence, which would selectively hybridize to the *V. harveyi* but not the *E. coli* ACP gene. The region of limited identity between residues 18 and 25 was chosen. Among nine amino acids in this region (Fig. 13A), six have two possible codons and both codons were chosen at a 1:1 ratio for oligonucleotide synthesis. However, only

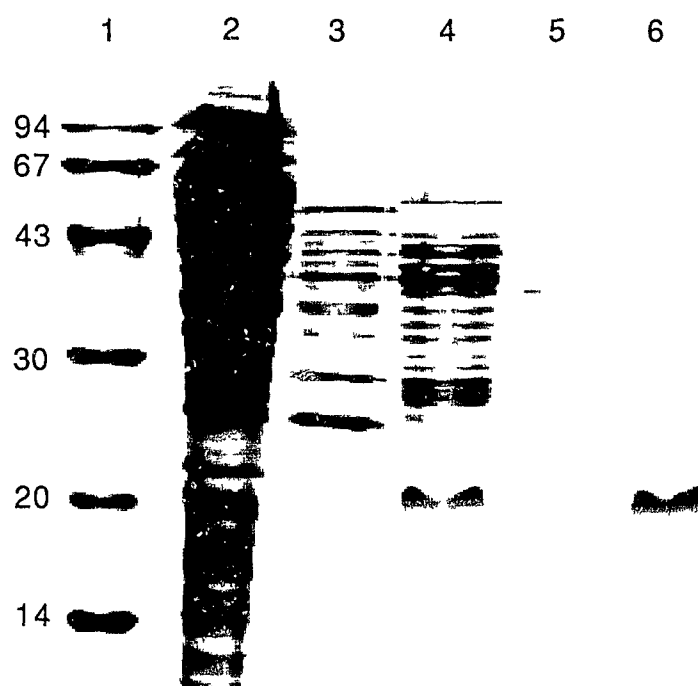


Fig. 11. SDS-PAGE of *V. harveyi* proteins at various stages of ACP purification. Samples were separated in 15% (w/v) acrylamide (4% acrylamide stacking gel) and visualized by Coomassie Blue staining. The running buffer consisted of 0.05 M Tris, 0.4 M glycine and 0.1% (w/v) SDS. *Lane 2*, cell-free lysate (500 μ g); *lane 3*, 2-propanol supernatant (55 μ g); *lane 4*, DEAE-Sepharose pool (55 μ g); *lane 5*, Sephacryl S-200 pool (12.6 μ g); *lane 6*, DEAE-5PW pool concentrated by acid precipitation (16 μ g). *Lane 1* contains protein markers: phosphorylase b, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; α -lactalbumin, 14.4 kDa.

	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75
<i>E. coli</i>	STIEERVKKIIGEQLGVKQEEVTNNASFVEDLGADSLDTVELVMALEEEFDTEIPDEEAEKITT VQAAIDYINGHQA														
<i>V. harveyi</i>	SNIEERVKKIIVEQLGVDEAEVKNEASFVDDL GADSLDTVELVMALEEEFDTEIP														

Fig. 12. N-terminal sequence of *V. harveyi* acyl carrier protein in comparison to the *E. coli* sequence. The N-terminal amino acid sequence of purified *V. harveyi* ACP was determined by Edman degradation using an Applied Biosystems 473A Sequencer.

A) Synthesized oligonucleotide probe AP-1:

E. coli ACP sequence (residues 18-26): Lys Gln Glu Glu Val Thr Asn Asn Ala

V. harveyi ACP sequence (residues 18-26): Asp Glu Ala Glu Val Lys Asn Glu Ala

Possible *V. harveyi* DNA sequence
deduced from ACP protein sequence:

GA ^T _C	GA ^A _G	GC ^T _A	GA ^A _G	GT ^T _A	AA ^A _G	AA ^T _C	GA ^A _G	GC
------------------------------	------------------------------	------------------------------	------------------------------	------------------------------	------------------------------	------------------------------	------------------------------	----

Synthesized oligonucleotide probe AP-1:

GA ^T _C	GA ^A _G	GCA	GA ^A _G	GTT	AA ^A _G	AA ^T _C	GA ^A _G	GC
------------------------------	------------------------------	-----	------------------------------	-----	------------------------------	------------------------------	------------------------------	----

(Corresponding *E. coli* DNA sequence: AAG CAG GAA GAA GTT ACC AAC AAI GC)

B) Synthesized oligonucleotide probe AP-2:

Synthesized oligonucleotide probe AP-2:

AAGCTTCTTTCGTTGAC

(Corresponding *E. coli* DNA sequence: AIGCTTCTTTCGTTGAA)

Fig. 13. Sequence of synthesized oligonucleotide probes and comparison to the corresponding *E. coli* ACP gene sequence. Nucleotides which must be different between *V. harveyi* and *E. coli* are underlined.

one codon was chosen for Ala and Val although four possible codons exist for each; the choice of nucleotide at the third position of these codons was made on the basis of the corresponding codons in the *E. coli* ACP gene (Fig. 13). Thus, the comprehensive pool of synthetic probe AP-1 contains 64 different oligonucleotides. The maximum possible identity between any synthetic oligonucleotide and the corresponding region of the *E. coli* ACP gene [178] is 70% while the minimum identity with the *V. harveyi* gene is 77%. More than one third of the synthetic oligonucleotides have sequences that are 85% or more identical to that of the *V. harveyi* ACP gene.

As expected, hybridization of ^{32}P -labeled AP-1 with Pst-digested *E. coli* genomic DNA or MR24 plasmid DNA (containing the *E. coli* ACP gene) was not observed (Fig. 14). However, several double digests of *V. harveyi* genomic DNA yielded a single hybridizing band (Fig. 14), indicating that only the *V. harveyi* ACP gene can be recognized by the AP-1 probe under these conditions. Among these, the ACP gene was localized on a 1.4-kb EcoRI-BamHI fragment since only the double digestion of EcoRI and BamHI, but not the single digestion of either EcoRI or BamHI, resulted in a single 1.4-kb band hybridizing to ^{32}P -labeled oligonucleotide probe AP-1. Therefore, DNA of approx. 1.4 kb from EcoRI-BamHI digestion was extracted from an agarose gel and was ligated to EcoRI-BamHI-digested pUC18 at a ratio of 1:1 (w/w). A recombinant plasmid pUS1, which has a 2.8-kb fragment containing the *acpP* region inserted in pUC18 vector, was detected by screening 5,000 colonies with [^{32}P]oligonucleotide probe AP-1 (Fig. 15). Restriction mapping indicated that the ACP gene was on a 1.4-kb EcoRI-EcoRI fragment instead of the expected 1.4-kb EcoRI-BamHI fragment. EcoRI-digestion of *V. harveyi* genomic DNA did not reveal any hybridization except in the high molecular weight region (data not shown), indicating that the DNA was poorly hydrolyzed by EcoRI alone. This might explain why a 1.4-kb hybridizing band was not observed in Southern hybridization after EcoRI digestion. Fortunately, this 1.4-kb EcoRI-EcoRI fragment was ligated to a 1.4-kb EcoRI-BamHI fragment to form a 2.8-kb EcoRI-BamHI fragment which was

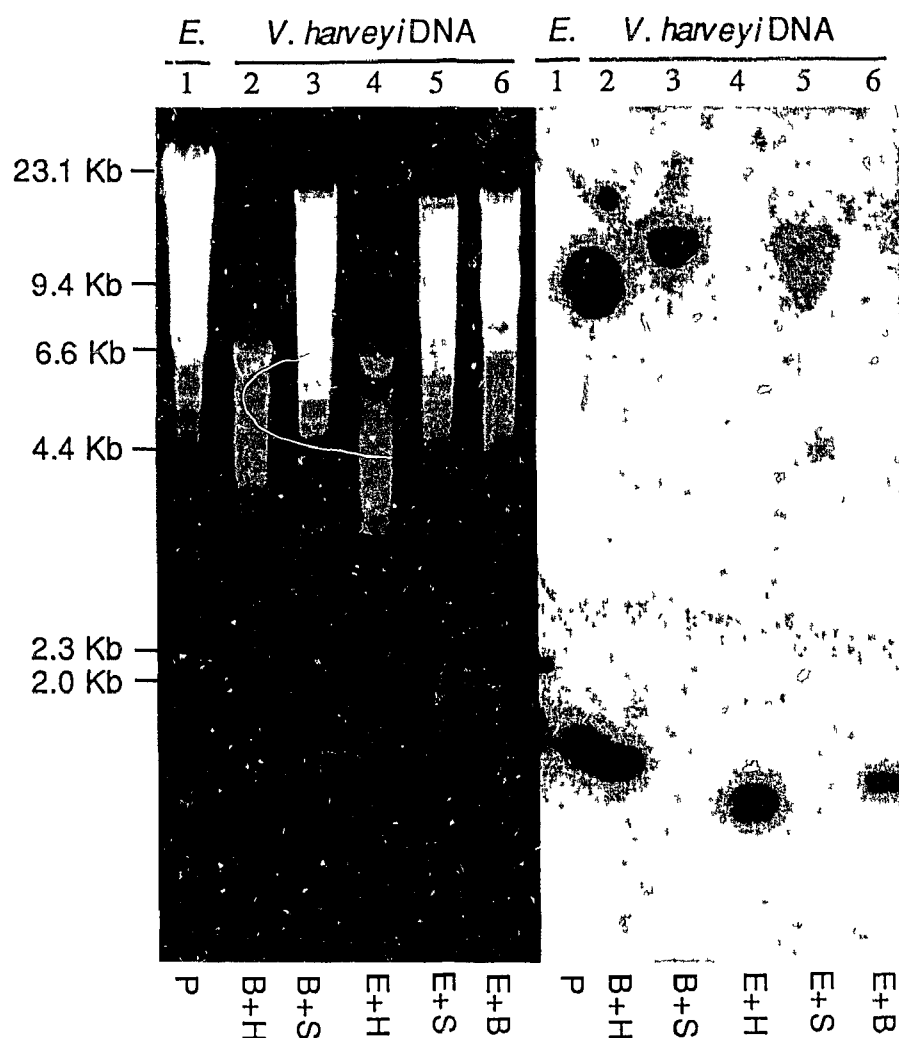


Fig. 14. Southern hybridization of *V. harveyi* or *E. coli* DNA with ^{32}P -labeled synthesized oligonucleotide probe AP-1. *E. coli* genomic DNA was extracted from *E. coli* strain MR24. Since the strain contains the recombinant plasmid pMR24 encoding the *E. coli* ACP gene, lane 1 (E.) includes both *E. coli* genomic DNA and pMR24 DNA. Lanes 2-6 contain restricted *V. harveyi* genomic DNA. DNA hydrolysis, separation and Southern hybridization were performed as described in Method D3. Restriction enzymes used in DNA hydrolysis are: PstI (P), BamHI (B), HindIII (H), SalI (S) and EcoRI (E).

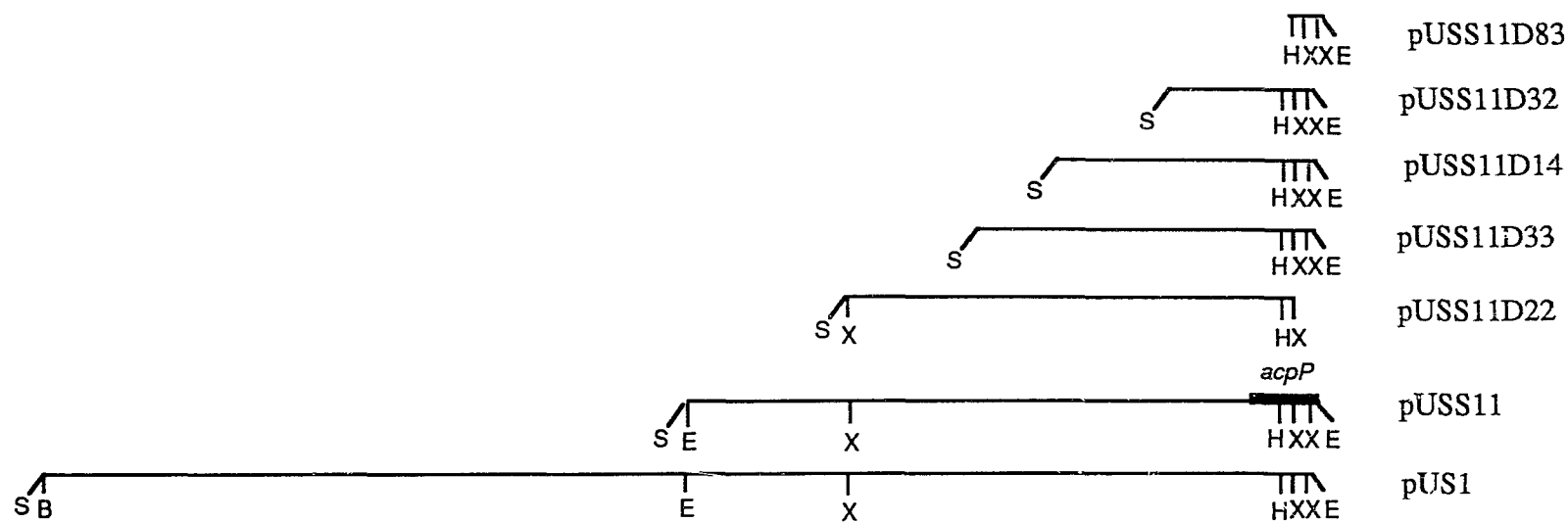


Fig. 15. Aligned partial restriction maps of recombinant plasmids used in this work. The *V. harveyi* insert portion of recombinant plasmids which carry *V. harveyi* ACP gene (*acpP*) is shown. The plasmid pUS1 was constructed by inserting a 2.8-kb BamHI-EcoRI *V. harveyi* chromosomal DNA fragment into pUC18 digested with same enzymes. The plasmid pUSS11 was constructed by ligating the 1.4-kb EcoRI-EcoRI fragment of pUS1 with EcoRI-digested pUC18. The plasmid pUSS22 was constructed by ligating 1.1-kb XbaI-XbaI fragment of pUSS11 with XbaI-digested pUC18. The plasmid pUSS83 was constructed by ligating a 71-bp HindIII-EcoRI fragment of pUSS11 with pUC18 digested with same enzymes. Other plasmids were constructed by nested deletion of pUSS11. The SphI (S) site is on the vector, and the EcoRI (E), HindIII (H), XbaI (X), BamHI (B) sites are either on the insert fragment or sites linking the insert fragment and vector. Only pUSS11 is labeled with a black bar to indicate position of the ACP gene.

successfully cloned with EcoRI-BamHI-digested vector. Another recombinant plasmid pUSS11, which was used for further subcloning and sequencing, was constructed by ligating the 1.4-kb EcoRI-EcoRI fragment of pUS1 with EcoRI-digested pUC18 (Fig. 15). Although colonies should theoretically contain recombinant plasmids with the insert in both directions, ten colonies screened all showed inserts with the ACP gene oriented in opposition to the direction of *lacZ* promoter on the vector. Sequence data from pUSS11 suggested that both pUSS11 and pUS1 contain only two-thirds of the ACP gene from the 5' end.

To clone the remaining 3' end of the *V. harveyi* ACP gene, a second oligonucleotide probe (AP-2, 18 mer) was synthesized based on the *V. harveyi* ACP nucleotide sequence determined above. AP-2 (5' AAGCTTCTTTTCGTTGACG 3'), which has two mismatched bases relative to the corresponding *E. coli* sequence (Fig. 13), was intentionally chosen downstream of a predicted HindIII site in the *acpP* gene for screening. HindIII was used for digestion of genomic and vector DNAs so that *V. harveyi* inserts lacking the 5'-region of the *acpP* gene could be selected because the presence of the 5'-region was found to result in low copy numbers of intracellular recombinant plasmids. The cloning strategy for the 3' end of the gene was otherwise similar to that used to clone the 5' end as described above. Since Southern hybridization with probe AP-2 revealed a 3 kb hybridization band among HindIII-digested *V. harveyi* genomic DNA, DNA of approx. 3 kb from HindIII digestion was extracted from an agarose gel and was ligated with HindIII-digested pUC118 vector at a 5:1 (w/w) ratio. A number of recombinant plasmids were selected for DNA sequencing and one of them containing the 3' end of the *V. harveyi* ACP gene was named pUO1 (Fig. 16); pUO1 was expressed at a normal copy number (data not shown) and had a 3 kb insert.

4. Gene structure and sequence.

The insert portion of pUSS11 was partially sequenced, as indicated in Fig. 16. The sequence of region I is similar to part of *E. coli fabD* (62% identity, Fig. 17, panel A);

Panel A



Panel B

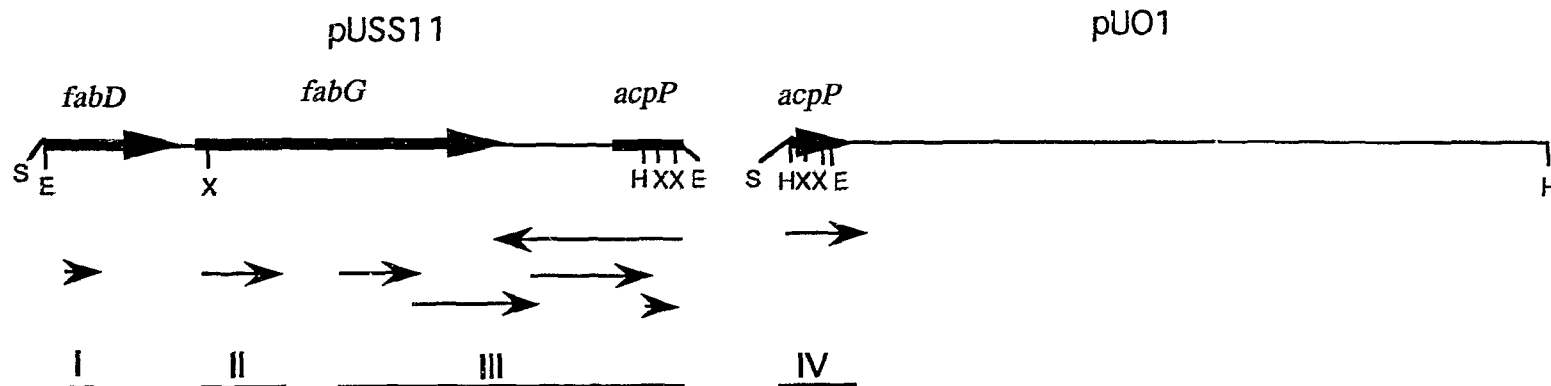


Fig. 16. Physical map of the *fabD-fabG-acpP* region and diagram of strategy used for DNA sequencing. *Panel A:* *E. coli fabD-fabG-acpP* region [178]. The gene positions are indicated with solid bars and the direction of transcription is indicated with arrows on the bar. *Panel B:* the insert portions of recombinant plasmids pUSS11 and pUO1 are shown. The strategy used for DNA sequencing is shown underneath the plasmid map with arrows indicating the extent and direction of sequencing reactions. The gene positions deduced from partial DNA sequencing and comparison between *V. harveyi* and *E. coli* DNA sequences are indicated with solid bars and the direction of genes is indicated with arrows on the bar. The SphI (S) site is a vector site, and the EcoRI (E), HindIII (H), XbaI (X) sites are either sites on the insert fragment or sites linking the insert fragment and vector. The bottom line shows regions which have been sequenced.

