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ESCHERICHIA COLI LONG-CHAIN FATTY ACID UPTAKE: MOLECULAR AND BIOCHEMICAL INVESTIGATION OF PROTEINS INVOLVED IN FATTY ACID UPTAKE ACROSS THE CELL ENVELOPE

By

JAE-HO YOO, B.Sc

A Thesis Submitted to the School

of Graduate Studies in Partial Fulfillment of

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McMaster University

FATTY ACID UPTAKE ACROSS THE *ESCHERICHIA COLI* CELL ENVELOPE

DOCTOR OF PHILOSOPHY (2001) McMASTER UNIVERSITY

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TITLE: *Escherichia coli* Long-Chain Fatty Acid Uptake: Molecular and Biochemical Investigation of Proteins Involved in Fatty Acid Uptake Across the Cell Envelope

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Abstract

E. coli can utilize long-chain fatty acids (LCFAs) as its sole carbon and energy source. This requires the coordinated expression of several proteins involved in the uptake and metabolism of LCFAs. The genes encoding these proteins are unlinked but regulated by the global repressor protein FadR, which makes up the *fad* regulon. FadR binds specific DNA operator sites located within *fad* gene promoters when levels of fatty acyl-coenzyme A are low and dissociates when levels are high. At least two proteins are required for LCFA uptake, FadL and FadD. FadL is an outer membrane protein thought to be involved in LCFA permeation across the outer membrane (OM) while FadD is a cytoplasmic fatty acyl-CoA synthetase. Tsp is a periplasmic protease that has recently been implicated in LCFA uptake but its precise role is unknown. The focus of my research was to investigate FadL and FadD function and determine the role of Tsp in *E. coli* fatty acid uptake.

Although FadL is proposed to facilitate permeation of LCFAs across the *E. coli* OM, there has never been any direct demonstration of this activity. Since uptake assays report the net accumulation of LCFAs due to binding, transport and metabolism, they represent an indirect approach to measuring FadL activity. We therefore developed a novel method for monitoring LCFA permeation across the OM. This assay allowed the first direct demonstration of FadL activity and insight into its mechanism of action. Permeation of the radiolabeled photoreactive LCFA analogue, 11-*m*-diazirinophenoxy

[11-^{3}H] undecanoate, was tracked by targeting a protein with affinity for the fatty acid probe, to the periplasm. The target protein consisted of rat intestinal fatty acid binding protein fused to the C-terminus of maltose-binding protein. *E. coli* cells expressing the fusion protein were incubated with the photoreactive fatty acid, photoactivated and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis/fluorography. Permeation was then assessed based on the level of fusion protein labeling. The *E. coli* cell line used for this assay did not express fatty acyl-CoA synthetase, which allowed us to uncouple LCFA permeation and metabolism. Expression of FadL significantly increased labeling of the fusion protein. Treatment of *E. coli* with the outer membrane-permeabilizing reagent EDTA, also increased the labeling of the fusion protein to an even greater level. FadL facilitated permeation of LCFAs appeared to reach a plateau in a relatively short time period. The plateau did not represent equilibrium of fatty acids across the OM since *E. coli* permeabilized with EDTA displayed higher levels of fusion protein labeling. Our data suggest that FadL is regulated *in vivo* and exists in open and closed states. This hypothesis is supported by the demonstration that *E. coli* are sensitive to relatively low concentrations of LCFAs when the OM is permeabilized but unaffected by FadL overexpression.

The role of Tsp in LCFA uptake has not been well defined. To determine if the periplasmic protease played a direct role in uptake, we investigated various ways in which Tsp might participate. Photoaffinity labeling with the photoreactive fatty acid suggested that Tsp has low affinity for fatty acids and is therefore unlikely to represent a periplasmic fatty acid binding protein. Preliminary evidence had suggested a link

between Tsp protease and FadD cleavage, but our data demonstrated that Tsp is not required for FadD proteolysis. We then investigated the possibility that Tsp was mediating its effects on LCFA uptake through FadL. There were no apparent differences in the expression levels or LCFA affinity of endogenous FadL between *tsp* mutants and wild type *E. coli*. However, FadL overexpression experiments revealed that *tsp* mutants overexpressed the OM protein at reduced levels relative to wild type. This suggested that the OM of *tsp* mutants were abnormal. We also observed differences in the protein labeling patterns from photoaffinity labeling experiments that showed a pair of 33- and 35-kDa proteins was labeled more intensely in *tsp* mutants. The labeled proteins resided in the outer membrane and likely represented the highly abundant porins. Similar sized proteins were also labeled more intensely when wild type *E. coli* were treated with an OM permeabilizing reagent or when FadL was expressed. These results implied that the increased labeling of these proteins was related to changes in OM permeability and therefore OM structure. These data therefore indicated that Tsp is required for normal outer membrane function and plays an indirect role in LCFA uptake. We suggest that the effects of the *tsp* mutation on LCFA uptake are related to changes in outer membrane permeability, although we are uncertain how Tsp is involved in OM integrity.

Purification of FadD, the *E. coli* fatty acyl-CoA synthetase, is a difficult endeavor since low yields of unstable enzyme samples are typically obtained. Discrepancies in the description of native FadD complicate matters further. To overcome some of these technical difficulties, we developed overexpression systems for FadD and a FadD fusion protein. The FadD fusion protein was purified to high levels and free of degraded

enzyme, which was a problem encountered when purifying FadD. Several studies had described native FadD, as a 42-50-kDa enzyme. More recent evidence suggested however, that FadD was a 62-kDa protein based on sequencing and expression data from the cloned *fadD* gene. We report that FadD enzyme is cleaved by the outer membrane protease OmpT, producing a 43-kDa carboxyl-terminal and a 19-kDa amino-terminal fragment, which remain tightly associated. OmpT appeared to be responsible for cleavage of FadD since it was not cleaved when incubated with whole cells or membrane fractions from *E. coli* that did not express the protease. Cleavage of the cytoplasmic enzyme by the outer membrane protease *in vitro*, indicated that only the 62-kDa form of FadD is physiologically relevant. OmpT-mediated proteolysis of FadD showed that the fatty acyl-CoA synthetase undergoes conformational changes in the presence of specific substrates including oleate, which inhibited cleavage. Other data also indicated that the region surrounding the cleavage site is not essential for enzyme function and mobile, behavior indicative of linker domains. These data demonstrated that OmpT is a useful tool for limited proteolysis of FadD and suggests that the OmpT cleavage site represents a linker domain.

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LIST OF PUBLICATIONS

Manuscripts submitted

1. Determination of the native form of FadD, the *Escherichia coli* fatty acyl-CoA synthetase and characterization of limited proteolysis by OmpT. (Submitted to Biochemical Journal).

Manuscripts in preparation

1. The *E. coli* outer membrane protein FadL, does not behave as a simple channel for long-chain fatty acids *in vivo* (In preparation).

2. Tsp is required for normal outer membrane function in *E. coli* (In preparation).

Abstracts

1. *Investigation of long-chain fatty acid uptake in Escherichia coli and 3T3 adpiocytes.* Presented at Annual Research Day conference, McMaster University, 1998

2. *Monitoring outer membrane permeability towards a photoreactive long-chain fatty acid: FadL facilitates permeation of long-chain fatty acids across the cell envelope of Escherichia coli.* Presented at Antimicrobial Research Center Symposium, McMaster University, 2000

3. *A novel method for directly monitoring protein mediated and non-protein mediated long-chain fatty acid permeation across the outer membrane of Escherichia coli.* Presented at International Symposium of Nucleic Acids and Signal Transduction Symposium (In honor of H.G. Khorana), Brandeis University, Waltham, 2000

LIST OF ABBREVIATIONS

ABC transporter	ATP-binding cassette transporter
AMP	adenosine 5'-monophospate
APMSF	(4-amidinophenyl)methanesulfonyl fluoride
ATP	adenosine 5'-triphosphate
BCA	bicinchoninic acid
Brij	polyoxyethylene 20 cetyl ether
BSA	bovine serum albumin
°C	degrees, Celsius
C-terminal	carboxyl terminal
Ci	curie
CoASH	coenzyme A
DAP	11-*m*-diazirinophenoxy
DNase	deoxyribonuclease
E.	*Escherichia*
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetate
FABP	fatty acid binding protein
FACS	fatty acyl-CoA synthetase
$FADH_2$	reduced flavin adenine dinucleotide

FPLC	Fast protein liquid chromatography
fab, Fab	fatty acid biosynthetic
fad, Fad	fatty acid degradation
g	gram
g	acceleration of gravity
H^+	proton
hr	hour
Ig	immunoglobulin
IFABP	intestinal fatty acid binding protein
IPTG	isopropyl β-D thiogalactopyranoside
pI	isoelectric point
K_d	dissociation constant
kDa	kiloDalton
K_m	Michaelis constant
l	liter
LCFA	long chain fatty acid
LPS	lipopolysaccharide
μ-	micro-
M	molar
m-	milli-
MBP	maltose binding protein
MCFA	medium-chain fatty acid

min	minutes
mol	moles
MME	minimal media E
MSH	mercaptoethanol
N-terminal	amino terminal
NADH	reduced dinicotinamide adenine dinucleotide
NP-40	ethylphenyl-polyethylene glycol
O.D.	optical density
OM	outer membrane
p-	pico
PAGE	polyacrylamide gel electrophoresis
pK_a	negative $\log_{10}$ of acid dissociation constant
PMSF	phenylmethane sulfonyl fluoride
RNase	Ribonuclease
rpm	revolutions per minute
SA	serum albumin
SCFA	short-chain fatty acid
SDS	sodium dodecyl sulfate
sec	seconds
tris	tris-(hydroxylmethyl)aminomethane
V_{max}	maxium rate

A. INTRODUCTION

A.1 Role of long chain fatty acids in prokaryotic and eukaryotic physiology

Fatty acids are found in all living organisms and represent the simplest class of lipids (Mathews and van Holde, 1990; Zubay et al., 1995). Unlike other biomolecules, fatty acids are not polymerized but are constituents for other more complex lipids (Magnuson et al., 1993). Fatty acids also represent a source of metabolic energy for eukaryotic and prokaryotic cells (Black and DiRusso, 1994; Mangroo et al., 1995b; DiRusso et al., 1999). It is also increasingly apparent that fatty acids and their metabolites are essential for cell signaling (Henry and Cronan Jr, 1992; DiRusso et al., 1993; Shrago et al., 1995; Færgmean and Knudsen, 1997; DiRusso et al., 1999; Grimaldi et al., 1999). Fatty acids are amphipathic molecules and possess a free carboxylate group and a hydrocarbon chain (Mathews and van Holde, 1990; Zubay et al., 1995). The hydrocarbon chains of most biological fatty acids are found with an even number of carbon atoms. Unsaturated fatty acids have one (mono-unsaturated) or more double bonds (polyunsaturated), while saturated fatty acids do not contain any double bonds. These lipids are also categorized based on the length of the hydrocarbon chain, with very long chain fatty acids (VLCFAs) classified as fatty acids containing 19 or more carbon atoms, long chain fatty acids (LCFAs) containing 12 to 18 carbon atoms, medium chain fatty acids (MCFAs) containing 7 to 11 carbon atoms and short chain fatty acids (SCFAs)

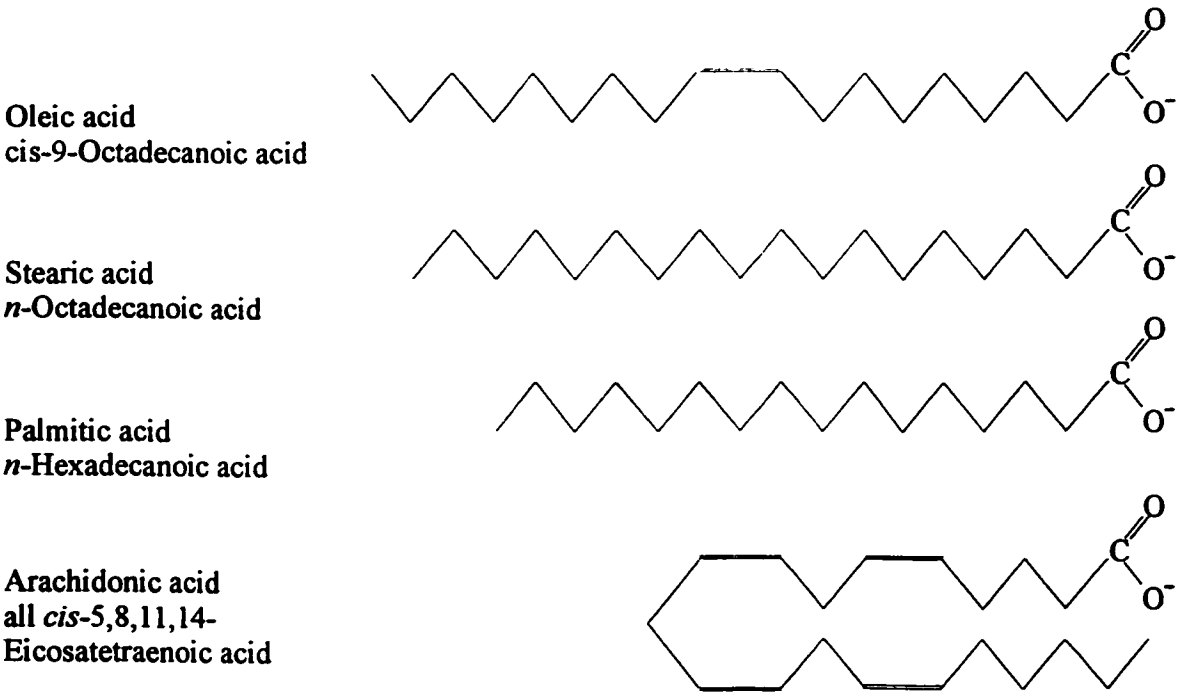
containing 4 to 6 carbon atoms (Neidhart et al., 1996). It is the variation in the number and position of double bonds, and length of the hydrocarbon chain that are important in determining the properties of a specific fatty acid (Figure 1).

Under most physiological conditions, fatty acids are present in the esterified form and not in the free form (Mathews and van Holde, 1990; Zubay et al., 1995). They are usually components of other more complex lipids such as glycerophospholipids, sphingolipids, triglycerides, eicosanoids, cholesterol and waxes. As will be evident in the next few sections, fatty acids serve various roles and functions.

A.1.1 Structural roles

Biological bilayer membranes are non-covalent macromolecular complexes comprised of various proteins, carbohydrates and lipids. The major lipid for most biological membranes are phospholipids, which include glycerophospholipids and sphingolipids (Figure 2). These lipids usually consist of a polar or charged chemical group linked to either a glycerol or sphingosine backbone that is also attached to a long chain fatty acid (Mathews and van Holde, 1990; Zubay et al., 1995). Fatty acids constitute the non-polar region of phospholipids, which due to the thermodynamics of hydrocarbon chains in an aqueous environment, causes the spontaneous formation of bilayer membranes (Zubay et al., 1995). As seen in figure 2, the charged or polar groups vary widely in size and charge, while the attached fatty acids vary in length and number of double bonds. The hydrophilic chemical group and the type of fatty acids present

(A)



(B)

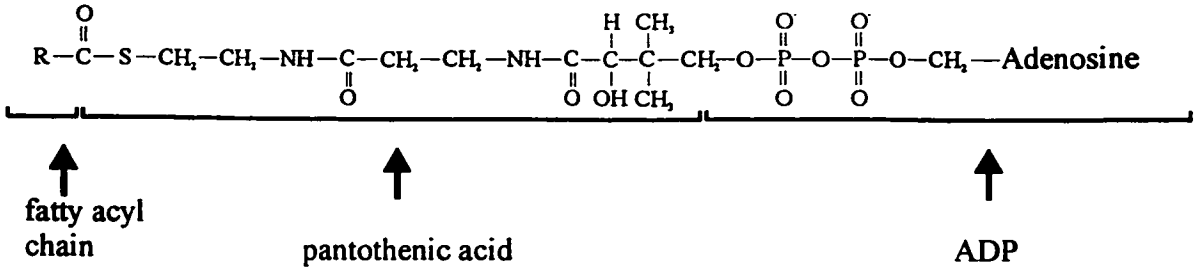
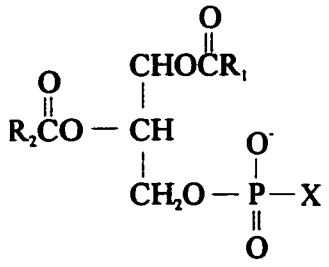


Figure 1. Common fatty acids and structure of fatty acyl-Coenzyme A. (A) Examples of mono-unsaturated long chain fatty acid (LCFA), oleic acid; saturated LCFA, stearic acid; saturated medium chain fatty acid, palmitic acid; polyunsaturated very long chain fatty acid, arachidonic acid (Adapted from Zubay *et al*, 1995 pg. 384). (B) Structure of a fatty acyl CoA molecule with R representing the hydrocarbon chain of the fatty acid (Adapted from Stryer, 1988 pg. 322).

(A) glycerophospholipids



R_1, R_2= hydrocarbom

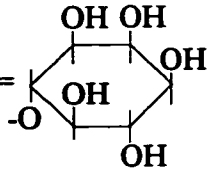
phosphatidylcholine → X= $-O-CH_2CH_2N(CH_3)_3^+$

phosphatidylethanolamine → X= $-O-CH_2CH_2NH_3^+$

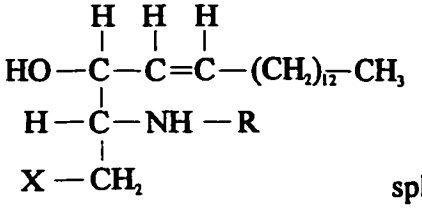
phosphatidylserine → X= $-O-CH_2CHNH_3^+$ (CO_2^-)

diphosphatidylglycerol (cardiolipin) → X= $-O-CH(CH_2OH)CH_2-OH$

phosphatidylinositol → X=



(B) Sphingolipids



sphingosine → R = H X = H

ceramide → R = hydrocarbon X = H

sphingomyelin → R = hydrocarbon X = phosphocholine

(C) Lipopolysaccharides (LPS)

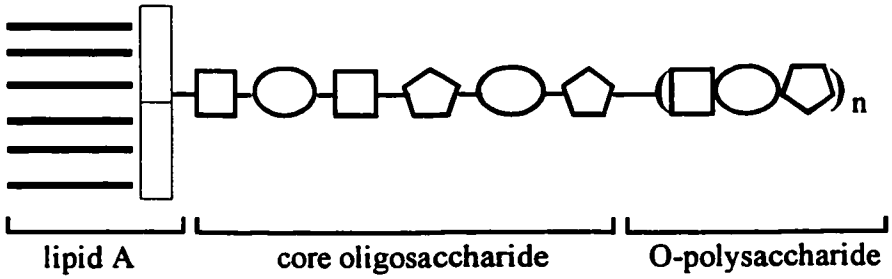


Figure 2. Lipids found in biological membranes. Structure of glycerophospholipids (A), sphingolipids (B) and lipopolysaccharide, LPS (C). (A) Chemical structure representing phosphoglycerol backbone and different functional groups from common phospholipids. (B) Chemical structure representing the common backbone of sphingolipids and the different functional groups. (Adapted from Zubay *et al*, 1995 pg. 383-384) (C) Lipid A consists of a *N*-acetylglucosamine disaccharide ester amine linked to various saturated fatty acids. Core oligosaccharide consists of components such as *N*-acetylglucosamine, galactose, glucose, 2-keto-3-deoxy-octanoic acid and L-glycero-mannoheptose. O-polysaccharide contains a repeating sequence of saccharides that can include abequose, mannose, L-rhamnose and glucose and are often branched (Adapted from Brock and Madigan, 1991, pg. 58).

determine the properties of a specific phospholipid and therefore the properties of the macromolecular structure formed by them. Most biological membranes are primarily composed of glycerophospholipids, although the composition of the glycerophospholipids between animals and bacteria varies widely. The most abundant phospholipid in animal membranes is phosphatidylcholine while phosphatidylethanolamine is more prominent in the plasma membrane of bacteria (Zubay et al., 1995). Cholesterol is another type of lipid found in biological membranes and mostly in the plasma membrane of animals. Fatty acids can be esterified to cholesterol reducing the polarity of the lipid and allowing it to be packaged in lipoproteins for cholesterol storage and transport in animals (Zubay et al., 1995). Cholesterol and sphingolipids are not usually found in bacteria, whereas, lipopolysaccharides (LPS) are membrane lipids unique to gram-negative bacteria (Neidhart et al., 1996). LPS are large, anionic, complex molecules composed of unusual linked sugar groups and multiple saturated fatty acids (usually 6 to 7) attached via amide linkages to *N*-acetylglucosamine disaccharide (Figure 2) (Brock and Madigan, 1991; Hancock, 1991; Neidhart et al., 1996). The acylated *N*-acetylglucosamine, commonly known as lipid A, elicits strong immunoresponses in animals (Brock and Madigan, 1991). The outer leaflet of outer membranes is composed of packed LPS molecules and Mg^{2+} ions (Nikaido and Vaara, 1985; Hancock, 1991; Vaara, 1992; Neidhart et al., 1996). These cations are situated between the lipid molecules and serve to reduce the repulsive forces generated by packing of LPS molecules (Hancock, 1984; Vaara, 1992).

Phospholipid and LPS molecules spontaneously form bilayer membrane structures in water to shield the hydrocarbon chains from the aqueous environments. Formation of liposomes is more entropically favorable than free phospholipids in aqueous solution. This is due to the ordered network of water molecules that must surround the fatty acyl chains for each phospholipid molecule in the "free" form (Zubay et al., 1995). A membrane bilayer structure sandwiches and shields the fatty acyl chains from the aqueous environment and allows water molecules to remain in a disordered state. The packing of the hydrocarbon chains in the membrane bilayer also creates a barrier for large polar or charged molecules, which cannot efficiently penetrate the hydrophobic core of these membranes.

Biological membranes are fluid structures with phospholipids and other lipids readily able to diffuse along the plane of the bilayer (Pjura et al., 1984; Mathews and van Holde, 1990; Zubay et al., 1995). Depending on the chemical state of free fatty acids, they can also diffuse across the membrane ("flip flop") (Kamp and Hamilton, 1993; Higgins, 1994). Protonated fatty acids thus are able to diffuse along and across the bilayer membrane efficiently (Pjura et al., 1984), while the unprotonated form is mostly restricted to lateral diffusion. Therefore, under acidic conditions, fatty acids can readily diffuse across phosphoglycerolipid bilayer membranes (Kamp and Hamilton, 1992).

Some proteins are acylated with fatty acids, which are thought to aid in the localization of these proteins to membranes (Zubay et al., 1995). An example of an acylated protein is the *E. coli* murein lipoprotein, an abundant 7.2-kDa protein located in the periplasm involved in anchoring the peptidoglycan to the OM (Neidhart et al., 1996).

Murein lipoprotein is covalently linked to the peptidoglycan and acylated with three fatty acids that penetrate into the inner leaflet of the OM (Hancock, 1991; Neidhart et al., 1996). In eukaryotes, there are many examples of proteins that require fatty acylation for normal function, including caveolin (Trigatti et al., 1999), heterotrimeric G-protein α-subunits (Duronio et al., 1992) and src (Duronio et al., 1992). Post-translational modification of protein therefore represents another important function for fatty acids in biological systems.

A.1.2 Signaling

In addition to their structural roles, fatty acids and metabolites are also essential for cellular signaling. Fatty acyl-CoA molecules are essential for fatty acid metabolism (Henry and Cronan Jr, 1992; DiRusso et al., 1993), while fatty acids and metabolites also have important roles in the regulation of metabolic and differentiation pathways in mammalian, yeast and bacterial cells (Tomoda et al., 1991; Shrago et al., 1995; Færgmean and Knudsen, 1997; Grimaldi et al., 1999).

The *E. coli* transcription factor FadR, plays an essential role in fatty acid synthesis and degradation (DiRusso et al., 1993; Black and DiRusso, 1994; DiRusso et al., 1999). FadR is a 27-kDa protein with a helix-turn-helix motif that is thought to recognize specific DNA operator sites (DiRusso et al., 1999). DNA bound FadR represses the transcription of enzymes involved in the degradation of fatty acids (Black and DiRusso, 1994) but activates transcription of enzymes involved in fatty acid synthesis (fab regulon). FadR binds DNA as a homodimer, consistent with the DNA

binding elements displaying dyad symmetry (DiRusso et al., 1999). Induction of the *E. coli* β-oxidation pathway requires the presence of fatty acids containing 12 or more carbon atoms in the growth medium and expression of the cytoplasmic long chain fatty acyl-CoA synthetase, FadD. FadR possesses fatty acyl-CoA binding activity, which when occupied, causes the repressor to dissociate from its DNA operator sites (Henry and Cronan Jr, 1992; Raman and DiRusso, 1995). Synthesis of fatty acyl-CoA molecules is therefore pivotal in dictating which metabolic pathway will prevail in a specific environment. FadR and fatty acid metabolism have also recently been implicated in stationary-phase survival of *E. coli* (Farewell et al., 1996).

Various hormones, signaling molecules and transcription factors are involved in the mobilization of fatty acids in mammalian cells (Grimaldi et al., 1999). Fatty acids themselves are also thought to induce the expression of proteins and enzymes involved in differentiation of preadipocyte cells to adipocyte (fat) cells (Grimaldi et al., 1999). It is thought that saturated and unsaturated fatty acids bind and activate the peroxisome proliferator activated receptor (PPAR) family of transcription factors required for adipocyte differentiation (Grimaldi et al., 1999). Fatty acids also induce the proliferation of peroxisomes in yeast, which are major sites of β-oxidation.

The very long chain polyunsaturated fatty acid, arachidonic acid, is a precursor to a diverse class of signaling molecules, the eicosanoids (Zubay et al., 1995). These lipids have potent physiological properties and represent an important class of hormones in animals. Other cellular processes such as ion channel activity and subcellular glucose transporter localization are also thought to be regulated by fatty acids and metabolites.

A.1.3 Fatty acid metabolism

Fatty acids also represent a primary source of energy for animals and bacteria. Certain types of bacteria such as *E. coli* and *Mycobacterium tuberculosis* thrive in environments where there are high levels of fatty acids, as in the mammalian gut (Nikaido and Vaara, 1985) and in the lung fluid (Cole et al., 1998), respectively. Liver and adipose cells carry out most of the LCFA metabolism in animals, and fatty acids are stored as triglycerides in specialized intracellular structures called lipid droplets in adipose cells (Zubay et al., 1995). The pathways for metabolism of fatty acids in bacteria, yeast and animals have been well characterized and appear to share common mechanisms for synthesis and degradation of fatty acids (DiRusso et al., 1999).

Homeostasis of fatty acid metabolism is essential in animals and bacteria. In humans, disruptions in lipid metabolism are common symptoms for many human diseases such as diabetes, heart disease and obesity (DiRusso and Black, 1999; Berk and Stump, 1999). Disruptions in the metabolism of sphingolipids have also been implicated in genetic diseases such as Tay-Sachs and Fabry's disease (Mathews and van Holde, 1990; Zubay et al., 1995). Fatty acids are also inherently toxic to bacteria due to their detergent properties, their ability to disrupt membrane pH gradients and other mechanisms (Borst et al., 1962; Sheu and Freese, 1973; Freese et al., 1973; Fay and Farias, 1975; Fay and Farias, 1977; Knapp and Melly, 1986; Cherrington et al., 1991; Roe et al., 1998). In fact, organic acids are commonly used as preservatives in the food

industry (Freese et al., 1973). As discussed later, the outer membrane of *E. coli* plays a pivotal role in preventing fatty acid toxicity (Sheu and Freese, 1973).

A.1.3.1 Fatty acid anabolism

Fatty acids are synthesized *de novo* by prokaryotic and eukaryotic cells (Black and DiRusso, 1994; DiRusso et al., 1999). Although the fatty acid biosynthetic pathways used by these cells exhibit some differences, they also share similar themes as well. In mammalian cells, a single multimeric protein complex (Nikaido, 1994a) is responsible for synthesis of fatty acids whereas in *E. coli*, separate enzymes are utilized (Type II system) (Magnuson et al., 1993). In both cell types, separate pathways and different enzymes/cofactors are used for fatty acid synthesis and degradation. The reaction sequences for fatty acid biosynthesis are also similar in both cell types and proceed by stepwise addition of two carbon units every cycle (Figure 3). The first committed step in the biosynthesis of fatty acids is the production of malonyl CoA from acetyl-CoA by acetyl-CoA carboxylase (Magnuson et al., 1993). Subsequent steps involve condensation of malonyl-CoA with another molecule of acetyl CoA followed by a reduction step and a dehydration step. The final step involves another reduction reaction, resulting in the addition of two carbon units to the original malonyl CoA. These steps are then repeated until the desired length saturated fatty acid is formed. In all of these steps, the fatty acid intermediates are coupled to acyl carrier protein (ACP) through a thioester bond with the coenzyme, phosphopantetheine.

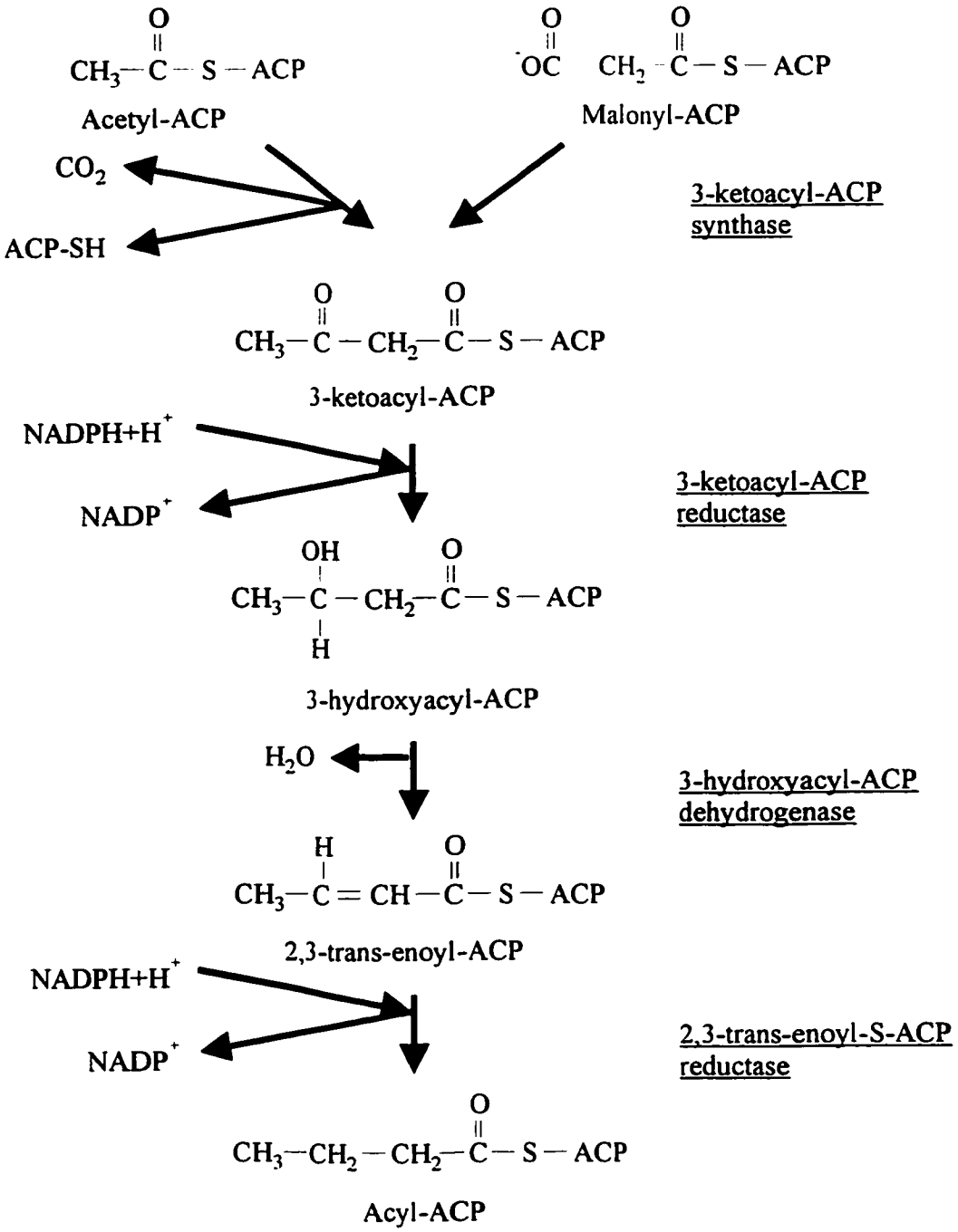


Figure 3. Biosynthesis of a saturated fatty acid. Biosynthetic pathway for the synthesis of a saturated fatty acid. The acyl-ACP end product reacts with another malonyl-ACP molecule, until the desired length fatty acid is synthesized. Saturated fatty acids can also be substrates for fatty acyl-CoA desaturases, which synthesize unsaturated fatty acids. ACP-acyl-carrier protein (Adapted from Zubay *et al*, 1995 pg. 422).

A.1.3.2 Fatty acid catabolism

Fatty acids are in a higher reduced state and less oxygenated than other carbon sources and therefore provide a high amount of metabolic energy per unit mass/volume. In fact acetyl-CoA, the breakdown product of carbohydrates, is a precursor for fatty biosynthesis. In animals and plants, fatty acids are esterified to glycerol, forming triglycerides that act as the primary sources of stored energy. β-oxidation is the primary route for fatty acid catabolism in both eukaryotes and prokaryotes (Figure 4) (Klein et al., 1971; Black and DiRusso, 1994; DiRusso et al., 1999). Oxidation of fatty acids proceeds like synthesis, two carbon atoms at a time. Before oxidation can proceed, the fatty acids must be metabolically activated by thioesterification with Coenzyme A. This reaction is catalyzed by fatty acyl-CoA synthetase (Groot et al., 1976) with different fatty acyl-CoA synthetases displaying different specificities for fatty acids of a certain length. In *E. coli*, the majority of fatty acyl-CoA molecules are metabolized by β-oxidation and minor amounts are used for phospholipid synthesis (DiRusso et al., 1999). One round of β-oxidation in *E. coli* results in the formation of one acetyl-CoA molecule (see figure 4). A β-oxidation cycle begins with abstraction of hydrogen from fatty acetyl-CoA by fatty acyl-CoA dehydrogenase (fadE), which also results in the production of a reduced flavin adenine dinucleotide ($FADH_2$). The unsaturated fatty acyl-CoA is then hydrated by enoyl-CoA hydratase (fabB) and then oxidized by 3-hydroxyacyl-CoA dehydrogenase.

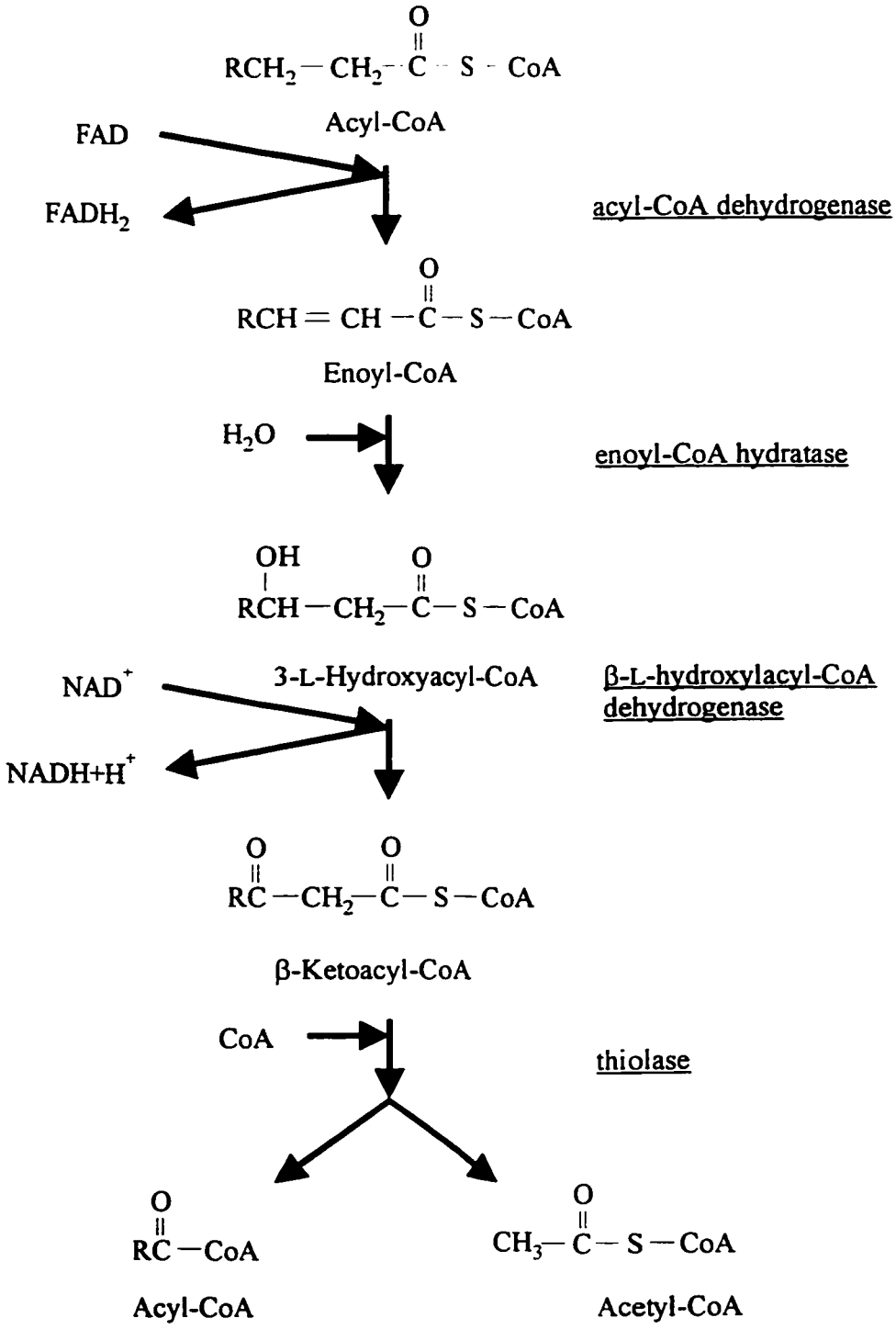


Figure 4. β-oxidation of fatty acids. β-oxidation of a saturated fatty acid. The end product acyl-CoA is further oxidized until two molecules of acetyl-CoA are produced by thiolase. Oxidation of unsaturated fatty acids requires enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase (Adapted from Zubay *et al*, 1995 pg. 415).

This step results in the generation of 3-ketoacyl-CoA and a reduced dinicotinamide adenine dinucleotide (NADH). 3-ketoacyl-CoA subsequently undergoes thiolytic cleavage, resulting in a fatty acyl-CoA molecule two carbon groups shorter, and ready for another round of β-oxidation. One cycle of β-oxidation therefore results in the formation of a $FADH_2$, a NADH, H^+, acetyl-CoA and a fatty acyl-CoA less two carbon groups. In *E. coli*, enzymes involved in β-oxidation form a multimeric complex composed of a 4-subunit heterodimer of two α and two β subunits (Black and DiRusso, 1994; Zubay et al., 1995). The *E. coli* β-oxidation pathway is an inducible system and requires fatty acyl-CoA molecules for derepression of FadR (Henry and Cronan Jr, 1992; DiRusso et al., 1993; Black and DiRusso, 1994; DiRusso et al., 1999). FadR regulates several unlinked genes of the fad (fatty acid degradative) regulon involved in the uptake and metabolism of LCFAs. At least two proteins are thought to be required for LCFA uptake, FadL and FadD (DiRusso and Black, 1999). FadL is thought to facilitate permeation of these carbon sources across the OM (Kumar and Black, 1991), while FadD converts free LCFAs into fatty acyl-CoA thioesters, making the transit of these substrates unidirectional (Klein et al., 1971). MCFAs and SCFAs are also metabolized by the fad regulon but they themselves cannot induce it since FadR has low affinity for these fatty acyl thioester CoA derivatives (Black and DiRusso, 1994).

A recent report also characterized medium and long-chain fatty acid metabolism in the gram-positive bacteria, *Streptomyces coelicolor* A3(2). Interestingly, the

metabolism of fatty acids by these bacteria differs in many respects compared to *E. coli* (Banchio and Gramajo, 1997).

A.1.3.3 Long chain fatty acid (LCFA) uptake

To facilitate the import of nutrients across the cytoplasmic membrane, specific membrane proteins are required, especially if these substances are large and hydrophilic. However, the role of membrane proteins in the transport of hydrophobic compounds such as LCFAs is not readily apparent.

A.1.3.3.1. Diffusion-mediated LCFA uptake

Diffusion of a permeable substance across the glycerophospholipid membrane is a nonspecific and nonsaturable process, with the rate of diffusion largely dependent on the concentration gradient and hydrophobicity (Higgins, 1994). Charged or large polar molecules do not efficiently diffuse across the plasma membrane and require specific transport proteins. However, the role of plasma membrane proteins in eukaryotic and prokaryotic LCFA uptake is controversial, which stems from the fact that glycerophospholipid bilayer membranes do not inhibit the permeation of hydrophobic compounds (Kamp and Hamilton, 1992; Kamp and Hamilton, 1993; Higgins, 1994; Mangroo et al., 1995b). Conversion of fatty acids to fatty acyl-CoA thioesters sequesters these molecules inside the cell since the CoA group adds size and charge, making efflux negligible. Based on this, there is no obvious requirement for a membrane protein to facilitate uptake of LCFAs. Although some amount of fatty acid diffusion occurs across

cell membranes, certain groups argue that membrane proteins are required for uptake of LCFAs at physiological concentrations (Berk and Stump, 1999). *E. coli* cells are unique compared to eukaryotic cells since the former have an outer membrane that is impermeable to most hydrophobic and amphipathic compounds, and therefore absolutely requires a membrane protein for LCFA uptake.

Protonated fatty acids readily diffuse across phospholipid membranes. The carboxyl groups of LCFAs have pK_a values of 3-4 in aqueous solution, which are thought to shift to 7-8 when dissolved in the membrane (Kamp and Hamilton, 1992). This suggests that when fatty acids are dissolved in the membrane, almost half of the molecules are protonated and able to diffuse across the membrane (Kamp and Hamilton, 1992; Kamp and Hamilton, 1993; Mangroo et al., 1995b; Trigatti and Gerber, 1996). It also suggests that desorption of fatty acids from the membrane is a slow process and the plasma membrane may act as a reservoir for free fatty acids.

A.1.3.3.2. Protein mediated LCFA uptake

Most membrane transport proteins are classified as channel/pore or carrier-type transporters. The former class of membrane proteins allow solute diffusion down an electrochemical gradient and do not require an energy source. Unlike simple diffusion, facilitated diffusion is saturable and specific, since proteins are involved in the process. Carrier-type proteins can actively transport molecules against their electrochemical gradient and accumulate solutes inside the cell using energy sources such as ATP or ion gradients. The role of *E. coli* membrane proteins in LCFA uptake is discussed in section

A.4, while the subsequent paragraphs focus on mammalian proteins implicated in LCFA uptake.

Several proteins have been implicated in LCFA transport in mammalian and yeast cells (Abumrad et al., 1993; Trigatti and Gerber, 1995; Faergeman et al., 1997; Hirsch et al., 1998; DiRusso and Black, 1999). Some of these proteins are thought to represent bona fide transport proteins (Abumrad et al., 1993; Faergeman et al., 1997; Hirsch et al., 1998), while others are thought to indirectly facilitate permeation of fatty acids across the plasma membrane (Trigatti and Gerber, 1995; Mangroo et al., 1995b). Some of the evidence suggesting that proteins are involved in mammalian fatty acid uptake includes saturation of uptake (Trigatti et al., 1991), induction of uptake during differentiation of adipocytes (Trigatti et al., 1991) and sensitivity of uptake to protease treatment (Mahadevan and Sauer, 1974). At least three proteins have been implicated as possible LCFA transporters: the 43-kDa $FABP_{PM}$ (Fatty acid binding protein at the plasma membrane), the 88-kDa FAT (Fatty Acid Transporter) (Abumrad et al., 1993) and the 63-kDa FATP (Fatty Acid Transport Protein) (Hirsch et al., 1998). These proteins were identified from hepatocytes or adipocytes, cells that play important roles in the metabolism and mobilization of LCFAs. None of these proteins however, have been shown to be directly involved in LCFA transport.

While some groups have proposed direct roles for membrane proteins in LCFA uptake (Berk and Stump, 1999), others have suggested that these proteins play peripheral roles (Trigatti and Gerber, 1996). Serum albumin (SA) is a serum protein that has multiple fatty acid binding sites and functions in part as a carrier for free fatty acids in the

blood stream. SA has also been suggested to facilitate LCFA uptake by directly delivering and releasing fatty acids to specific cells via a SA receptor (Trigatti and Gerber, 1995; Mangroo et al., 1995b). Certain mammalian cells also express high levels of fatty acid binding proteins (FABPs), which are small soluble cytoplasmic proteins. FABPs are abundant in cells focused on fatty acid metabolism such as heart, adipocyte, liver and intestinal cells. They contain a single binding site for LCFAs with affinity constants between 0.1 and 2 μM (Richieri et al., 1998). It is not clear however, what their roles in fatty acid metabolism are. Some have suggested that FABPs act as "sinks" for LCFAs, while others have suggested that they are involved in intracellular trafficking of fatty acids. It has also been proposed that desorption of fatty acids from the plasma membrane is a rate limiting step in uptake and FABPs are involved in desorption of these fatty acids (Mangroo et al., 1995b; Trigatti and Gerber, 1996).

A.2 Structure of gram-negative cell envelope

Gram-negative bacteria such as *E. coli* and *S. typhimurium* have a complex cell envelope structure (Figure 5) that consists of an outer and inner membrane (Nikaido and Vaara, 1985; Hancock, 1991; Neidhart et al., 1996; Duong et al., 1997). Separating these two membranes is the compartment called the periplasm which contains the cell wall or peptidoglycan layer. The cell envelope is a formidable barrier for various large macromolecules that are hydrophilic or hydrophobic in nature, including toxic molecules such as antibiotics, detergents, bile salts and hydrolytic enzymes

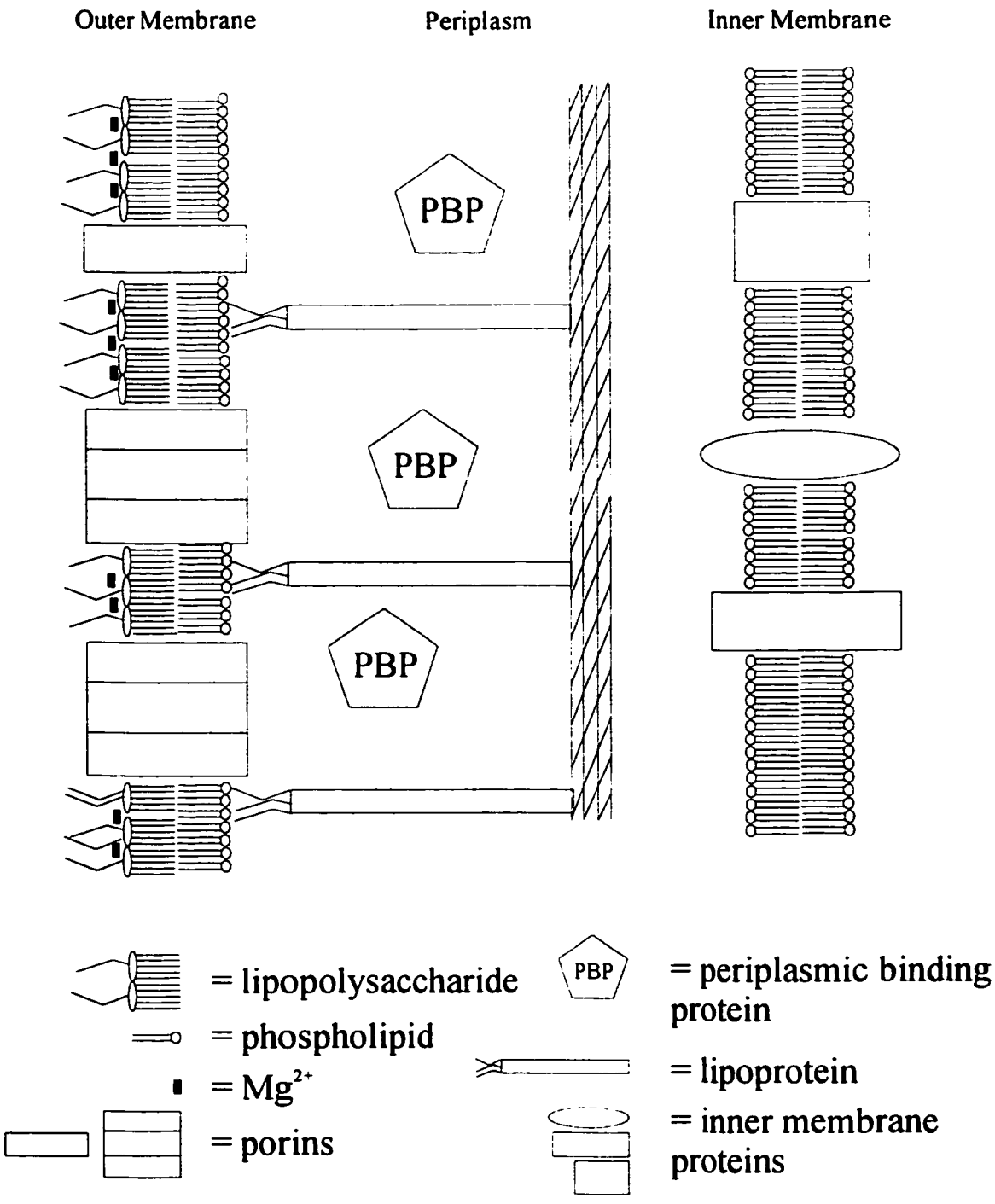


Figure 5. Structure of *E. coli* cell envelope. Schematic of *E. coli* cell envelope consisting of the outer membrane, periplasmic space and the inner or plasma membrane (Adapted from Hancock, 1991). The crossed bar in the periplasm represents peptidoglycan.

(Nikaido and Vaara, 1985; Vaara, 1992). An implication of this property is that systems are required to specifically transport nutrient molecules across the cell envelope.

A.2.1 Outer membrane

The outer membrane (OM) is structurally and functionally distinct from glycerophospholipid bilayer membranes (Nikaido and Vaara, 1985; Hancock, 1991). Unlike the inner or cytoplasmic membrane, the OM is an effective barrier against hydrophobic molecules including long chain fatty acids (Sheu and Freese, 1973). This property is attributed to lipopolysaccharides (LPS) that comprise the outer leaflet of OMs (figure 5) (Nikaido and Vaara, 1985; Neidhart et al., 1996).

The OM has been an important focus of scientific research, since this structure is essential for protecting gram-negative bacteria from many hazardous natural and synthetic compounds, such as antibiotics and detergents (Nikaido and Vaara, 1985). Gram-positive bacteria, which do not have an OM, are generally more sensitive to these type of compounds. Gram-negative bacteria can be sensitized to some of these toxic compounds by exposure to specific chemicals such as EDTA (Sheu and Freese, 1973; Freese et al., 1973; Hancock, 1984; Vaara, 1992). EDTA chelates Mg^{2+} ions that are essential for bridging negatively charged LPS molecules together in the OM. Disruption of this ionic network is thought to increase levels of glycerophospholipids in the outer leaflet of OMs, leading to increased permeation of hydrophobic compounds (Neidhart et al., 1996). Disruption of specific genes involved in; OM integrity, OM protein expression or lipopolysaccharide (LPS) biosynthesis can also lead to sensitivity to the

aforementioned compounds (Hancock, 1984; Black et al., 1987; Hancock, 1991; Silber and Sauer, 1994).

Although LCFAs can act as the sole carbon source, this class of molecules is unique because they are also toxic to bacteria if the OM has been disrupted (Sheu and Freese, 1973; Freese et al., 1973). Fatty and other organic acids are thought to uncouple the proton gradient at the cytoplasmic membrane of *E. coli* and also compromise membrane integrity (Borst et al., 1962; Freese et al., 1973; Fay and Farias, 1975; Knapp and Melly, 1986; Cherrington et al., 1991; Roe et al., 1998). The OM therefore acts as a barrier for LCFAs, which is essential for preventing cell toxicity. Utilization of a protein to mediate and regulate the permeation of LCFAs across the OM may allow the uptake of this carbon source despite its toxic properties.

A.2.2 Periplasm and cell wall

The periplasm contains the peptidoglycan (cell wall) and is filled with various proteins and oligosaccharides that make the periplasmic space viscous and gel-like (Neidhart et al., 1996). Many of the proteins present in the periplasm are involved in nutrient detection and transport. Various proteases are also present in this compartment, some of which are thought to degrade abnormal or unstable periplasmic proteins (Strauch and Beckwith, 1988). Breakdown of large biomolecules into smaller ones also occurs here so that they can be transported across the cytoplasmic membrane by specific transporters (Neidhart et al., 1996). There are no typical energy sources in the periplasm,

but a proton gradient is situated across the cytoplasmic membrane, which is used to actively transport biomolecules against their concentration gradient.

The peptidoglycan layer in the periplasm is essential for cell stability and ensuring that osmotic forces do not rupture the cells. It is composed of a complex network of covalent $\beta 1 \rightarrow 4$ linked sugars and peptides. As bacterial cells grow, the peptidoglycan undergoes constant synthesis and degradation to accommodate cell growth and division (Neidhart et al., 1996). The composition of the peptidoglycan from gram-positive bacteria tend to be very diverse, whereas the peptidoglycan of gram-negative bacteria tend to be similar (Nikaido, 1994b). This has been attributed to the fact that the OM shields the cell wall in gram-negative bacteria from glycosidic enzymes. Since gram-positive bacteria do not have an OM, variations in the composition of the peptidoglycan are thought to increase resistance towards lytic enzymes (Nikaido, 1994b).