A. Nucleotide sequence of pUSS11 region I:

```

1 caccacaacttcggttatcaacaacgttgatgtggcggctgaaactgatcctgcaaaaa 60
  |||||
738 caccaacagttcctgttgaataacgttgatgtgaaatgcgaaaccaatggtgatgcc 797
  |||||
      tcaaagac 68
      |||||
      tccgtgac 805

```

B. Nucleotide sequence of pUSS11 region II:

```

1 tctagaaygtaagatcgctctagtgcaggcgcaagtaggggcattggctcgtcgattgc 60
  |||||
84 ttttgaaggaaaaatcgcaactggtaaccgggtgcaagccgcggaattggccgcgaattgc 143
  |||||
      tgaacttcttgttgaacgcggtgcaacggtaatcggtactgcgacttctgaa---ggcggcca 120
      |||||
      tgaaacgctcgcagcccgtggcgggaaagttattggcactgcgaccagtgaataatggcgctga 205
      |||||

ggcgatcagtgagtaggtgaaaacggtaaggtctagcacttaacgtactgacgt 180
  |||||
ggcgatcagtgattatttaggtgccaacggcaaggtctgatgttgatgtgaccgacc 265
  |||||

```

Fig. 17. Partial nucleotide sequence of pUSS11 and pUO1 and alignment of the sequence with corresponding *E. coli* sequences. The *V. harveyi* sequence is shown above the *E. coli* sequence (gray background). Vertical bars indicate the nucleotides identical between the two species. The position of the plasmid regions are shown in figure 16. *Panel A*, nucleotide sequence of pUSS11 region I. The *E. coli* sequence and numbering for this part of the *fabD* region is from [142]. *Panel B*, nucleotide sequence of pUSS11 region II. *Panel C*, nucleotide sequence of pUSS11 region III. *E. coli* sequence between *fabG* and *acpP* are not shown. *Panel D*, nucleotide sequence of pUO1 region IV. The *E. coli* sequence and numbering for the *fabG* and *acpP* regions in Panel B, C and D are from [178].

tgagtcctatcgaagcaacactaaaaaccatcaatgatgagtttggtgca 229
 ggcatctatcgaatctgttctggaaaaattcgcgcagaatttggtgaa 254

C. Nucleotide sequence of pUSS11 region III:

1 gcgctggctcgattcatcaacgtaggttctgttggtgactatgggtaacgctgggtcaaa 60
 464 gtcattggtcgtattatcactatcgggttctgttggtggtaccatgggaaatggcggtcagg 523

caactatgcagcagcaaaagcgggtgtgattggtttcactaaatcaatggctcgtgaag 120
 ccaactacgctgcggcgaaagcgggttgatcggcttcagtaaatcactggcgcgcgaag 583

tggtttctcgtggtgtaacagtgaacacggttgctccagggtttcatcgaaactgacatga 180
 ttgcgtcacgcggtattactgtaaacggttggtgctcgggctttattgaaacggacatga 643

ctaaggcgtataatgacgaccaacgtgcagcaacggtgtcgaacgtgccagcaggtcgtc 240
 cacgtgcgctgagcgtatgaccagcgtgcgggtatcctggcgcaggttcctgcgggtcgcc 703

taggtgaccctcgtgaaattgcatcagcagttgtattccttgcttcacctgaagcggctt 300
 tcggcggcgccacaggaaatcgccaacgcggttgcatcctggcatccgacgaagcagctt 763

acattactggtgaaactttgcatgtaaatggtggcatgtacatggtttaatgcacagttc 360
 acatcacgggtgaaactttgcatgtgaacggcgggatgtacatggtctga 814

gtgcaaaataagctattttactttatgaaaaatagcctatagttgctgttaactgtcatt 420
 aagttgtcatgctcatgcgcaagatttggtgcatgatttaagtcaaaaatgtattgaaatt 480
 cggttaaattcgctaattttgtggtttgaccagcaaggaccccccttgcaaaacttcaatag 540

Fig. 17.

ttcgaataaactacggaatcatcgcattaggcgaaatctgtaaaggaaaagaaaaatgag 600
 atgag 1028

caacatcgaagaacgcgttaaagaaaatcattggtgaacagctaggtgtagacgaagcaga 660
 cactatcgaagaacgcgttaagaaaattatcggcgaacagctgggcgttaagcaggaaga 1088

agttaaaacgaagcttctttcgttgacgatctaggtgctgattctctagacactgtaga 720
 agttaccaacaatgcttctttcgttgaaagacctgggcgcggattctcttgacaccgttga 1148

gctagtaatggctctagaagaggaattc 748
 gctggtaatggctctggaagaagagttt 1176

D. Nucleotide sequence of pUO1 region IV:

1 aagcttctttcgttgacgatctaggtgctgattctctagacactgtagagctagtaatgg 60
 1100 atgcttctttcgttgaaagacctgggcgcggattctcttgacaccgtcgagctggtaatgg 1159

ctctagaagagguattcgacactgagattcctga'tgaagaagcagagaaaatcactactg 120
 ctctggaagaagagtttgatactgagattccggacgacgaagctgagaaaatcaccaccg 1219

ttcaagctgcaatcgactacgtaaacagcgctcag taattatctctcccagg 152
 ttcaggctgccattgattacatcaacggccaccaggcgtaa 1260

Fig. 17.

region II is homologous to the 5'-terminus of *E. coli fabG* (68% identity, Fig. 17, panel B). The sequence of region III revealed a noncoding region of 245 bp between *fabG* and *acpP* (Fig. 17, panel C), compared to the 210 bp noncoding region in *E. coli* [178]; the two noncoding regions are not homologous. Based on the physical map of pUSS11 and comparison of sequences with the corresponding *E. coli* region (Fig. 16, panel A), the arrangement of these genes in *V. harveyi* are as indicated in Figure 16 (panel B). Overall, pUSS11 contains the 3'-end of *fabD* followed by the whole open reading frame of *fabG* and the 5'-end of *acpP*. Sequencing pUO1 revealed that the plasmid contains the 3'-end of *acpP* (Fig. 16) with a 77 base pair overlap with pUSS11 (Fig. 17, panel D).

5. The ACP gene and deduced protein sequence.

The complete DNA sequence of the *V. harveyi acpP* gene is highly homologous to that of *E. coli* (79% identity) (Fig. 17, C and D) and encodes a protein of 77 amino acids (Fig. 18). Although the coding region for ACP is only 234 base pair long, it contains several 6-base restriction enzyme sites, ie. single EcoRI, HindIII, AccI, and HincII sites and two XbaI sites (Fig. 18, top panel). The deduced protein sequence (Fig. 18, bottom panel) agrees exactly with the amino acid sequence determined by protein sequencing (Fig. 12), except that the first methionine is missing in the latter, indicating that the N-terminal methionine is removed by post-translational processing [91]. Therefore, *V. harveyi* apo-ACP contains 76 amino acids with 22 acidic and 5 basic amino acid residues; its calculated molecular weight is 8395.05 daltons and the calculated isoelectric point is pH 3.99. The deduced *V. harveyi* ACP amino acid sequence was aligned with other bacterial or algal ACP sequences (Fig. 19). *E. coli* ACP has highest homology with *V. harveyi* ACP (86% identical) while others have less identity (Table IV), consistent with the fact that *V. harveyi* and *E. coli* are most closely related to each other among these species [244]. The sequence similarity of ACP between Gram-negative bacteria and the alga *Cryptomonas* is much higher (55-65%) than that between Gram-negative bacteria and the Gram-positive bacterium *Saccharopolyspora erythraea* (32-40%).

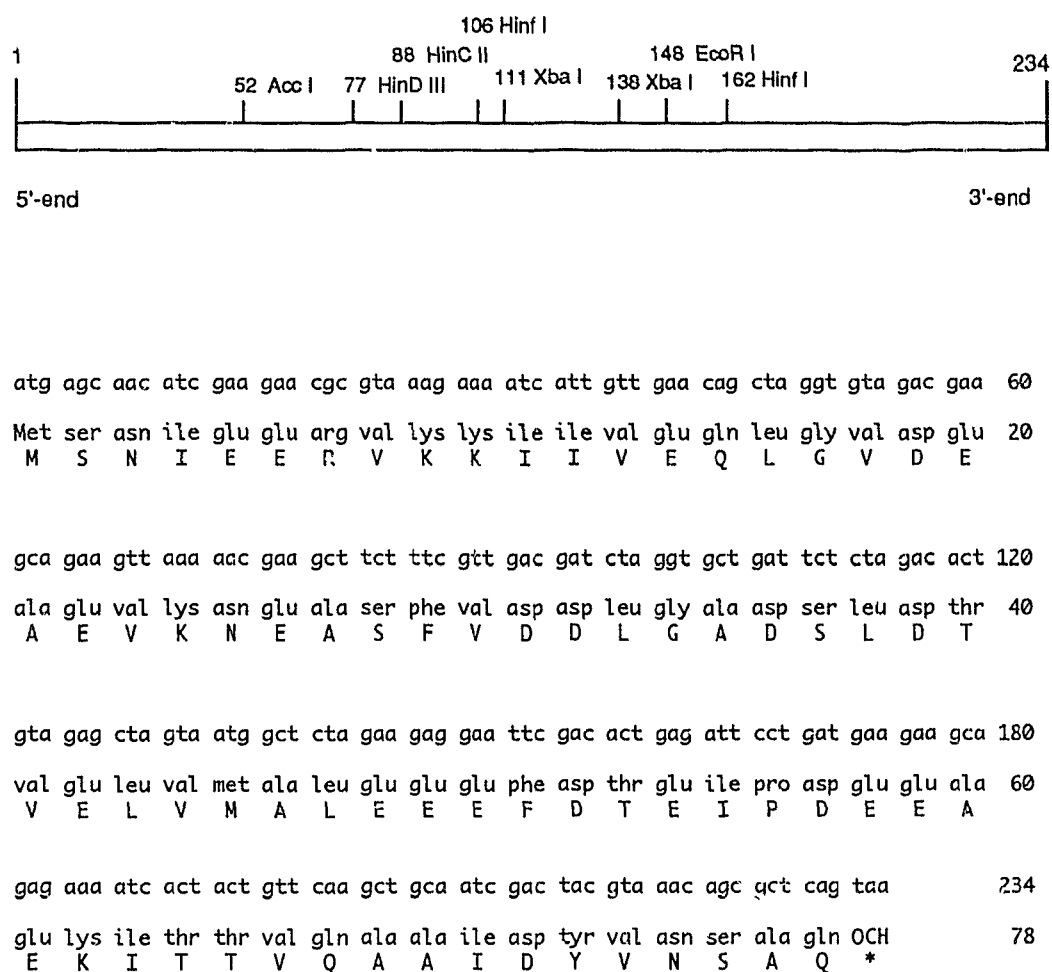


Fig. 18. Physical map and sequence of *V. harveyi acpP*. *Top panel*, a partial list of restriction enzyme sites in *acpP* is shown. The blank bar represents the whole *acpP* gene. *Bottom panel*, nucleotide and deduced amino acid sequence of the *V. harveyi acpP* gene. Both three letter and one letter abbreviations of amino acid are displayed.

?

	10	20	30	40	50	60	70
<i>Vibrio harveyi</i>	SNIEERVKKIIVEQLGVD-EAEVKNEASFVDDL <u>GADSL</u> DTVELVMALEEEFDTEIPDEEA EKITT VQA AIDYVNSAQ						
<i>Escherichia coli</i>	·T·····G·····K-QE·T·N·····E····· <u>I</u> ·····I·GH·A						
<i>Rhizobium meliloti</i>	·D·A·····VIDH·····AEK·SEG···I····· <u>I</u> ·····F·····GV·····DA·DS·L··GD·VKFIEK··A						
<i>Rhodobacter sphaeroides</i>	·D·AD·····V·H···E-·EK·TETT·I····· <u>I</u> ·····F·····GI·····DA·T·Q·FGD·P						
<i>Anabaena variabilis</i>	SQ·ETF·K·····VI···S·ENPDT·TP·····AN··Q· <u>I</u> ·····I·····A·····V·?I·						
<i>Cryptomonas</i>	NEQE·F·K·QT·S·····KSQ·TKD·N·AN····· <u>I</u> ·····I·A·NI·····DA·Q·SNL·Q·V·FISQKVAA						
<i>Saccharopolyspora erythraea</i>	MDRKE·F··IEQVLA····IP-AEQITE··DLRE···M· <u>I</u> ·L····S···D·VGM RVEQSQL·G·E··GHVMELTLDL...						

Fig. 19. Sequence comparison of *V. harveyi* and other ACPs. The amino acid sequence of *V. harveyi* ACP, deduced from the DNA sequence, is aligned with ACP sequences of *Escherichia coli* [178], *Rhizobium meliloti* [172], *Rhodobacter sphaeroides* [172], *Anabaena variabilis* [64], *Cryptomonas* [240] and *Saccharopolyspora erythraea* [182]. Residues identical to *V. harveyi* ACP are indicated by dots. The conserved serine (position 36), to which phosphopantetheine group is attached, is underlined.

Table IV
Sequence similarity between *V. harveyi* ACP and other ACPs*

	<i>V. harveyi</i>	<i>E. coli</i>	<i>A. variabilis</i>	<i>R. sphaeroides</i>	<i>R. meliloti</i>	<i>Cryptomonas</i>
<i>E. coli</i>	86					
<i>A. variabilis</i>	70	72				
<i>R. sphaeroides</i>	65	64	62			
<i>R. meliloti</i>	62	62	62	80		
<i>Cryptomonas</i>	55	57	64	57	55	
<i>S. erythraea</i>	38	40	36	35	36	32

* Numbers listed in the Table are percentage of identical amino acids between individual pairs of sequences, calculated from the sequence alignment in Fig. 19.

B. Characterization of *V. harveyi* ACP and acyl-ACP.

Purification of *V. harveyi* ACP allowed us to further characterize structural and functional aspects of this protein, including its acylation with fatty acid, its structural properties by gel electrophoresis, and its role and expression using immunochemical approaches.

1. Preparation of acyl-ACP using *V. harveyi* acyl-ACP synthetase.

Acyl-ACPs of different FA chain lengths were prepared to study the effect of acylation on ACP structure and to obtain acyl-ACP standards for metabolic investigations. Although *E. coli* acyl-ACP synthetase has been used routinely for ACP acylation in the past [101], previous work in our laboratory has indicated that *V. harveyi* acyl-ACP synthetase may also be useful in preparation of acyl-ACP substrates for biochemical study [26].

To determine the potential of partially purified *V. harveyi* acyl-ACP synthetase for the preparation of acyl-ACPs of different chain length, conditions for optimal acylation of *E. coli* ACP (Sigma product) with [³H]14:0 was investigated using a filter disk assay. By counting the purified radiolabeled acyl-ACP products (free of radiolabeled fatty acid) in a liquid scintillation spectrometer both directly and on a filter disk, counting efficiencies of 16% for ³H-labeled acyl-ACPs and of 75% for ¹⁴C-labeled acyl-ACPs were determined for the filter disk assay. Figure 20 shows the effect of acyl-ACP synthetase and myristic acid concentrations on acyl-ACP formation as a function of time when the ACP concentration was fixed at 70 μM. Although the initial acylation rate increased as a function of enzyme concentration, the final yield of acyl-ACP was independent of enzyme concentration, and 90% acylation was reached within 3 h with as little as 0.5 units/ml of acyl-ACP synthetase (Fig. 20A). Incubation for up to 24 h did not decrease the final yield of acyl-ACP regardless of the amount of enzyme used, indicating the absence of significant acyl-ACP esterase or transferase activity in partially purified acyl-ACP synthetase preparations [212]. The rate and extent of acyl-ACP formation was relatively independent

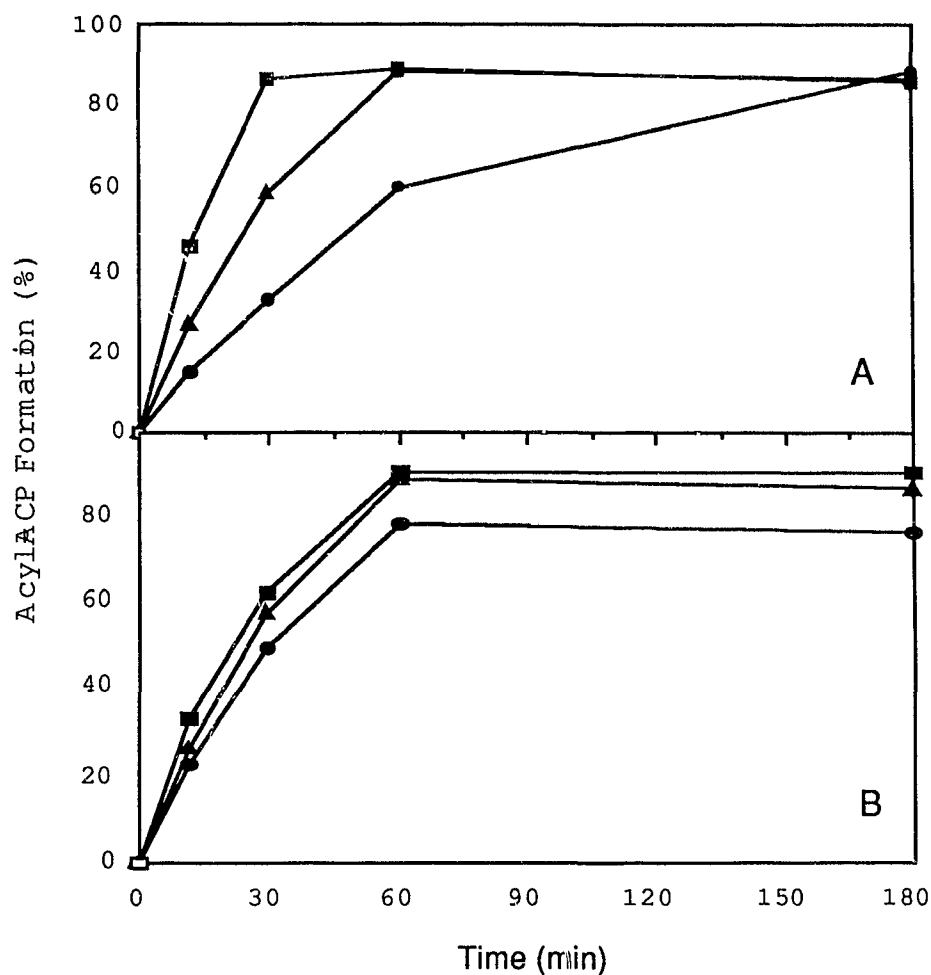


Fig. 20. Effects of enzyme and fatty acid concentration on myristoyl-ACP formation by *V. harveyi* acyl-ACP synthetase. *Panel A:* *E. coli* ACP (190 μ g, 70 μ M final concentration) was incubated with [3 H]myristic acid (440 μ M, 1 Ci/mmol) and ATP in the presence of (●) 0.5, (▲) 1.0, and (■) 1.5 units per ml of acyl-ACP synthetase in a total volume of 0.3 ml as outlined in the text. *Panel B:* ACP (190 μ g) was incubated with (●) 180 μ M, (▲) 440 μ M, and (■) 710 μ M [3 H]myristic acid (1 Ci/mmol) and ATP in the presence of 1.0 unit per ml of acyl-ACP synthetase. At the times indicated, duplicate 10 μ l samples were withdrawn and [3 H]myristoyl-ACP was quantitated using the filter disk assay. Results are expressed as percent conversion of ACP to acyl-ACP.

of fatty acid concentration at fatty acid to ACP molar ratios of six or greater, while some decrease in acylation was noted at lower fatty acid concentrations (Fig. 20B).

V. harveyi acyl-ACP synthetase is capable of utilizing a broad range of fatty acid substrates for acyl-ACP formation. Although incubation time for maximal acylation was dependent on the fatty acid species, conversion of ACP to acyl-ACP after 6 hours incubation was greater than 84% for all saturated fatty acids tested between C₆ and C₁₄ carbons (Table V). The yield of acylation with 16:0 was lower than with shorter chain fatty acids, which may be due to the higher K_m and lower maximal velocity of this enzyme for 16:0 [26], but increased yields could be obtained by increasing the reaction time (Table V). Partial acylation (about 20%) with 18:0 and no appreciable formation of 4:0-ACP were observed under these conditions. Therefore, *V. harveyi* acyl-ACP synthetase appears to be a useful tool for acyl-ACP (C₆ to C₁₆) preparation.

Further separation of acyl-ACP from free fatty acid substrate by DEAE-Sepharose chromatography and from unacylated ACP by octyl-Sepharose chromatography [191] usually resulted in some loss in small scale preparations ($\leq 350 \mu\text{g}$), but the resulting yield of product was still greater than 60%. After DEAE-Sepharose chromatography, acyl-ACP could be concentrated by acid precipitation at pH 4 and was free of labeled fatty acid substrate. However, if products of the acylation reaction were directly precipitated at pH 4 without prior chromatography, only 50% of the radiolabeled free fatty acid was removed, indicating that chromatography on DEAE-Sepharose is necessary to completely separate acyl-ACP from labeled fatty acid substrate.