A.2.3 Inner membrane

The inner or cytoplasmic membrane of *E. coli* is composed mostly of proteins (60-70%) and phospholipids (30-40%) such as, phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL)(Black and DiRusso, 1994). Various membrane proteins involved in transport are also found here. The electron transport chain is also located in the cytoplasmic membrane with the acidic membrane potential situated at the periplasmic face (Harold, 1986).

Unlike the OM, the inner membrane does not express open channels and is not permeable towards small hydrophilic molecules. The inner membrane therefore contains

many proteins involved in transport of small and large hydrophilic compounds (Nikaido and Saier Jr, 1992). Much like the eukaryotic cytoplasmic membrane, the *E. coli* inner membrane is permeable towards hydrophobic compounds. If the OM is compromised, toxic hydrophobic compounds can enter the periplasm and exert its deleterious effects on the cell. However, inner membrane proteins have been described that recognize and actively pump some of these compounds out of the cell (Nikaido, 1994c).

A.3 *E. coli* transport systems

A.3.1 Transport across the outer membrane

Many compounds required for growth, such as carbohydrates, fatty acids, iron complexes and nucleosides cannot cross the outer membrane. Proteins are expressed in the OM however, that mediate the diffusion or transport of these nutrient molecules (Schirmer and Rosenbusch, 1991; Hancock, 1991; Nikaido and Saier Jr, 1992; Nikaido, 1994b). Most of these proteins are classified as porins or related channel proteins and appear to share a common structural feature (see below) (Jap and Walian, 1990; Cowan et al., 1992; Nikaido, 1994b; Schirmer et al., 1995). These OM proteins are commonly classified into three general classes: i) non-specific or "classical" porins ii) specific-porins and iii) specific transporters (Nikaido and Saier Jr, 1992). The "classical" porins such as OmpF and OmpC, permit the diffusion of mostly hydrophilic compounds that fall under a specific exclusion size. These channel proteins generally do not exhibit strong preferences for any specific class of substrates (Jap and Walian, 1990), which distinguish

them from the specific-porin group. The latter class of channel proteins possesses binding sites for specific substrates or related compounds and facilitates their diffusion. The third class of OM proteins is characterized by their very high affinities for substrates and the requirement for an energy source. The substrates for this class of OM proteins are typically in low abundance and are actively accumulated inside the cell.

Based on X-ray crystallography, the OM proteins from all three classes seem to form β-stranded channels or β-barrels (Cowan et al., 1992; Hofnung, 1995; Ferguson et al., 1998). Non-specific porins form β-barrel channels, while the specific-porins form similar structures plus a binding site(s) located within the lumen of the channel (Hofnung, 1995; Schirmer et al., 1995). The specific transporters are similar to the specific porins but in addition, contain a protein domain used to gate access to a channel (Killmann et al., 1993; Ferguson et al., 1998).

A.3.1.1 Non-specific porins

The OM is permeable to most small hydrophilic compounds that are 600-Da or less. The permeability and size exclusion properties of the OM are due in part to the expression of the "classical" porins. In *E. coli*, these include OmpF, OmpC and PhoE (Jap and Walian, 1990; Nikaido and Saier Jr, 1992; Nikaido, 1994b). Each of these outer membrane proteins forms homotrimers with each subunit containing a single channel. Charged amino acid side chains line the aqueous channels, which make it unfavorable for hydrophobic substances to pass through (Jap and Walian, 1990; Cowan et al., 1992; Nikaido, 1994b). These porins are expressed at high levels (up to 2% of total cell protein

(Neidhart et al., 1996)) and permit diffusion of molecules down their electrochemical gradient. Although generally considered to be non-specific, some of them display slight preferences for molecules of a specific charge (Bauer et al., 1988; Cowan et al., 1992; Karshikoff et al., 1994). OmpA is another porin expressed at high levels in the OM of *E. coli* but is found in a monomeric form and is inefficient in facilitating permeation of solutes (Sugawara and Nikaido, 1994; Nikaido, 1994b). The function of this porin is puzzling considering it is purported to have a channel diameter larger than the other non-specific porins (Nikaido, 1994b). It has been proposed that OmpA exists in open and closed states *in vivo*, accounting for the low levels of permeation (Sugawara and Nikaido, 1994).

Regulation of porin activity has not been well characterized, although some studies have shown that porins are voltage regulated, *in vitro* (Jap and Walian, 1990). It is thought however, that membrane potentials cannot be generated across the OM *in vivo*, due to its permeability (Nikaido, 1994b). Although negatively charged oligosaccharides in the periplasm can create a Donnan potential, it was shown not to affect porin activity (Sen et al., 1988).

A.3.1.2 Specific porins

The members of the second class of channel proteins, the specific-porins, contain binding sites for specific substrates and facilitate their diffusion across the OM. Most of these substrates represent nutrients such as maltose (Hofnung, 1995), maltodextrin,

nucleosides (Maier et al., 1988) and sucrose. All of these molecules are too large to diffuse efficiently through the classical porins.

The best characterized specific porin is LamB, which specifically facilitates diffusion of maltose and maltodextrins (Nikaido, 1994a; Hofnung, 1995). Structural determination of this protein using X-ray diffraction revealed that LamB adopts a β-barrel structure, much like the non-specific porin OmpF (Hofnung, 1995; Schirmer et al., 1995).

A.3.1.3 High affinity transport proteins

The third class of OM channel proteins is comprised of high affinity receptors. Ligands for these transport proteins are normally found at very low concentrations in their surroundings, such as iron siderophores (Killmann et al., 1993; Jiang et al., 1997; Braun, 1998) and vitamin B_{12}. The low levels of these nutrients require that *E. coli* have high affinity receptors for these ligands, which are actively transported into the cell. Active transport by these proteins requires the cytoplasmic membrane protein TonB, which transduces protonmotive energy (Skare et al., 1993). TonB is an integral membrane protein with a large domain that is thought to extend through the periplasmic space and physically interact with the receptor proteins, to facilitate ligand release (Skare et al., 1993; Ferguson et al., 1998).

A.3.2 Role of periplasm binding proteins in transport

Efficient transport of some sugars and amino acids also requires periplasmic binding proteins. These are soluble proteins that bind their respective ligands with high affinity (0.1-1 μM) in the periplasm and facilitate their transport across the inner membrane (Nikaido, 1994a; Neidhart et al., 1996). Some of the better-characterized binding proteins include maltose-binding protein and histidine-binding protein. The periplasmic binding proteins work in conjunction with specific multimeric inner membrane protein complexes to transport substrates into the cell (Nikaido, 1994a) (see next section). To date, no periplasmic binding proteins have been identified for LCFAs (Mangroo and Gerber, 1992; Azizan and Black, 1994).

A.3.3 Transport across the inner membrane

The *E. coli* inner membrane contains various transport proteins involved in uptake of nutrients and ions (Nikaido and Saier Jr, 1992). These transport proteins are specific and display very high affinities and can be classified based on the type of energy source used for activity (Nikaido and Saier Jr, 1992). These are; 1) facilitators, 2) ion driven transporters, 3) ATPase ion transporters, 4) periplasm binding protein dependent transporters and 5) group translocation systems. There is only a single known example of a facilitator cytoplasmic membrane protein in *E. coli*, GlpF, which facilitates the diffusion of glycerol. Ion-driven transporters couple ion gradient movement across the

inner membrane to drive the transport of solutes in the same or opposite direction, commonly referred to as symporters and antiporters respectively (Harold, 1986). These membrane proteins commonly use H^+ or Na^+ flux to drive transport of sugars and amino acids into the cell. The third class of transporters are similar to the P-type ATPases found in eukaryotes and utilize ATP hydrolysis to pump ions such as K^+ and Mg^{2+} (Nikaido and Hall, 1998). Periplasm binding proteins deliver their substrates to inner membrane protein complexes called periplasmic permeases. The periplasmic permeases directly interact with their respective binding proteins to transport their substrates. Specific subunits of the periplasmic permeases share homology to ABC (ATP-binding cassette) transport systems found in yeast and mammals, which use ATP hydrolysis to transport their substrates (Nikaido, 1994a). The last class of inner membrane transporters is the group translocation systems, which is best characterized by the phosphoenolpyruvate (PEP) - dependent phosphotransferase system (PTS). Sugars are transported and phosphorylated by PTS, effectively trapping the sugar substrates inside the cell and allowing accumulation of these molecules. Phosphorylation involves a series of phosphotransfers among various subunits of PTS, which is initiated by the PEP molecule (Nikaido and Saier Jr, 1992).

A.4 *E. coli* long chain fatty acid transport

Like other nutrients, long chain fatty acid (LCFA) uptake requires passage through two membranes and the periplasmic space. Two proteins, FadL and FadD, are

absolutely required for uptake, although other proteins such as the periplasmic protease Tsp have also been implicated (Azizan and Black, 1994; DiRusso et al., 1999).

Uptake of substances into a cell is frequently regulated to ensure that resources are not wasted, and levels of specific molecules are not depleted or accumulated to excessive levels. Expression of the OmpF and OmpC porins are regulated by changes in the surrounding environment, specifically osmolarity and temperature (Neidhart et al., 1996). It is postulated that expression of these porins is regulated to decrease permeation of toxic compounds into the cell, such as bile salts from the human gut, its natural environment (Nikaido, 1994b). Based on the inherent toxicity of LCFAs, it is also possible that uptake of these biomolecules is carefully coordinated.

A.4.1 FadL

FadL is a 43-kDa outer membrane protein that is thought to represent a specific channel for LCFAs (Black et al., 1987; Kumar and Black, 1991; Mangroo and Gerber, 1992; Kumar and Black, 1993; Black and Zhang, 1995; Azizan et al., 1999) and also act as a receptor for T_2 bacteriophage (Morona and Henning, 1986). FadL has been implicated in the binding and transport of LCFAs across the outer membrane based on the following observations: i) FadL is required for growth of *E. coli* on LCFAs, ii) LCFA uptake is reduced in *E. coli* cells treated with FadL antibodies, iii) expression of cloned FadL in *E. coli* deficient in this OM protein increases uptake and iv) expression of FadL increases the LCFA binding capacity of *E. coli* cells deficient in FadL (Ginsburgh et al., 1984; Nunn, 1986; Nunn et al., 1986; Black, 1990).

The *fadL* gene has been sequenced (Said et al., 1988; Black, 1991) and found to share significant homology with the *H. influenza* outer membrane protein P1 (Black, 1991), but not to other *E. coli* OM proteins. The first 27 amino acids of FadL encode an OM signal sequence and a signal peptidase recognition sequence which are normally found in proteins targeted to the OM (Black, 1991; Duong et al., 1997). FadL also possesses extensive stretches of hydrophobic amino acids, which may be related to formation of a hydrophobic channel. It was also reported that regions upstream of the *fadL* transcriptional start site contained DNA sequences that could be recognized by the transcription factors, catabolite gene activator protein and FadR (Said et al., 1988; Black, 1991). Another study has suggested that *fadL* transcription is also regulated by osmoregulation (Higashitani et al., 1993).

FadL has high affinity for long chain fatty acids (LCFAs) (Black, 1990; Mangroo and Gerber, 1992), which has been characterized with the use of a photoreactive LCFA analogue (Mangroo and Gerber, 1992). These studies showed that FadL bound LCFAs specifically and with high affinity (K_d 63 nM) (Mangroo and Gerber, 1992). FadL protein has also been purified (Black et al., 1987) and shown to migrate as a 33-kDa protein when solubilized with SDS at 25° C and migrate as a 43-kDa protein when solubilized by boiling in SDS. This heat modifiable behavior is also seen in other OM proteins and is thought to be related to a common structural feature of these proteins.

Studies performed by Black *et al* have proposed that FadL is composed of separate functional domains, with the carboxyl end responsible for facilitating LCFA transport and the amino end involved in binding and transport (Kumar and Black, 1991;

Kumar and Black, 1993). They also suggested that LCFA transport and binding were independent functions and not coupled. Although FadL is expected to form a β-barrel channel, little else is known about the actual structure of the protein. FadL seems to represent an atypical OM protein as it exhibits properties that are common to specific channels and high affinity receptors. FadL appears to belong to the specific channel class since it is reported to function in a TonB-independent manner (Kumar and Black, 1993; Neidhart et al., 1996). However, FadL affinity for LCFAs is unusually high (sub-μM) compared to the other specific channels. Receptor proteins utilize protonmotive force transduced by the cytoplasmic membrane protein TonB to mediate the release of the tightly bound ligand (Skare et al., 1993). Since FadL does not appear to directly use or have access to energy sources, it is unclear why it has such a high affinity-binding site if its role is to facilitate diffusion. This brings up the simple but important question of the relationship between LCFA binding and permeation.

FadL represents the only known protein thought to be required for LCFA permeation across a membrane (Mangroo et al., 1995b). However, FadL activity has never been directly demonstrated and not surprisingly, not much is known about its mechanism of action. Present methods used to measure LCFA uptake are not sensitive enough to detect FadL mediated permeation. It was therefore imperative that a system be developed to specifically monitor LCFA translocation across the OM. Development of such an assay could facilitate functional studies of FadL and help to determine the relationship between binding and transport, which may give insight into the mechanism of FadL function.

A.4.2 Tsp

Tsp (Silber et al., 1992) or Prc, is an *E. coli* periplasmic serine-type protease (Keiler and Sauer, 1995) that recognizes protein substrates with specific hydrophobic carboxyl termini "tails"(Hara et al., 1989; Hara et al., 1991; Silber et al., 1992; Keiler and Sauer, 1996). Tsp is involved in processing of penicillin-binding protein 3 (Hara et al., 1989) and the inner membrane protein TonB. PBP3 is involved in cell septation (Fraipont et al., 1994; Goffin and Ghuysen, 1998) but is unknown what effect or role processing has on its function. *E. coli* deficient in Tsp protease filament and die when grown in hypotonic media at elevated temperatures, suggesting that it is also required for thermal and osmotic stress response (Hara et al., 1991; Bass et al., 1996). The *tsp* gene has been cloned (Hara et al., 1991; Silber et al., 1992) and found to share regions of homology with inter-photoreceptor binding proteins (IRBP), which bind hydrophobic ligands such as cholesterol, retinods and fatty acids in photoreceptor cells (Pepperberg et al., 1993).

Tsp was also identified in a study using transposon mediated random mutagenesis, to identify proteins located in the cell envelope that were required for growth on LCFAs (Azizan and Black, 1994). FadL was identified in this screen in addition to two other cell envelope proteins, TolA and Tsp. TolA is a periplasmic protein required for outer membrane integrity and was identified in this screen as it protects cells from the toxic effects of long chain fatty acids and detergents. Disruption of the *tsp* gene did not inhibit growth on LCFAs but caused the *E. coli* cells to exhibit an initial growth

lag and exhibit changes in LCFA uptake energetics. These observations suggested that Tsp was not absolutely required for growth on LCFAs but somehow participated in their uptake and/or metabolism. Although these observations implicated the protease in LCFA uptake, it was not clear how Tsp was involved or if it played a direct or indirect role. It was proposed that Tsp might represent a periplasmic fatty acid binding protein based on the presence of the IRBP-like domains (Azizan and Black, 1994). It was also possible that Tsp was exerting its effects on LCFA uptake through FadL or FadD.

A.4.3 FadD

FadD, encoded by the *fadD* gene, is an *E. coli* fatty acyl-coenzyme A synthetase (FACS) with substrate specificity for medium and long-chain fatty acids (Kameda and Nunn, 1981; Black and DiRusso, 1994; DiRusso et al., 1999). FadD activates fatty acids for metabolism by catalyzing the formation of fatty acyl-coenzyme A (LCFA-CoA) thioesters (equations 1 and 2) (Groot et al., 1976), which are substrates for β-oxidation and phospholipid biosynthesis (DiRusso et al., 1999). Since LCFA-CoA molecules cause derepression of FadR, FadD also plays a pivotal role in regulation of lipid catabolism in *E. coli*.

$$R\text{-}COO^- + ATP + \overset{Mg^{2+}}{\leftrightarrows} R\text{-}CO\text{-}AMP + PP_i \qquad (1)$$

$$R\text{-}CO\text{-}AMP + CoASH \leftrightarrows R\text{-}CO\text{-}SCoA + AMP + H^+ \qquad (2)$$

FadD is a soluble protein that is thought to reside in the cytosol. However, FadD is activated *in vitro* by bacterial membranes and Triton X-100 detergent and can be co-fractionated with inner membrane isolation preparations (Mangroo et al., 1995b). Work from our laboratory has previously proposed that LCFA uptake in *E. coli* may be regulated by the translocation of FadD from the cytosol to the cytoplasmic membrane (Mangroo et al., 1995b).

Animal, plant and yeast cells express multiple forms of FACS (Knoll et al., 1994; Johnson et al., 1994; Iijima et al., 1996; Fujino et al., 1997; Fulda et al., 1997). In contrast, *fadD* represents the only FACS gene in *E. coli*, although FadD protein isoforms had been reported (Samuel et al., 1970; Kameda and Imai, 1985; Black et al., 1998). Purified native FadD had typically been reported to have an apparent molecular mass ranging from 42- to 50-kDa (Kameda and Nunn, 1981; Kameda et al., 1985; Kameda and Imai, 1985). Early work from our laboratory had also indicated that partially purified monomer FadD behaved as a 45-kDa protein. In contrast, the *fadD* gene sequence predicts a 62-kDa protein which was verified by expression studies using the cloned *fadD* gene (Black et al., 1992). This discrepancy in the molecular weight of *E. coli* FACS has never been addressed.

FadD is required for LCFA uptake (Klein et al., 1971) and has been proposed to represent the rate-limiting step (Mangroo and Gerber, 1993). Earlier studies had shown that if FadD was absent, LCFA uptake could not be observed, suggesting that uptake was coupled to metabolism. Thioesterification of LCFAs not only activates them for metabolism, it also sequesters them in the cell due to the presence of the bulky and

charged CoA group. *E. coli* cells are typically starved for uptake assays to ensure that radiolabeled substrates are not diluted by endogenous sources. It was discovered that starvation reduced the levels of LCFA uptake and inclusion of lactate reversed this effect (Mangroo and Gerber, 1993). The mechanism of this starvation effect and reversal by lactate has not been elucidated.

The *fadD* gene has been sequenced (Black et al., 1992; Fulda et al., 1994) and regions were identified that appear to be homologous to regions found in other FACS (Black et al., 1992; Abe et al., 1992; Fulda et al., 1994; Black et al., 1998; DiRusso et al., 1999). Based on sequence homologies and molecular modeling, a partial protein structure (starting at Thr^{213}) for FadD has been proposed (DiRusso et al., 1999) but experimental data describing the structural organization of FadD is lacking.

A.4.4 Other proteins implicated in transport

Some studies have also implicated *E. coli* inner membrane proteins in LCFA transport. A 26.5-kDa inner membrane protein was proposed to represent an H^+/fatty acid co-transporter and found to increase LCFA uptake in spheroplasts containing FadD (Kameda et al., 1987). Unpublished work from our laboratory showed however, that inner membrane vesicles isolated from *E. coli* grown on minimal media using glucose or fatty acid as a carbon source, exhibited LCFA uptake (Mangroo et al., 1995a). Uptake by these vesicles only required trapped FadD, ATP and CoA, indicating that induction of the *fad* regulon did not induce the expression of any plasma membrane proteins involved in LCFA uptake. The gene for this protein has yet to be identified and other studies using

genetic screens and photoaffinity labeling have failed to identify any plasma membrane proteins required for LCFA uptake or binding (Azizan and Black, 1994; Mangroo et al., 1995b).

A.5 Objectives and rationale

FadL and FadD, the two proteins absolutely required for LCFA uptake have been cloned, sequenced and purified. Preliminary evidence from several studies has started to unravel some information regarding structure-function relationships in these proteins. However, the mechanisms utilized by these proteins to transport LCFAs are poorly understood. We want to further characterize each protein, in anticipation that it may lead to a better understanding of the mechanism of *E. coli* LCFA uptake. The role of Tsp in LCFA uptake is also unclear and needs to be investigated. It is hoped that each of the above investigations will lead to greater insight into how *E. coli* utilizes this transport machinery to transport LCFAs across the cell envelope.

The mechanism of FadL mediated LCFA permeation is unknown. It is difficult to investigate this without a specific system to directly assay FadL function. Using photoaffinity labeling (Bayley, 1983; Brunner, 1993) with the photoreactive LCFA, 11-*m*-diazirinophenoxy[11-^{3}H]undecanoate (Figure 6), we developed a novel method for directly monitoring permeation of a LCFA across the OM. The toxic properties of LCFAs suggest that FadL may utilize a unique mechanism for transporting these nutrients across the OM and not behave as a simple open channel. We therefore want to use this assay to

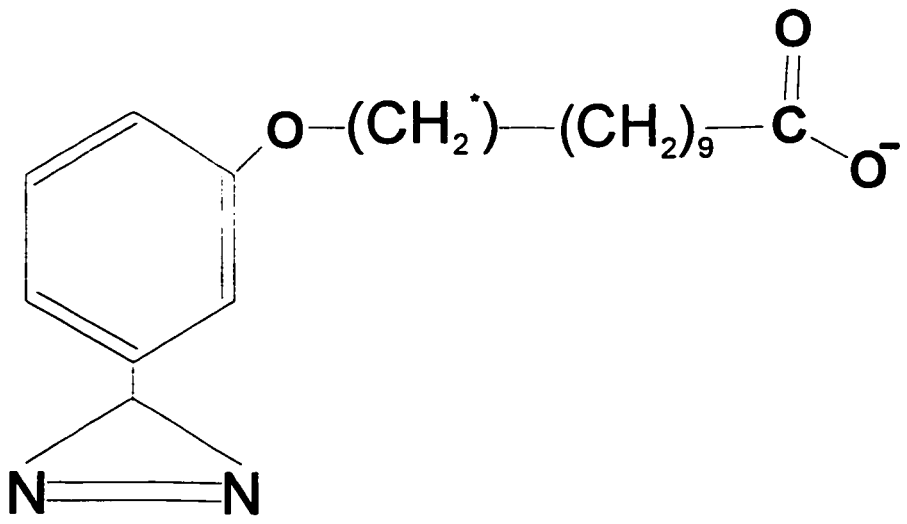


Figure 6. Structure of photoreactive fatty acid, 11-*m*-diazirinophenoxy-[11-^{3}H] undecanoate. Exposure of the diazirinophenoxy group to 360 nm radiation results in the formation of a carbene (Bayley, 1983). The LCFA analogue was synthesized as described in LeBlanc *et al*, 1982. * represents tritium. (Adapted from Mangroo PhD thesis, 1992 pg. 28)

understand how FadL functions to facilitate the diffusion of a carbon and energy source that is potentially toxic to the cell.

We also examined the role(s) that Tsp may play in LCFA uptake. It is possible that Tsp represents a periplasmic binding protein for LCFAs or Tsp may be mediating its effects on uptake through either FadL or FadD. It is also possible that Tsp is not directly involved in LCFA uptake and that disruption of this gene has indirect effects.

There appear to be discrepancies in the literature concerning the molecular weight of FadD and the existence of isoforms. The nature of these different forms of FadD and their relationship to each other has remained unclear and will be investigated. Although FadD is arguably the best characterized fatty acyl-CoA synthetase studied thus far, not much is known about the structure of this enzyme. Since a better understanding of enzyme structure can lead to a better understanding of enzyme function, we want to develop a purification scheme for the enzyme to perform structure-function analysis.

B. MATERIALS AND METHODS:

B.1 Materials

Amplify fluorography reagent, Aqueous counting scintillant, ECL chemiluminesence reagent and horseradish peroxidase conjugated to goat anti-rabbit immunoglobulin G (Ig) were purchased from Amersham. Yeast extract and tryptone were obtained from BDH Laboratories. All electrophoresis reagents and equipment were purchased from Bio-Rad. Tris-(hydroxymethyl) aminomethane (Tris) was obtained from Boehringer Mannheim. Chloroform was purchased from Caledon Laboratories. Hydrochloric acid, Oleic acid, potassium hydrogen pthalate, scintillation vials and sulphuric acid were from Fisher. Disposable cuvettes and pipette tips were from Canlab. XAR-5 X-ray film was obtained from Kodak. Amylose resin, restriction enzymes, T_4 DNA ligase, Calf intestinal alkaline phosphatase (CIAP) and Vent polymerase were purchased from New England Biolabs. [9-10 ^{3}H] oleic acid, Solvable tissue and gel solubilizer were obtained from NEN Research Products. BCA protein assay kit was obtained from Pierce. Adenosine 5'-triphosphate (ATP), bromophenol blue, ampicillin, Brij 58, Bovine serum albumin ((BSA) from fraction V and essentially fatty acid free)), Coenzyme A (CoASH), deoxyribonuclease (Dnase), ethylenediaminetetraacetate (EDTA), isopropyl β-D thiogalactopyranoside (IPTG), Ribonuclease (RNase), Triton X-100 and M9 minimal media salts were obtained from Sigma.

Table 1. Bacterial strains and plasmids.

Bacterial Strains Used:	*Relevant phenotypes*
N-4830-1	encodes a temperature sensitive λ phage repressor
EDL1	*fadL*, *fadD*, *fadR*, fad regulon constitutively induced
ML308	wild type *E. coli*
RS3338	*fadR fadL*
X90	wild type with respect to Tsp
KS1000	*tsp* (X90)
BL21(DE3)	*ompT*
K27	*fadD*
JM105	$ompT^+$
W3110	$ompT^+$
XL-1 Blue	$ompT^+$

Plasmids Used:	*Description*
pKK223-3**	expression vector, P_{tac} promoter, Amp^R
pACYC177	low copy plasmid, no multiple cloning site, Amp^R, Kan^R
pMAL-p2	maltose binding protein fusion expression plasmid, P_{tac} promoter,

	MBP has leader sequence for periplasmic targetting, Amp^R
pLEL	expresses FadL, contains native *fadL* promoter, pACYC177 based, Amp^R, Kan^R
pKK101	His^+ tagged Tsp overexpression plasmid, Amp^R
pMD62	overexpresses full length FadD fused to MBP, pMAL-p2 based, Amp^R
pMDC45	expresses ΔN172FadD (last 386 C-terminal amino acids) fused to MBP, pMAL-p2 based, Amp^R
pj8	overexpresses FadD, pKK223-3** based, Amp^R
ptj8	expresses ΔN172FadD, pKK223-3** based, Amp^R
pPMIFABP	The intestinal fatty acid binding protein (IFABP) gene fused to 3' end of the maltose binding protein gene (with periplasm targeting sequence) in the expression plasmid pMAL-p2, Amp^R. Under the control of a P_{tac} promoter.

Stocks of bacterial cultures were grown in LB media and stored at –80° C with 15% glycerol. ML308 was obtained from the American Type Culture Collection and pMAL-P2 was obtained from NEB. N-4830-1 was kindly provided by Dr. D.W. Andrews. pDOC55 was kindly provided by (O'Connor and Timmis, 1987). pN132 plasmid, anti-FadL antibody and the EDL1 *E. coli* cell line was kindly provided by Dr. P.N. Black. The *E. coli* strains KS1000 and X90, was kindly provided by Dr. R.T. Sauer and pKK223-3** was kindly provided by Dr. G.D. Wright. The plasmid containing the intestinal fatty acid binding protein (IFABP) gene, was kindly provided by Dr. J.C. Sacchettini. The FadD antibody raised against the 45-kDa C-terminal FadD fragment was provided by O. H. Cheng.

B.2. Methods

B.2.1 Cell growth

Unless noted otherwise, bacteria were grown aerobically at 37° C with shaking in LB media or M9 minimal media salts (1%) (Sigma Chem. Co., St. Louis) supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5μg/ml thiamine with either glucose (0.4%), maltose (0.4%) or KOH neutralized oleic acid (5mM in 0.5 % Brij 58 detergent). When required, ampicillin was added to the media to a final concentration of 150 μg/ml. Cell number was estimated by measuring optical density at 600 nm with a UV/Vis. spectrophotometer (Cary).

The N-4830-1 *E. coli* cell line expresses a temperature sensitive λ repressor. When these cells were used to express FadL, they were grown at 30° C for the permissive temperature and switched to 42° C for the non-permissive temperature.

Growth curves for the effects of EDTA and long chain fatty acids (LCFA) were generated following the procedure reported by (Sheu and Freese, 1973). Cultures were inoculated from frozen stocks and grown to saturation overnight. The saturated cultures were then subcultured into fresh media, typically M9 minimal media + 0.4% glucose and grown to mid-log. At this point the cells were harvested by centrifugation at 10 000 *g* for 10 minutes and washed by resuspending the cells in M9 minimal media and centrifugation again at 10 000 *g* for 10 minutes. The harvested cells were resuspended to an optical density of 2.0 at 600 nm with M9 minimal media containing various

combinations of 1mM EDTA and/or desired amounts of fatty acid. The cells were then incubated at 37° C with shaking for two minutes. 9 volumes of M9 minimal media were then added and the cultures grown at 37° C with shaking. At specified times, samples were taken out to be measured for optical density at 600 nm.

B.2.2 Construction of expression plasmids

For the construction of the expression plasmids below, common molecular biology techniques were performed following procedures given by (Sambrook et al., 1989).

Construction of FadL protein expression vector

The *fadL* gene was subcloned from the FadL expression plasmid pN132, provided by Dr P.N. Black. A DNA fragment containing the *fadL* gene was isolated from pN132 by treatment with BamHI. For the high expression vector (pDOC93), the *fadL* insert was isolated and ligated to pDOC55 (O'Connor and Timmis, 1987) that was linearized with *Bam*HI. For the low expression vector (pLEL), the insert was isolated and ligated to pACYC177 (Chang and Cohen, 1978) that was linearized with *Bam*HI. The ligation reaction mixtures were then transformed into *E. coli* that were made competent, by $CaCl_2$ treatment. Colonies were picked and plasmids isolated, so that they could be treated with restriction enzymes and screened for orientation of the *fadL* gene. The desired plasmids were subsequently stored at –80° C in 10 mM Tris-HCl pH 8.0. The

plasmids were subsequently used to transform competent RS3338 (*fadR fadL*) and tested for their ability to grow on oleate minimal media.

Construction of maltose binding protein-Intestinal fatty acid binding protein (MBP-IFABP) fusion protein expression vector.

The rat IFABP gene was isolated by PCR amplification from a plasmid containing the gene, kindly provided by James C. Sacchettini. The sense primer, 5' - CCG GAA TTC ATG GCA TTT GAT GGC ACT TGG - 3' and the antisense primer 5' - TTT CTG CAG TCA CTA TTC CTT AAA GAT CCG - 3' were designed and used for PCR. The primers contained the restriction sites *Pst*I and *Eco*RI, which flank the IFABP gene and allowed us to ligate into the pMal-p2 plasmid. The DNA insert containing the IFABP gene and pMAL-p2 was treated with *Pst*I and *Eco*RI. The restriction enzyme treated insert and plasmid were purified, combined and incubated with T_4 DNA ligase. Ligation of the DNA insert containing the IFABP gene into pMal-P2, facilitated fusion of IFABP, in frame, to the C-terminus of maltose binding protein. The ligation reaction mixtures were transformed into *E. coli* that were made competent, by $CaCl_2$ treatment. Colonies were picked and plasmids isolated so that they could be treated with restriction enzymes and screened for pMAL-P2 containing the IFABP gene.

Construction of FadD and MBP-FadD expression vectors

Genomic DNA for PCR amplification of the *fadD* gene was isolated from the *E. coli* strain ML308 using the procedure reported by (Marmur, 1963).We constructed the

following PCR primers based on the published nucleotide sequence of the *fadD* gene by (Black et al., 1992). The 5' primer used was 5'- TGC TCT AGA AGG AGA TAT ACA CAT GAA GAA GGT TTG GCT TAA CCG - 3', which contained a *Xba*I site, a ribosome binding site and an ATG translation initiation codon while the 3' PCR primer used was 5'- TGA CAT AAG CTT ATT ATT GTC CAC TTT GGC GCG C -3' which contained a *Hin*DIII site to facilitate cloning. The PCR primers were used to isolate the *fadD* gene and ligated into the expression plasmid, pKK223-3** (Brosius and Holy, 1984). The ligation reaction mixtures were then transformed into competent *E. coli*. Colonies were picked and plasmids isolated so that they could be treated with restriction enzymes and screened for pKK223-3** containing the *fadD* gene (pJ8). Transformation of a *fadD* mutant cell line K27, with a low expression vector containing the cloned *fadD* gene allowed growth on oleate minimal media, indicating that the cloned gene was able to complement the *fadD* phenotype.

To construct the full length FadD-MBP fusion plasmid (pMD62) and truncated FadD-MBP fusion plasmid (pMDC45) the following sense primers were designed, 5' – CCG GAA TTC ATG AAG AAG GTT TGG CTT AAC C – 3' and 5' – CCG GAA TTC CGT TTG GTG CCG AAA TAC C – 3' respectively. Both primers contained an *Eco*RI site that would fuse the full length and truncated *fadD* genes, in frame, to the C-terminus of MBP. The antisense strand used to clone FadD was used for both constructs. The FadD overexpression plasmid pJ8, was used as the template for PCR to generate DNA inserts containing full length and truncated *fadD*. Subsequent steps were identical to that

described for construction of the MBP-IFBAP fusion protein, except the pMAL-P2 plasmid and DNA inserts were treated with *Eco*RI and *Hin*DIII enzymes.

Double transformation of EDL1 with FadL and MBP-IFABP expression vectors

EDL1 cells were made competent by calcium chloride treatment and transformed with pPMIFABP vector using ampicillin resistance as selection. These cells were then made competent again by treatment with calcium chloride and transformed with the pLEL expression vector, using ampicillin and kanamycin resistance to select for double transformants. Other transformations were performed with the control plasmids pACYC177 and pMAL-p2 to test the EDL1 genotype. The double transformants used for the permeation assay EDL1(pPMIFABP)(pLEL), were typically cultured in minimal media E (Cowan et al., 1992) with 0.4 % maltose as a carbon source. To induce expression of MBP-IFABP, the aforementioned cells were grown to an optical density of 0.5 at 660 nm and induced with 0.1 mM IPTG for 45 minutes before harvesting.

B.2.3 Preparation of bacterial whole cells and cell lysates for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

When analyzing whole cell *E. coli*, cultures were spun in a microfuge for two min. at maximum speed. The pellets were resuspended with 1X SDS-PAGE sample buffer (62.5 mM Tris-HCl pH6.8, 5% SDS (w/v), 10% β-mercaptoethanol (v/v), 10% glycerol (w/v) and 0.01% bromophenol blue) to 2.4×10^9 cells/ml and immediately boiled for four minutes before analysis by SDS-PAGE (Sambrook et al., 1989).

Cell lysates were prepared by using a modified method by (Witholt et al., 1974), which involved spinning *E. coli* cells at 10, 000 *g* for 10 min at 4° C and resuspension of the pellets with 200 mM Tris-HCl pH 8.0, 1 mM EDTA to 2×10^{10} cells/ml. Lysozyme was added to a final concentration of 100 μg/ml and the suspension incubated at 25° C for 20 minutes with periodic mixing. Three volumes of an ice-cold solution containing 100 μg/ml DNase 1 and 10 mM magnesium sulfate was added and mixed, before incubation at 37 ° C for a further 20 min. The resulting cell lysate typically contained 2 mg/ml cell protein.

B.2.4 SDS-PAGE analysis/ fluorography

SDS-PAGE was performed as described by (Laemmli, 1970). Separating gels typically contained 7.5-15 % polyacrylamide (acrylamide:*N,N'*-methylenebisacrylamide ratio of 37.5) , 375 mM Tris-HCl pH 8.8, 0.1% SDS and 1% glycerol. Stacking gels contained 4% polyacrylamide, 125 mM Tris-HCl pH 6.8 , 0.1% SDS and 1% glycerol. Protein samples were diluted with 2X SDS-PAGE sample buffer (62.5 mM Tris-HCl pH6.8, 5% SDS (w/v), 10% β-mercaptoethanol (v/v), 10% glycerol (w/v) and 0.01% bromophenol blue). Electrophoresis was performed at 200 V in running buffer, 24.9 mM Tris-Glycine pH 8.3 and 0.1% SDS and the gels stained with Brilliant Coomassie Blue stain. For autoradiography, gels were soaked in Amplify (Amersham) reagent and subsequently dried and exposed against Kodak XAR X-ray film at –80° C.

B.2.5 Immunological methods

Immunoblotting was performed as described by (Burnette, 1981). Protein samples were transferred from a SDS-polyacrylamide gel onto nitrocellulose in transfer buffer consisting of 25 mM Tris, 192 mM glycine, 20% methanol and 0.1% SDS (w/v), pH 8.3. The blots were subsequently incubated with the appropriate primary rabbit antibody and secondary anti-rabbit, Horse Radish Peroxidase (HRP)-linked goat antibody. The blots were washed thoroughly and treated with ECL reagent (Amersham) and exposed against X-ray film (Kodak XAR).

B.2.6 Photoaffinity labeling

Photoaffinity labeling was carried out as previously described (Trigatti et al., 1991; Mangroo and Gerber, 1992; Gerber et al., 1993). In brief, bacterial cell lysates were prepared as described above and typically diluted to a total protein concentration of 1.0 mg/ml in 50 mM Tris-Cl, pH 7.0 and kept on ice. Cell lysates were prewarmed at 37°C for 4 minutes and a equal volume of 11-DAP-[11-^{3}H] undecanoate solution preincubated at 37°C were mixed and incubated at 37°C for a further 2 minutes. These samples were then photolyzed by exposure to a beam from an efficient 200 or 1000 watt Xenon-Mercury arc lamp for 2-5 seconds. The samples were then analyzed by SDS-PAGE, stained with Coomassie brilliant blue stain, soaked in Amplify (Amersham), dried and placed against X-ray film (Kodak XAR) at $-80°C$ before being developed.

B.2.7 Cell Fractionation

Isolation of total membrane and soluble fractions from E. coli

To obtain total membrane and cell supernatant fractions, bacterial cells were lysed according to methods described above. The crude cell lysate then underwent centrifugation at 100 000 *g* for one hour with the resulting pellet representing total membranes and the supernatant representing the soluble fraction. The protein concentrations for each fraction were then determined by using the instructions provided by the vendor for the BCA protein assay kit (Pierce).

Isolation of periplasm fractions from E. coli

We utilized two methods for isolating periplasm fractions from *E. coli*, chloroform extraction and osmotic shock. Chloroform extraction of the periplasmic fractions was performed based on the method by (Ames et al., 1984). In brief 1.5 ml cultures of cells were spun in a microcentrifuge for one minute and most of the supernatant discarded. 15 µL of chloroform was added, vortexed and left to sit at room temperature for 15 minutes. 75 µL of 100 mM Tris-HCl pH 8.0 was then added and the sample centrifuged for 15 min at maximum speed. The supernatant containing the periplasmic fractions was then saved and used for subsequent analysis. Osmotic shock treatment was also used to isolate the periplasm fractions as described by (Neu and Heppel, 1965)

B.2.8. Permeation assay

For the permeation assay, EDL1 (pPMIFABP) cells co-transformed with either pACYC177 or pLEL were grown in minimal media E (MME) minimal media containing 0.4 % maltose at 37° C. When these cells reached a OD_{600} of 0.5, IPTG was added to a final concentration of 0.1 mM and the cultures allowed to grow for an additional 45 min. At this point, the cultures were placed on ice and collected in a microfuge at 12,000 *g* for 2 minutes and re-suspended with MME media at the original volume of culture. Some of these samples were measured on a spectrophotometer at OD_{600}, to determine final cell number and the cells re-suspended with MME media so that the cell concentration was 1.2×10^9 cells/ml. Cells were kept on ice and when ready incubated at 37° C for four minutes, before being incubated with a two times stock solution of 11-*m*-diazirinophenoxy[11-^{3}H]undecanoate solution for a set amount of time. These samples were then exposed to a 200 watt Mg-Xe lamp for 5 seconds, through a 0.05% K^+-pthalate chemical filter. The samples were placed on ice until they were centrifuged in a microfuge for 90 seconds at maximum speed and washed once with a similar volume of MME minimal media. The pellets were resuspended with 1X SDS-PAGE sample buffer to a cell concentration of 2.4×10^9 cells/ml and heated in a water bath for 4 minutes at 90° C before analysis by SDS-PAGE.

The level of MBP-IFABP labeling was measured following the technique described by (Dougherty et al., 1996). Briefly, labeled samples were first separated by SDS-PAGE/autoradiography. Sections of the dried gel were cut that contained the radiolabled MBP-IFBAP and placed into scintillation vials. To each vial, 500 µL of dH_2O

was added and 60 minutes later a equal volume of Solvable gel solubilizer (DuPont NEN) was also added. The samples were then incubated at 50° C for three hours and allowed to cool at room temperature, before adding 5 ml of scintillation fluid. 50 μL of glacial acetic acid was also added and the samples vortexed. The samples were usually allowed to sit for 1-2 days before counting or until consistent counts could be obtained.

To verify that the counts obtained from the above procedure corresponded to real differences in levels of labeling, samples from a single labeling reaction were serial diluted with SDS-PAGE sample buffer and equal volumes of sample loaded. Radioactivity from labeled MBP-IFABP was then determined and plotted against the amount of sample present. When plotted, a 1:1 linear relationship was apparent between the amount of radioactivity present versus the amount of sample loaded (data not shown).

B.2.9 Permeabilization of *E. coli* cells with EDTA

E. coli cells were permeabilized with Tris-EDTA based on the method described by (Sheu and Freese, 1973). The desired cells were typically grown to mid-log phase and harvested by centrifugation at 10 000 *g* at 4° C for 10 minutes. These cells were washed once with M9 minimal media salts and resuspended with 100 mM Tris-HCl pH 8.0, 1 mM EDTA to a volume 1/10 of the desired cell density. Control cells were resuspended with an equivalent amount of M9 minimal media. The cells were then incubated on ice for 5 minutes and then incubated at 37° C for two minutes with shaking. These cells were then diluted with 9 volumes of M9 minimal media and used as needed.

B.2.10 Purification of MBP-FadD

BL21(DE3) *E. coli* transformed with the MBP-FadD expression plasmid were grown in LB media and induced at mid-log phase with 1 mM IPTG for 90 min. Soluble fractions from total membrane pellets resuspended with buffer C (25 mM Tris pH 7.5, 25 mM NaCl, 0.25 % Triton X-100) were isolated from these cells in a manner similar to that described for isolation of cleaved FadD (see above). The supernatant containing MBP-FadD were then incubated with amylose resin and eluted. The samples from the first 10 mM maltose elution were kept and used as a source of purified MBP-FadD.

B.2.11 Purification of MBP fused to the 19-kDa N-terminal FadD fragment

This was achieved by preparing cell lysate from IPTG induced *E. coli* BL21 (DE3)(pMD62) and subsequent isolation of the soluble fraction from total membranes resuspended with Triton X-100. The soluble fractions were then incubated with whole *E. coli* JM105 cells, which express OmpT, for a set time after determining a time course for complete cleavage. The reaction was stopped by centrifugation and the supernatant then incubated with amylose resin and washed several times before eluting with 10 mM maltose to obtain MBP-N-terminal FadD.

B.2.12 Partial purification of FadD

E. coli JM105 transformed with the FadD expression plasmid were grown to a cell density of 4.8 X 10^8 cells/ml and induced with 1 mM IPTG for 75 minutes. The cultures were then centrifuged for 10 minutes at 10,000 *g* and 4°C, and washed once with

cold 50 mM potassium phosphate buffer, pH 7.5 (buffer A). The cells were resuspended in buffer A and passed through a French Press cell three times. PMSF in isopropyl alcohol and EDTA were added to the cell lysate to a final concentration of 1 mM, immediately after passage through the French Press. Cell debris and unlysed cells were cleared by centrifugation at 12,000 *g* for 15 min at 4°C. The crude cell extract was then centrifuged at 100,000 *g* for 60 minutes, the supernatant discarded and the total membrane pellet resuspended in cold buffer A, which was subsequently stored at –80°C. The total membrane pellets were resuspended with 100 mM Tris-Cl pH 7.5, 0.2 % Triton X-100 and 1 mM neutralized oleic acid and incubated on ice for 10 minutes. These samples were centrifuged at 100,000 *g* for 60 minutes at 4°C and the supernatant used a source of intact FadD.

B.2.13 Fatty acyl-CoA synthetase assay

Acyl-CoA synthetase activity was performed as described by (Kameda and Nunn, 1981) with some slight modifications. The reaction buffer consisted of the following: 200 mM Tris-HCl pH 7.5, 8 mM $MgCl_2$, 5 mM mercaptoethanol, 20 mM NaF, 0.1% Triton X-100 (w/v), 10mM ATP, 2.5 mM CoA and a specified amount of radiolabeled [9,10-^{3}H] oleic acid diluted with unlabeled oleic acid. Reaction samples containing enzyme and all the above components except CoA were preincubated at 37° C for 2 minutes. The reactions were initiated by adding CoA to the prewarmed samples and incubated at 37° C for a further 2 minutes. The reactions were then terminated by aliquoting 100 µL of the reaction mixture into 500 µL of DOLE solution, which

contained isopropyl alcohol/heptane/1 M sulfuric acid (40:10:1, v/v/v). Water (300 μL) and heptane (300 μL) were also added and mixed vigorously. The reactions were then left until phase separation had occurred and the upper organic phase subsequently removed. The aqueous phase was then extracted at least three times with diethyl ether before being analyzed for total radioactivity by scintillation counting. Background levels of radioactivity in the reactions were determined by performing a typical assay reaction in the absence of CoA and then used to subtract the counts obtained for the other samples.

B.2.14 OmpT proteolysis assay

Detergent-soluble membrane fractions prepared prior to application onto ion-exchange chromatography were used as substrate for the proteolysis assays. Total membrane and supernatant fractions used for testing protease activity were prepared by centrifugation of cell lysates at 100, 000 *g* for 60 min at 4° C (see above). These fractions were incubated with partially purified FadD (0.25 μg/μl in 25 mM sodium phosphate pH 7.0) and incubated at 37° C for 60 min. For proteolysis with whole cells, *E. coli* cultures were grown to mid-log in LB media, harvested and washed with 1 × M9 minimal media and resuspended to 0.25 μg/μl with 25 mM sodium phosphate pH 7.0. Typically, 5 μl of the bacterial suspension was incubated with 20 μl of the FadD substrate at 37° C for 60 min. These reactions were then centrifuged in a microfuge for one min. and the supernatant saved.

B.2.15 Miscellaneous

Protein concentrations were determined using the BCA protein assay as described by the vendor (Pierce) and electrophoresis was performed as described by (Laemmli, 1970). For N-terminal protein sequencing, FadD fragments were isolated from a SDS-PAGE gel in a manner similar to that described for preparation of antibodies, except the protein samples were transferred onto PDVF membrane. These samples were then sent to the protein sequencing facility at Hospital for Sick Children, Toronto, Ontario, Canada for analysis. Oleic acid was neutralized with 1.1 equivalents of KOH in 47.5 % ethanol. T_7 RNA polymerase used in the proteolysis assay was obtained from Fermentas.

C. RESULTS AND DISCUSSION

C.1 FadL

C. 1.1 Proof of principle permeation experiment

FadL has never been directly shown to mediate permeation of fatty acids in the absence of metabolism. Filtration assays measure long chain fatty acid (LCFA) accumulation, representing the net processes of LCFA binding, transport, activation and metabolism (Trigatti and Gerber, 1996). Although these assays are relatively simple to perform and do not require special equipment, there are many inherent limitations with this technique. To observe *E. coli* LCFA uptake with filtration assays, the β-oxidation pathway needs to be fully induced. This method does not reveal FadL mediated LCFA permeation in the absence of metabolism and therefore cannot be used to specifically monitor FadL activity.

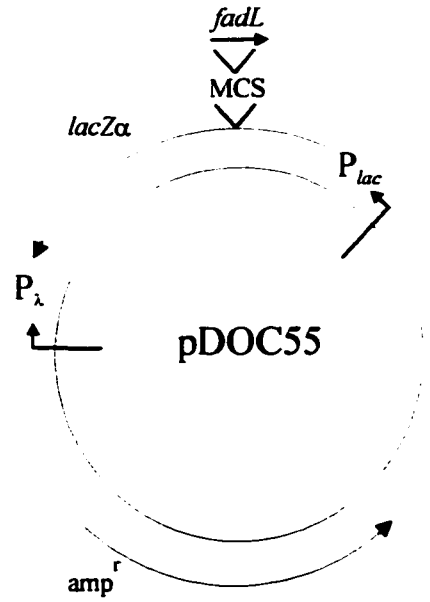
Our goal was the development of an assay system to specifically monitor FadL mediated LCFA permeation across the outer membrane using the radiolabeled photoreactive LCFA, 11-diazirinophenoxy-[11-^{3}H]-undecanoate (Figure 6) (Gerber et al., 1993). The photoreactive fatty acid is recognized and metabolized as a LCFA in eukaryotes and prokaryotes and can be used to identify and characterize membrane bound and soluble fatty acid binding proteins (Leblanc et al., 1982; Trigatti et al., 1991; Mangroo and Gerber, 1992; Gerber et al., 1993; Trigatti and Gerber, 1996). Permeation

of the photoreactive fatty acid across the *E. coli* outer membrane was to be monitored based on the level of intracellular protein labeling, *in vivo*. The results shown below represent a proof of principle experiment to test whether such an assay was feasible which will form the basis of a permeation assay to investigate the mechanism of FadL activity.

C. 1.2. Expression of plasmid encoded FadL

A constitutive FadL expression system was constructed so that the outer membrane (OM) protein could be expressed under non-inducing conditions. The *fadL* gene was subcloned into the highly repressible, expression plasmid pDOC55 (see figure 7 (A)), since overexpression of OM proteins can be toxic to *E. coli* (O'Connor and Timmis, 1987). The pDOC55 plasmid contains two promoters, P_{lac} and P_{λ}, oriented towards each other and flanking the multiple cloning site. Depending on the orientation of the inserted gene, one promoter serves to increase transcription while the other represses it. We inserted the *fadL* gene downstream of the λ phage promoter so that the P_{lac} promoter transcribes in the opposite direction. Activation of the P_{lac} promoter represses transcription through antisense mRNA and physical interference of transcription in the sense direction (O'Connor and Timmis, 1987). N-4830-1 cells express a temperature sensitive λ repressor and were grown at the non-permissive temperature. The immunoblot

(A)



(B)

fadL plasmid	-	-	+	+		-	-	+	+
IPTG	-	+	-	+		-	+	-	+
T.S. lamda repressor	-	-	-	-		+	+	+	+
lane	1	2	3	4		5	6	7	8

(C)

FadL plasmid	+	-	+	-
growth media	LB	LB	Glu	FA
lane	1	2	3	4

Figure 7. Diagram of pDOC55 plasmid and expression of FadL from pDOC55 based plasmid containing the *fadL* gene. **(A)** Diagram of pDOC55 plasmid and location of cloning site and promoters. MCS-multiple cloning site. (Adapted from O'Connor and Timmis, 1987). **(B)** Immunoblot analysis with anti-FadL antibody of the effects of IPTG and the λ phage repressor on FadL expression. XL1-Blue *E. coli* (lane 1-4) and N-4830-1 *E. coli* (lanes 5-8), which express a temperature sensitive λ phage repressor (grown at a non-permissive temperature), were used to express FadL. **(C)** Immunoblot analysis with anti-FadL antibody comparing FadL expression levels from plasmid and native sources. LB – LB media, Glu – M9 media + glucose, FA- M9 media + oleate.

in figure 7 (B) shows lower levels of FadL expression in cells expressing the λ phage repressor (N-4830-1) or cells grown in the presence of IPTG. Immunoblotting also revealed that overexpression of FadL was highest in LB media when compared to expression in glucose minimal media (Figure 7(C), lane 1 vs. 3). It was also apparent that expression of FadL from the plasmid was several-fold higher than induced native levels (Figure 7(C), lane 1 vs. lane 4). It was decided that cells transformed with the FadL expression plasmid would be cultured in LB media to ensure that we would observe FadL activity at the highest levels, in case sensitivity was a problem.

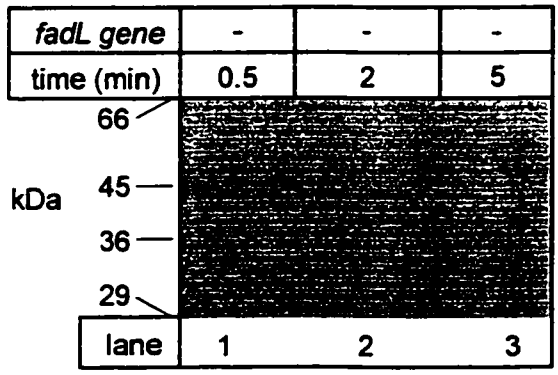
To verify that the cloned *fadL* gene was functional, the expression plasmid was transformed into *E. coli* mutants deficient in FadL. Transformation of these mutants with the plasmid allowed the cells to grow on oleate minimal media indicating that the cloned *fadL* gene was functional (data not shown).

C.1.3. Photolabeling of plasmid encoded FadL

We proceeded to characterize the LCFA binding activity of plasmid encoded FadL, by photoaffinity labeling *E. coli* cells transformed with the FadL expression plasmid. SDS-PAGE/fluorography analysis of these cells indicated labeling of a 43-kDa protein in cells transformed with the *fadL* gene (Figure 8). This indicated that the cloned *fadL* gene product was functional with respect to LCFA binding activity.