After optimal conditions for the synthesis of *E. coli* acyl-ACP were determined, *V. harveyi* acyl-ACPs were also prepared using purified *V. harveyi* ACP and ³H- or ¹⁴C-labeled fatty acids on a smaller scale (20 to 100 μl reactions) under similar conditions. Quantitative acylation of *V. harveyi* ACP was also achieved with fatty acids C₆ to C₁₄ and these labeled *V. harveyi* acyl-ACPs were used as acyl-ACP standards in further experiments.

Table V
 Acylation of ACP with fatty acids of different chain
 lengths by *V. harveyi* acyl-ACP synthetase

Fatty Acid	Acyl-ACP (%) ^a
6:0	91
8:0	90
10:0	84
12:0	86
14:0	91
16:0	89 ^b

^a. Each reaction mixture (0.9 ml) contained Tris-HCl (0.1 M, pH 7.8), MgCl₂ (10 mM), ATP (10 mM), DTT (1 mM), *E. coli* ACP (400 µg, 50 µM), acyl-ACP synthetase (0.45 units) and 1-¹⁴C-fatty acids (250-300 µM). After incubation for 6 hours at 37°C, labeled acyl-ACP was quantitated using the filter disk assay (mean of triplicate 10 µl samples) and expressed as a percent of the initial ACP concentration. For each sample, acylation was also verified visually by SDS-PAGE and fluorography.

^b. Incubation time for the 16:0 reaction was 24 h.

2. Analysis of ACP and acyl-ACP derivatives by SDS-PAGE.

The products of enzymatic acylation of ACP were analyzed by SDS-PAGE and fluorography. As indicated in Fig. 21, acylation with fatty acids of different chain length caused increased migration of *E. coli* ACP in SDS-PAGE (Fig. 21, panel A) which corresponded to radiolabeled bands in fluorograms (Fig. 21, panel B). The 20 kDa ACP band disappeared upon acylation, further indicating that acylation is quantitative under these conditions.

Despite the apparent similarities in the properties of *E. coli* and *V. harveyi* ACP during purification and as substrates for acyl-ACP synthetase, significant differences between acylated derivatives of these two proteins were noted in SDS-PAGE (Fig. 22). In particular, *V. harveyi* acyl-ACP exhibited a much greater variation of electrophoretic mobility as a function of fatty acid chain length. As shown in Fig. 22 and noted previously [110], migration of *E. coli* acyl-ACP increased with fatty acid chain length to C₁₀ and remained fairly constant with longer chains. In contrast, although migration of *V. harveyi* acyl-ACP was also maximal for C₁₀, longer fatty acid derivatives exhibited substantially decreased electrophoretic mobility under these conditions (Fig. 22).

Further studies indicated that migration of acyl-ACPs in SDS-PAGE is affected by buffer components. The gel running buffer we routinely used in SDS-PAGE contains 50 mM Tris, 384 mM glycine and 0.1% SDS. This buffer ("high salt" electrophoresis buffer) is double the concentration of Tris and glycine relative to Laemmli electrophoresis buffer (25 mM Tris-HCl, 192 mM glycine and 0.1% SDS) [129]. While migration of *V. harveyi* ACP or *E. coli* ACP showed little difference between the two running buffers, the migration of some acyl-ACPs was significantly affected. As indicated in Figure 23 (A and B), *V. harveyi* 8:0-, 12:0- and 14:0-ACP migrated slower in Laemmli running buffer than in the "high salt" electrophoresis buffer, while mobility of 16:0-ACP was unchanged. In contrast to *V. harveyi*, *E. coli* acyl-ACPs exhibited little difference between the two electrophoresis running buffers. Changing the running temperature did not result in

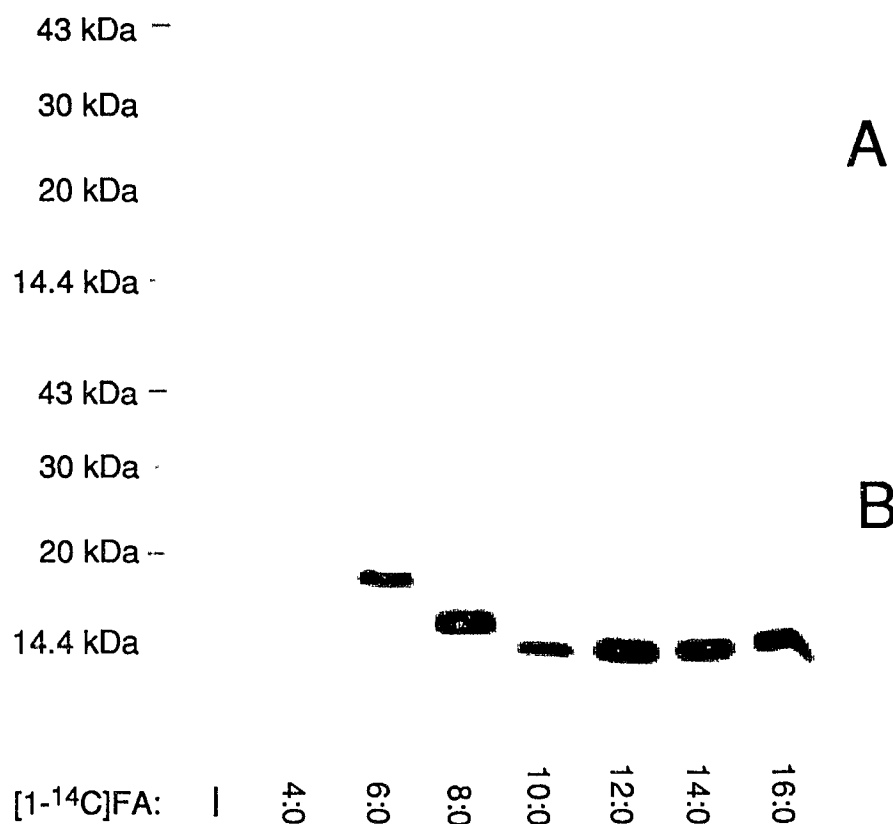


Fig. 21. SDS-PAGE analysis of *E. coli* acyl-ACP products synthesized using *V. harveyi* acyl-ACP synthetase. ACP from *E. coli* (2.4 μ g) was incubated for 12 h with acyl-ACP synthetase (0.01 units), ATP, and 1- 14 C-labeled fatty acids (80 μ M) in a total volume of 20 μ l as indicated (no fatty acid was present in the first lane). After addition of SDS sample buffer, half of the reaction mixture was separated by SDS-PAGE. The Coomassie Blue staining profile is shown in Panel A while a fluorogram (7 d exposure) is shown in panel B (labeling intensities reflect different specific radioactivities of fatty acid substrates). FA, fatty acid.

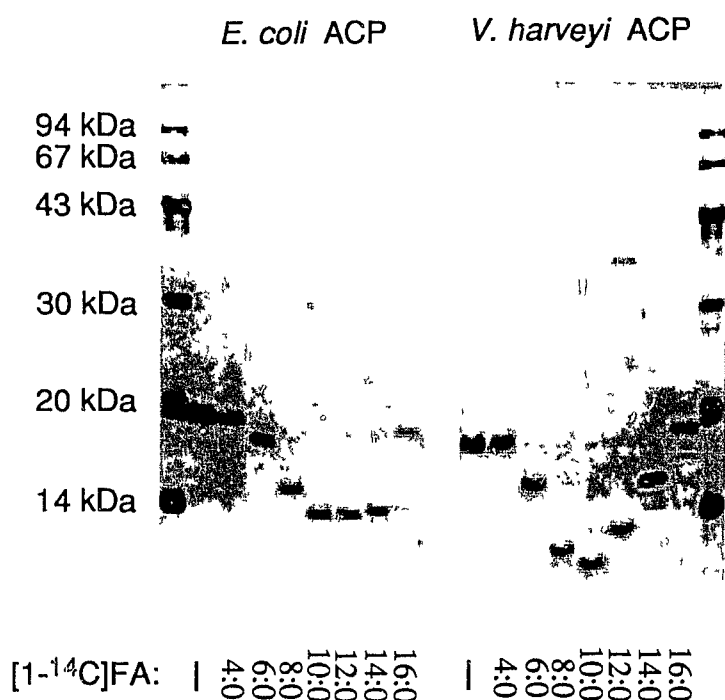


Fig. 22. Acylation of *E. coli* and *V. harveyi* ACP with various fatty acids catalyzed by *V. harveyi* acyl-ACP synthetase. ACP (2.4 μ g) from either *E. coli* or *V. harveyi* was incubated for 12 h with acyl-ACP synthetase (0.01 units), ATP, and 1-¹⁴C-labeled fatty acids (80 μ M) in a total volume of 20 μ l as indicated. After addition of SDS sample buffer, half of the reaction mixture (or ACP alone, 1.2 μ g) was separated by SDS-PAGE and stained with Coomassie Blue. The outer lanes contain protein markers as described in Fig. 11.

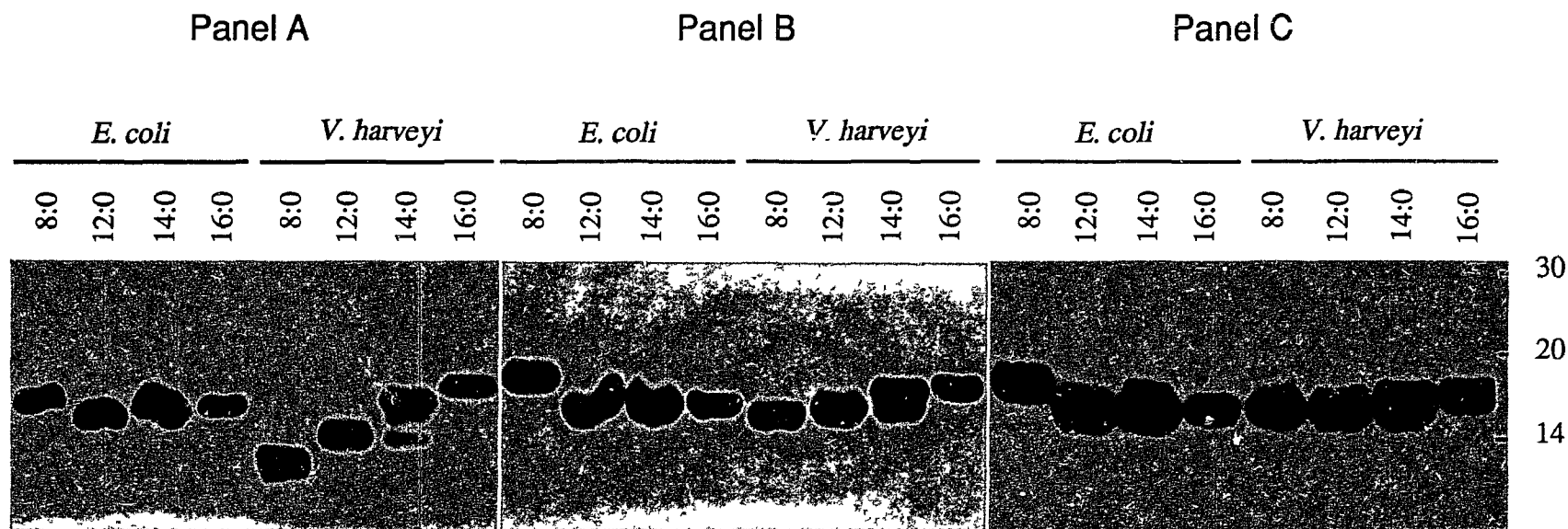


Fig. 23. Fluorogram of SDS-PAGE analysis of *E. coli* and *V. harveyi* acyl-ACP under different electrophoresis conditions. Acyl-ACP was prepared as described in Fig. 22 and separated on 15% polyacrylamide gels using either 50 mM Tris-base, 384 mM glycine, 0.1% SDS (A) or 25 mM Tris-base, 192 mM glycine, 0.1% SDS (B and C) as electrophoresis buffer. The SDS-PAGE was performed in a Mini-PROTEAN II dual slab cell at 200 volts either at 4°C (A and B) or at room temperature (C). Migration of protein markers (kDa) is shown at right, while the source of ACP and the acyl group attached is shown at the top. A 5 d exposure is shown.

significant differences in migration of either *E. coli* or *V. harveyi* acyl-ACPs (Fig. 23, B and C). Therefore, SDS-PAGE was routinely performed in high salt electrophoresis buffer at 4°C for reproducible results.

3. Biological activity of enzymatically synthesized *V. harveyi* 14:0-ACP.

To confirm that enzymatic acylation of ACP resulted in biologically active acyl-ACP, partially purified *V. harveyi* bioluminescence-specific transferase was used to hydrolyze the 14:0-ACP product of acylation. Transferase is responsible for cleavage of 14:0-ACP to form 14:0 for reduction to myristaldehyde *in vivo* and is functionally inactive in M17 mutants [159]. As shown in Fig. 24, a 30-50% ammonium sulfate fraction containing this enzyme from wild type cells catalyzed complete cleavage of [³H]14:0-ACP within 1 h (a higher MW band of unknown origin occasionally noted in acyl-ACP batches was also cleaved), while no significant cleavage was noted in the presence of the M17 fraction. Similar results were obtained when [³H]14:0 released from [³H]14:0-ACP by the transferase fraction was monitored by liquid scintillation spectrometer following hexane extraction [27] (data not shown). This experiment shows that enzymatically synthesized *V. harveyi* 14:0-ACP is a functional substrate for *V. harveyi* bioluminescence-specific transferase.

4. Detection of intracellular *V. harveyi* ACP by western blot.

Given the chain length-dependent mobility of acyl-ACPs in SDS-PAGE, western blot analysis has the potential to identify and quantitate intracellular ACP intermediates and provide important information about the composition of the ACP pool [174]. A polyclonal rabbit antiserum was raised against purified *V. harveyi* ACP; western blots of cell extracts indicated that this immune serum recognizes both *V. harveyi* and *E. coli* ACP (Fig. 25, panel A). *V. harveyi* ACP displayed two bands of equal intensity on western blot while *E. coli* ACP in cell extracts exhibited only a single band that migrated slower than *V. harveyi* ACP (Fig. 25). If anti-*V. harveyi* ACP serum was preincubated with purified *E. coli*

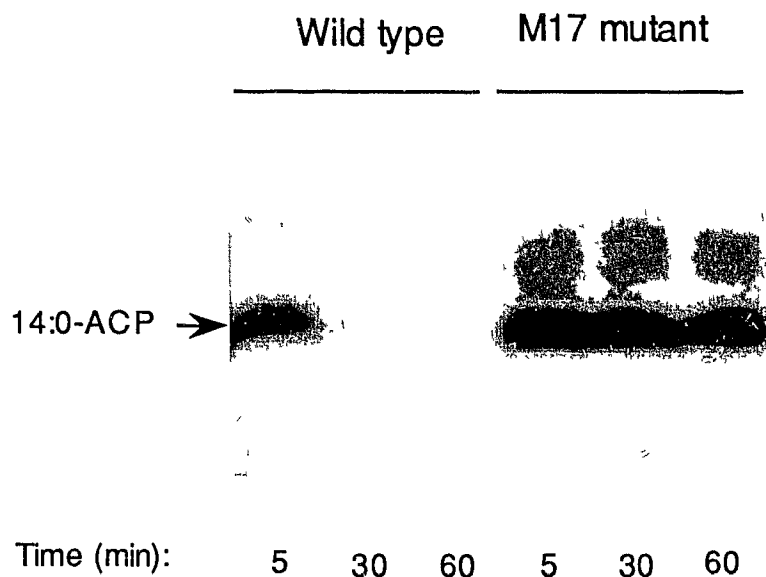


Fig. 24. Cleavage of [^3H]myristoyl-ACP by the *V. harveyi* bioluminescence-specific acyltransferase. Ammonium sulfate fractions (30-50% saturated) containing the acyltransferase were prepared from *V. harveyi* cell-free extracts of wild type or the M17 mutant as described in the Method C3. These fractions (3 μl) were incubated with *V. harveyi* [^3H]myristoyl-ACP (70 pmol in a total volume of 30 μl) in 1 M phosphate buffer (pH 7) for 5 min, 30 min, or 60 min as indicated. The reaction mixture was separated by SDS-PAGE; a fluorogram of exposure 11 days is shown.

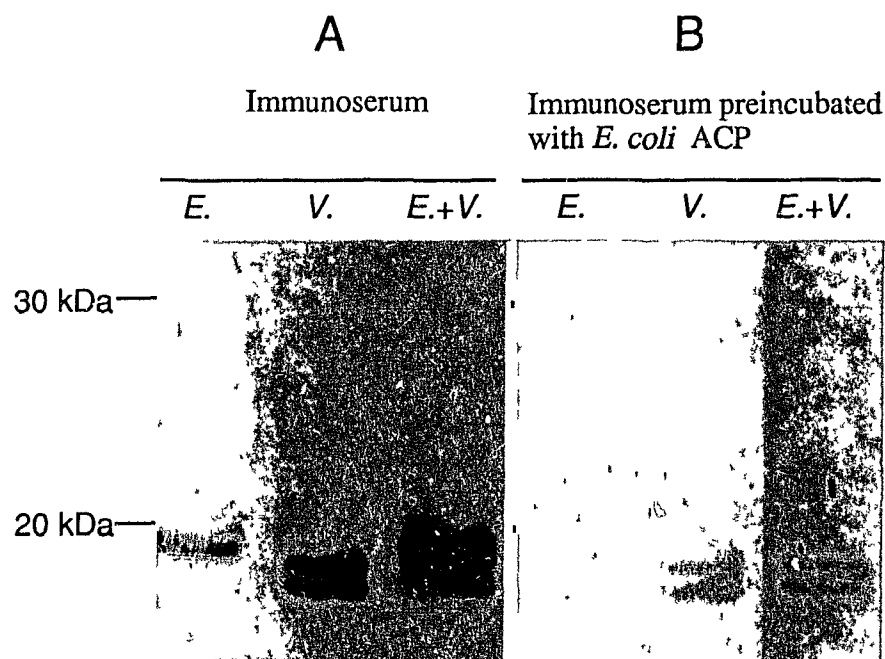


Fig. 25. Western blot analysis of intracellular *V. harveyi* and *E. coli* ACP. *E. coli* and *V. harveyi* were grown in complex medium and cell lysate of *E. coli* (5 μ g of protein, lane E.), *V. harveyi* (5 μ g of protein, lane V.) or both (10 μ g of protein with 5 μ g of each, lane E.+V.) was separated by 15% mini-SDS-PAGE. After electrophoresis, protein was electrophoretically transferred to nitrocellulose membrane, incubated with anti-*V. harveyi* ACP immunoserum (A) or with the same immunoserum which had been pre-incubated with *E. coli* ACP (B) as described in Method E2. After incubation with the secondary antibody (goat anti-rabbit IgG alkaline phosphatase conjugate), immunoreactive bands were visualized by developing the membrane in a colour development reagent which contains bromo-chloroindolyl phosphate and nitro blue tetrazolium as substrates for alkaline phosphatase.

ACP, only *V. harveyi* ACP was detected (Fig. 25, panel B), indicating the presence of *V. harveyi* epitope-specific antibodies in the immune serum. Immunoreactive bands corresponding in migration to acyl-ACPs (C₆ to C₁₆) were not observed in the present experiments, and attempts to visualize acyl-ACP by increasing (5-fold) the amount of cell lysate loaded on the gel resulted in high background and anomalous protein bands on western blot, so further attempts to quantitate acyl-ACP intermediates by this method were discontinued.