To ensure that labeling of FadL was specific, an oleic acid competition experiment was also performed. This was tested by repeating the labeling experiments

(A)



(B)

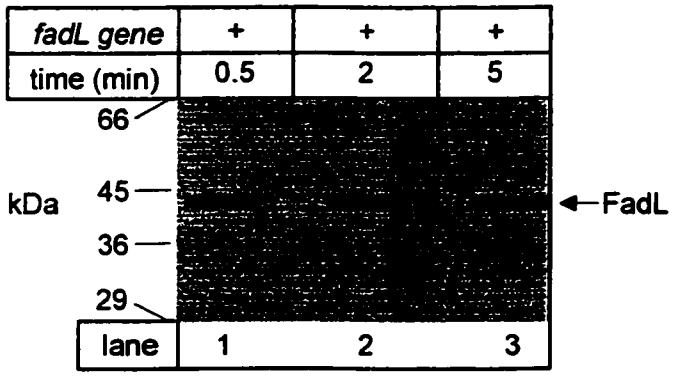


Figure 8. Time-dependent photoaffinity labeling of *E. coli* transformed with FadL expression vector. SDS-PAGE/fluorography analysis of *E. coli* cells transformed with pDOC55 plasmid **(A)** or pDOC55 plasmid with the *fadL* gene **(B)**, incubated with 2 μM photoreactive fatty acid for various times before being photolyzed and boiled in SDS-PAGE sample buffer.

with *E. coli* cells pre-incubated with various concentrations of oleate, and then labeled with a fixed concentration of photoreactive fatty acid. Based on this experiment, LCFA binding by plasmid encoded FadL was shown to be specific (Figure 9). All of the above results show that FadL protein expressed from the plasmid is functional.

C.1.4. Photolabeling of intracellular *E. coli* FABPs *in vitro* and *in vivo*

Permeation of the photoreactive fatty acid across the outer membrane was to be assessed based on the level of protein labeling. A periplasmic protein with affinity for the photoreactive fatty acid was preferable, although previous photolabeling studies carried out in our laboratory failed to identify any periplasmic proteins possessing appreciable affinity for the probe. It was possible however, that there were proteins present in the cytoplasm that had affinity for photoreactive fatty acid. To determine this, *E. coli* cells were lysed and fractionated into total membrane and soluble fractions, and then photolabeled. As seen in figure 10, we observed several proteins in the soluble fraction (representing cytoplasmic and periplasmic proteins) displaying affinity for the photoreactive fatty acid. It was not known if these labeled proteins were of high affinity and low abundance or low affinity and high abundance. The most prominently labeled band (as pointed out by the bottom arrow in figure 10) migrated below the 29-kDa protein marker, while a 45-kDa protein represented the next most intensely labeled band (top arrow). The 45-kDa protein did not appear to represent cross contamination by FadL since this band was also seen in cells not expressing FadL (Figure 10(A)) and appeared to

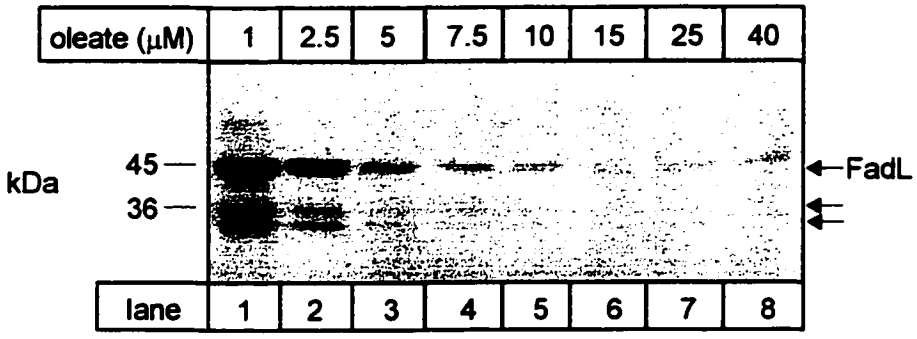


Figure 9. Oleate competition with photoreactive fatty acid for labeling of FadL. SDS-PAGE/fluorography analysis of *E. coli* cells expressing plasmid encoded FadL incubated with the indicated levels of oleate, before incubation with 10 μM photoreactive fatty acid for one minute followed by photolysis. The two lower bands represent labeled outer membrane proteins.

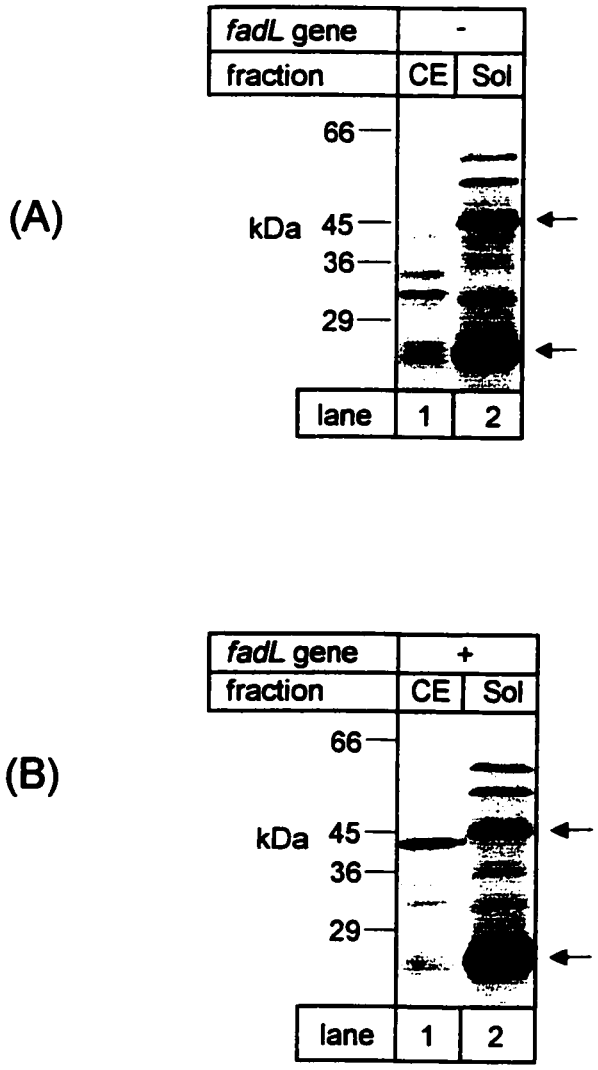
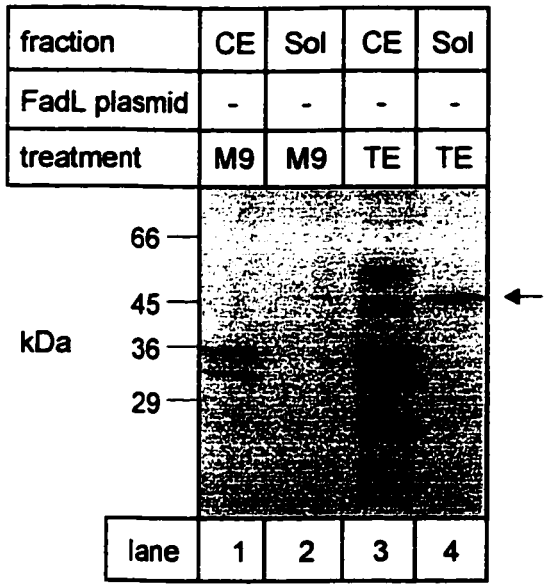


Figure 10. Photoaffinity labeling of total membrane and soluble fractions from *E. coli*. Total membrane and soluble fractions were prepared from *E. coli* transformed with either the pDOC55 plasmid alone **(A)** or pDOC55 containing *fadL* gene **(B)**. These fractions were then incubated with 10 μM photoreactive fatty acid, photolabeled and analyzed by SDS-PAGE/fluorography. The arrows identify the two most intensely labeled bands. CE – cell envelope or total membrane fraction, Sol – soluble fraction

be slightly larger than the OM protein. If FadL facilitated permeation of photoreactive fatty acids into the cell, we anticipated that one or both of these proteins would be labeled.

To test this, whole *E. coli* cells were incubated with photoreactive fatty acid and photolabeled. After labeling, the cells were harvested, lysed and fractionated, so that the soluble fractions could be analyzed by SDS-PAGE/fluorography (Figure 11, (A) and (B) lane 2). The results revealed faint labeling of a soluble 45-kDa protein when FadL was expressed and similar in size to the labeled protein from the soluble fraction in figure 10. Even though the 45-kDa protein was not the most intensely labeled protein *in vitro* (Figure 10), it was the most intensely labeled protein *in vivo* (Figure 11). It was surprising that we only observed intense labeling of the 45-kDa protein considering the intensity of labeling of the smaller protein *in vitro* (Figure 10 (A) and (B) lane 2, bottom arrow). This may be due to the localization of the 45-kDa protein that allows it to have greater access to photoreactive fatty acid, *in vivo*. To verify that labeling of the 45-kDa protein was due to permeation of the photoreactive fatty acid, a control experiment was also performed using EDTA, an outer membrane-permeabilizing reagent (Hancock, 1984). EDTA disrupts the permeability barrier of bacterial outer membranes by chelating Mg^{2+} ions that are essential for stabilizing the OM (Sheu and Freese, 1973; Hancock, 1984; Nikaido and Vaara, 1985; Vaara, 1992). It is thought that disruption of the OM allows more glycerolphospholipids to occupy the outer leaflet of the OM increasing permeability towards hydrophobic compounds. *E. coli* cells

(A)



(B)

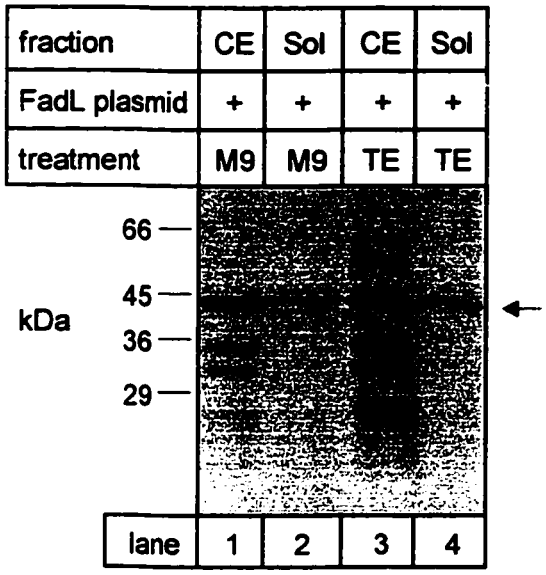


Figure 11. SDS-PAGE analysis of cell envelope and soluble fractions from *E. coli* cells photoaffinity labeled with photoreactive fatty acid. *E. coli* cells transformed with either the pDOC55 plasmid alone **(A)** or pDOC55 containing *fadL* gene **(B)** were treated with M9 minimal media (M9) or Tris-EDTA (TE) buffer (see below). These cells were then incubated with photoreactive fatty acid and photolabeled before lysis with lysozyme. The arrows identify the most intensely labeled protein from the soluble fraction. The lysates were then fractionated into cell envelope (CE) and soluble fractions (Sol).

were therefore permeabilized with EDTA and incubated and labeled with photoreactive fatty acid. Treatment of *E. coli* with EDTA also facilitated labeling of a soluble 45-kDa protein in the absence of FadL (Figure 11 (A), lane 4). This indicates that labeling of the 45-kDa protein *in vivo*, is likely due to permeation of the photoreactive fatty acid into the cell and binding by the soluble protein. FadL mediated labeling of the 45-kDa protein appeared to level off before or at the shortest incubation time period of 10 seconds (data not shown), which suggested that permeation of the photoreactive fatty acid quickly reached a plateau. The above data appeared to represent the first direct demonstration of FadL activity, but upon closer examination, we had to be cautious in our interpretation of these results. As explained below, it appeared that we could not use this system to specifically assay FadL function without additional modifications of the assay.

Observations from our FadD studies presented later, suggested that the labeled 45-kDa protein represented *E. coli* fatty acyl-CoA synthetase. This enzyme has been described in several studies as a soluble 42-50-kDa protein. Expression of FadD would indicate that metabolism of the photoreactive fatty acid is occurring since work from our laboratory had previously shown that *E. coli* metabolizes the photoreactive fatty acid if the *fad* regulon is induced (Mangroo and Gerber, 1992). As mentioned previously, we wanted to observe fatty acid permeation in the absence of metabolism to specifically assay FadL activity. If FadD was being labeled, it meant that fatty acid labeling was occurring in the cytoplasm and we were not exclusively measuring OM permeation. In addition, it was also difficult at times to distinguish the labeled 45-kDa protein from labeled 43-kDa FadL. This potentially made it difficult to accurately determine the level

of intracellular protein labeling. The possibility also remained that permeation of the photoreactive fatty acid was not specifically due to FadL but due to non-specific permeation caused by FadL overexpression. Although the permeation assay was not well characterized and could not be used to specifically assay FadL activity, it indicated that a fatty acid permeation assay using protein labeling could be feasible.

C.1.5. Effect of long chain fatty acids (LCFAs) on permeabilized *E. coli*

An interesting observation obtained from the permeation experiments was the observation of lower levels of FadL-mediated protein labeling when compared to EDTA-mediated protein labeling (Figure 11, compare lanes 2 to 4 in (B)). Our data indicated that permeation of fatty acids via FadL plateaus to a lower level than that seen with diffusion-mediated permeation. In the absence of metabolism, it was expected that FadL would facilitate diffusion of fatty acids down their concentration gradient and allow protein labeling to approach EDTA- mediated levels. EDTA treatment represents a control for the maximal level of protein labeling possible at a given concentration of photoreactive fatty acid and should occur when levels of the probe are equal across the outer membrane. However, a protein labeling time course indicated that FadL mediated labeling did not approach the intensity of EDTA-mediated labeling even after extended periods of incubation time (up to two minutes). These results appeared to indicate that FadL may not function as a simple channel for LCFAs. This was assuming though that the photoreactive fatty acid was not being metabolized, which at this point was uncertain.

Work by Sheu *et al.* showed that low concentrations of LCFAs are toxic to *E. coli* if their outer membranes (OMs) are disrupted (Sheu and Freese, 1973). This was demonstrated by permeabilizing *E. coli* with EDTA and exposing the cells to low concentrations of LCFAs. Permeabilization inhibited growth of these cells only when fatty acids were present. They discovered that even cells previously grown on fatty acid minimal media were sensitive to LCFAs when treated with EDTA. This was worthy of note, since *fad* induced *E. coli* grow on much higher concentrations of fatty acid. Sensitivity of permeabilized *fad* induced cells to LCFAs indicates that the *fad* regulon does not encode any proteins that protect the cell from the toxic effects of LCFAs inside the cell. This illustrates that even though *E. coli* can utilize LCFAs as a sole carbon source, they are intrinsically toxic to bacteria.

These studies therefore suggested that protein labeling caused by EDTA permeabilization represented fatty acid levels that were inhibitory for growth of *E. coli*. This was confirmed when growth curve experiments were performed with *E. coli* cells treated with EDTA in a similar manner to the photolabeling experiments (Figure 12).

If FadL simply facilitated the diffusion of fatty acids down their concentration gradient, we would expect the level of fatty acids inside the cell to equilibrate with the external concentration in the absence of metabolism. We therefore investigated if overexpression of FadL would sensitize *E. coli* to LCFAs. This was carried out by initially growing the cells in a non-fatty acid medium to ensure that the *fad* regulon was not induced. LB media was used since the highest expression levels were obtained

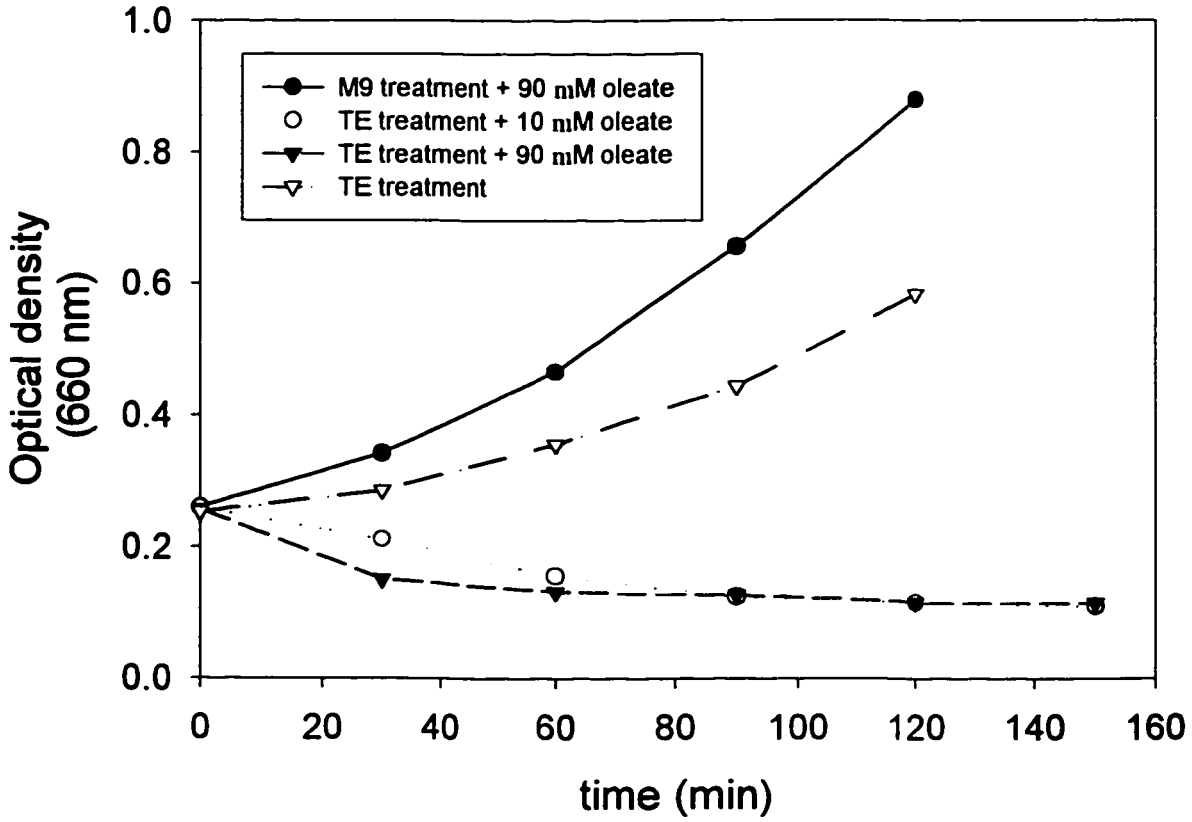


Figure 12. Effect of Tris-EDTA and M9 media treatment on growth of *E. coli* in minimal media containing long chain fatty acids (LCFAs). Growth curves were generated following the procedure reported by (Sheu & Freese 1973) Cultures were inoculated from frozen stocks and grown to saturation overnight. The saturated cultures were then subcultured into fresh media, typically M9 minimal media + 0.4% glucose and grown to mid-log. At this point the cells were harvested by centrifugation at 10 000 *g* for 10 minutes and washed by resuspending the cells in M9 minimal media and centrifugation again at 10 000 *g* for 10 minutes. The harvested cells were resuspended to an optical density of 2.0 at 600 nm with M9 minimal media containing various combinations of 1mM EDTA and/or desired amounts of fatty acid. The cells were then incubated at 37° C with shaking for two minutes. 9 volumes of M9 minimal media was then added and the cultures grown at 37° C with shaking. At specified times, samples were taken out to be measured for optical density.

(Figure 7(C)) with this media, and β-oxidation activity in this media is low (3-5% of fully induced cells (Black and DiRusso, 1994)). As shown in figure 13, EDTA treatment inhibited *E. coli* growth in the presence of oleate whereas overexpression of FadL had no affect on growth in the presence of oleate. This indicated that FadL did not facilitate the diffusion of LCFAs to levels seen in EDTA permeabilized cells even though FadL was overexpressed. This was consistent with the differences seen in the labeling of the 45-kDa protein when FadL and EDTA were used to facilitate permeation of the photoreactive fatty acid (Figure 11). Based on the above results and observations, we hypothesized that FadL did not function as a simple channel to facilitate LCFA diffusion. Instead, FadL activity appeared to be regulated so that toxic levels of LCFAs are not reached inside the cell.

C.1.6. LCFA permeation assay

Our goal now was to create a new assay that could address some or all of the limitations of the previous assay to test the hypothesis that FadL activity is regulated. Figure 14 is a schematic of how the assay was set up and all the components used. Our experimental approach now involved targeting a protein with affinity for the photoreactive fatty acid, to the periplasm. This would allow direct monitoring of photoreactive fatty acid flux, across the OM. The protein used to monitor permeation consisted of rat intestinal fatty acid binding protein (IFABP) fused to the C-terminus of

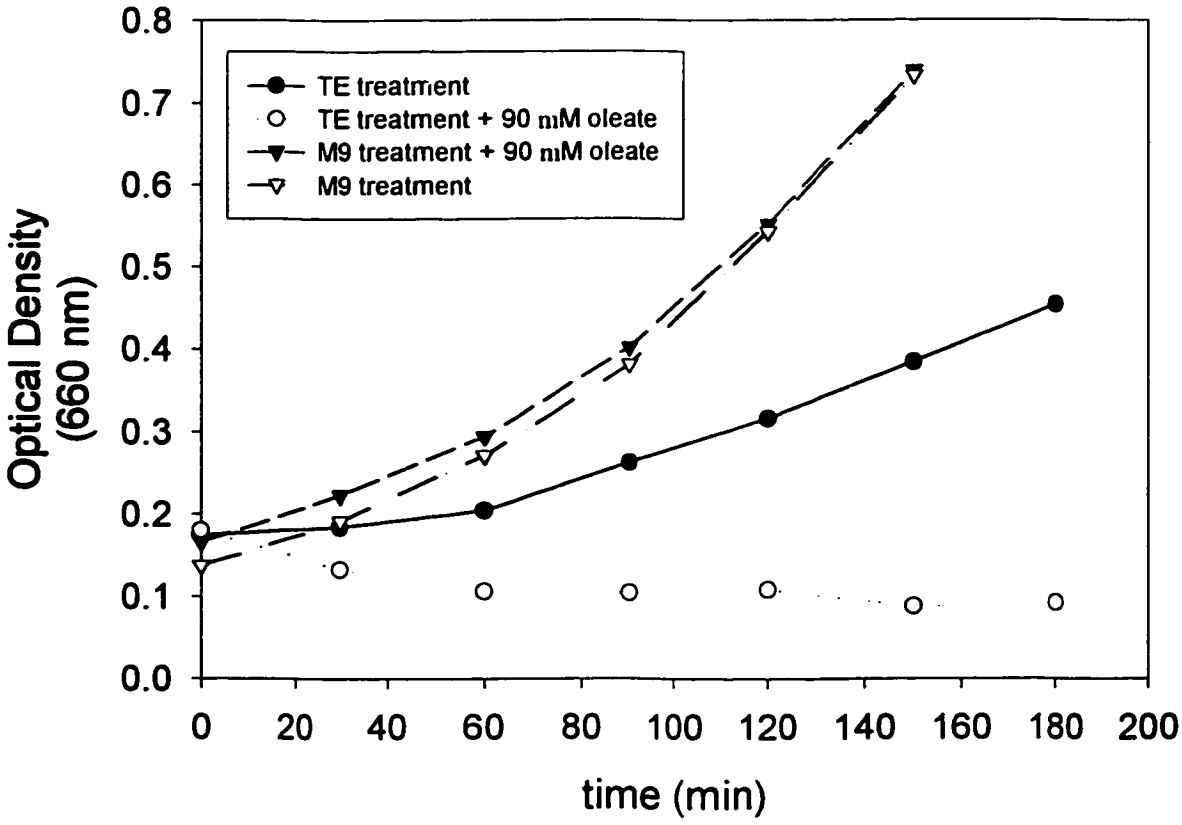


Figure 13. Effect of FadL overexpression on the growth of *E. coli* in minimal media containing long chain fatty acid (LCFA). The growth curves for this figure were prepared as described in figure 12, except the *E. coli* cells used were initially grown in LB media and overexpressed FadL protein.

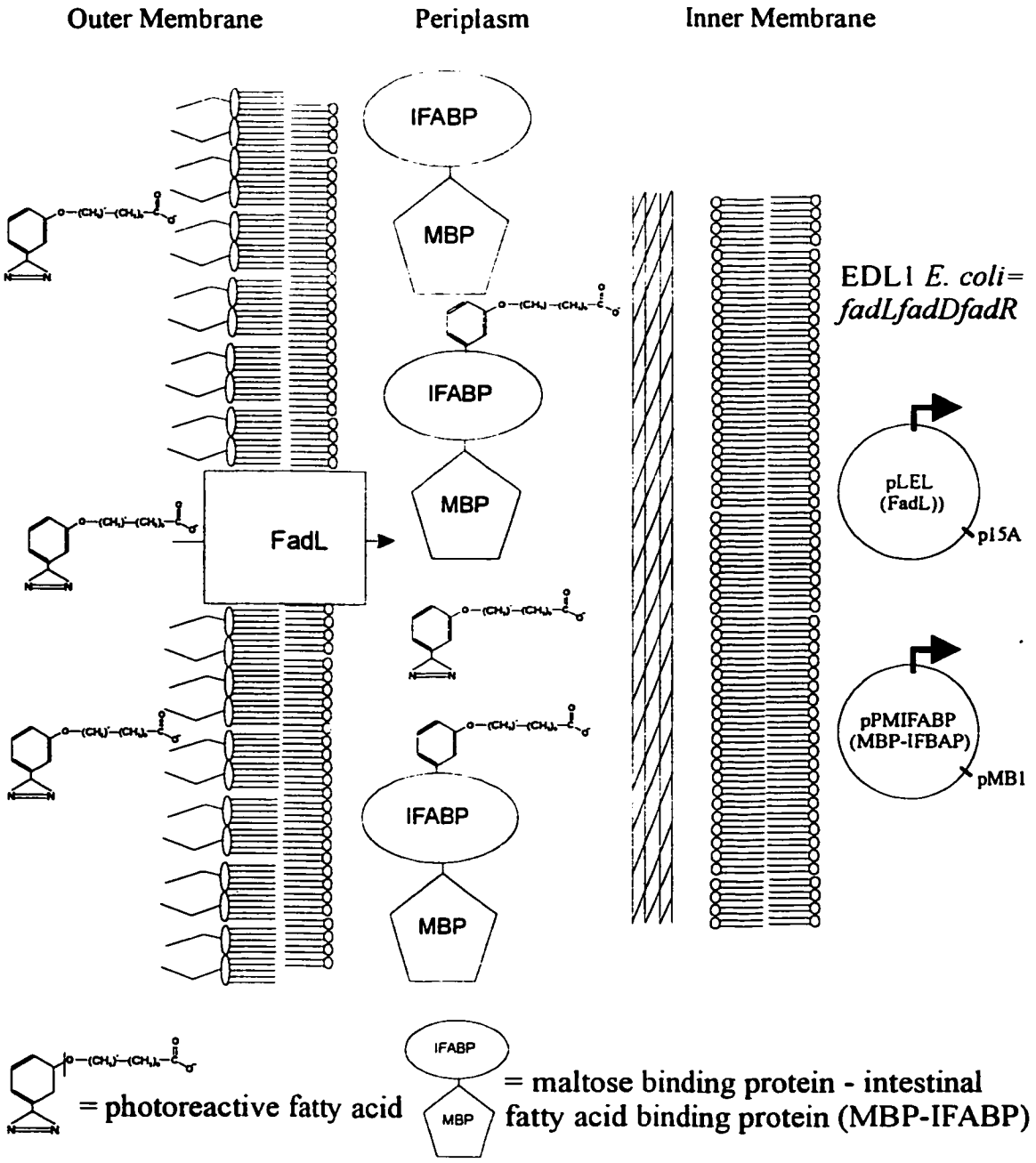


Figure 14. Schematic of photoreactive fatty acid permeation assay. The *E. coli* cell line EDL1 (*fadL fadD fadR*), was transformed with the FadL expression plasmid (pLEL) and the rat intestinal fatty acid binding/maltose binding protein expression plasmid (pPMIFABP). These cells were grown to mid-log, induced to express the MBP-IFABP fusion protein, harvested, incubated with photoreactive fatty acid, photolabeled and the cells analyzed by SDS-PAGE/fluorography. See figure 15 for the construction of pLEL and pPMIFAB.

maltose-binding protein (MBP) (Figure 15 (B)). It was expected that the fusion protein would be targeted to the periplasmic space, since the targeting sequence of MBP is intact (Blondel and Bedoulle, 1990). *E. coli* cells transformed with the plasmid and induced to express the fusion protein were incubated with photoreactive fatty acid and analyzed by SDS-PAGE/fluorography. Liquid scintillation counting was then used to assess the level of fusion protein labeling. Another plasmid containing the *fadL* gene was co-transformed into the same cells and used to express FadL at levels approaching native amounts (Figure 15(A)). Both plasmids were transformed into the *E. coli* cell line EDL1 which were *fadL fadD fadR*. The *fadL* genotype was desired so that analysis of FadL would not be complicated by endogenous protein and the *fadD* genotype was required so that the photoreactive fatty acid would not be metabolized. The *fadR* genotype was needed for expression of the FadL from the plasmid since the *fadL* insert contained its native promoter (Figure 15(A)).

C.1.6.1. Expression of MBP-IFABP and FadL

Rat intestinal fatty acid binding protein (IFABP) is a member of the cytoplasmic fatty acid binding protein family and possesses a single, high-affinity (K_d = 0.2 μM) binding site for LCFAs (Lowe et al., 1987; Sacchettini and Gordon, 1993). Recombinant IFABP was expressed and purified to high levels in *E. coli* (Lowe et al., 1987) and was thus chosen as the target for the photoreactive fatty acid in our assay. Maltose binding protein was used to target IFABP to the periplasm and ensure that it would be retained in

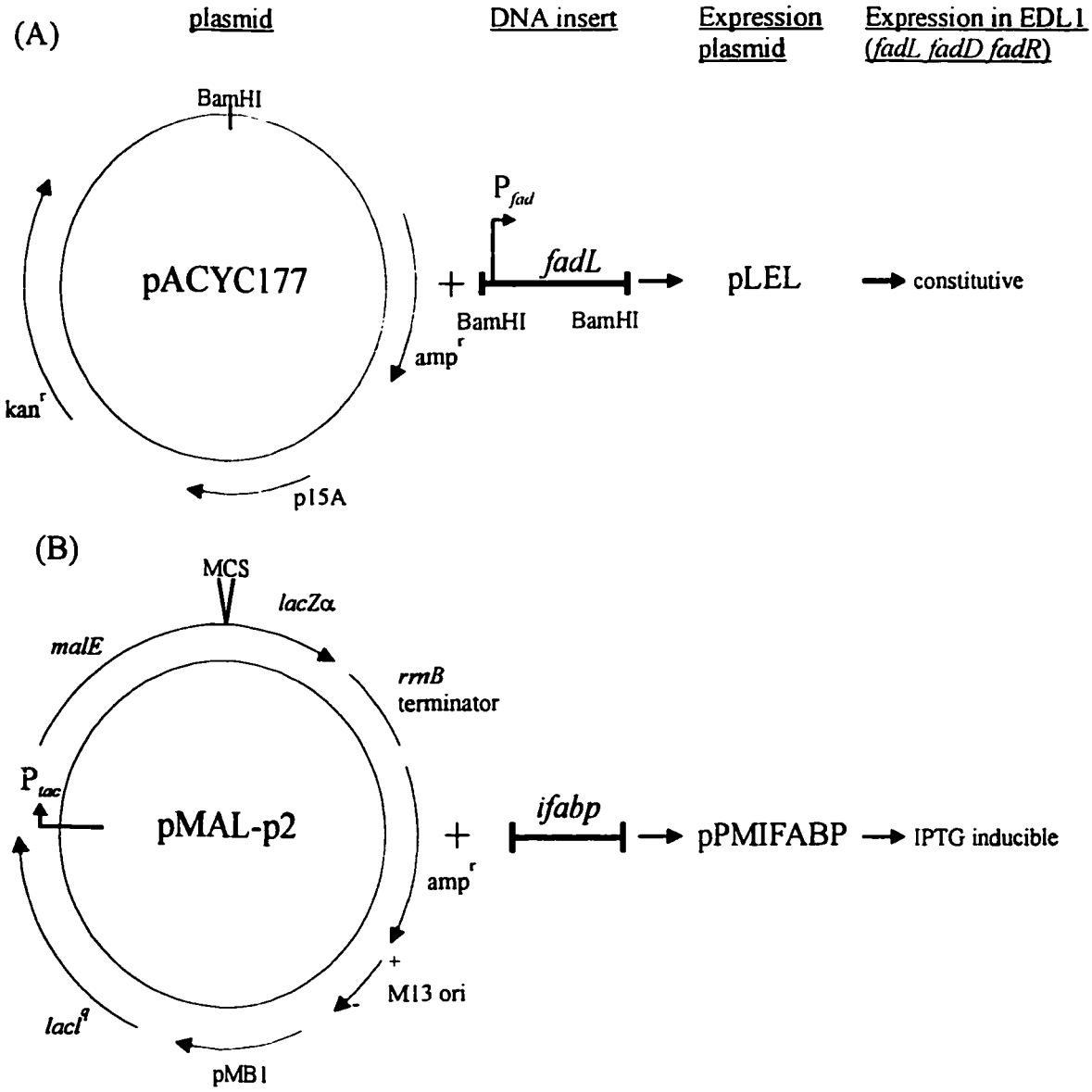


Figure 15. Construction of MBP-IFABP and FadL expression vectors for the photoreactive fatty acid permeation assay Construction of the FadL expression plasmid. The DNA insert containing the *fadL* gene was ligated into the BamHI site of pACYC177 (Chang and Cohen, 1978). P_{fad} is the native *fadL* promoter and regulated by FadR. (B) Construction of the MBP-IFABP expression plasmid. The DNA insert containing the gene for IFABP (*ifabp*) was ligated into the multiple cloning site of pMAL-p2. P_{tac} is regulated by the $lacI^q$ repressor.

the periplasm and not "leak" out of the cell, as it is only 15-kDa in size. Figure 16 (A) shows the induced expression of a 60-kDa protein when the MBP-IFABP expression plasmid was induced by IPTG. The molecular weight of the induced protein was consistent with it being MBP-IFABP, since IFABP is 15-kDa (Lowe et al., 1987) and processed MBP has a molecular weight of 43-kDa (Duplay et al., 1984).

For the FadL expression system, a pACYC177-based vector was used based on two criteria: it was compatible with the MBP-IFABP fusion plasmid and it replicated at low copy numbers (Sambrook et al., 1989). This meant that expression of both the fusion protein and FadL in the same cell was possible and low levels of FadL expression could be attained. It is apparent from figure 16(A) that co-expression of FadL had no visible effects on MBP-IFABP expression. Since the DNA insert containing the *fadL* gene retained its native promoter, expression of FadL was therefore regulated by FadR and catabolite repression (Said et al., 1988; Black, 1991). We therefore expected that FadL would be constitutively expressed since EDL1 are *fadR* mutants and cells used in the assay were grown in maltose minimal media. The immunoblot in figure 16 (B) showed FadL expression levels from the plasmid were comparable to wild type levels (wild type *E. coli* grown in oleate minimal media). Based on this, any effects of FadL expression on LCFA permeation would not be due to overexpression effects.

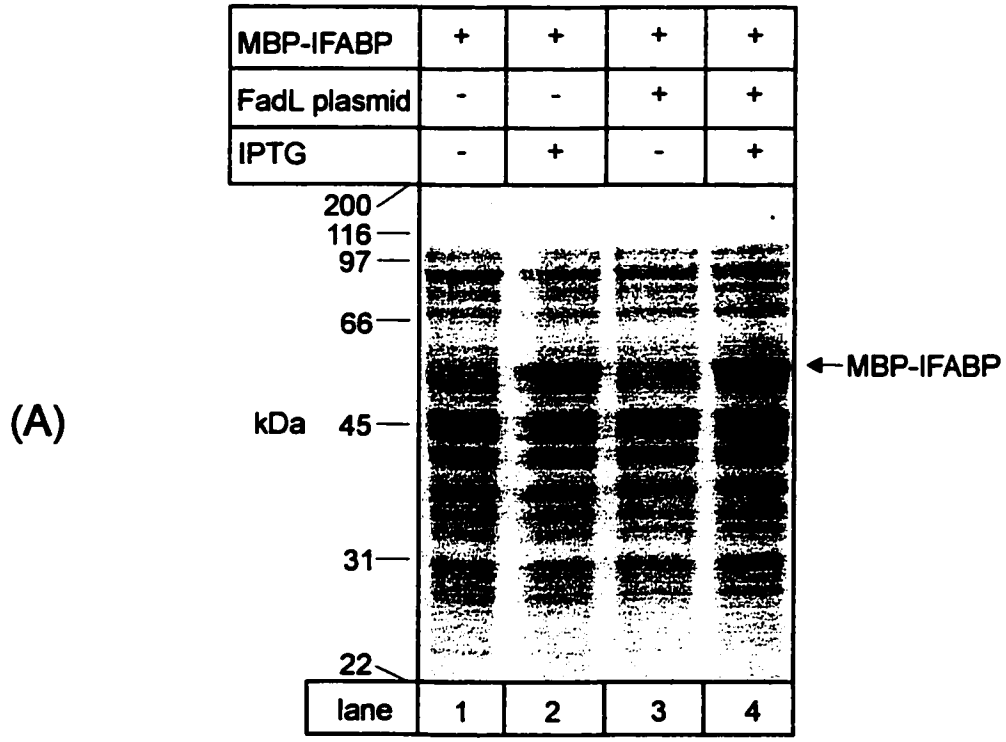


Figure 16 (A). Expression of maltose binding protein – intestinal fatty acid binding protein (MBP-IFABP) and FadL in EDL1 *E. coli.* SDS-PAGE of EDL1 cells co-transformed with the MBP-IFABP and FadL expression vectors, induced with IPTG. The gel was stained with Coomassie Blue dye and the arrow denotes the induced expression of the fusion protein.

(B)

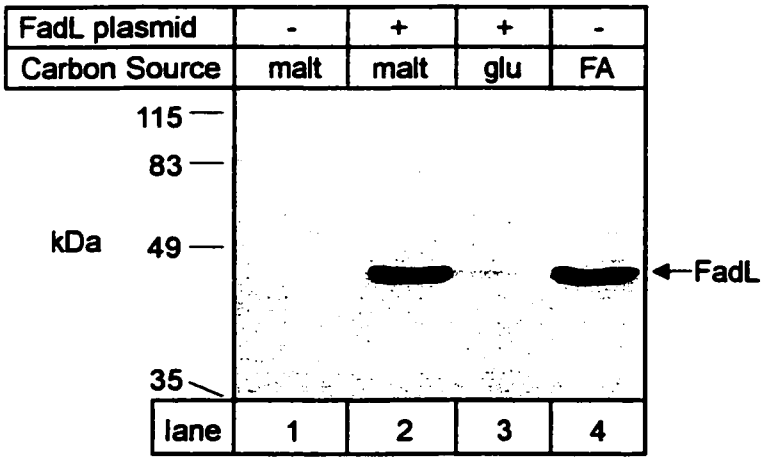


Figure 16 (B). Expression of maltose binding protein – intestinal fatty acid binding protein (MBP-IFABP) and FadL in EDL1 *E. coli.* Immunoblot with anti-FadL antibody of *E. coli* cells transformed with the low expression FadL plasmid, pLEL, grown in minimal media using maltose (malt), glucose (glu) or oleate (FA) as carbon sources. JM105 cells transformed with the MBP-IFABP fusion plasmid, were made competent and transformed with either pACYC177 ((-), lanes 1,4) or pLEL((+), lanes 2,3) containing the *fadL* gene. These transformants were selected based on their ability to grow on LB containing ampicillin (100 μg/ml) and kanamycin (20 μg/ml). These cells were harvested at mid-log, boiled with SDS-PAGE sample buffer, loaded onto a SDS-polyacrylamide gel, transferred onto nitrocellulose and probed with rabbit anti-FadL antibody.

C.1.6.2. Characterization of MBP-IFABP

To determine if MBP-IFABP displayed affinity for the photoreactive fatty acid *in vitro*, cell lysates were prepared from EDL1 cells expressing the fusion protein and photolabeled. As shown in figure 17, several proteins in the cell lysate exhibited affinity for the photoreactive fatty acid. The most intensely labeled band corresponded to a 60-kDa protein that was only present in cell lysates containing MBP-IFABP. This showed that IFABP had retained LCFA binding affinity after fusion to MBP. Although the fusion protein was the most intensely labeled protein *in vitro*, it does not always necessarily correspond to labeling patterns seen *in vivo* as seen in our previous results.

It was essential that MBP-IFABP was targeted to the periplasm, since we only wanted to assay permeation across the OM. The MBP fusion plasmid pMAL-p2 was initially characterized to ensure that the maltose binding protein itself was correctly targeted and localized to the periplasm. This was verified by SDS-PAGE analysis of periplasm fractions from osmotic shocked (Neu and Heppel, 1965) and chloroform treated *E. coli* (Ames et al., 1984) transformed with pMAL-p2 (data not shown). To confirm targeting of the fusion protein, periplasm fractions were isolated from chloroform treated EDL1 cells co-expressing FadL and MBP-IFABP. Immunoblotting of the periplasm fractions with anti-MBP antibody revealed the detection of two bands (Figure 18(A)). The smaller, faint band likely represents native processed MBP (43-kDa), as these cells were grown in maltose minimal media. Detection of this band showed that

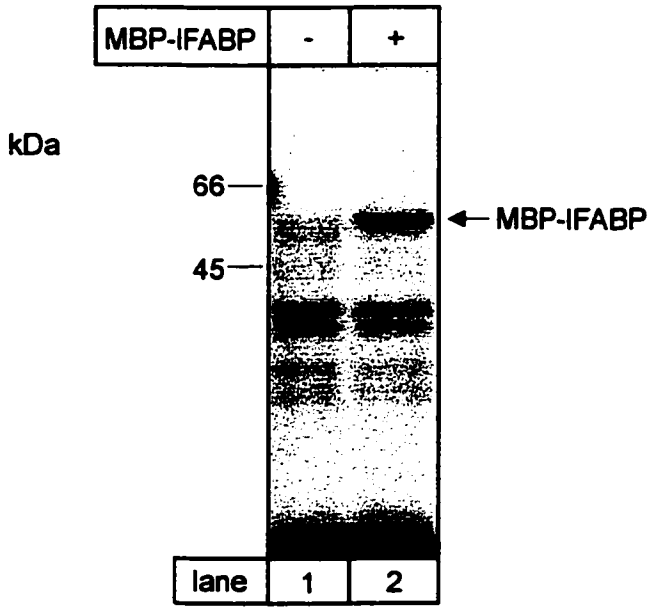


Figure 17. Photoaffinity labeling of *E. coli* cell lysates containing MBP-IFABP fusion protein. SDS-PAGE/fluorograph of EDL1 *E coli* cell lysates expressing MBP-IFABP fusion protein, photolabeled with photoreactive fatty acid.

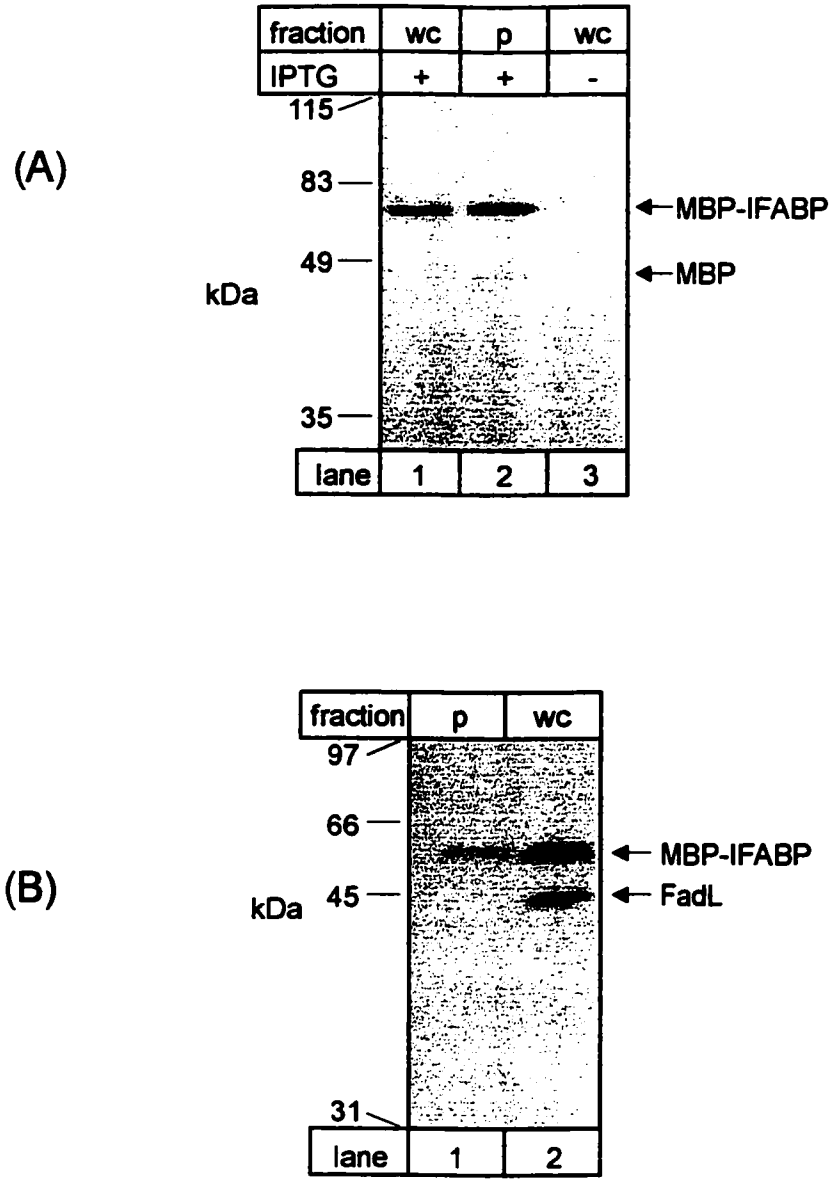


Figure 18. Isolation of MBP-IFABP from the periplasm. **(A)** Immunoblot analysis with anti-FadL antibody and **(B)** SDS-PAGE/fluorograph of periplasm samples isolated from EDL1 cells expressing FadL and MBP-IFABP using chloroform treatment (Ames *et al*, 1984). These cells were previously labeled with photoreactive fatty acid. In figure (A), a three times equivalent of periplasm sample was loaded compared to the whole cell sample. wc-whole cell, p-periplasm

periplasm proteins were being extracted since MBP is targeted to the periplasm. The larger, more intense band represents overexpressed MBP-IFABP since it is only detected in cells induced to express the fusion protein (Figure 18, lane 1 versus lane 3). Our results indicated that we were not getting quantitative extraction of MBP-IFABP from the *E. coli* cells, using chloroform treatment or osmotic shock. Chloroform treated EDL1 cells overexpressing MBP alone also gave incomplete extraction (data not shown), suggesting that this effect is not specific for the fusion protein. We attributed the retention of these periplasmic proteins to inefficiencies in the isolation techniques used and/or expression of the fusion protein in the cytoplasm. If some of the MBP-IFABP was present in the cytoplasm, it was possible that some of the labeling observed may have also occurred in the cytoplasm. However, only a single protein band was observed in the whole cell labeling experiments. Proteins that are targeted to the periplasm normally contain a signal sequence ($\approx$ 26 amino acids) that is cleaved upon import into the periplasm. Fusion protein molecules unable to reach the periplasm would not have its signal sequence cleaved resulting in the presence of two species of MBP-IFABP, processed and unprocessed, with a difference in molecular weight of about 3-kDa (Blondel and Bedoulle, 1990). Based on staining and immunoblotting, there were no apparent differences in molecular weight between the fusion protein observed *in vivo* and the form present in the periplasmic fraction (Figure 18 (A)(B)). This suggests that MBP-IFABP in the periplasm fraction represents the *in vivo* form and most of the visible fusion protein is cleaved *in vivo*. To confirm this, periplasm fractions containing MBP-IFABP were treated with Factor Xa to separate MBP from IFABP and compared to processed MBP.

Both bands appeared similar in weight, indicating that the MBP moiety from MBP-IFABP is processed and therefore targeted to the periplasm (Blondel and Bedoulle, 1990)(data not shown).

It is thought that smaller proteins are much more efficiently translocated as a MBP fusion protein than larger proteins (Blondel and Bedoulle, 1990). In fact, osmotic shock and chloroform treatment of *E. coli* cells expressing the larger maltose binding protein-FadD fusion protein (MBP-FadD) did not reveal any fusion protein in the periplasm fractions (data not shown).

C.1.6.3. Effect of FadL expression on MBP-IFABP labeling *in vivo*

The next step involved the actual testing of the assay by determining the effect of FadL expression on photoreactive fatty acid permeation and MBP-IFABP labeling. The assay was performed by photolabeling EDL1 cells expressing FadL and MBP-IFABP with photoreactive fatty acid. EDL1 cells expressing only MBP-IFABP were used as a control for background labeling. It was expected that MBP-IFABP would only be labeled if FadL was co-expressed and figure 19 (A) showed this to be the case. The apparent molecular weight of the two bands was consistent with the upper band representing MBP-IFABP and the lower band, FadL. To confirm that the upper band represented MBP-IFABP, we isolated periplasm fractions from EDL1 cells expressing FadL and MBP-IFBAP photolabeled with the LCFA analogue. Analysis of the periplasm fraction

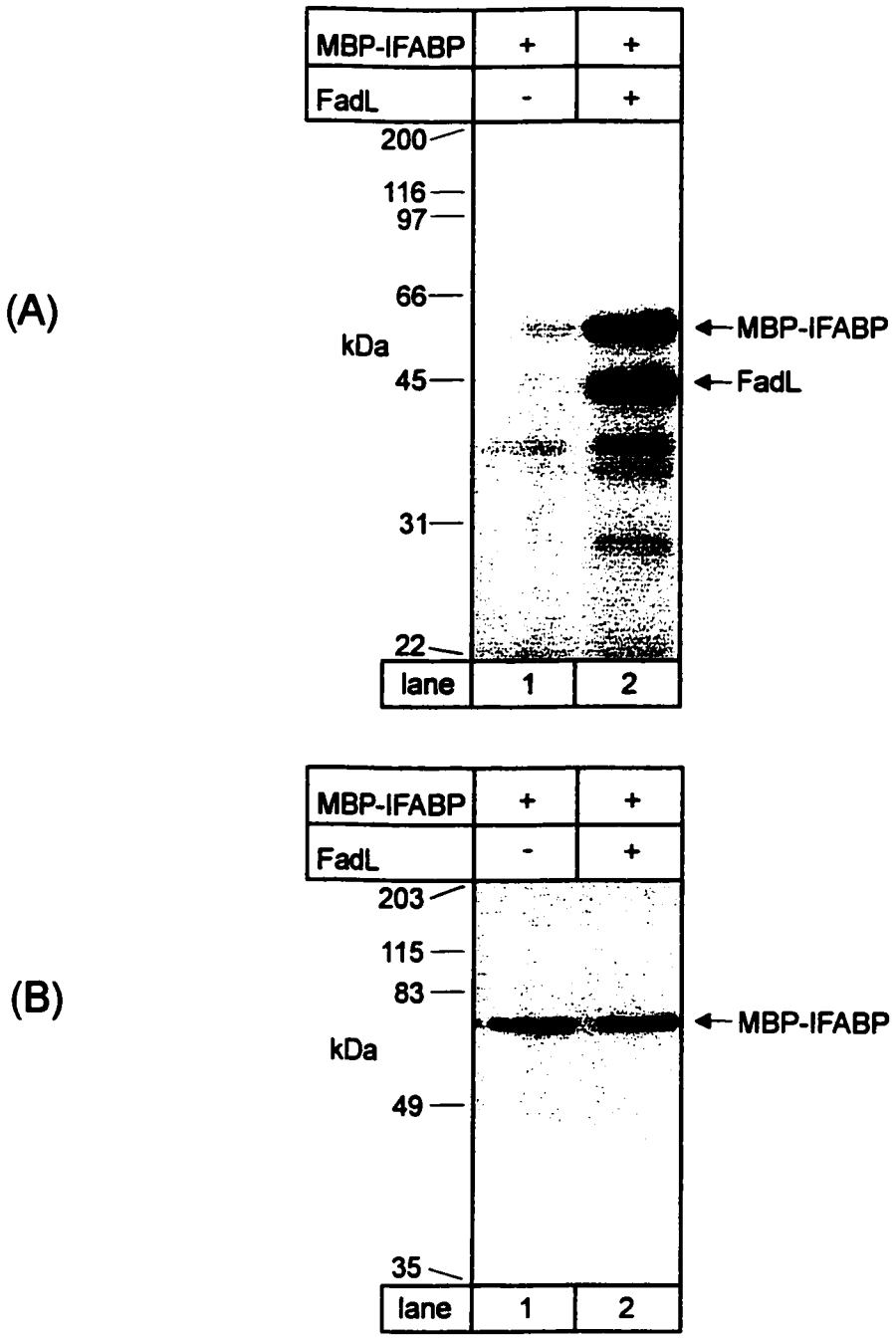


Figure 19. Effect of FadL expression on labeling of MBP-IFABP *in-vivo.* **(A)** SDS-PAGE/fluorograph of EDL1 cells expressing the MBP-IFABP fusion protein co-transformed with FadL expression vector or the vector alone, photolabeled with photoreactive fatty acid. **(B)** Immunoblot of samples analyzed in **(A)** with anti-MBP antibody.

by SDS-PAGE/fluorography revealed a single band in the periplasm fraction (Figure 18(B), lane1)). The extraction of the labeled band by chloroform treatment showed that the labeled protein was present in the periplasm. The lower FadL band seen in whole cells was not seen in the periplasmic fraction as expected since FadL is an outer membrane protein.