5. Association of ACP and acyl-ACP synthetase in cell extracts.

V. harveyi acyl-ACP synthetase is quite stable during early stages of purification by Cibacron Blue and gel filtration chromatography but exhibits significant loss of activity upon further purification [26]. Moreover, this enzyme exhibits a MW of 500,000 by gel filtration in cell extracts while purified acyl-ACP synthetase appears to be a 62 kDa protein [61]. To investigate whether ACP might be associated with an acyl-ACP synthetase complex *in vivo*, ACP immunoprecipitated from *V. harveyi* cell extracts was monitored for enzyme activity (Fig. 26). Prior to adding antiserum, cell extracts were pre-cleared with preimmune serum to remove proteins that might bind nonspecifically to the immune complex or to fixed *Staphylococcus aureus* Cowan I (SAC) [85]. Thirty percent of the acyl-ACP synthetase activity in the cell extracts was removed by immunoprecipitation with anti-ACP serum while no appreciable enzyme activity was removed by treatment with preimmune serum. Some of the precipitated acyl-ACP synthetase activity could also be eluted from the immunoprecipitate complex using 0.5 M phosphate buffer, which has been shown to elute acyl-ACP synthetase from ACP-Sepharose affinity columns [61]. More than 4% of initial enzyme activity in lysates was recovered from anti-ACP immune complex while less than 0.4% of activity was eluted in the control (Fig. 26). Thus, it appears that a significant fraction of acyl-ACP synthetase may be bound to ACP in cell extracts, and can be isolated using antibodies which recognize non-competing epitopes of ACP.

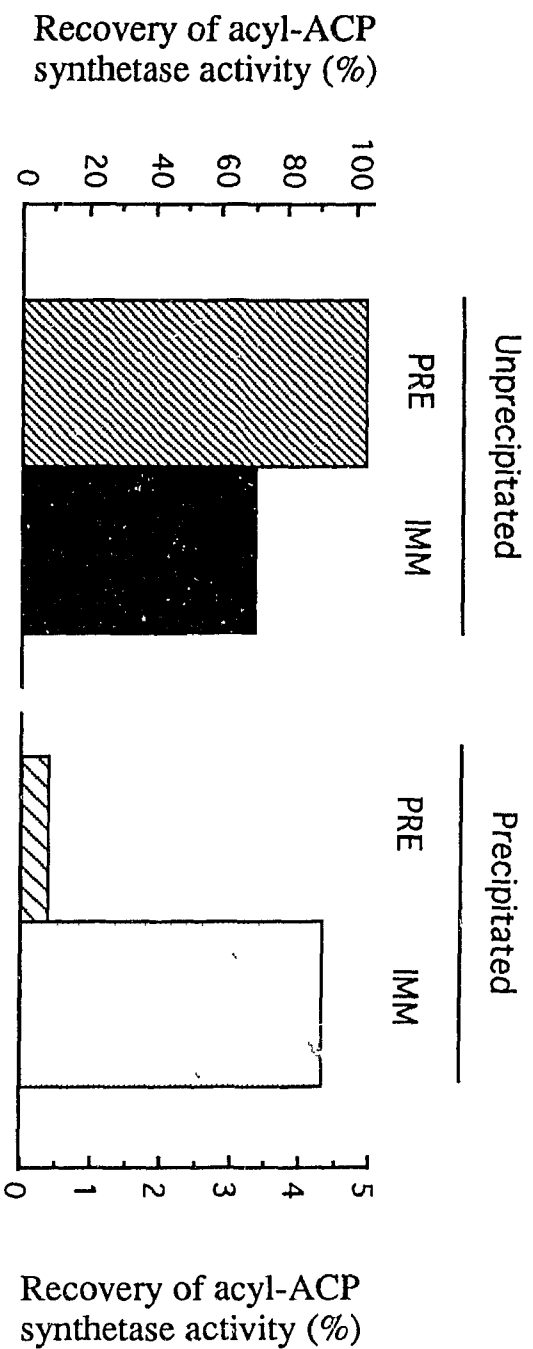


Fig. 26. Co-precipitation of *V. harveyi* acyl-ACP synthetase by anti-ACP immune serum. *V. harveyi* cell lysate (110 µl), pre-cleared with preimmune serum (20 µl) and SAC, was divided into two equal portions. One was incubated with 10 µl of preimmune serum (PRE) and the other was incubated with 10 µl of anti-*V. harveyi* ACP immune serum (IMM). After incubation, antibody or antibody-antigen complex was precipitated by incubating with 10 µg of washed SAC pellet followed by centrifugation, and the remaining acyl-ACP synthetase activity in the supernatant (Unprecipitated) was analysed. Acyl-ACP synthetase which was co-precipitated with SAC pellet was eluted from the pellet by suspending the pellet in 10 µl of 0.5 M phosphate buffer (pH 7.5) containing 2 mM DTT and 10% glycerol (v/v) at 0°C followed by centrifugation. The eluted acyl-ACP synthetase activity in the supernatant (Precipitated) was analysed. Acyl-ACP synthetase activity in all samples was measured as [³H]14:0-ACP formed/min, and converted to the percentage of activity in the lysate.

6. *Photobacterium phosphoreum* ACP.

In previous study [26], acyl-ACP formation was not detected when [^3H]14:0 and ATP were incubated with extracts of another luminescent bacterium, *Photobacterium phosphoreum*. To determine whether this might reflect the absence of acyl-ACP synthetase activity and/or an altered ACP in this species, cell lysates of *P. phosphoreum* were incubated with *V. harveyi* acyl-ACP synthetase in the presence of ATP and labeled fatty acid (Fig. 27). The results clearly indicate that while no acylation occurs in the absence of added acyl-ACP synthetase, an endogenous ACP capable of reaction with the *V. harveyi* enzyme is present in *P. phosphoreum*. Indeed, no differences in the migration of *V. harveyi* and *P. phosphoreum* 14:0-ACP were noted when both were present in the same sample (Fig. 27). Using acylation by *V. harveyi* acyl-ACP synthetase to monitor ACP concentration, *P. phosphoreum* ACP was purified to homogeneity and acyl-ACPs were synthesized. Interestingly, purified ACP exhibited a doublet on SDS-PAGE and two acyl-ACP bands were noted for fatty acyl chains of 8:0, 12:0 and 14:0 by both protein staining and fluorography. As with *V. harveyi* acyl-ACP (Fig. 22), migration on SDS-PAGE decreased substantially at chain lengths greater than C_{12} (Fig. 28).

C. Exogenous fatty acids are activated to form acyl-ACP intermediates in *V. harveyi*.

Previous work from this laboratory has indicated that exogenous fatty acids can be taken up and elongated in *V. harveyi*, suggesting that ACP may play a direct role in activation of exogenous fatty acid. To test this hypothesis, conditions were developed to separate acyl-ACP species by PAGE and to confirm their identity using immunoprecipitation with anti-ACP serum.

1. Identification of acyl-ACPs labeled *in vivo* with [^3H] β -alanine and [^3H]14:0.

The M17 mutant of *V. harveyi* was chosen for this study to avoid potential interference of the luminescence-specific myristoyl-ACP transferase in the detection of

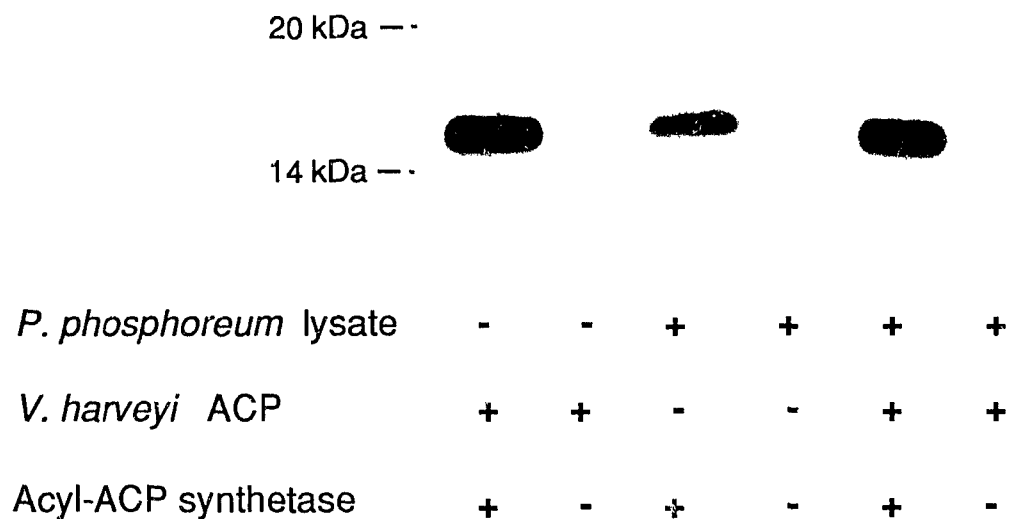


Fig. 27. Detection of *P. phosphoreum* ACP by acylation using *V. harveyi* acyl-ACP synthetase. *P. phosphoreum* cell-free lysate was prepared as described in the text for *V. harveyi*. As indicated, samples (20 μ l) containing *V. harveyi* ACP (1.1 μ g), *P. phosphoreum* lysate (150 μ g), or both were incubated with [3 H]myristic acid and ATP in the presence or absence of *V. harveyi* acyl-ACP synthetase for 20 min under standard assay conditions. Samples were separated by SDS-PAGE and a fluorogram of 4 d exposure is shown.

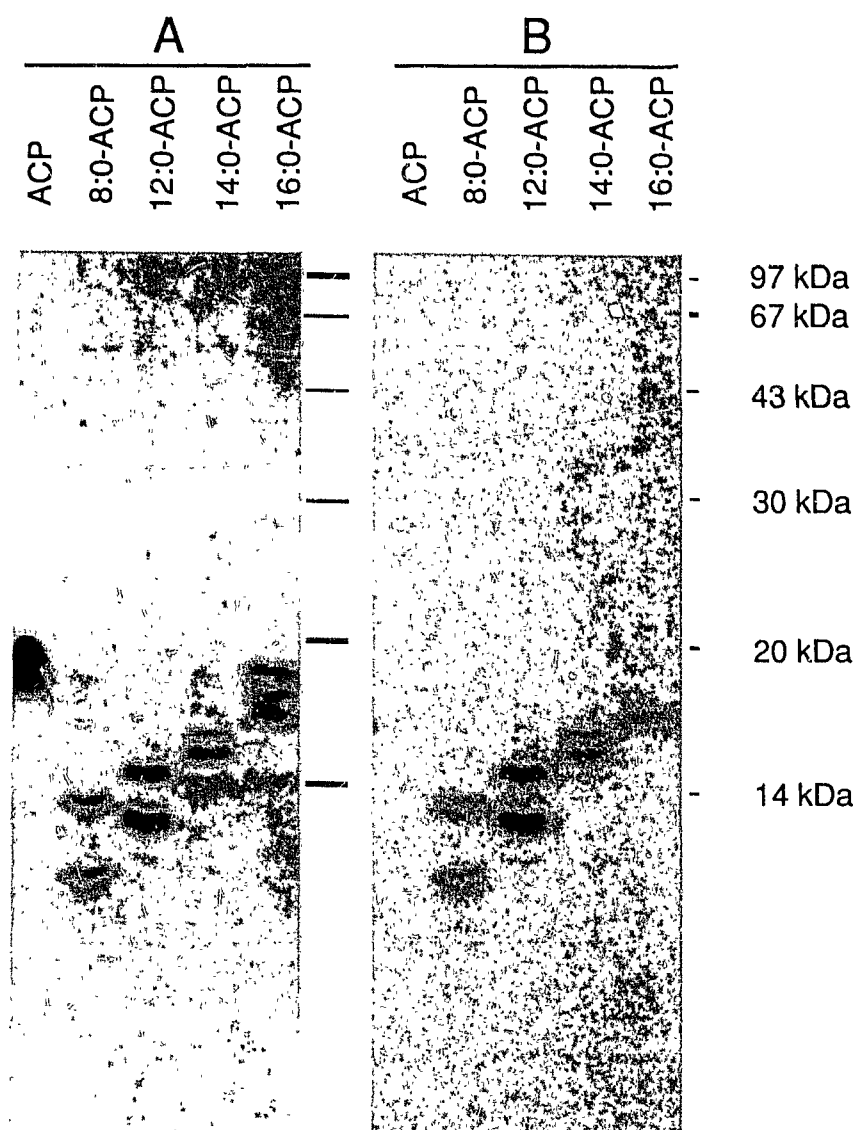


Fig. 28. SDS-PAGE analysis of purified *P. phosphoreum* ACP and acyl-ACP products. Purified *P. phosphoreum* ACP (12 μ g) was incubated overnight with *V. harveyi* acyl-ACP synthetase (0.015 units), ATP, and the indicated $1\text{-}^{14}\text{C}$ -labeled fatty acids (80 μ M) in a total volume of 30 μ l. After addition of SDS sample buffer, half of the reaction mixture (or ACP alone, 6 μ g) was separated by SDS-PAGE. The Coomassie Blue staining profile is shown in Panel A while a fluorogram is shown in panel B. The migration of protein standards is shown on the right.

acyl-ACP intermediates. In addition, most experiments were performed in minimal medium to maximize incorporation of exogenous precursors into acyl-ACP and thus reduce fluorography time.

As shown in Fig. 29, incubation of M17 cells with [^3H]14:0 resulted in the labeling of proteins which were immunoprecipitated with anti-ACP serum and co-migrated with authentic acyl-ACP standards (C_8 to C_{14}) on SDS-PAGE. Incubation of cells with [^3H] β -alanine (a specific precursor of the pantotheine prosthetic group of ACP) also resulted in the labeling of medium to long chain acyl-ACPs, as well as higher MW bands corresponding to unacylated ACP and short chain ($\leq \text{C}_8$) acyl-ACPs (Fig. 29). While the above experiment demonstrates that exogenous [^3H]14:0 can label acyl-ACP in *V. harveyi*, Fig. 29 also illustrates that most of the [^3H]14:0-labeled lysate product which migrates in this region of the gel was not immunoprecipitated with anti-ACP serum. In contrast, immunoprecipitation was much more effective with [^3H] β -alanine as precursor. The non-precipitable [^3H]14:0-labeled bands were further identified as lipopolysaccharide (see below, section C6).

2. Chain length analysis of acyl-ACPs labeled with [^3H]14:0 *in vivo*.

Due to interference by co-migration of [^3H]14:0-labeled lipopolysaccharide in the Laemmli SDS-PAGE system, we explored other gel systems for further investigation of *V. harveyi* acyl-ACP intermediates. Post-Beittenmiller et al [174] have reported that long chain spinach acyl-ACP derivatives can be separated in 20% polyacrylamide gels with 2.5 or 5.0 M urea. After trying different urea concentrations (2 M, 3 M and 4 M), we observed that *V. harveyi* acyl-ACP standards of chain length from C_8 to C_{16} are best resolved in 15% polyacrylamide gels containing 2 M urea (Fig. 30), where their migration as a function of chain length is reversed relative to that in SDS-PAGE (Fig. 22). Decreased resolution in 15% polyacrylamide gels was observed when urea concentration was raised to 3 M or more (data not shown). When the above gel system was used to separate proteins from M17 cells incubated with [9,10- ^3H]14:0, labeled bands were again observed which

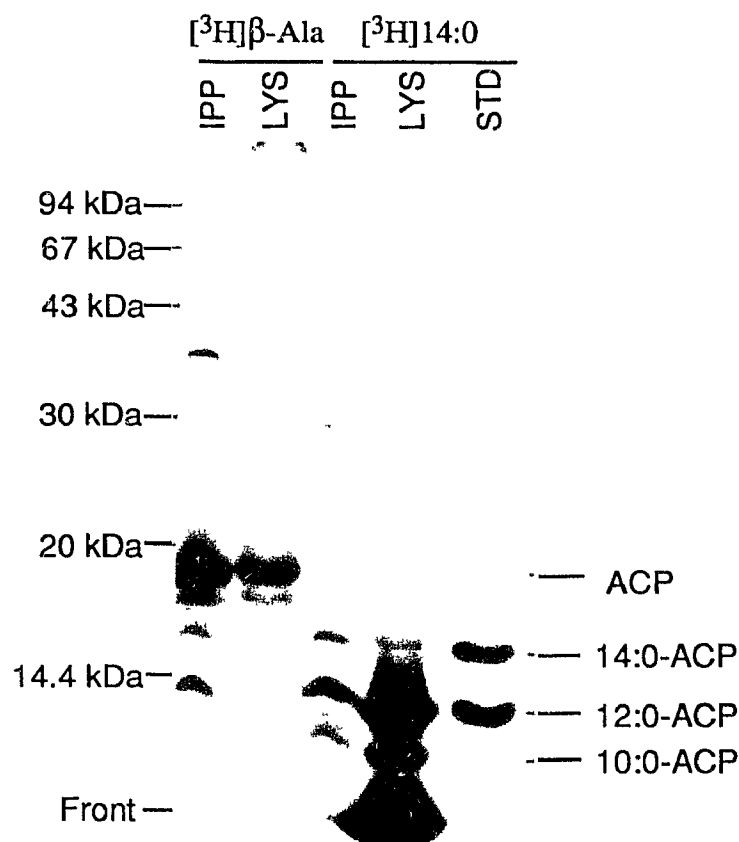


Fig. 29. SDS-PAGE of *V. harveyi* M17 proteins labeled with [^3H] β -alanine or [^3H]14:0 in minimal medium. *V. harveyi* M17 cells (1 ml) were labeled with 0.3 μM [^3H] β -alanine (87 Ci/mmol) during growth (40 hours to $A_{660} = 2.5$) in minimal medium at 27°C. Twenty μl of unlabeled 14:0 (0.2 mM) was added to cell culture for 10 min incubation prior to lysis. For [^3H]myristic acid labeling, cells were grown similarly and 1 ml was incubated with 20 μl of 0.2 mM [^3H]14:0 (20 Ci/mmol) for 10 min. Cells were lysed and treated with either anti-ACP serum or preimmune serum as described in Method D3. Lysates (LYS, 2 μl), immunoprecipitates (IPP) from 10 μl of lysate, and ^{14}C -acyl-ACP standards (STD) were fractionated by SDS-PAGE as described in Method A6. The migration of ACP and standard molecular mass proteins (kDa) are also indicated. A fluorogram of a 20 days exposure is shown.

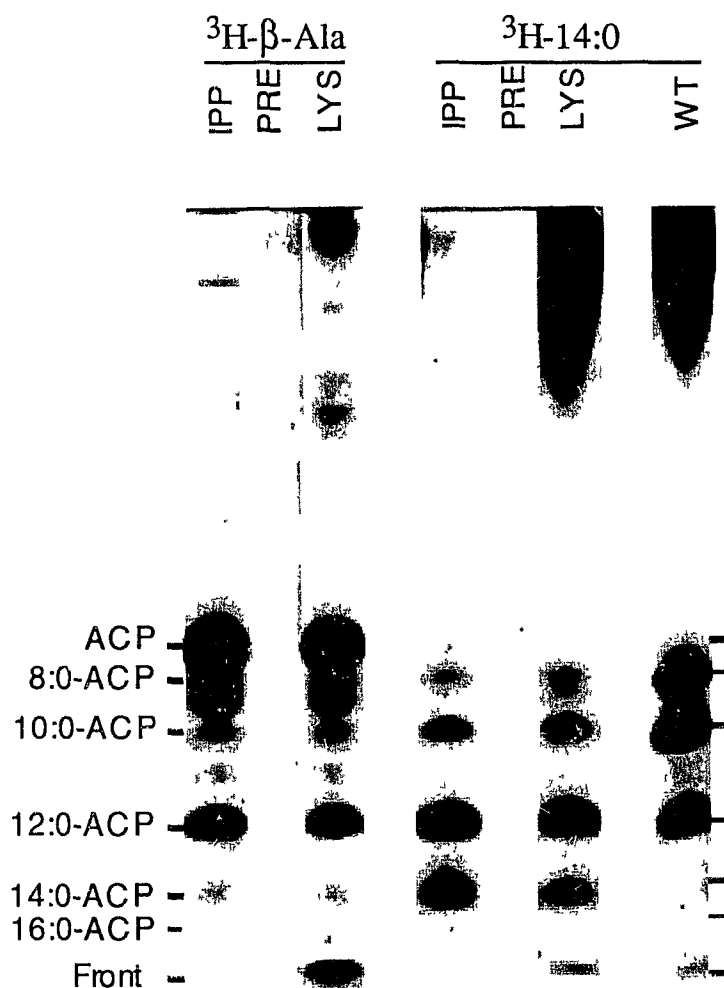


Fig. 30. Urea-PAGE of *V. harveyi* proteins labeled with ^3H β -alanine or ^3H 14:0 in minimal medium. *V. harveyi* M17 cells were labeled with ^3H β -alanine or ^3H 14:0 in minimal medium as described in the legend to Fig. 29 except that no unlabeled 14:0 was added during ^3H β -alanine labeling. The wild type strain of *V. harveyi* (WT) was also labeled with ^3H 14:0 under identical conditions. Cells were lysed and treated with either anti-ACP serum (IPP) or preimmune serum (PRE) as described in the text. Four μl of cell lysate (LYS, WT) or immunoprecipitates from 15 μl of lysate (IPP, PRE) were fractionated by 2.5 M urea-PAGE. A fluorogram of 30 days exposure is shown.

comigrated with acyl-ACP standards and [^3H] β -alanine-labeled bands, while labeled phospholipid and lipopolysaccharide migrated poorly into the urea separating gel (Fig. 30, LYS). Both [^3H]14:0- and [^3H] β -alanine-labeled bands were immunoprecipitated by anti-ACP serum (IPP), but not by preimmune serum (PRE). To avoid possible metabolism or degradation of acyl ACPs in routine analysis, cell lysates were heated at 80 °C to remove most other proteins (no effect on acyl-ACP was noted) and frozen in liquid nitrogen or fractionated by urea-PAGE immediately after preparation. Repeated freezing and thawing of lysate or prolonged incubation of lysate and antiserum during immunoprecipitation was found to alter the acyl-ACP profile to a small extent. Although in most of our experiments radiolabeled cells were washed once with minimal medium before lysis to remove any possible radiolabeled contamination from the medium, we found that this wash was unnecessary and was omitted in some of the later experiments.

Relative incorporation of [^3H]14:0 label into individual acyl-ACP species was quantitatively measured by densitometric scanning of fluorograms following urea-PAGE. Surprisingly, only about one-third of the [^3H]14:0-derived label associated with acyl-ACP was found as 14:0-ACP in M17 cells under these conditions. Over 95% of the fatty acid label associated with acyl-ACP was found in four species: 14:0-ACP ($33.0 \pm 7.9\%$), 12:0-ACP ($39.8 \pm 4.4\%$), 10:0-ACP ($14.4 \pm 5.4\%$) and 8:0-ACP ($8.5 \pm 2.4\%$) (mean $\pm$ SD of 8 samples) when M17 cells were labeled in late exponential phase ($A_{660} = 1.6$ to 2.5). Essentially no labeling of 16:0-ACP ($< 1\%$) was observed with [^3H]14:0 as precursor. Labeled acyl-ACP bands originating from [^3H]14:0 disappeared upon chasing 15 min with unlabeled 14:0, indicating that these are metabolically-active intermediates (data not shown).

Incorporation of [^3H]14:0 into acyl-ACP and lipids as a function of time was also examined (Fig. 31). While incorporation of fatty acid into the phospholipid and lipopolysaccharide fractions of M17 cells in minimal medium generally increased with time to 20 minutes, the amount of label in acyl-ACP was constant between 5 and 10 min and

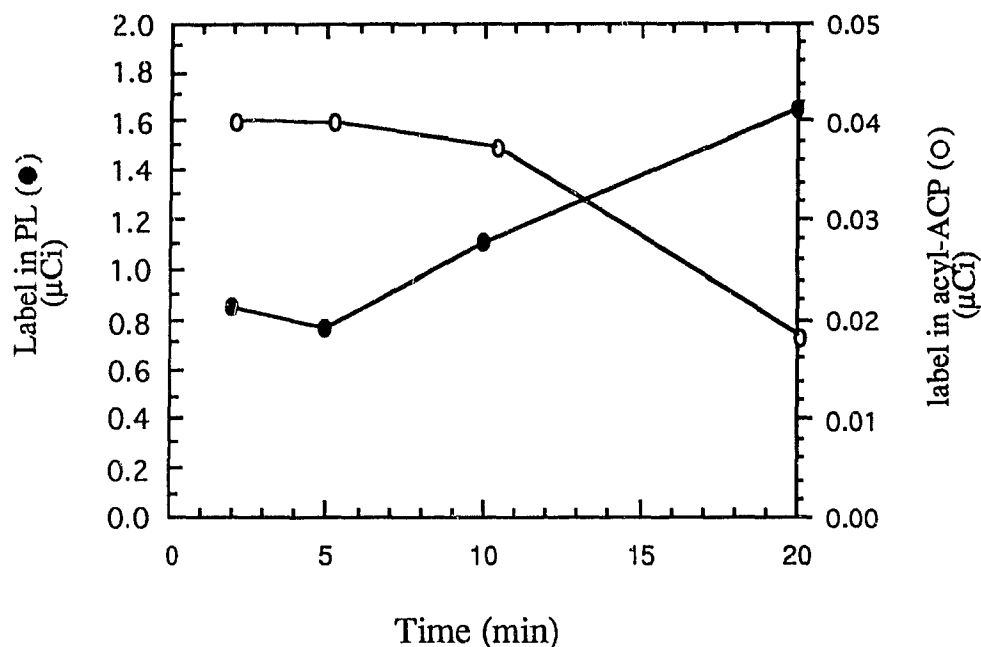


Fig. 31. Incorporation of radioactivity from $[^3\text{H}]14:0$ into phospholipid and acyl-ACP by *V. harveyi* M17 cells in minimal medium. M17 cells (5 ml, $A_{660} = 2.1$) were incubated with 100 μl of 0.2 mM $[^3\text{H}]14:0$ (20 Ci/mmol) in minimal medium at 27°C. One ml of cell culture was removed at the indicated times and divided into two equal fractions. The cell pellet from one 0.5 ml fraction was lysed without washing with medium as described in Method A3 and proteins in the extract were separated by 15% Urea-PAGE. Radiolabeling of acyl-ACP bands was quantitated by scanning densitometry of fluorograms. A lipid extract from the remaining half of the sample was analysed by TLC developed in chloroform/methanol/acetic acid/water (25:15:4:2, v/v), and radiolabel in phospholipid (PL) was quantitated by scanning as described in Method A5.

decreased thereafter: label in total acyl-ACPs decreased from 5% of that in phospholipid at 5 min to about 1% at 20 min. There was no significant change in the relative labeling of individual acyl-ACP species over this period (data not shown).

3. Labeling of acyl-ACP intermediates does not arise primarily from total degradation of [^3H]14:0 to [^3H]acetyl-CoA.

Labeling of acyl-ACPs with [9,10- ^3H]14:0 could theoretically be due either to direct acylation of ACP with ^3H -fatty acid or to de novo synthesis from [^3H]acetyl-CoA, which might arise from β -oxidation of [9,10- ^3H]14:0 (specifically during degradation of [^3H]6:0-CoA to [^3H]4:0-CoA). In light of the considerable chain shortening of [^3H]14:0 that occurs prior to acyl-ACP formation (Fig. 30), the latter possibility was investigated by incubating *V. harveyi* M17 cells with either [^3H]14:0 and unlabeled acetate, or unlabeled 14:0 and [^3H]acetate in minimal medium under chemically equivalent conditions (Fig. 32). The relative labeling of different acyl-ACP species with [^3H]14:0 should be identical to that with [^3H]acetate if exogenous [^3H]14:0 is totally degraded to [^3H]acetyl-CoA and then resynthesized to form ^3H -labeled acyl-ACP. However, the observed labeling pattern from [^3H]14:0 was significantly different from that with [^3H]acetate as precursor. In the former case, 12:0-ACP was the major acyl-ACP band representing 46% of the total label associated with acyl-ACPs, and short chain acyl-ACPs ($\leq \text{C}_6$) were not detected. In contrast, ACP and short chain acyl-ACPs comprised 52% of total acyl-ACP label from [^3H]acetate while 12:0-ACP was less than 10% (Fig. 32). Moreover, the temporal pattern of incorporation was different, with [^3H]14:0-labeling of long chain acyl-ACP decreasing with longer incubation times (as noted above) while the opposite was observed for [^3H]acetate (Fig. 32). This experiment indicates that labeling of acyl-ACPs arises mainly from ^3H -labeled acyl groups which are partial degradation products of the parent [9,10- ^3H]14:0 chain. It was also noted that unlabeled acetate inhibited the incorporation of radioactivity from [^3H]14:0 into acyl-ACP *in vivo*, although it did not affect the profile of radiolabeled acyl-ACPs.

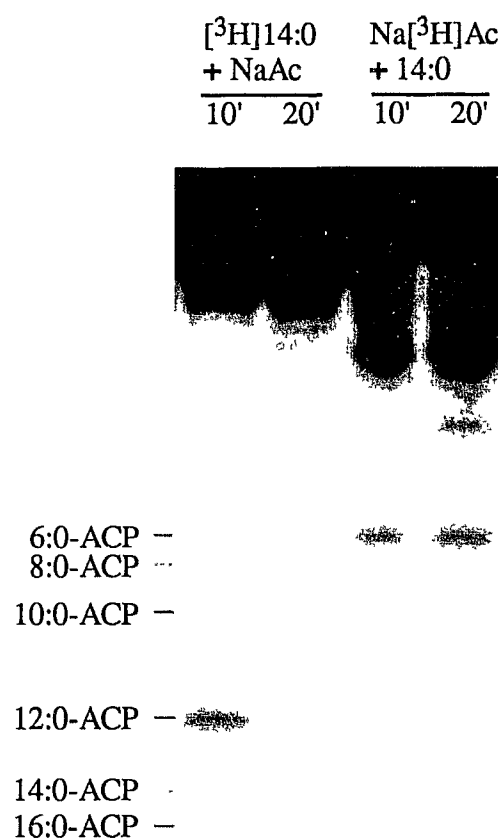


Fig. 32. Urea-PAGE of *V. harveyi* M17 proteins labeled with $[^3\text{H}]14:0$ or sodium $[^3\text{H}]$ acetate in minimal medium. *V. harveyi* M17 cells ($A_{660} = 1.6$) were incubated for 10 or 20 min in minimal medium (1 ml) containing either 5 μM unlabeled 14:0 and 66 μM $[^3\text{H}]$ acetate or 5 μM $[^3\text{H}]14:0$ and 66 μM acetate as indicated. Radiolabeled cells were lysed as described in Method A4 and 5 μl of cell lysate was analyzed by urea-PAGE. A fluorogram of 80 days exposure is shown.

4. The distribution of radiolabeled acyl-ACP intermediates is affected by cerulenin.

V. harveyi M17 cells were incubated with [^3H]14:0 or [^3H] β -alanine in the presence or absence of cerulenin, which blocks fatty acid synthesis by irreversibly inhibiting 3-ketoacyl-ACP synthase [49]. Unlabeled 14:0 alone (not shown) or with acetate (Fig. 33B) did not affect the labeling pattern of ACP derivatives with [^3H] β -alanine, indicating that these precursors do not significantly perturb the mass composition of the acyl-ACP pool at the concentrations used (Fig. 33A and B). In contrast, cerulenin had dramatic effects on the mass of acyl-ACPs, resulting in an overall 5-fold increase in acyl-ACP (C_8 to C_{14}) labeled with [^3H] β -alanine. Label was increased for both 8:0-ACP (25-fold) and 10:0-ACP (16-fold), while label on 14:0-ACP was virtually undetectable in the presence of cerulenin even upon much longer fluorographic exposure; comparable changes were also noted with [^3H]acetate as precursor (data not shown). The accumulation of 8:0- and 10:0-ACP and depletion of 14:0-ACP suggests that elongation of 10:0-ACP and 12:0-ACP may be particularly sensitive to inhibition by cerulenin while longer products, such as 14:0-ACP, are quickly removed by other biosynthetic pathways. With [^3H]14:0 as precursor, however, there was no change ($\sim 2\%$ decrease) in overall label incorporation into acyl-ACPs (C_8 to C_{14}) in the presence of cerulenin, but labeling of 14:0-ACP was substantially decreased (6-fold) while label on both 8:0-ACP and 10:0-ACP increased 6-fold, although much less than with [^3H] β -alanine labeling (Fig. 33C) or with [^3H]acetate (data not shown). These results suggest that long chain acyl-ACPs labeled with [^3H]14:0 have not completely equilibrated with the mass pool (labeled with [^3H] β -alanine) under these conditions.

5. The distribution of radiolabeled acyl-ACP intermediates was affected by acyl-ACP transferase and growth medium composition.

The radioactivity from [^3H]14:0 can also be incorporated into acyl-ACPs of luminescent wild type *V. harveyi* cells (Fig. 30, WT). However, a pronounced shift to

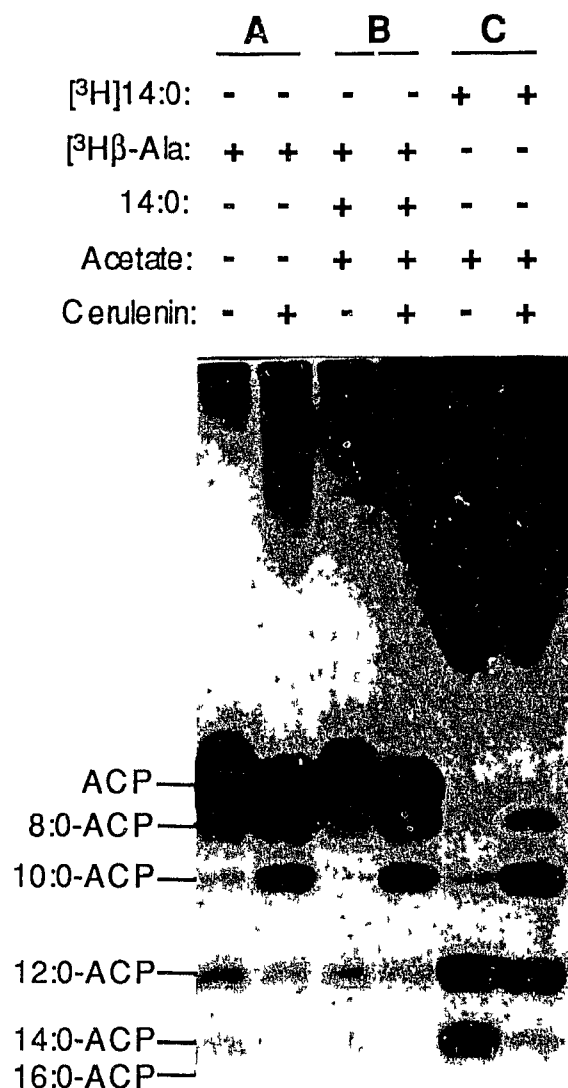


Fig. 33. Urea-PAGE of *V. harveyi* proteins labeled with [³H]β-alanine or [³H]14:0 in the presence or absence of cerulenin. Parts A and B: *V. harveyi* M17 was grown in minimal medium (1.2 ml) containing 0.3 μM [³H]β-alanine (87 Ci/mmol); for part B, unlabeled 14:0 (3.3 μM) and Na-acetate (55 μM) were also added for 10 min prior to harvest. Part C: cells in minimal medium were incubated with [³H]14:0 (4.2 μM, 20 Ci/mmol) and 55 μM unlabeled acetate for 10 min prior to harvest. Where indicated, cerulenin (10 μg/ml) was also added for 1 hour prior to harvest and lysis at A₆₆₀ = 2.8, as described as in Method A4. Cell lysates (10 μl) were fractionated by urea-PAGE; a fluorogram of 10 days exposure is shown.

shorter chain length intermediates was observed, with increases in labeled 8:0-ACP (10-fold) and 10:0-ACP (6-fold) relative to M17 cells and the complete absence of 14:0-ACP (Fig. 30). These differences suggest that activity of the 14:0-ACP transferase in the wild type strain depletes 14:0-ACP and also influences the composition of other labeled acyl-ACP intermediates. On the other hand, relatively more 14:0-ACP was generally labeled with [^3H]14:0 in M17 cells approaching stationary phase (data not shown), suggesting that the distribution of intermediates is also influenced by the rate of cell growth. Little or no 16:0-ACP was observed under any conditions.

To ensure that acyl-ACP formation from exogenous fatty acid was not an artifact of restricted growth in minimal medium, *V. harveyi* cells were incubated with [^3H]14:0 in complex medium before and after induction of luminescence (Fig. 34). Although extremely long exposure times were necessary under these conditions, 12:0-ACP and 14:0-ACP were observed in both wild type (Fig. 34) and M17 (not shown), with labeling in 14:0-ACP predominant in preinduced wild type cells. Under similar conditions, no labeled bands were observed in this molecular weight region when *E. coli* was incubated with [^3H]14:0. This result is consistent with all known evidence that exogenous fatty acids are not activated to biosynthetic acyl-ACP intermediates in this species.

6. Exogenous [^3H]14:0 is also incorporated into lipid A.

The experiment shown in Figure 29 demonstrates that most of the [^3H]14:0-labeled products in *V. harveyi* cell extracts which co-migrates acyl-ACP in SDS-PAGE were not immunoprecipitated with anti-ACP serum. In contrast, anti-ACP antibody was much more effective in precipitating [^3H]β-alanine-labeled products migrating in this region. The non-precipitable [^3H]14:0-labeled bands were characterized further by SDS-PAGE and thin layer chromatography. As shown in Fig. 35 (lane A), a labeled doublet corresponding in migration to the above bands was observed by SDS-PAGE and fluorography after incubation of M17 cells with [^3H]14:0 in complex medium (acyl-ACPs are not visualized under these conditions and short exposure times). These bands could not be extracted by

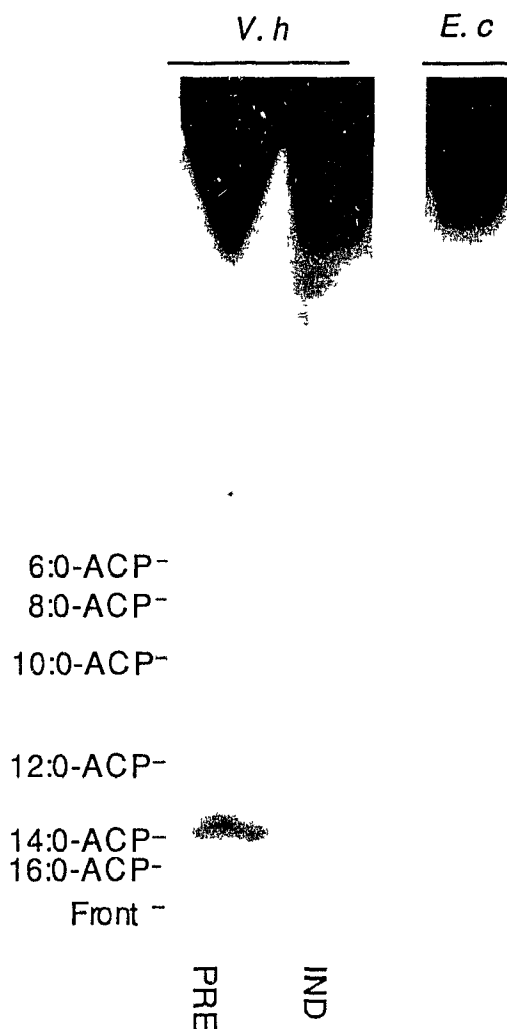


Fig. 34. Urea-PAGE of *V. harveyi* and *E. coli* proteins labeled with [^3H]14:0 in complex medium. *V. harveyi* wild type cells were grown in complex medium and 1 ml samples were incubated with 20 μl of 0.2 mM [^3H]14:0 for 10 min before (PRE, $A_{660} = 0.37$) or after (IND, $A_{660} = 1.75$) the induction of bioluminescence. *E. coli* cells were labeled at $A_{660} = 0.80$ under identical conditions. Equivalent amounts of cell lysate protein, adjusted based on A_{660} at the time of harvest, were separated by urea-PAGE. A fluorogram of 50 days exposure is shown.