Immunoblotting with the anti-MBP antibody (Figure 19(B)) and treatment of EDL1 cells with EDTA (Figure 21), showed that the lower levels of MBP-IFABP labeling in the absence of FadL were not due to differences in the expression levels or intrinsic LCFA binding activity of the fusion protein respectively. It appeared that the increased labeling in the presence of FadL was due to increased permeation of photoreactive fatty acid. These results show that the assay had behaved as expected and demonstrated for the first time, FadL activity *in vivo*.

FadL mediated permeation was further characterized by determining the time course of permeation. The shortest time period that could be performed accurately and consistently was five seconds and even at this time point, there was no significant difference in the level of MBP-IFABP labeling when compared to labeling after 120 seconds of incubation (figure 20(A)) or even 5 minutes (data not shown). To analyze MBP-IFABP labeling quantitatively, the labeled proteins were eluted from rehydrated gel slices and their radioactivity determined (Figure 20(B)). Consistent with the fluorographs, scintillation counting also indicated that labeling was relatively constant after five seconds. Since time points below five seconds could not be measured, the section of the graph depicting time dependent protein labeling could not be generated and we were

therefore unable to determine initial rates of permeation. Expression of FadL at native levels appeared to result in a three to four fold increase in the level of fatty acid present in the periplasm versus cells that did not express FadL. The low level of labeling observed in cells without FadL likely represented permeation of fatty acids through the porins or other OM hydrophobic pathways (Plesiat and Nikaido, 1992). Although a definite increase in LCFA permeation was evident when FadL was expressed, it appeared that this permeation occurred very quickly (≤ 5 seconds) followed by a very slow level of permeation as indicated by the plateau. The plateau in FadL mediated labeling could be attributed to labeling of all the MBP-IFABP protein molecules or equilibrium of the photoreactive fatty acid across the OM before the five seconds.

C.1.6.4. Effect of EDTA on MBP-IFABP labeling *in vivo*

To address if the plateau in protein labeling (Figure 20) was due to equilibrium of the photoreactive fatty acid across the OM or complete labeling of all the fusion protein molecules, EDL1 cells expressing the fusion protein were permeabilized with EDTA and photolabeled. EDTA treatment permeabilizes the OM to hydrophobic compounds and should therefore reveal the maximal amount of MBP-IFABP labeling possible at a given concentration of photoreactive fatty acid. Figure 21(A) and (B) showed that protein labeling due to EDTA treatment was much higher than FadL mediated labeling (about a 4-5 fold increase), indicating that labeling facilitated by FadL did not represent equilibrium levels or complete labeling of the fusion protein. To ensure

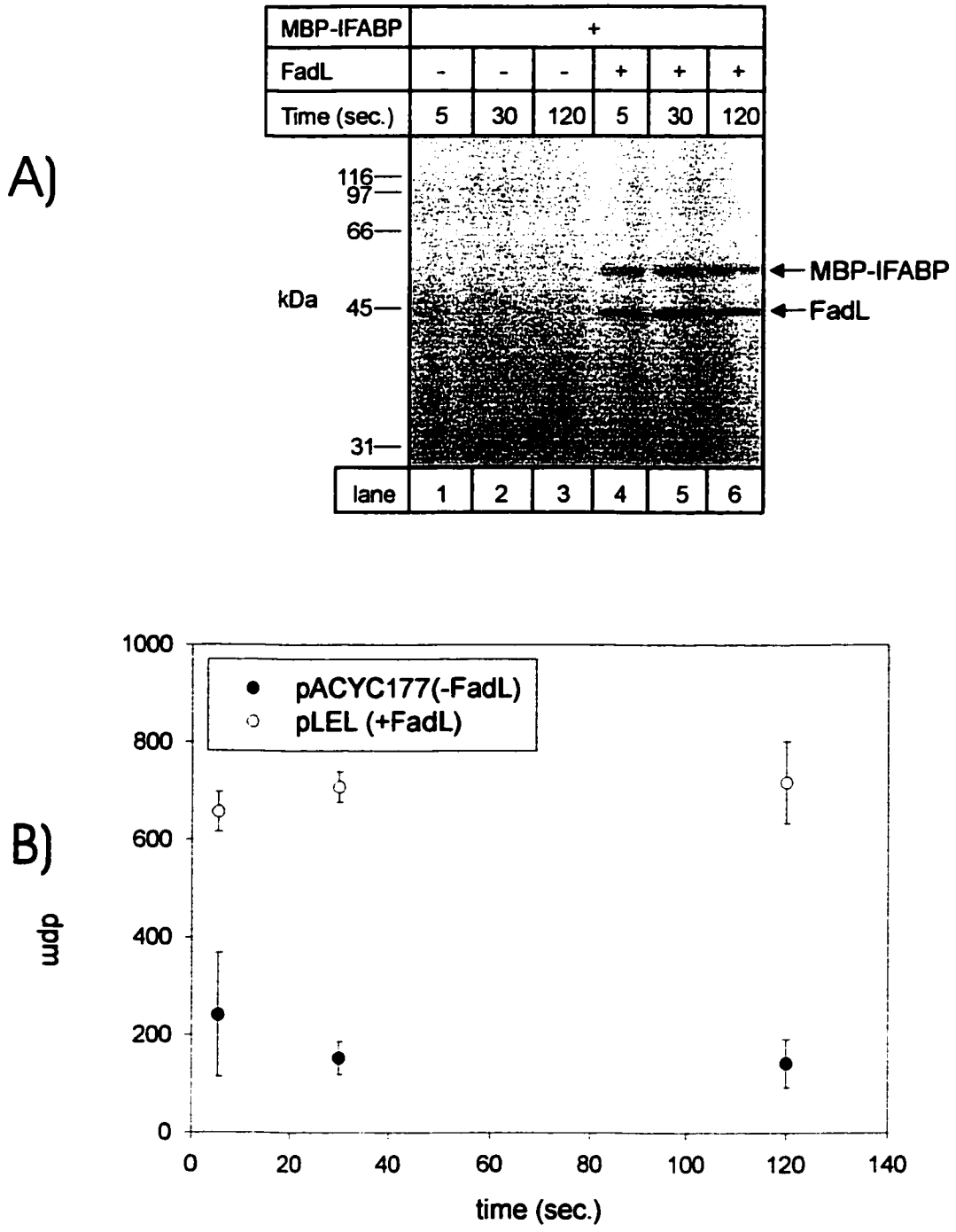


Figure 20. Time course of MBP-IFABP labeling **(A)** SDS-PAGE/fluorography of EDL1 cells expressing MBP-IFABP fusion protein co-transformed with FadL expression vector or vector alone, incubated with photoreactive fatty acid for various times. **(B)** Scintillation counts of excised MBP-IFABP labeled bands from SDS-PAGE shown in (A). These values and their respective standard deviations were calculated from four separate experiments and background subtracted.

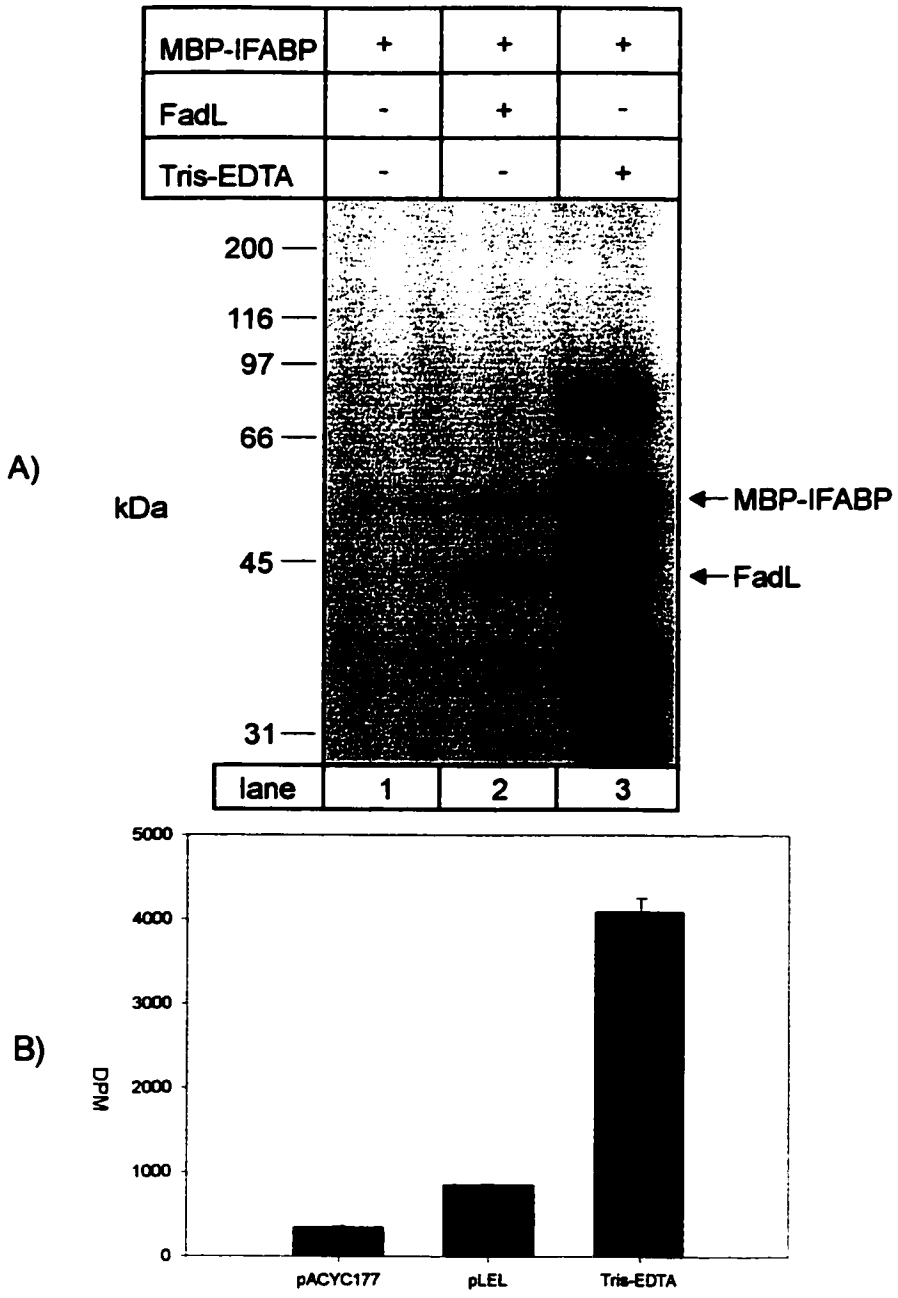


Figure 21. Effect of EDTA treatment on LCFA permeation and concentration dependent labeling of MBP-IFABP. **(A)** SDS-PAGE/fluorograph of EDL1 cells expressing MBP-IFABP fusion protein co-transformed with the FadL expression vector or vector alone after treatment with EDTA or minimal media and labeling with photoreactive fatty acid. **(B)** Graph of scintillation counts from excised labeled MBP-IFABP bands from SDS-PAGE shown in (A). The values shown represent average values from three different labeling experiments and were background subtracted. **(C)** is on the next page.

C)

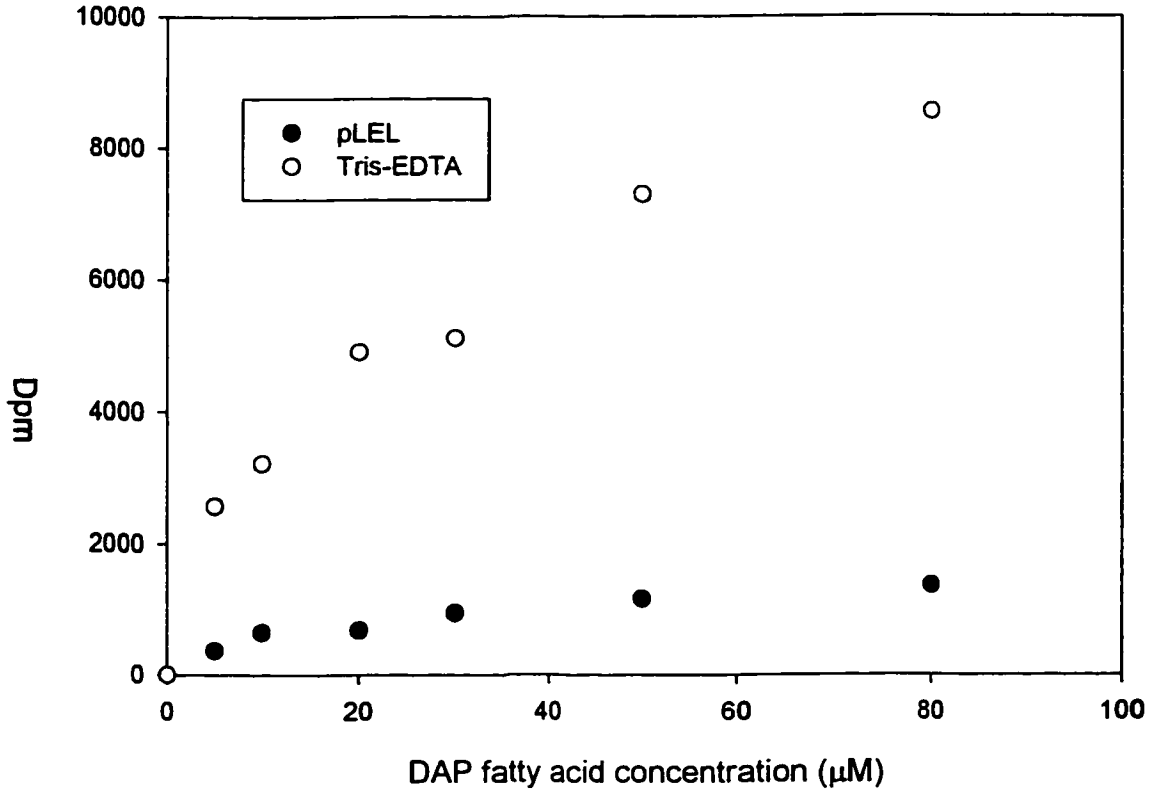


Figure 21(cont.). Effect of EDTA treatment on LCFA permeation and concentration dependent labeling of MBP-IFABP. **(C)** cells expressing MBP-IFABP fusion protein co-transformed with the pACYC177 vector (open circle) were treated with Tris-EDTA and incubated with the denoted concentrations of photoreactive LCFA for 2 minutes at 37 C. These cells were then photolabeled and analyzed by SDS-PAGE/fluorography and the level of fusion protein labeling determined as described in materials and methods. The experiment was repeated with EDL1 cells expressing FadL and the fusion protein without treatment with Tris-EDTA. The experiment was performed several times and the above represented a typical plot.

that the permeation assay was responsive to different concentrations of photoreactive fatty acid, a concentration-dependent labeling experiment was performed. The results shown in figure 21(C) indicated that in *E. coli* cells permeabilized by EDTA, MBP-IFAP labeling was proportional to the concentration of photoreactive fatty acid present. It was also evident from this plot that MBP-IFABP labeling was unaffected at higher concentrations of photoreactive fatty acid in *E. coli* cells expressing FadL, as compared to the EDTA treated *E. coli* cells.

Regulation of porin activity has not been well characterized but voltage-dependent gating of porins has been demonstrated *in vitro* (Buechner et al., 1990; Jap and Walian; 1990) and it has also been shown that pH can affect porin activity (Todt et al., 1992). OmpA porins and other OM proteins have also been proposed to exist in open and closed conformations (Benz and Hancock, 1981; Buechner et al., 1990; Sugawara and Nikaido, 1994), to account for their slow level of permeation activity despite their wide channels (Benz and Hancock, 1981; Sugawara and Nikaido, 1994). Our results are also consistent with FadL existing in open and closed states.

C.1.7 Monitoring outer membrane permeability using the LCFA permeation assay

Gram-negative bacteria are intrinsically resistant towards most hydrophobic and amphipathic compounds including certain antibiotics (Nikaido, 1994c). Changes in the sensitivity of bacteria to these compounds, can reflect changes in OM permeability. This property has been exploited to monitor qualitative changes in OM permeability, caused by chemical permeabilization or mutation of genes involved in OM integrity or LPS

biosynthesis (Freese et al., 1973; Hancock, 1984; Nikaido and Vaara, 1985; Hancock, 1991; Nikaido, 1994c). However, monitoring quantitative changes in OM permeability is difficult and complicated by systems in *E. coli* that pump toxic compounds including antibiotics, out of the cell (Nikaido, 1994c). Figure 21 indicated that MBP-IAFBP labeling was sensitive to changes in OM permeability (due to EDTA permeabilization) and suggests that the photoaffinity permeation assay represents an alternative assay for directly monitoring general changes in OM permeability.

C.1.8. Conclusions

A novel method has been developed to directly monitor long-chain fatty acid (LCFA) permeation across the cell envelope of *E. coli*. This assay allowed the first direct demonstration of FadL-facilitated fatty acid permeation across the cell envelope. *E. coli* treated with the OM permeabilizing reagent EDTA also displayed increased labeling, demonstrating that this assay could be useful for monitoring general changes in OM permeability. Permeation of fatty acids through FadL occurs quickly but plateaus to a level well below equilibrium. Although FadL is required for fatty acid permeation, it also does not allow fatty acid concentrations to reach toxic levels. FadL appears to sense when levels of fatty acids are too high and inhibits further influx. The data therefore indicates that FadL may exist in open and closed states, to regulate fatty acid permeation.

Previous studies have shown that there is a coupling between LCFA uptake and metabolism since both FadL and FadD are required for LCFA uptake. Although the mechanism of this coupling is unknown, it now seems logical that these two processes

should be linked, since permeation of fatty acids into the cell in the absence of metabolism would lead to toxic buildup of fatty acids.

Previous observations suggested that LCFA uptake (FadL) and metabolism (FadD) are coupled such that uptake can not occur without FadD (Klein et al., 1971; Black and DiRusso, 1994). We show here however, that a certain level of LCFA permeation via FadL can occur in the absence of FadD and metabolism. The data also suggests that coupling of LCFA uptake and metabolism is mediated solely through FadL and does not involve both proteins.

We propose that FadL is normally in an open state to facilitate diffusion of LCFAs. When internal levels of fatty acid start to rise, FadL somehow senses this and restricts further permeation until levels start to come down due to metabolism. How FadL senses the levels of fatty acid in the periplasm is unknown, although it is possible that the high affinity LCFA binding site of FadL is actually involved in sensing LCFA concentration. This suggests that understanding the relationship between FadL mediated LCFA binding and permeation is important in testing the above model.

C.1.9. Future investigations

FadL affinity for LCFAs can be accurately determined using the photoreactive fatty acid (Mangroo and Gerber, 1992). The next step should involve determining the relationship between LCFA binding and permeation. The LCFA binding and permeation assays will allow the testing of the above hypothesis.

If our hypothesis is correct, disrupting FadL LCFA binding may keep the channel in an open state and actually increase permeation. An implication of this situation is that such FadL mutants may be lethal for *E. coli* if they are grown in media containing fatty acids. We made an initial attempt to identify regions or amino acids in FadL that are essential for regulating LCFA permeation. *In vitro* chemical mutagenesis was performed with plasmids containing the *fadL* gene to generate random mutants that might be defective in regulation. The mutated plasmids were transformed into *E. coli* and plated onto glucose minimal media plates. These plates were then replica plated onto fatty acid minimal media plates and the plates screened for colonies that could not grow on fatty acid media. Initial attempts did not identify any mutants but only a very small number of colonies were screened (< 2000 colonies). As a control, cells were treated with EDTA and plated onto fatty acid and glucose plates, and colonies were only observed in the latter plates. Refinement of this technique and further screening may identify the desired FadL mutants.

C.2 Tsp

Periplasmic permeases are commonly used by *E. coli* to transport nutrient molecules across the cytoplasmic membrane, and consist of three components: an outer membrane protein for facilitating diffusion across the OM, a periplasmic binding protein and a cytoplasmic multisubunit membrane protein complex (Nikaido, 1994a). The cytoplasmic membrane protein complex exhibits similarities to the eukaryotic ABC-type transporters (Nikaido, 1994a) and works in conjunction with a corresponding periplasmic binding protein to transport substrates across the cytoplasmic membrane. Although it was thought that *E. coli* does not use this type of system to transport long chain fatty acids (LCFAs), Black and colleagues implicated the periplasmic protease Tsp, in LCFA transport (Azizan and Black, 1994). The protease was identified in a transposon based random mutagenesis screen for cell envelope proteins required by *E. coli* for growth on LCFAs. They characterized Tsp deficient *E. coli* and discovered that these mutants exhibited two phenotypes, growth lag in fatty acid minimal media and alterations in LCFA uptake energetics (also see introduction A.5.2).

Tsp is a serine-type endoprotease (Keiler and Sauer, 1995) which displays specificity for proteins with specific hydrophobic C-terminal "tails". The protein substrates are usually cleaved after alanine or valine residues at discrete sites throughout the protein. The only clearly defined function attributed to Tsp is processing of penicillin binding protein 3 (Hara et al., 1989; Fraipont et al., 1994; Goffin and Ghuysen, 1998) to

its mature form and processing of TonB. Recently, a study showed Tsp or a related protease is also part of a system in *E. coli* involved in degrading polypeptides from mRNA lacking stop codons (Jentsch, 2001).

The role of Tsp in fatty acid uptake was unknown. If Tsp was directly involved in *E. coli* LCFA uptake, it was reasoned that the protease would be involved in one of the following ways, 1) It represented a periplasmic LCFA binding protein 2) It was responsible for FadD cleavage, or 3) It influenced FadL function.

C.2.1. Photolabeling of periplasmic fractions and overexpressed Tsp

Data suggesting that Tsp could be a periplasmic fatty acid binding protein included the presence of regions in Tsp that shared homology with the inter-photoreceptor binding protein, which are involved in binding hydrophobic ligands including fatty acids (Pepperberg et al., 1993). A group led by R.T. Sauer suggested that the IRBP-like domain of Tsp was involved in binding hydrophobic C-terminal protein substrates (Silber et al., 1992), while P.N. Blacks group postulated that this region was involved in binding fatty acids (Azizan and Black, 1994).

To investigate Tsp affinity for LCFAs, an IPTG inducible Tsp overexpression plasmid pKK101 kindly provided by R.T. Sauer, was transformed into the KS1000 cell line, which contained a deleted *tsp* gene (Silber and Sauer, 1994). Figure 22 (A) shows SDS-PAGE analysis of periplasmic fractions isolated from these cells. The addition of IPTG to these cells induced the expression of a protein close to the expected weight of

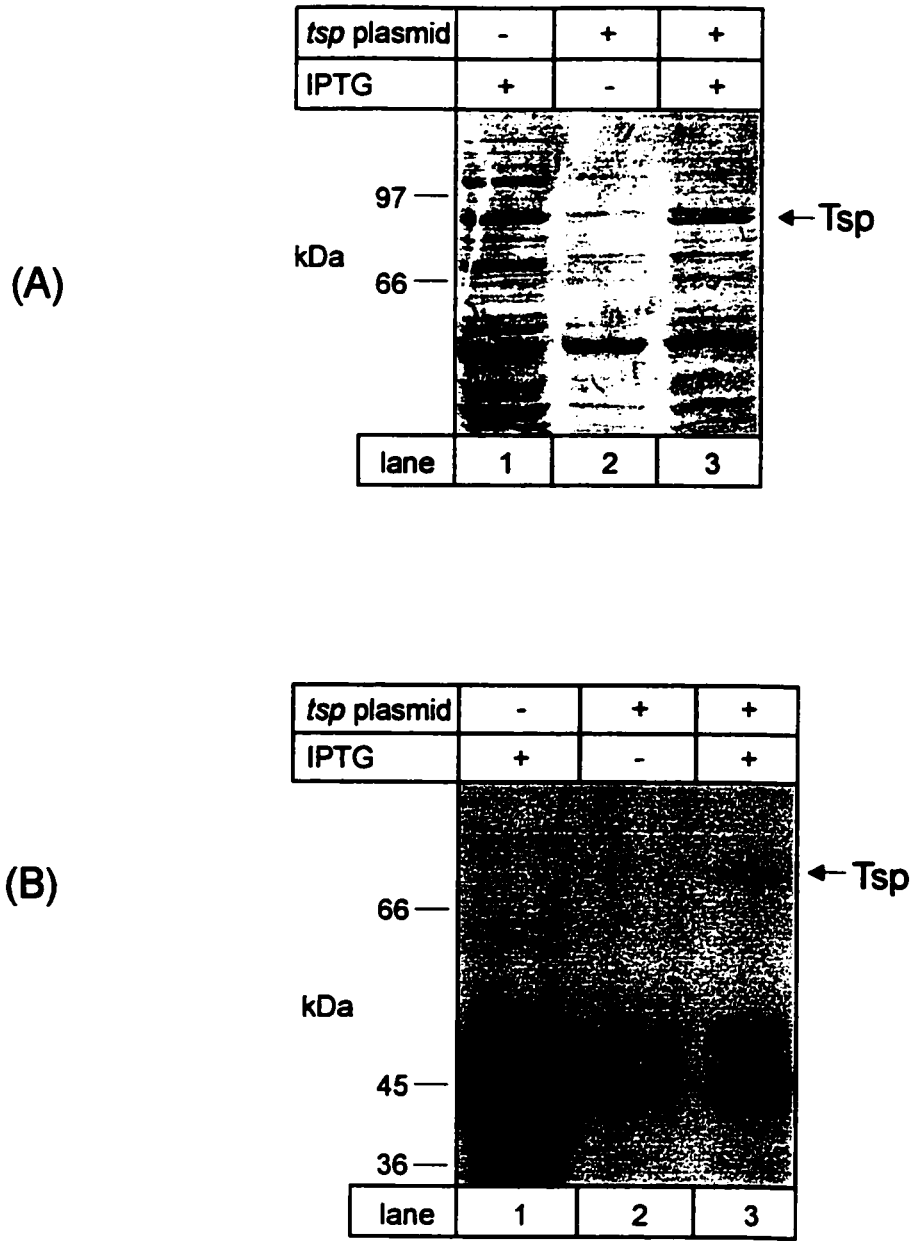


Figure 22. Expression and photolabeling of Tsp protease in the *tsp* deficient *E. coli* cell line KS1000. The *tsp* gene inactivated cell line, KS1000 was transformed with the Tsp expression plasmid +/- IPTG. Periplasm fractions were isolated using osmotic shock from these cells and untransformed KS1000 cells **(A)** SDS-PAGE analysis of periplasmic fractions stained with Coomassie Blue dye. **(B)** SDS-PAGE/fluorograph of periplasmic fractions labeled with 9 μM photoreactive fatty acid.