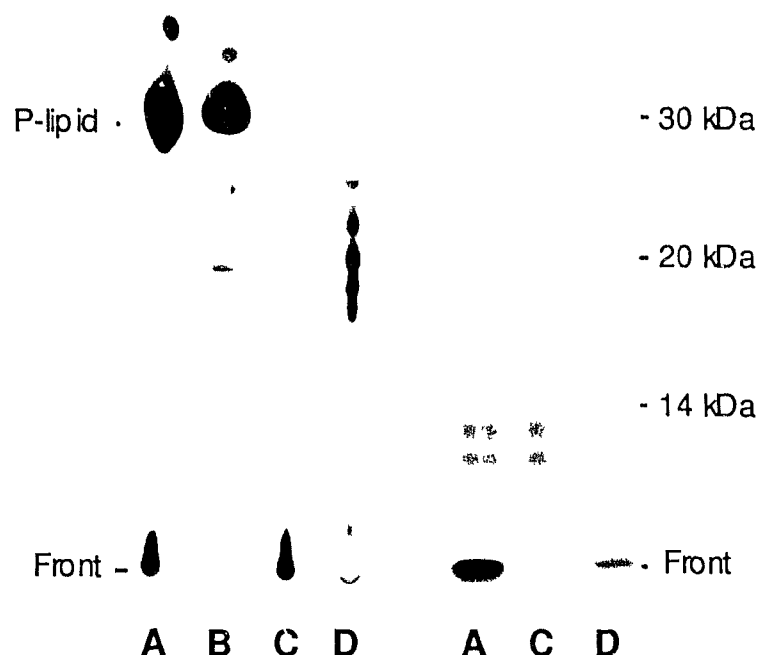


Fig. 35. Comparison of [^3H]14:0-labeled products in *V. harveyi* cells by SDS-PAGE and thin layer chromatography. *V. harveyi* M17 ($A_{660} = 1.2$) in 1 ml of complex medium was incubated with 20 μl of 0.2 mM [^3H]14:0 (20 Ci/mmol) for 10 minutes. One half of the washed cell pellet was used to prepare cell lysate (A) in 125 μl of RIPA buffer; 2.0 μl and 0.5 μl portions were analyzed by SDS-PAGE and TLC developed in chloroform/methanol/water/ammonia (40:25:4:2, v/v), respectively, as described in Method E5 and A6. The other half of the cell pellet was used to prepare a lipid extract (total volume 2.2 ml); 6 μl of this lipid extract (B) was used for TLC analysis. Half of the insoluble residue containing lipopolysaccharide (C) was resuspended in 68 μl of RIPA buffer by sonication, diluted with equal volume of 2XSDS sample buffer and analyzed by TLC (1 μl) or SDS-PAGE (4 μl). The other half of the insoluble residue (D) was heated in 68 μl of 0.2 N HCl, neutralized with NaOH, diluted with SDS sample buffer and analyzed by TLC (2 μl) and SDS-PAGE (4 μl). Fluorography exposure was 2 days for TLC and 1 day for SDS-PAGE.

chloroform/methanol and remained at the origin following TLC (lane C), while labeled products at the gel front were extracted by organic solvent and identified as glycerophospholipid by TLC (lane B). The labeled bands on SDS-PAGE were resistant to proteinase K treatment but were destroyed by acid hydrolysis of the chloroform/methanol-insoluble residue, resulting in the appearance of a series of labeled spots on TLC (lane D). This pattern is characteristic of the 4'-monophosphoryl lipid A degradation products released by acid hydrolysis of *E. coli* lipopolysaccharide, as reported by Galloway and Raetz [67]. Moreover, migration of these [³H]14:0-labeled products in this TLC system corresponded to those from samples prepared from both *E. coli* RR1 and *V. harveyi* labeled with ³²Pi, where virtually identical patterns were observed (J. Douglas and D. M. Byers, unpublished observations). These observations indicate that the lipid A component of outer membrane lipopolysaccharide can also be labeled with exogenous myristic acid in *V. harveyi*. Incorporation of radioactivity from [³H]14:0 into lipopolysaccharide was approximately 15% of that into phospholipid in *V. harveyi* (data not shown), and was not observed in *E. coli* as noted previously [20]. Incorporation of label from [³H]14:0 into lipopolysaccharide was decreased more than 80% when cells were labeled in the presence of cerulenin, while incorporation of label into phospholipid was unchanged (Fig. 36, lanes 1 and 2). However, cerulenin totally blocked incorporation of [³H]acetate into both lipopolysaccharide and phospholipid (Fig. 36, lanes 3 and 4) while it also caused accumulation of ³H-labeled medium chain acyl-ACPs inside the cells (Fig. 33). These observations suggest that cerulenin, which specifically targets KAS-I and KAS-II in fatty acid elongation, preferentially blocks incorporation of ³H-labeled fatty acid into lipid A. Presumably, this is due to requirement for condensation to form β -hydroxymyristic acid (two-thirds of lipid A acyl chains), while phospholipids can be effectively labeled directly with [³H]14:0 in the presence of cerulenin [24].

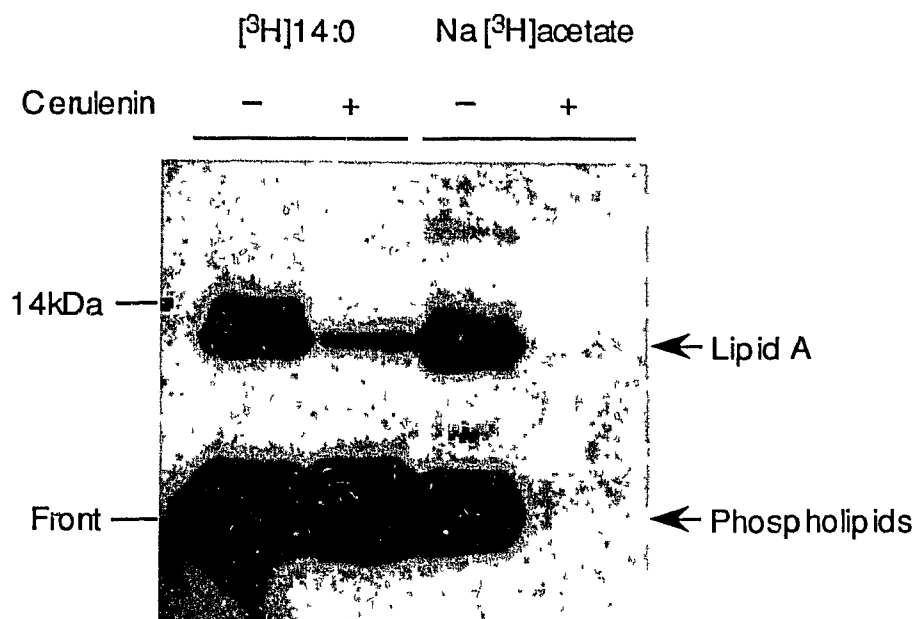


Fig. 36. SDS-PAGE of labeled products from incubation of *V. harveyi* M17 with $[^3\text{H}]14:0$ or $[^3\text{H}]$ acetate in the presence or absence of cerulenin. One ml of *V. harveyi* M17 ($A_{660} = 1.5$) was incubated with $2\ \mu\text{M}$ $[^3\text{H}]14:0$ (20 Ci/mmol) or $33\ \mu\text{M}$ $[^3\text{H}]$ acetate (22 Ci/mmol) in complex medium for 10 min. Where indicated, cerulenin ($10\ \mu\text{g/ml}$) was also added for 1 hour prior to harvest. Cell lysate was prepared as described in Method A4 and $1\ \mu\text{l}$ of lysate was separated by SDS-PAGE. A fluorogram of a 30 days exposure is shown.

D. Analysis of *V. harveyi* fatty acid transport.

Although exogenous [^3H]14:0 was shown to be activated to form acyl-ACP, the pathways involved in transport of this fatty acid across both cell membranes of *V. harveyi* remain unknown. Long chain FA transport in *E. coli* is mediated by at least two proteins, FadL and FadD. To investigate whether analogous proteins are essential for long chain FA transport in *V. harveyi*, cells were mutagenized with nitrosoguanidine and different approaches were used to screen possible mutations in long chain FA transport and activation.

1. *V. harveyi* growth and luminescence is affected by temperature.

V. harveyi cells were normally cultured at 27°C. However, in order to select strains with temperature sensitive mutant gene products, growth of *V. harveyi* cells at 37°C was investigated. As shown in Figure 37, wild type or M17 mutant cells of *V. harveyi* were incubated in complex medium at 27°C or 37°C, and cell growth was monitored by A_{660} while luminescence was measured as described in Method A2. These strains showed no difference in growth rate at 27°C until late exponential phase, where M17 cells exhibited slightly faster growth than wild type cells. At 37°C, M17 cells exhibited a slower growth rate than that at 27°C. However, wild type cells did not grow well at 37°C: a maximum of $A_{660} = 0.9$ was attained. Luminescence was not observed in either strain growing at 37°C. Because of the abnormal cell growth of wild type *V. harveyi* at 37°C, it appears that this temperature is not suitable for screening temperature-sensitive mutants.

2. Selection of *V. harveyi* FA transport mutants using 11-bromoundecanoate.

In *E. coli*, the *fadL* gene encodes an outer membrane protein which is necessary for long-chain fatty acid transport. *FadL* mutants can be enriched and isolated by incubating cells in medium containing 0.5 mM 11-bromoundecanoate, since transport of this bacteriocidal fatty acid analog is partially blocked when the *fadL* transport system is defective [202]. To explore whether *V. harveyi* has a similar outer membrane receptor

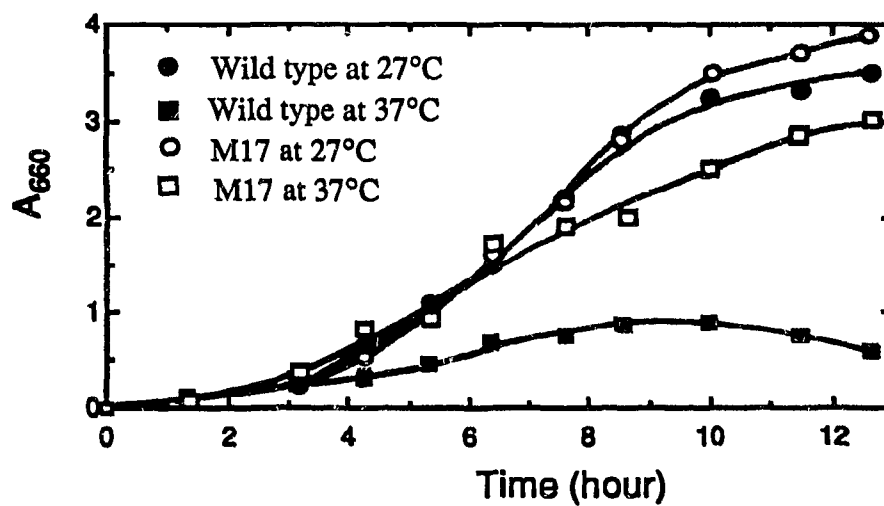


Fig. 37. Growth of wild type and M17 mutant of *V. harveyi* cells (wild type or M17) were subcultured and incubated in complex medium at different temperatures. Cell growth was monitored by measuring A_{660} of cell culture.

protein involved in FA transport, nitrosoguanidine-treated *V. harveyi* M17 or wild type cells were plated on minimal medium containing 0.3 mM 11-bromoundecanoate. Of 10^5 nitrosoguanidine-treated cells, no resistant colonies were obtained. Further studies with liquid *V. harveyi* cultures indicated that cell survival was almost complete when the concentration of 11-bromoundecanoate was lower than 40 μ M, while concentrations greater than 0.1 mM were lethal to *V. harveyi*. The lack of 11-bromoundecanoate-resistant cells raised questions as to whether *V. harveyi* lacks an outer-membrane fatty acid receptor or whether it merely has a low physical tolerance to medium chain fatty acid. Incubation of M17 cells with sodium decanoate (10:0) in minimal medium showed that although 10:0 stimulates *V. harveyi* cell growth at concentrations between 10 μ M and 0.3 mM, cell growth was inhibited at concentrations higher than 0.5 mM (data not shown). This observation was in marked contrast to *E. coli*, where up to 5 mM concentrations of long or medium chain fatty acid are used in liquid cultures during fatty acid metabolic studies [202]. Considering the decreased tolerance to medium chain fatty acids in *V. harveyi*, the failure of nitrosoguanidine-treated *V. harveyi* to grow at concentrations of 11-bromoundecanoate used to select FA transport-defective *E. coli* mutants may be attributable in certain degree to the toxic effect of the fatty acid itself. If medium chain fatty acids can be transported into *V. harveyi* by protein-independent mechanisms, a low tolerance to these fatty acids would make it impossible to screen fatty acid receptor mutants with 11-bromoundecanoate. Therefore, the effect of this chemical was not further characterized.

3. Isolation and characterization of double luminescence mutants.

Although 14:0-ACP transferase is defective in the dark mutant M17 strain of *V. harveyi*, luminescence in this strain can be restored by addition of myristic acid or long chain aldehyde to the medium. A strategy to select FA transport mutants was devised based on the hypothesis that, if *V. harveyi* contains a fatty acid receptor necessary for 14:0 transport, a mutation in this receptor should cause M17 to lose its ability to emit light in the

presence of exogenous 14:0. This hypothesis was tested by selecting double luminescence mutants from nitrosoguanidine-treated M17 cells. Three of 2,000 colonies screened exhibited a qualitative lack of luminescence response when 14:0 was added to the medium. One of the double mutant strains, MBM1, may have defective luciferase activity because addition of dodecanal to the medium also did not stimulate luminescence. Two other mutants (MBM4 and MBM6) showed apparent normal luciferase activity since their luminescence in the presence of dodecanal was comparable to that of M17 (Fig. 38). Incubation with [^3H]14:0 in complex medium demonstrated that fatty acid was incorporated normally into both phospholipids and lipid A in all three double mutants, indicating that these mutants retained their ability to transport and activate fatty acids. However, both strains MBM1 and MBM4 had abnormal labeled patterns of lipopolysaccharide in SDS-PAGE compared to that of *V. harveyi* wild type or M17 (Fig. 39). Further characterization is necessary to elucidate the relationship between the altered lipopolysaccharide patterns and the loss of 14:0-stimulated luminescence in MBM1 and MBM4.

4. Selecting mutations by tritium suicide.

The tritium suicide technique is another approach that was used in an attempt to isolate *V. harveyi* long chain FA transport mutations. This technique has been used previously to isolate several *E. coli* strains which are unable to incorporate fatty acids into phospholipids normally [90]. When nitrosoguanidine-treated M17 cells (3.5 ml, $A_{660} = 0.25$) were incubated with 200 μCi [^3H]14:0 (20 Ci/mmol) at 27°C, about 60 μCi of [^3H]14:0 was taken up by the 5×10^8 cells in 30 min. As *V. harveyi* cells are unable to survive in complex medium when stored at 4°C, washed cells were stored at -70°C and survival was periodically checked by plating dilutions and colony counting. After 10 weeks, the survival of [^3H]14:0-treated M17 cells had decreased by 10^5 -fold while the titer of viable control cells remained constant (Fig. 40). At this stage, about 100 [^3H]14:0-treated colonies were grown in liquid culture and analyzed for incorporation of [^3H]14:0 into phospholipid as described in Method A5. The results indicated that all cells retained the

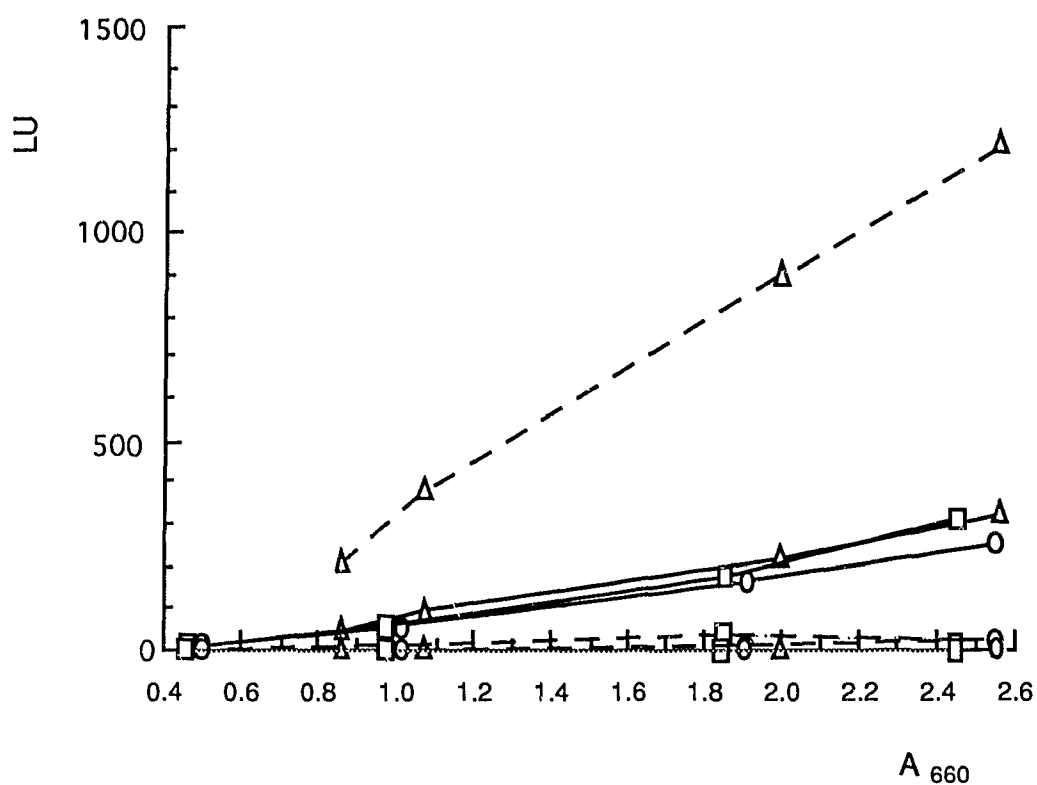


Fig. 38. Cell growth and bioluminescence of double luminescent mutants derived from M17. Colonies of double dark mutants screened as described in Methods were incubated in 50 ml complex medium at 27°C. Cell growth was monitored by measuring A_{660} of the cell culture. The bioluminescence of parent strain M17 (Δ), MBM4 ($\circ$) and MBM 6 ($\square$) alone (dotted line), in the presence of myristic acid (dashed line) and in the presence of dodecanal (solid line) was measured as described in Method A2.



Fig. 39. SDS-PAGE of $[^3\text{H}]14:0$ -labeled products in *V. harveyi* mutant cells. *V. harveyi* M17 or double dark mutants ($A_{660} = 1$ to 1.5) in 0.6 ml of complex medium were incubated with 3 μM $[^3\text{H}]14:0$ (20 Ci/mmol) for 10 min. Labeled cells were washed and lysed in 50 μl RIPA buffer as described in Method A3. One μl of lysate was separated by SDS-PAGE and fluorography exposure was 2 day.

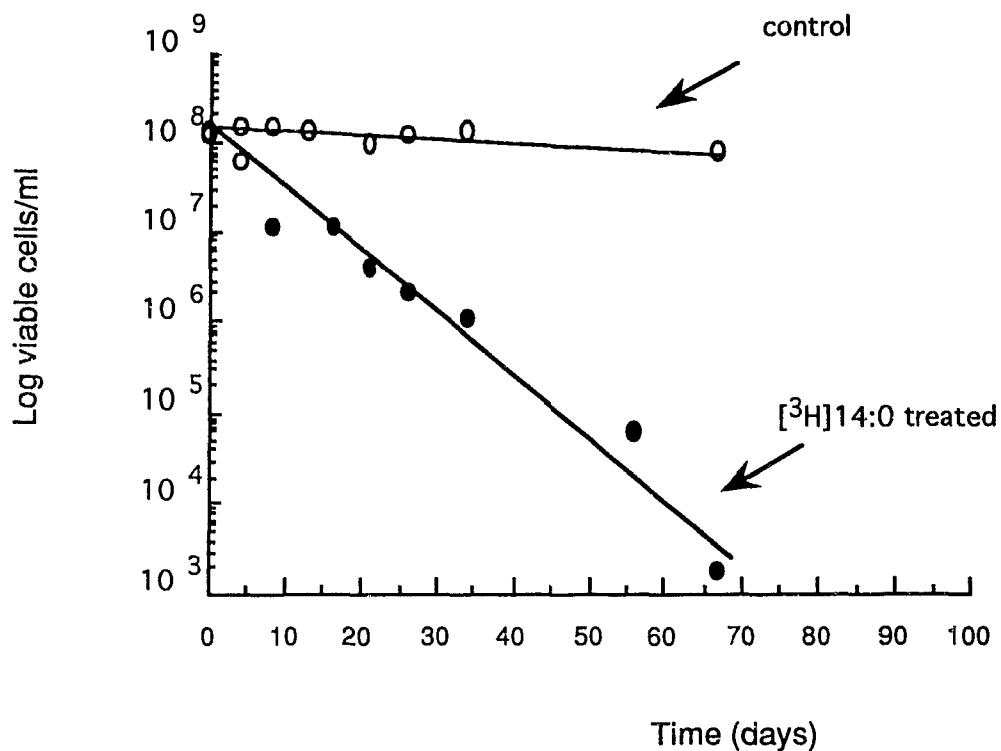


Fig. 40. Inactivation of *V. harveyi* M17 after incorporation of [³H]14:0. Nitrosoguanidine-treated *V. harveyi* M17 cells were cultured in minimal medium to $A_{660}=0.3$ and 3.6 ml cell culture were treated with [³H]14:0 or unlabeled 14:0 as described in Method F3. After washing, cells were resuspended in 5 ml complex medium containing 20% glycerol and stored in aliquots at -70°C . Periodically, the radiolabeled or control batch was diluted and plated on complex medium and cell survival was measured by colony counting.

ability to incorporate [^3H]14:0 into phospholipid at normal levels relative to untreated M17 cells (data not shown). This observation contrasts to previous suicide experiments performed with [^3H]oleate in *E. coli* showing that when viable cells decreased from 10^7 to 10^3 , all surviving cells were defective in their ability to grow on oleate as the sole carbon source and exhibited dramatically decreased ability to incorporate oleate into lipids [90]. The above experiments indicate that incorporation of 14:0 into phospholipid is retained in nitrosoguanidine-treated *V. harveyi* mutants.

Section V. Discussion.

A. Characterization of *V. harveyi* ACP and acyl-ACP.

The luminescent bacterium *V. harveyi* provides an attractive system for the study of the structure and function of ACP. In addition to its usual roles in bacterial FA and complex lipid synthesis, ACP appears to be involved in providing 14:0 for aldehyde synthesis and in activation and elongation of exogenous fatty acid in this organism [24]. Purification and characterization of ACP are essential steps towards a complete understanding of its function in these processes. The present experiments describe the purification of ACP and cloning of the ACP gene from *V. harveyi*. Homogeneous ACP has been obtained in yields of 30-60 mg/kg wet weight of cells.

V. harveyi ACP in cell lysates exhibited two bands of equal intensity on Western blots (Fig. 25). Although purified *V. harveyi* ACP displayed a single band on SDS-polyacrylamide gel stained with Coomassie Blue (Fig. 11), Western blots of this product also revealed two bands which comigrated with the intracellular doublet, but the lower molecular weight band was much more prominent (data not shown). Similar observations reported for purified *R. meliloti* ACP were attributed to ACPs with and without N-terminal methionine [172]. However, the two ACP bands observed could also be products encoded by separate ACP genes, or derived from post-translational modification or specific degradation by protease. Alternatively, the higher molecular weight band could be a *V. harveyi* short chain acyl-ACP, such as acetyl-ACP or malonyl-ACP. It has been observed that malonyl-ACP is the major ACP derivative in *E. coli* and migrates slower than ACP in conformationally-sensitive gel electrophoresis. However, it is unclear whether malonyl-ACP can be distinguished from ACP in SDS-PAGE. More experiments are necessary to further identify these intracellular ACP isoforms.

Analysis of the primary structure of *V. harveyi* ACP has revealed that this protein is one residue shorter (76 amino acids) and slightly more acidic (pI = 3.99) than *E. coli* ACP

(77 amino acids, pI = 4.1). *V. harveyi* ACP contains an apparent phosphopantetheine binding site (Ser-36) by comparison to the corresponding *E. coli* region (Fig. 19). Compared to all other ACPs of known sequence, the primary structure of *V. harveyi* ACP shows highest similarity (86% identical) with *E. coli* ACP (Table IV), consistent with the close relationship between these bacterial species. Eleven residues are different between the two ACPs, among which two (Asp-30 and Val-72 in *E. coli* ACP) are replaced by similar amino acids (Glu and Ile, respectively) in *V. harveyi*. The sequence between residues 31 and 71, which includes the phosphopantetheine prosthetic group attachment site, are identical in *V. harveyi* and *E. coli*, while the region from residues 3 to 17 displays only a single amino acid difference (Fig. 19).

As discussed in the Introduction, the dynamic model of *E. coli* ACP based on NMR structural data contains three long α -helices between residues 3-14 (helix-I), 37-51 (helix-II), and 65-75 (helix-III). Helices I and II are linked by a long loop region. To examine the predicted positions of amino acid replacements in *V. harveyi* ACP, the coordinates for one of the two *E. coli* ACP dynamic states (structure B) [122, 123] were obtained from the Protein Data Bank [1,10] at the Brookhaven National Laboratory and the tertiary structure was displayed using the Insight II program (Biosym Technologies, San Diego, CA) on an Iris Indigo XS-24 workstation. In Fig. 41, the amino acid residues which are different between *V. harveyi* and *E. coli* ACP are highlighted in green on the purple backbone of the *E. coli* ACP model. This model clearly shows that most of these differences are clustered in the loop region between helix-I and helix-II or at the C-terminus. All but three (Val-12 in helix-II; Val-72 and Ser-74 at the end of helix-III) of the amino acid residues in the three helices are identical between the two ACPs, indicating that *V. harveyi* ACP may have a secondary structure similar to *E. coli* ACP. Since α -helix accounts for 50% of the amino acid residues in *E. coli* ACP and is very important in maintaining the overall protein tertiary structure, a similar secondary structure in *V. harveyi* and *E. coli* ACP may indicate that these proteins have similar overall tertiary structures.



Fig. 41. Protein structural model of *E. coli* ACP. The tertiary model is reproduced based on the coordinates from the Brookhaven Protein Data Bank for one of the two *E. coli* ACP dynamic states (structure B) [123]. The peptide backbone is shown in purple while the amino acid residues which are different between *V. harveyi* and *E. coli* ACP are highlighted in green. From left to right, helices II, I and III are shown, respectively. Serine-36 which is the phosphopantetheine binding site is labeled in red while two proposed fatty acid binding sites, Ile-54 and Ala-59, are labeled in blue.

Predicted similar structures of *V. harveyi* and *E. coli* ACP would suggest that these proteins might be able to substitute for each other in many functions. This is indeed supported by several lines of evidence. First, previous work in our laboratory has shown that both ACPs are substrates for *V. harveyi* acyl-ACP synthetase which has identical K_m values of 20 μ M for these substrates [61]. Second, both *V. harveyi* and *E. coli* acyl-ACP can be cleaved by *V. harveyi* acyltransferase, product of the *luxD* gene. This enzyme specifically cleaves 14:0-ACP to provide 14:0 for aldehyde synthesis in *V. harveyi* [27,60]. Recombinant *E. coli* which contains expressed *luxABCDE* gene products emits light without addition of 14:0 or long chain aldehyde, indicating that *E. coli* 14:0-ACP is used by *V. harveyi* acyltransferase *in vivo*. The present work also demonstrates that *E. coli* 14:0-ACP can be effectively cleaved by *V. harveyi* acyltransferase *in vitro* (Fig. 24). Third, recent experiments by Dr. Eugene Kennedy and coworkers have shown that *V. harveyi* ACP (purified in our laboratory) is also able to activate *E. coli* transglucosylase (A. Weissborn and E.P. Kennedy, personal communication). *E. coli* ACP had been shown to differ from other bacterial or plant ACPs, ie. *Rhizobium melioli*, *Rhodobacter sphaeroides* and spinach, because of its unique ability to activate of *E. coli* transglucosylase in MDO synthesis [172]. We also believe that the similarity of *V. harveyi* and *E. coli* ACPs is reflected in the anomalous migration of both proteins in SDS-PAGE and in unstable expression of the *V. harveyi* ACP gene in *E. coli* (see below). Since *E. coli* ACP has been so extensively characterized, purification and characterization of the *V. harveyi* protein should provide helpful clues for further dissecting the specific structural features involved in specialized roles, such as the *E. coli* transglucosylase reaction.

Although *V. harveyi* and *E. coli* ACPs are highly homologous, significant differences between these two proteins have also been observed. After incubation of anti-*V. harveyi* ACP immune serum with excess *E. coli* ACP, antibodies clearly remain which specifically recognize epitopes that are present only on *V. harveyi* ACP (Fig. 25),

indicating that anti-*V. harveyi* ACP antibody may be useful in defining structural and functional differences between the proteins.

V. harveyi and *E. coli* ACPs and acyl-ACPs also exhibit some differences in SDS- and native-PAGE. Both ACPs exhibit anomalously slow mobility in SDS-PAGE (Fig. 22) with an apparent mass of 20 kDa, compared to the calculated molecular mass of 8.8 kDa. Anomalous migration of *E. coli* ACP has been attributed to decreased SDS binding due to its high negative charge density (charge to mass ratio = -0.0017) and lack of hydrophobic amino acids [186]. Indeed, anomalous migration of some acidic proteins can be reversed upon chemical modification which neutralizes acidic side chains. Most proteins bind 1.4 grams of SDS per gram of protein, which is thought to be mainly due to interactions between SDS and hydrophobic amino acids [183]. SDS normally contributes 90% of the negative charge of the SDS-protein complex. Thus, the charge to mass ratio of a typical SDS-protein complex is constant (-0.0020), and migration of the complex is empirically related to the mass of the protein through the degree of asymmetry (ie. length) of the rod-shaped SDS-protein micelle [241].

Like the *E. coli* protein, *V. harveyi* ACP also lacks hydrophobic amino acids and has an even more negative charge to mass ratio (-0.0019). Thus, the above hypothesis might suggest decreased migration of *V. harveyi* ACP, despite its smaller size. However, *V. harveyi* ACP migrated faster than *E. coli* ACP in SDS-PAGE (Fig. 22). Indeed, other facts also do not fit with the hypothesis that anomalous migration of ACP is solely due to decreased SDS binding. Previous experiments have shown that *Rhodobacter sphaeroides* ACP exhibits SDS-PAGE behaviour more typical of its true size, despite a high acidic amino acid content [37,172].

A native gel electrophoresis system which was identical to SDS-PAGE except without SDS was also used to analyse ACP. *E. coli* and *V. harveyi* acyl-ACPs (C₆ to C₁₆) were not resolved in this gel system and exhibited similar mobility to *E. coli* ACP, while *V. harveyi* ACP migrated slower than acyl-ACP (data not shown). The failure to

separate *E. coli* ACP and acyl-ACP may be due to the temperature (4°C) of the experiment. Future native gel analysis of ACP and acyl-ACP should be performed at 37°C as described by Rock and Cronan [43,187].

The effect of fatty acylation is also not consistent with SDS binding as the predominant factor influencing the anomalous migration of acyl-ACP in SDS-PAGE. Increased migration of *E. coli* ACP upon acylation was originally attributed to increased SDS-binding by the more hydrophobic acyl-ACPs [110]. However, this cannot explain why *V. harveyi* acyl-ACP exhibits decreased mobility with chain lengths greater than C₁₀. No difference in mobility between long chain acyl-ACPs of different chain length was observed for either *E. coli* or *V. harveyi* ACP in native-PAGE, suggesting no major alterations in conformation or hydration occur with increasing chain length above C₁₀. Moreover, preliminary octyl-Sepharose binding studies indicate that *V. harveyi* acyl-ACPs are, if anything, slightly more hydrophobic than *E. coli* acyl-ACPs (D. M. Byers, unpublished observations).

For the above reasons, we favor a model in which ACP or acyl-ACP migration in SDS-PAGE is a complex function of both conformational and SDS binding factors. We suggest that acylation with chain lengths up to C₁₀ may permit some compaction of the molecule, even in the presence of SDS, resulting in increased electrophoretic mobility. At longer chain lengths, which affect *V. harveyi* and *E. coli* ACP differently, migration could be subject to more influences, such as conformational destabilization, SDS binding, or hydration. Future studies involving other hydrodynamic approaches and direct SDS binding analysis will be required to test more refined models of acyl-ACP structure and behaviour on SDS-PAGE.

Comparing the amino acid sequences of *V. harveyi* and *E. coli* ACP to that of other bacterial ACPs (Fig. 19), a high degree of similarity (>90%) is observed from residues 3 to 14 and from 31 to 50, suggesting that all of these bacterial ACPs may possess helices-I and II in their secondary structures. Beyond residue 58, however, *R. meliloti* and *R.*

sphaeroides ACP are less similar to *E. coli* and *V. harveyi* ACP, indicating that the former pair may have different secondary and tertiary structures in this region. In contrast to *E. coli* ACP, *R. sphaeroides* ACP has only 69 amino acid residues which may not be long enough to form a stable α -helix at the C-terminus.

B. The *V. harveyi* ACP gene.

Purification and protein sequencing of *V. harveyi* ACP was an essential step in the isolation of the corresponding gene. Due to the toxic effect of ACP overexpression, cloning of the *E. coli* ACP gene in *E. coli* proved unusually difficult [99]. A high frequency of spontaneous deletions in the *E. coli* ACP gene cloned in M13 vectors was also reported [113,178]. The high degree of homology between *V. harveyi* and *E. coli* ACP also created problems for cloning of the *V. harveyi* ACP gene in *E. coli* in the present work. Our initial cloning attempts were based on the strategy of using *V. harveyi* ACP-specific antibodies (prepared as described above by preincubation of immune serum with *E. coli* ACP) to detect expression of *V. harveyi* ACP. Using this approach, *V. harveyi* genomic DNA fragments (about 4 kb) generated by *Sau*3A cleavage and cloned into pUC18 vector yielded a positive clone in the first round of screening of transformed *E. coli* cells. However, the positive signal was not further observed in the second round of screening after transfer of this colony to a fresh LB plate, suggesting that expression of the *V. harveyi* ACP gene may have also inhibited *E. coli* cell growth.

Therefore, our strategy was changed to screen the cloned ACP gene instead of expressed ACP. Expression of this gene in recombinant *E. coli* might be due either to the *lacZ* promoter of the pUC vector or to internal *V. harveyi* promoters if the latter are recognized by *E. coli* transcriptional enzymes. On the other hand, if *E. coli* cannot recognize internal *V. harveyi* promoters, the *V. harveyi* ACP gene might not be expressed if inserted into pUC vector in opposite orientation to the *lacZ* promoter, and the silent cloned ACP gene should not affect host cells. Thus, a synthesized oligonucleotide probe (AP-1) was used to screen recombinant plasmids which could contain the ACP gene in

either orientation. Considering the inhibition of cell growth by expressed ACP, construction of a *V. harveyi* genomic library is not suitable for ACP gene cloning, as cells which express such a toxic gene would grow much slower in liquid medium than other cells and would not be represented after several generations. Therefore, size-selected restriction fragments of *V. harveyi* genomic DNA, which showed positive hybridization to the oligonucleotide probe on Southern blots, were isolated and cloned into pUC vectors.

A recombinant plasmid pUS1 containing two thirds of the *V. harveyi* ACP gene in an orientation opposite to the *lacZ* promoter was identified by screening with the AP-1 probe. Western blot analysis of lysates of recombinant *E. coli* containing pUS1 using anti-ACP immune serum did not reveal any new bands, compared to control cells without insert. This experiment indicated that the cloned ACP gene fragment cannot be transcribed from *V. harveyi* promoters in *E. coli*, that the insert did not contain an internal promoter, or that antibodies do not recognize the protein fragment derived from this region. However, a putative promoter sequence (TATAGT, position 398 to 403 in Fig. 17C) similar to the Pribnow box (TATAAT) is located between *fabG* and *acpP*, and a presumptive -35 region (TTTACT, position 377 to 382 in Fig. 17C) is also found 22 bp upstream of the TATAGT sequence. This suggests that the *V. harveyi* ACP gene may be transcribed by *E. coli* RNA polymerase using an internal *V. harveyi* promoter. The purine-rich Shine-Dalgarno sequence, normally located 10 bp upstream of the translation initiation site, is also observed in the upstream region of the *V. harveyi* ACP gene (AAGGAAA, position 584 to 590 in Fig. 17C).

Nevertheless, some observations indicated that the host cells were affected by the cloned ACP fragment. In contrast to strains containing pUC vector without inserts, lower plasmid copy numbers were observed with recombinants containing two-thirds of the *V. harveyi* ACP gene including the 5'-end and the upstream region (Fig. 15, pUS1 to pUS32). Deletion of the 5'-end and its upstream region in pUSS83 caused intracellular plasmid copy numbers to return to normal levels. Another strain (UO1), which contains

half of the *V. harveyi* ACP gene encoding the 3'-end in recombinant plasmid pUO1, also exhibited normal intracellular copy plasmid numbers. The relationship between low plasmid copy number and the corresponding regions around the 5'-end of the *V. harveyi* ACP gene remains unknown and needs to be further analyzed. To further characterize the *V. harveyi* ACP gene, assembly of the complete gene from the two overlapping segments (pUO1 and pUSS11) and expression in *E. coli* should be attempted in future studies.

Although not fully developed in the present work, evidence was obtained that organization of *V. harveyi* FA synthase genes is quite similar to that in *E. coli*. The *V. harveyi* ACP gene appears to be located downstream of genes with considerable sequence similarity to *E. coli fabD* and *fabG*, which encode malonyl-CoA-ACP transacylase and a 3-ketoacyl-ACP reductase, respectively [178]. The isolation and sequencing of additional *V. harveyi* FA synthetic genes should offer further insight into the mechanism and regulation of fatty acid metabolism in this organism.

C. Acyl-ACP preparation using *V. harveyi* acyl-ACP synthetase.

The present study clearly indicates that partially purified *V. harveyi* acyl-ACP synthetase is very useful for preparative synthesis of acyl-ACPs with chain lengths from 6:0 to 16:0. Acyl-ACP formation was found to be unaffected over the range of ACP concentrations tested in the present experiments (13-70 μM); this range spans the K_m of the enzyme for both *V. harveyi* and *E. coli* ACPs (20 μM) [26]. This is in contrast to the reaction catalyzed by *E. coli* acyl-ACP synthetase, which prefers ACP concentrations less than 15 μM for maximum acylation [190]. Using *V. harveyi* luminescence-induced acyl-ACP transferase, we have shown that *V. harveyi* acyl-ACP synthetase forms biologically active thioester derivatives of ACP with no further side reaction. Moreover, this enzyme has been used to monitor the purification of *V. harveyi* and *Photobacterium phosphoreum* ACP. The activity of *V. harveyi* acyl-ACP synthetase with ACP substrates from three different bacterial species is consistent with the high degree of ACP structural homology among different organisms and suggests that the *V. harveyi* enzyme may be useful for a

wider range of bacterial and plant species. Indeed, it has recently been shown that *V. harveyi* acyl-ACP synthetase will fatty acylate the D-alanyl carrier protein (Dcp) from *Lactobacillus casei* (F. Neuhaus and D.M. Byers, unpublished observations).

The only other enzyme that has been described with the ability to directly ligate long chain fatty acids to ACP is *E. coli* acyl-ACP synthetase/2-acylglycerolphosphatidylethanolamine acyltransferase, an 81 kDa inner membrane enzyme that uses tightly bound ACP as an intermediate in the activation and transfer of fatty acids to form phosphatidylethanolamine. That enzyme has been employed for the preparation of acyl-ACP derivatives to study the reactions of bacterial [40,71,78,222] and plant [149,173,174] fatty acid metabolism, phospholipid synthesis [20], lipopolysaccharide synthesis [98] and fatty acylation of proteins [96]. We believe that *V. harveyi* acyl-ACP synthetase also offers a useful tool for fatty acylation for a number of reasons. First, a suitable enzyme preparation that is stable for several months can be obtained after two simple purification steps. This preparation appears to be free of contaminating activities that utilize acyl-ACP [223], allowing long reaction times under conditions in which ACP is the major protein component. Second, *V. harveyi* acyl-ACP synthetase is a soluble enzyme and exhibits optimal activity at low ionic strength and in the absence of detergents. This is in contrast to *E. coli* acyl-ACP synthetase, which requires Triton X-100 for solubility and high concentrations of LiCl for release of acyl-ACP [38]. Third, the extent of acyl-ACP formation with the *V. harveyi* enzyme appears to be largely independent of ACP concentration and the scale of the preparation (compare Figs. 20, 21 and Table V). Finally, *V. harveyi* acyl-ACP synthetase quantitatively (>85%) acylates ACP using fatty acids from 6 to 16 carbons in length. This range overlaps that of the *E. coli* enzyme, which provides maximum yields with 14 to 18 carbon fatty acids, although some acylation with 8:0 to 12:0 is also noted [180,191,222]. Thus, preparation of medium to long chain fatty acyl-ACP derivatives can be performed using a single procedure, rather than a combination of chemical and enzymatic methods as reported previously [48].

D. Exogenous 14:0 can be partially degraded prior to activation to form acyl-ACP.

The purification of *V. harveyi* ACP and preparation of acyl-ACP standards have provided important tools for further exploring the role of ACP in activation of exogenous fatty acids. The present study has clearly shown that acyl chains derived from exogenous 14:0 can be activated to form acyl-ACP and lipid A in *V. harveyi*. Acyl-ACPs labeled by incubation with [9,10-³H]14:0 were positively identified by (i) co-migration with authentic acyl-ACP standards in two different gel systems, (ii) co-migration with acyl-ACP labeled specifically with [³H]β-alanine, and (iii) immunoprecipitation with anti-*V. harveyi* ACP serum. To our knowledge, this is the first direct demonstration of activation of exogenous fatty acids to biosynthetic acyl-ACP intermediates in bacteria and provides strong evidence that elongation of these fatty acids observed previously in *V. harveyi* involves ACP [23,24].

The present results also provide further contrast to FA metabolism in *E. coli*, where exogenous fatty acids can be activated to both acyl-CoA and acyl-ACP at the inner cell membrane [194], but neither intermediate has access to pathways of FA synthesis. In *E. coli*, acyl-CoA is oxidized or incorporated directly into phospholipid by glycerol-3-phosphate acyltransferase [75], while acyl-ACP derived from exogenous fatty acid is a transient, tightly-bound intermediate in the reacylation of the 1-position of phosphatidyl ethanolamine and is not elongated [38,101]. *V. harveyi* also differs from *E. coli* in that fatty acids do not appear to induce enzymes responsible for their metabolism (ie. a *fad* - like operon) nor can they support growth in the absence of other carbon sources [24].

An unexpected result of this study, and one that would not be predicted from earlier metabolic labeling studies, is that exogenous 14:0 can be partially degraded to shorter chain lengths prior to acyl-ACP formation. An alternative explanation, that labeling of these acyl-ACPs with [³H]14:0 is mainly due to degradation of the fatty acid to form [³H]acetyl-CoA followed by de novo resynthesis, appears unlikely for several reasons. First, labeling with

[^3H]14:0 and [^3H]acetate under identical conditions resulted in completely different acyl-ACP profiles (Fig. 32): fatty acid label appeared exclusively in medium- to long-chain (C_8 to C_{14}) acyl-ACPs while acetate label appeared mostly in shorter chain derivatives ($\leq \text{C}_6$). These differences occurred without substantial influence of either 14:0 or acetate on acyl-ACP pool sizes (Fig. 33B). Moreover, only one-sixth of the label in [9,10- ^3H]14:0 would be expected to appear in acyl chains via recycling of acetate; the label in the 9-position of the fatty acid would be lost as $^3\text{H}_2\text{O}$ upon degradation, and two-thirds of the remaining label in [^3H]acetyl-CoA (ie. from the 10-position of [^3H]14:0) would be lost during resynthesis. This is clearly at odds with the rapid appearance of labeled acyl-ACP (5% of lipid label by 5 min) from [^3H]14:0, but not from [^3H]acetate itself (Fig. 32). Also, we found no evidence of general protein labeling from precursors derived from [^3H]14:0 via [^3H]acetate, whereas SDS-PAGE bands other than acyl-ACP were labeled with [^3H]acetate in a time-dependent manner. Finally, it has been shown previously that all of the fatty acid label from [1- ^{14}C]12:0 and [1- ^{14}C]14:0 appears in saturated acyl chains [24], with none in 16:1 and 18:1 which account for over 60% of the fatty acid in *V. harveyi* [23]. Both saturated and unsaturated fatty acids would be expected to be labeled with [1- ^{14}C]acetate. It should be noted that the experiment shown in Fig. 32 does not rule out possible conversion of [^3H]14:0 to a "hot" pool of [^3H]acetyl-CoA which is preferentially used for acyl-ACP synthesis; the specific radioactivities of acetyl-CoA and of lipid and protein products of acetate were not measured in this experiment.

A model of exogenous FA metabolism in *V. harveyi* that is consistent with the findings of this and earlier studies is presented in Fig. 42 [211]. Based on the appearance of labeled acyl-ACPs with chain lengths shorter than 14:0, as well as the observed formation of $^{14}\text{CO}_2$ from [1- ^{14}C]14:0 [24], we suggest that exogenous 14:0 may be initially activated to acyl-CoA (as in *E. coli*) and partially oxidized as CoA derivatives. These partially degraded acyl moieties can be transferred from acyl-CoA to ACP and can also be re-elongated by FA synthase. The latter step would be blocked by cerulenin

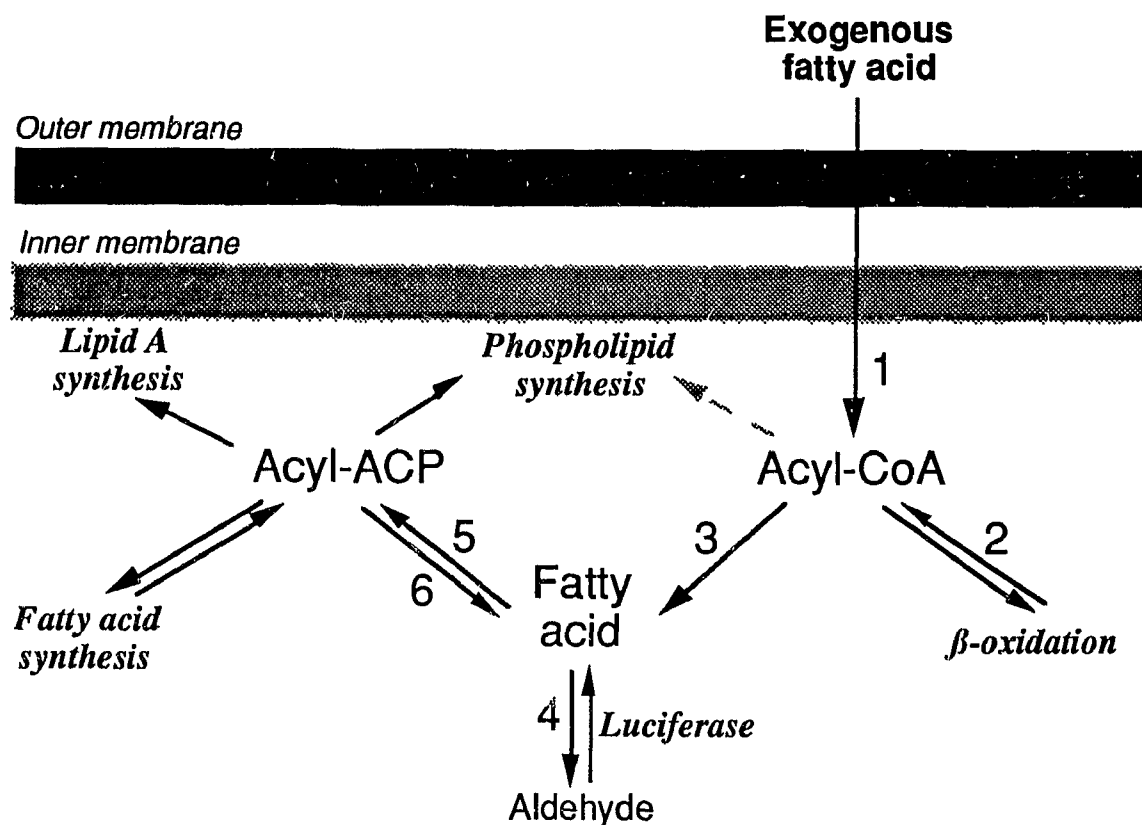


Fig. 42. Proposed model for the metabolism of exogenous fatty acid in *V. harveyi*. Exogenous fatty acid is transported and activated to form acyl-CoA (1) which can be oxidized to shorter chain lengths (2). Long chain acyl-CoA intermediates may also be hydrolyzed to free fatty acid (3) as a substrate for aldehyde synthesis by fatty acid reductase (4, 14:0 only) or activation by acyl-ACP synthetase to form acyl-ACP (5). In wild type bacteria (but not mutant M17), endogenous 14:0 for bioluminescence is provided by cleavage of 14:0-ACP by a specific transferase (6).

resulting in the observed shift of labeled acyl-ACPs to shorter chain lengths (Fig. 33). The dramatic decrease in labeled 14:0-ACP in the presence of cerulenin likely reflects its efficient use as an intermediate in phospholipid acylation (14:0 is about 10% of total *V. harveyi* fatty acid [23]) in the absence of competing endogenous 14:0-ACP. Note that our results do not rule out direct activation or transfer of at least some of the intact 14:0 chain to ACP (ie. without degradation). Indeed, 30% of labeled fatty acid derived from [1-¹⁴C]14:0 appears in *V. harveyi* phospholipids as 16:0 in complex medium [24]: this labeled 16:0 must arise from direct elongation of the 14:0 precursor, as the label would be lost after a single round of β -oxidation. Thus, degradation of exogenous fatty acid does not appear to be a prerequisite for transfer to ACP.

The mechanism of acyl chain transfer from acyl-CoA to acyl-ACP in *V. harveyi* has not been established. One possibility is direct transacylation by a condensing enzyme of FA synthase (β -ketoacyl-ACP synthase). This mechanism has been suggested for the *in vivo* acylation of ACP by exogenously-derived fatty acid in the angiosperm *Spirodela oligorrhiza*, based on inhibition by cerulenin and *in vitro* transfer of labeled acyl-CoA to ACP [149]. However, we favor involvement of a free FA intermediate (as shown in Fig. 42) for several reasons. First, acyl-ACP is labeled when *V. harveyi* cell extracts are incubated with [³H]14:0 (+ ATP), but not with [³H]14:0-CoA [239]. Second, cerulenin might be expected to decrease labeling of all acyl-ACPs by similar extents, rather than produce a shift in their chain length profile, if a single condensing enzyme was involved in acyl-CoA transfer. Third, a transient free FA pool (product of the luciferase reaction) does exist and is detectable under certain conditions in *V. harveyi* [23]. Finally, the presence of a soluble *V. harveyi* acyl-ACP synthetase, which activates a broad range of fatty acids (C₆ to C₁₆) to acyl-ACP *in vitro* [26,61,212], is consistent with activation of free fatty acid to form acyl-ACP *in vivo*.

The relative levels of acyl-ACP intermediates should reflect the balance of enzyme activities involved in their synthesis and utilization. In bioluminescent bacteria, 14:0-ACP

is the specific substrate of a luminescence-related transferase which provides endogenous 14:0 for aldehyde synthesis [27,60]. This study indicates that the presence of this enzyme in wild type *V. harveyi* dramatically affects acyl-ACP composition labeled with exogenous [^3H]14:0, as labeled 14:0-ACP was virtually absent in wild type compared to M17 cells incubated in minimal medium (Fig. 30). Labeled 14:0-ACP is also decreased in the presence of cerulenin, while 8:0-ACP and 10:0-ACP accumulate (Fig. 33). These results suggest that elongation of longer chain acyl-ACPs might be particularly sensitive to cerulenin and that 14:0-ACP (a suitable substrate for phospholipid acylation) is quickly removed under these conditions. The composition of the growth medium also affects the labeling of acyl-ACP with [^3H]14:0: in minimal medium, the balance appears to be shifted towards oxidation of 14:0 prior to ACP acylation, with acyl-ACPs as short as C₈ detected, while less degradation of 14:0 was noted in complex medium, where 12:0-ACP was the only shortened product observed. The absence of labeled 16:0-ACP from [^3H]14:0, even in complex medium, likely reflects the fact that 16:0-ACP is a preferred substrate for phospholipid acylation and does not accumulate as an intermediate to any appreciable extent.

Previous investigations examining the mass composition of acyl-ACP pools in both plants [174,196] and *E. coli* [39,109] have been valuable in providing insight into the rate determining steps in FA biosynthesis. The incorporation of exogenous FA precursors in *V. harveyi* should provide another dimension to this approach, particularly in studying the control of the synthesis of less abundant cell components that require acyl-ACP as acyl donors, such as secreted proteins [98] and lipid A. In *E. coli*, acyl groups in lipid A are derived from β -hydroxy-14:0-ACP, 12:0-ACP and 14:0-ACP [6]. While lipid A cannot be directly labeled with exogenous fatty acid in *E. coli* [20], it is effectively labeled with [^3H]14:0 in *V. harveyi* (Fig. 35). As 12:0-ACP and 14:0-ACP are the major labeled acyl-ACP intermediates, we would expect that all the major fatty acids of lipid A (12:0, β -hydroxy-14:0, and 14:0) should also be labeled to some extent. In any case, *V. harveyi*

should provide a good model to study the competition for acyl-ACP intermediates between different pathways and products, shedding light on, for example, the regulatory process which diverts about 20% of the acyl-ACP to the formation of lipid A rather than phospholipid [99].

E. FA transport across the *V. harveyi* cell membrane may be protein independent.

Although exogenous fatty acid was found to be activated to acyl-ACP in *V. harveyi*, little is known about FA transport in this organism. Since the mechanism of FA transport is closely linked to the activation of fatty acyl intermediates in *E. coli* and other organisms, several approaches were tried to identify proteins which may play key roles in long chain FA transport in *V. harveyi*.

FadL and acyl-CoA synthetase (FadD) have been shown to be involved in FA transport in *E. coli*. Defective FLP and FadD almost totally block incorporation of long chain fatty acid into lipids. However, the present work suggests that 14:0 transport in *V. harveyi* is not dependent on a single protein. Surviving cells (approx. 1 out of 10⁵) after incubation of nitrosoguanidine-treated M17 cells with [³H]14:0 in tritium suicide experiments all showed normal incorporation of 14:0 into lipids. In contrast to our results, tritium suicide experiments with [³H]18:1 in *E. coli* found that all surviving cells (1 out of 10⁴) were defective in their ability to grow on 18:1 [90]. Since *V. harveyi* cannot grow on fatty acid as a sole carbon source, FA transport was examined by measuring the incorporation of [³H]14:0 into lipids. The results suggest that transport of 14:0 is not absolutely dependent on a single critical protein, such as FLP in *E. coli*, for which mutants can be enriched using this approach. One possibility is that two or more transport mechanisms, possibly of overlapping fatty acid chain length specificity, may be present in *V. harveyi*. Mutations in all of these transport systems would be required to completely block FA uptake and lipid incorporation. In this case, a much larger population of cells would likely have to be mutagenized to generate appropriate double or triple mutants.

Another possibility is that a defective FA transport protein is lethal to *V. harveyi*. Finally, our results would also be consistent with an unfacilitated passive transport mechanism, where there is no gene or protein to respond to selective pressure by tritium suicide.

Screening nitrosoguanidine-treated M17 cells by measuring 14:0-stimulated luminescence also failed to select mutants in 14:0 transport and incorporation. Since only 2,000 colonies were characterized, it is conceivable that additional screening might yet be successful in identifying mutations in these processes. However, characterization of the three double luminescence mutants obtained did produce some interesting results. One strain (MBM1) could not be stimulated by dodecanal, probably due to defective luciferase, while the other two (MBM4 and MBM6) showed normal luciferase activity (Fig. 38). It is possible that an additional mutation in fatty acid reductase occurred in MBM4 and MBM6, resulting in loss of 14:0-stimulated luminescence in the two strains. However, anomalous lipopolysaccharide SDS-PAGE bands in two of the strains were also observed (Fig. 35). Although separate and unrelated mutations in lipopolysaccharide biosynthesis are not unexpected, the relationship between lipopolysaccharides and luminescence should be further characterized as preliminary studies have indicated that some other *V. harveyi* luminescence mutants, such as AFM [27], also show anomalous lipopolysaccharide banding patterns.

Other observations have also suggested that the mechanism of FA transport and utilization may differ in luminescent bacteria and *E. coli*. Experiments with 10:0 and 11-bromoundecanoate indicated that these medium chain fatty acids or FA analogues may cause physical disruption of *V. harveyi*. In contrast, *E. coli* can tolerate much higher concentrations of these compounds than *V. harveyi*. Moreover, other experiments in our laboratory have shown that no heat-modifiable *V. harveyi* membrane proteins are specifically recognized by anti-*E. coli* FLP antibodies on western blots (K. Johnson and D.M. Byers, unpublished observations). Interestingly, another luminescent bacterium, *P. phosphoreum*, is unable to use [^3H]14:0 for β -oxidation or lipid synthesis, possibly due to

lack of acyl-CoA synthetase in this bacterium. However, enzymes involved in fatty aldehyde synthesis are still radiolabeled by exogenous [^3H]14:0 [24], indicating that FA transport is independent of fatty acid activation and incorporation into lipid. *V. harveyi* is capable of both FA elongation and degradation, but these processes are not induced by fatty acid as with the Fad system in *E. coli* [24]. Thus, the presence of proteins analogous to acyl-CoA synthetase and FLP may not be necessary for other bacterial species with different requirements for fatty acid.

Even in *E. coli*, FLP and FadD have recently been shown to be unnecessary for transport of 14:0 and 16:0: these radiolabeled fatty acids did accumulate in *fadD* and *fadL* mutants and could be utilized in a recombinant protein N-myristoylation system provided that yeast acyl-CoA synthetase was also expressed in these strains [125]. These observations raise questions whether FLP is necessary for long chain FA transport per se or for targeting of long chain fatty acids to specific pathways of activation. *E. coli* does not maintain an intracellular free fatty acid pool, probably due to toxicity of these hydrophobic molecules, and the transport of exogenous fatty acid is normally coupled with its utilization by β -oxidation or incorporation into lipid. If the function of FLP is to deliver long chain fatty acid to acyl-CoA synthetase and 2-acylglycerolphosphoethanolamine acyltransferase, inhibition of measurable long chain FA uptake by mutations in FLP would be expected as alternate intracellular fates of fatty acid would not exist. However, it is possible that long chain fatty acid can be transported via other mechanisms, such as that for medium chain FA transport. Indeed, it was estimated that only 67% of the medium chain fatty acid 10:0 is transported via FadL in an *E. coli fadL*⁺ strain [143]. If a similar situation occurs in *V. harveyi*, enrichment of long chain FA transport mutants by tritium suicide would be more effective using a longer chain fatty acid, such as [^3H]18:1.

E. Future directions?

The nature of the two ACP bands observed on Western blots of *V. harveyi* extracts should be further explored. Removal of the phosphopantetheine group with

[ACP]phosphodiesterase before SDS-PAGE of cell extracts could be used to determine whether these bands arise from differences in protein sequence or phosphopantetheine modification. If the former is true, the relative expression of the two isoforms should be examined in detail under different physiological conditions.

Since exogenous fatty acid can be incorporated into phospholipid and lipid A via acyl-ACP in addition to the fatty acid biosynthetic pathway, further comparison of fatty acid and acetate labeling of phospholipid, lipid A and acyl-ACP might reveal details of the mechanism and regulation of fatty acid metabolism in *V. harveyi*. With acetate labeling, the labeling of protein and acyl moieties should be further analysed to determine the relationship between fatty acid and protein synthesis.

More research is necessary to explain how *V. harveyi*, *E. coli* and other bacteria are capable of regulating the fates of fatty acids in biosynthetic and degradative pathways. The bioluminescence system could provide a useful tool in these investigations. For example, genes involved in bioluminescence have been cloned and expressed in *E. coli*. Thus, it should be possible to construct an *E. coli* recombinant strain with *V. harveyi luxABCE*, but without *luxD* (14:0-ACP acyltransferase). Induced luminescence in such a strain would be expected to be dependent on exogenous 14:0. If this strain was also defective in FLP or FadD, it might indicate whether 14:0 uptake is independent of FLP and FadD for other metabolic fates in *E. coli*. Another approach will be to clone *V. harveyi* acyl-ACP synthetase and to determine whether its expression in *E. coli* wild type and transport-defective mutants affects fatty acid transport and metabolism, including the ability to elongate exogenous fatty acids.

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