Tsp, which was 73.4-kDa (Hara et al., 1991). Analysis of the stained gel also revealed more proteins were present in the periplasmic fractions of KS1000 cells not containing the Tsp plasmid (Figure 22 (A) compare lane 1 with lanes 2 and 3). This was consistent with other studies, which showed that high levels of intracellular proteins are released into the medium from *E. coli* deficient in Tsp (Hara et al., 1991). Our results show that the Tsp expression plasmid appears to complement this phenotype since less protein was visible in the periplasm fractions of transformed cells. The periplasm samples containing overexpressed Tsp were then incubated with photoreactive fatty acid and photolabeled, which revealed the labeling of several proteins (Figure 22 (B)). However, labeling was performed with higher concentrations of photoreactive fatty acid than normally used for specific FadL labeling (Mangroo and Gerber, 1992) and film exposure time had to be extended to observe these labeled proteins. A faint labeled band migrating above the 66-kDa protein marker could only be seen in periplasm fractions containing overexpressed Tsp, indicating that it likely represented labeled Tsp. Considering Tsp was overexpressed with respect to the other periplasmic proteins, the intensity of Tsp labeling was very low and appeared to be non-specific, which indicated that Tsp was an inefficient fatty acid binding protein. These results are consistent with previous work done by (Mangroo and Gerber, 1992) whom failed to identify any fatty acid binding proteins in the periplasm or the cytoplasmic membrane (Also using concentrations of photoreactive fatty acid several hundred-fold higher then that normally used to label FadL). These results suggest that Tsp does not represent a periplasmic fatty acid binding protein and the IRBP-like regions in Tsp are involved in binding hydrophobic polypeptide substrates.

C.2.2. Role of Tsp in FadD cleavage

Circumstantial evidence initially linked Tsp and FadD together since the protease was implicated in fatty acid uptake and cleavage of FadD by an unknown protease was observed. As discussed in the FadD section, the 62-kDa FadD enzyme is cleaved into 45- and 19- kDa fragments when *E. coli* are lysed. FadD however, does not possess a consensus Tsp cleavage sequence (Keiler and Sauer, 1996) and direct interaction between the two proteins seemed unlikely since they are located in different subcellular compartments. It was possible though that FadD, which is thought to associate with membranes may insert a region of the enzyme into the cytoplasmic membrane bilayer and become cleaved by the periplasmic protease.

To determine if Tsp protease was responsible for cleavage of FadD, we overexpressed FadD in the *tsp* mutant strain (KS1000) and isogenic wild type strain (X90) and prepared crude cell lysates. Analysis of these samples by SDS-PAGE showed that FadD proteolysis occurred in both the Tsp-deficient and wild type cell line, indicating that Tsp protease is not required for FadD cleavage (data not shown, also see FadD section).

C.2.3. Role of Tsp in FadL function

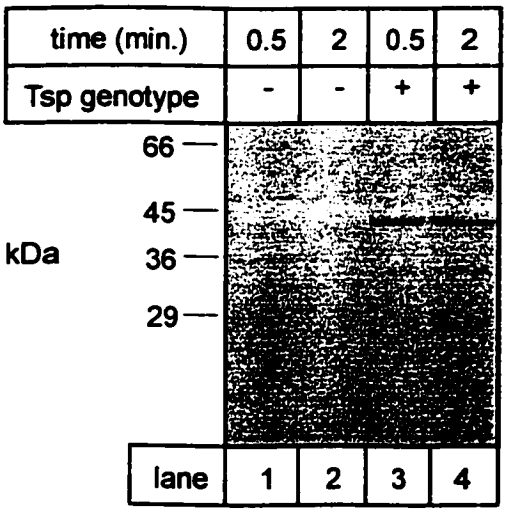
Tsp and FadL are located in a common subcellular location, which makes it possible for them to interact with each other. FadL function was therefore characterized in the context of a *tsp* background. Although FadL does not contain the consensus recognition sequence for Tsp (Keiler and Sauer, 1996), the C-terminus of FadL is

relatively hydrophobic and represented a potential substrate for Tsp. SDS-PAGE analysis and immunoblotting did not reveal any visible differences in the electrophoretic mobility of FadL expressed in wild type or *tsp* mutants (Figure 23 (A),(B)). This suggested that FadL was not a substrate for Tsp, although it was possible that proteolysis of FadL occurred at a site(s) very close to the ends of the protein making it difficult to be detected by SDS-PAGE analysis.

FadL has very high affinity for LCFAs (sub-micro molar) and has been suggested that another unknown component may be required for ligand release. It has been proposed that Tsp protease might be involved in facilitating the release of bound fatty acids from FadL (Azizan and Black, 1994). To determine FadL affinity for LCFAs in the context of the *tsp* background, *tsp* mutant and wild type cells overexpressing FadL were photolabeled (Figure 23 (A)). SDS-PAGE/fluorography analysis of these cells revealed much lower levels of FadL labeling in *tsp* mutants, which was either due to differences in FadL affinity for LCFAs or differences in the levels of FadL expressed. Immunoblotting of comparable samples with anti-FadL antibody verified the latter explanation Figure 23(B)). This showed that less FadL was overexpressed in *tsp* cells compared to wild type indicating that *tsp* mutants have a reduced ability to overexpress the outer membrane protein.

Figure 23 suggested that Tsp might be involved in modulating FadL expression. However, our results had to be interpreted with caution since we were not observing

(A)



(B)

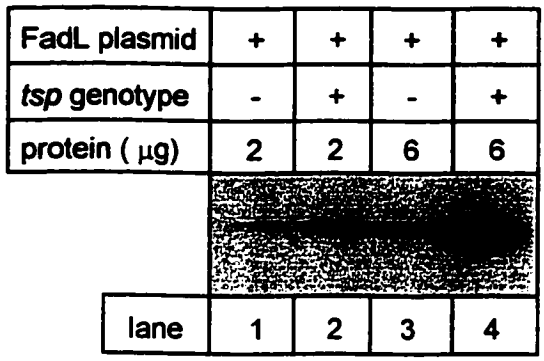
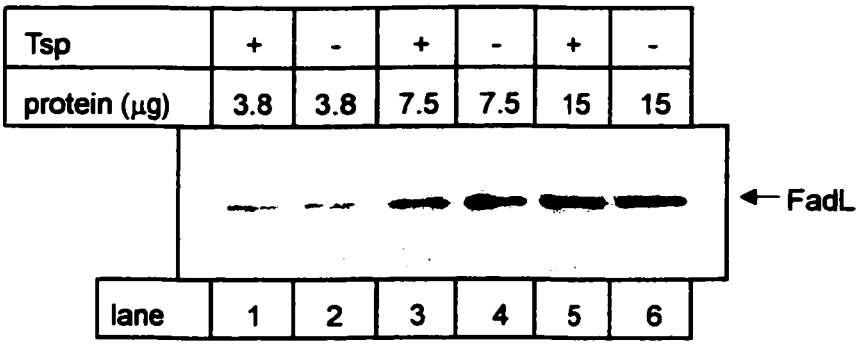


Figure 23. Photoaffinity labeling and immunoblotting of wild type (X90) and *tsp* deficient (KS1000) *E. coli* overexpressing FadL protein. **(A)** SDS-PAGE/fluorograph of *tsp* deficient (KS1000) and wild type (X90) *E. coli* cells transformed with a expression vector with (+) or without (-) the *fadL* gene, incubated and photolabeled with photoreactive fatty acid. **(B)** Immunoblot with anti-FadL antibody of KS1000 and X90 transformed with FadL expression vector.

native FadL expression. For this reason, the immunoblotting experiment was repeated using wild type and *tsp* mutant cells grown in oleate minimal media, so that endogenous FadL expression could be observed. Immunoblots of these cells (Figure 24(A)) revealed no apparent differences in the levels of FadL expressed between wild type and *tsp* cells. This suggested that Tsp was not exerting its effects on LCFA uptake by modulating native FadL expression. We then investigated the effect of the *tsp* mutation on FadL affinity for LCFAs *in vivo*. As Figure 24 (B) shows, there was no apparent difference in FadL affinity for LCFAs between wild type and *tsp* mutants. There were however, other differences in the labeling pattern seen between wild type (X90) and *tsp* mutant (KS1000) cells. Photoaffinity labeling of Tsp deficient cells revealed increased labeling of two proteins below the FadL band (Figure 24 (B), bottom two arrows and Figure 25 (A)). The reason behind the distinct labeling patterns was not readily apparent since the *tsp* mutant and wild type cells were genetically identical, other than the *tsp* genotype.

Interestingly, increased labeling of similar sized 32- and 35-kDa proteins were also observed during photoaffinity labeling experiments with *E. coli* cells permeabilized with EDTA or expressing FadL (Figure 25 (B) and (C), respectively). These results suggest that these proteins undergo increased labeling by photoreactive fatty acids due to increased permeation via FadL or chemical permeabilization. SDS-PAGE analysis of cell envelope fractions isolated from labeled cells indicated that the 32- and 35-kDa bands were cell envelope proteins (Figure 25 (B)). The apparent molecular

(A)



(B)

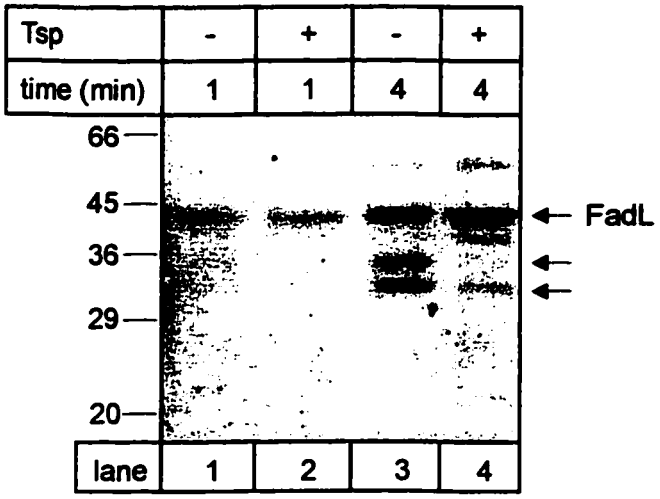


Figure 24. Immunoblot analysis and photoaffinity labeling of wild type and *tsp* deficient *E. coli* cells grown in fatty acid minimal media. **(A)** Immunoblot with anti-FadL antibody of *tsp* deficient (KS1000) and wild type (X90) *E. coli* grown in oleate minimal media. **(B)** SDS-PAGE/fluorograph of KS1000 and X90 cells grown in oleate minimal media labeled with photoreactive fatty acid.

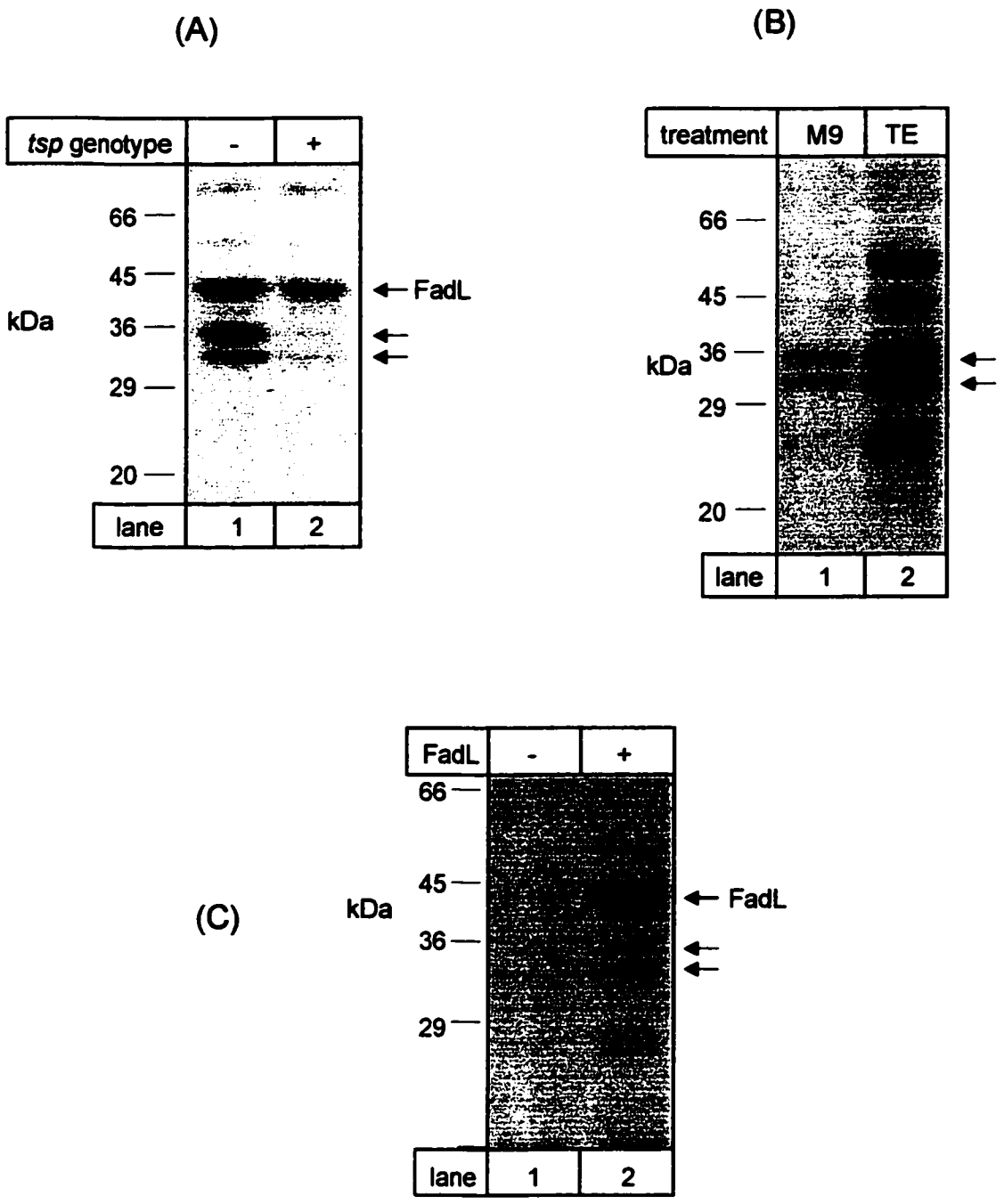


Figure 25. Effects of *tsp* mutation, EDTA permeabilization and FadL expression on labeling of the 35-and 32-kDa outer membrane proteins. **(A)** SDS-PAGE/fluorograph simlar to fig 24(B) **(B)** SDS-PAGE/fluorograph of cell envelope isolated from whole *E. coli* cells labeled with photoreactive fatty acid treated with M9 minimal media (M9) or Tris-EDTA (TE) **(C)** SDS-PAGE/fluorograph of *E. coli* cells transformed with vector or vector with *fadL* gene and labeled with photoreactive fatty acid.

weights suggested that they represented the OmpF/C (38,37-kDa) and OmpA (35-kDa) porins, two abundantly expressed OM proteins. Based on SDS-PAGE analysis and Coomassie Blue staining, there were no visible differences in the levels of cell envelope proteins expressed between wild type and *tsp* mutants (data not shown). This suggested that cell envelope proteins are labeled more intensely in *tsp* cells due to changes in OM permeability.

C.2.4. Role of Tsp in OM integrity

The results obtained thus far suggested that Tsp did not play a direct role in LCFA transport affirming the belief that only two proteins, FadL and FadD are required for LCFA transport. The only differences observed between wild type and *tsp* mutants were increased protein release into the medium, lower levels of FadL overexpression and increased labeling of outer membrane (OM) proteins in the *tsp* mutants. These observations implied that Tsp was required for maintaining OM integrity. Other studies have also suggested this role for Tsp as *E. coli* cells deficient in the protease are more sensitive to hydrophobic antibiotics (Seoane et al., 1992) and release large quantities of periplasmic proteins into the medium (Hara et al., 1991). Deficiency of Tsp protease did not appear to have drastic effects on OM permeability though since *tsp* mutants were not inhibited by LCFAs or detergent (Azizan and Black, 1994).

Since Tsp appeared to play an indirect role in LCFA uptake, it seemed the growth lag exhibited by *tsp* mutants in fatty acid minimal media might be a manifestation

of a disrupted OM. To test this, growth curves were repeated with *tsp* mutants in carbon sources other than oleate. As the graphs in Figure 26 (A) and (B) show, *tsp* mutants displayed growth lags in glucose minimal media and rich media (LB) respectively, indicating that mutation of *tsp* causes general growth defects.

C.2.5 Conclusion

Our data does not support a direct role for Tsp in LCFA uptake. Tsp appears to play a role in OM integrity since mutants have a reduced ability to overexpress FadL and increased permeability towards fatty acids. Our results are consistent with other studies, which proposed that *tsp* mutants had defects in OM structure. The growth lag exhibited by *tsp* mutants in fatty acid minimal media is a non-specific effect, as growth lags were also observed in other carbon sources. This general growth defect may also be a sign of a disrupted OM.

C.2.6. Future investigations

Immediate work should follow up on confirming the role of Tsp in OM integrity. This would include using the Tsp expression plasmid to verify that the effects of the *tsp* mutation are due to deficiencies in Tsp expression. This would involve repeating the growth curves and photoaffinity labeling experiments with *tsp* mutants transformed with the expression plasmid.

To verify that the Omp proteins are labeled in *tsp* mutants, immunoprecipitation

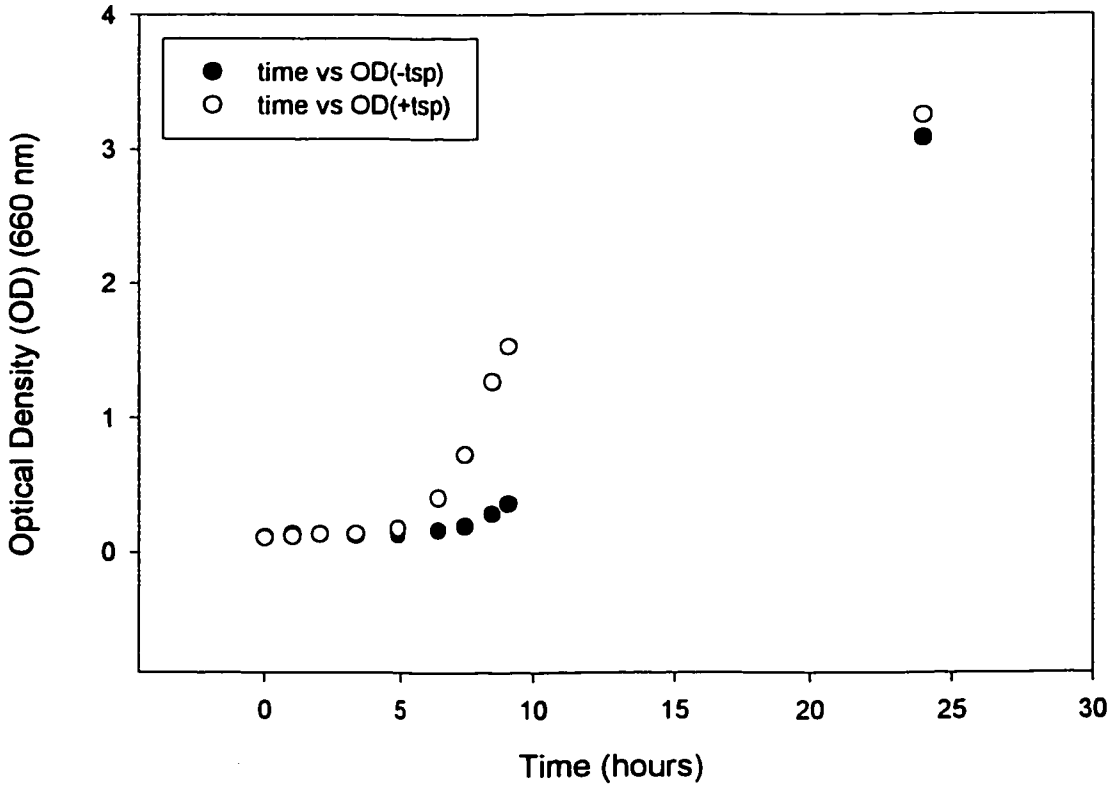


Figure 26. (A) Growth curves for wild type (X90) and *tsp* deficient (KS1000) *E coli* grown in glucose minimal media. Wild type (X90) and *tsp* mutant (KS1000) *E coli* were grown to mid-log in M9 minimal media + 0.5% glucose. They were washed once in M9 minimal media salts and resuspended in M9 minimal media + 0.5% glucose to similar O.D. and the growth monitored by O.D. at 600 nm.

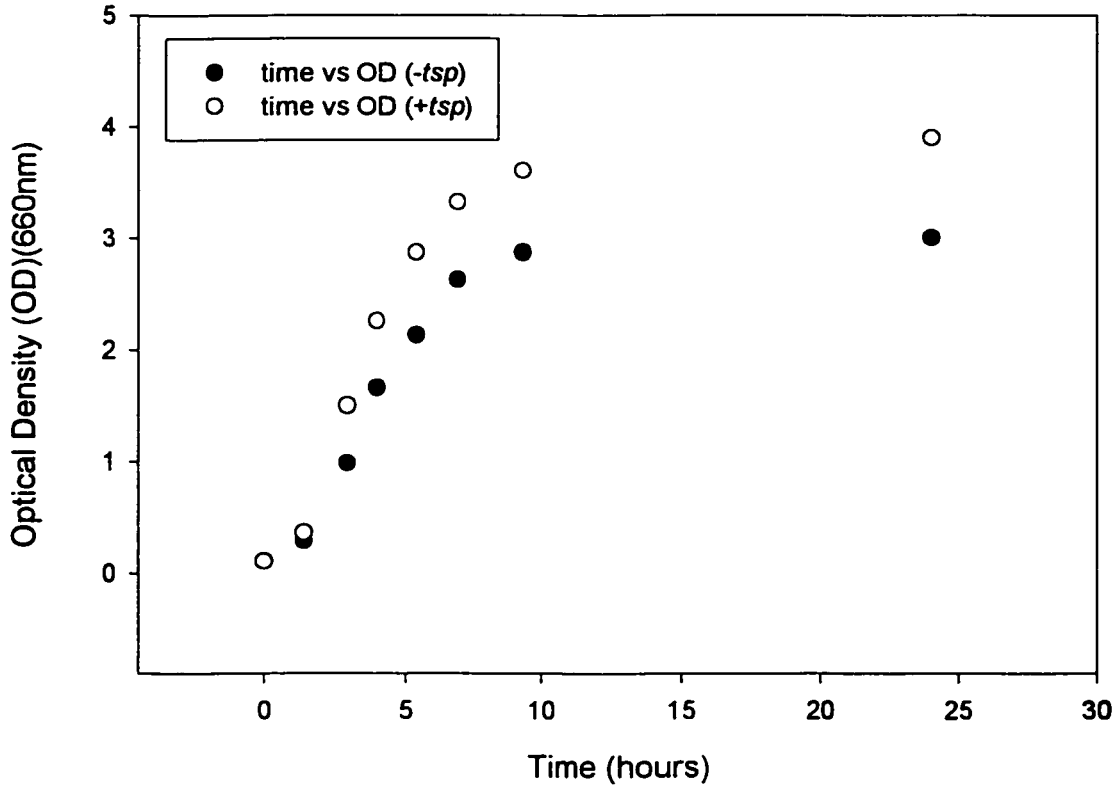


Figure 26. (B) Growth curves for wild type (X90) and *tsp* deficient (KS1000) *E. coli* grown in LB media. Wild type (X90) and *tsp* mutant (KS1000) *E. coli* were grown to mid-log in LB media. They were washed once in M9 minimal media salts and resuspended in LB to similar O.D. and the growth monitored by O.D. at 660 nm.

assays should be performed on labeled cell envelopes with antibodies raised against OmpF/C and OmpA. The fatty acid permeation assay developed in the FadL section should also be used to verify that *tsp* mutants have increased permeability to LCFAs. These experiments would further confirm the role of Tsp in OM integrity.

While the above proposed experiments only serve to confirm or verify the results already obtained, the physiological role of Tsp in OM integrity has yet to be addressed. Although Tsp processes penicillin-binding protein 3 *in vivo*, no phenotypes have been associated with unprocessed PBP3 (Hara et al., 1989). It would be interesting to see if *E. coli* expressing an un-cleavable form of PBP3 displays the same types of phenotypes associated with the *tsp* mutants. This would suggest that the effects of the *tsp* mutation are actually due to disruptions in PBP3 function and possibly peptidoglycan structure. If expression of un-cleavable PBP3 did not have similar effects, it would suggest that there is another unidentified substrate for Tsp that is required for OM stability.

C.3 FadD

Animal, plant and yeast cells express multiple forms of fatty acyl-CoA synthetase (FACS) (Suzuki et al., 1990; Knoll et al., 1994; Harington et al., 1994; Johnson et al., 1994; Iijima et al., 1996; Fujino et al., 1997; Fulda et al., 1997). Sequencing of the *Mycobacterium tuberculosis* genome revealed that the bacterium devoted a large number of genes to lipid metabolism, including 36 FadD homologues (Cole et al., 1998). In contrast, the *Escherichia coli* fatty acyl-CoA synthetase, FadD (fatty acid:CoA ligase, AMP-forming, EC 6.2.1.3), represents the only known fatty acyl-CoA synthetase in this organism (Kameda and Nunn, 1981; Black et al., 1992; Fulda et al., 1994).

However, several studies have described purified native FadD as a 42-50-kDa enzyme (Kameda and Nunn, 1981; Kameda et al., 1985; Kameda and Imai, 1985), while others have reported that FadD is a 62-kDa protein based on sequencing (Black et al., 1992; Fulda et al., 1994) and expression data from the *fadD* gene (Black et al., 1992). Complicating things further are reports of FadD isoforms distinguished based on isoelectric point (Kameda and Imai, 1985) and substrate specificity (Samuel et al., 1970). The nature of these different forms of FadD and their relationship to each other has remained unclear.

Recent studies have started to unravel information regarding the structure of FadD. The *fadD* gene possesses a region of homology with other FACS called the fatty

acyl-CoA synthetase (FACS) signature motif (Black et al., 1992). The FACS signature motif is a 25-amino acid consensus sequence present in all FACS that is thought to play a role in fatty acid substrate specificity and binding (Black et al., 1998; Black et al., 2000). FadD and other FACS also belong to the AMP-binding protein family which is composed of adenylate forming enzymes and is characterized by specific signature motifs (Black et al., 1992; Fulda et al., 1994).

C.3.1. Overexpression of FadD

A FadD overexpression system was constructed to overcome technical problems associated with purifying native fatty acyl-CoA synthetase from *E. coli* including enzyme instability and low yields of active protein. Figure 27(A) is a schematic of the IPTG-inducible plasmid used for FadD overexpression. Figure 27(B) shows the overexpression of FadD from *E. coli* transformed with the expression plasmid induced with IPTG and lysed by one of two methods. When cells were lysed by boiling (4 min) in the presence of 5 % SDS (SDS-PAGE sample buffer) and immediately analyzed by SDS-PAGE, an induced 62-kDa protein was observed (lane 3, arrow labeled "intact FadD" compare with lane 1). In contrast, when these cells were lysed with lysozyme following exposure to Tris-EDTA (see materials and methods), the 62-kDa band was not observed. Instead, two other polypeptides (45- and 19-kDa, labeled "Fragment 1 and 2" in figure 27(B), lane 4) appeared to be induced. We reasoned the incubation time required for the Tris-EDTA/lysozyme method led to proteolytic digestion of the 62-kDa FadD

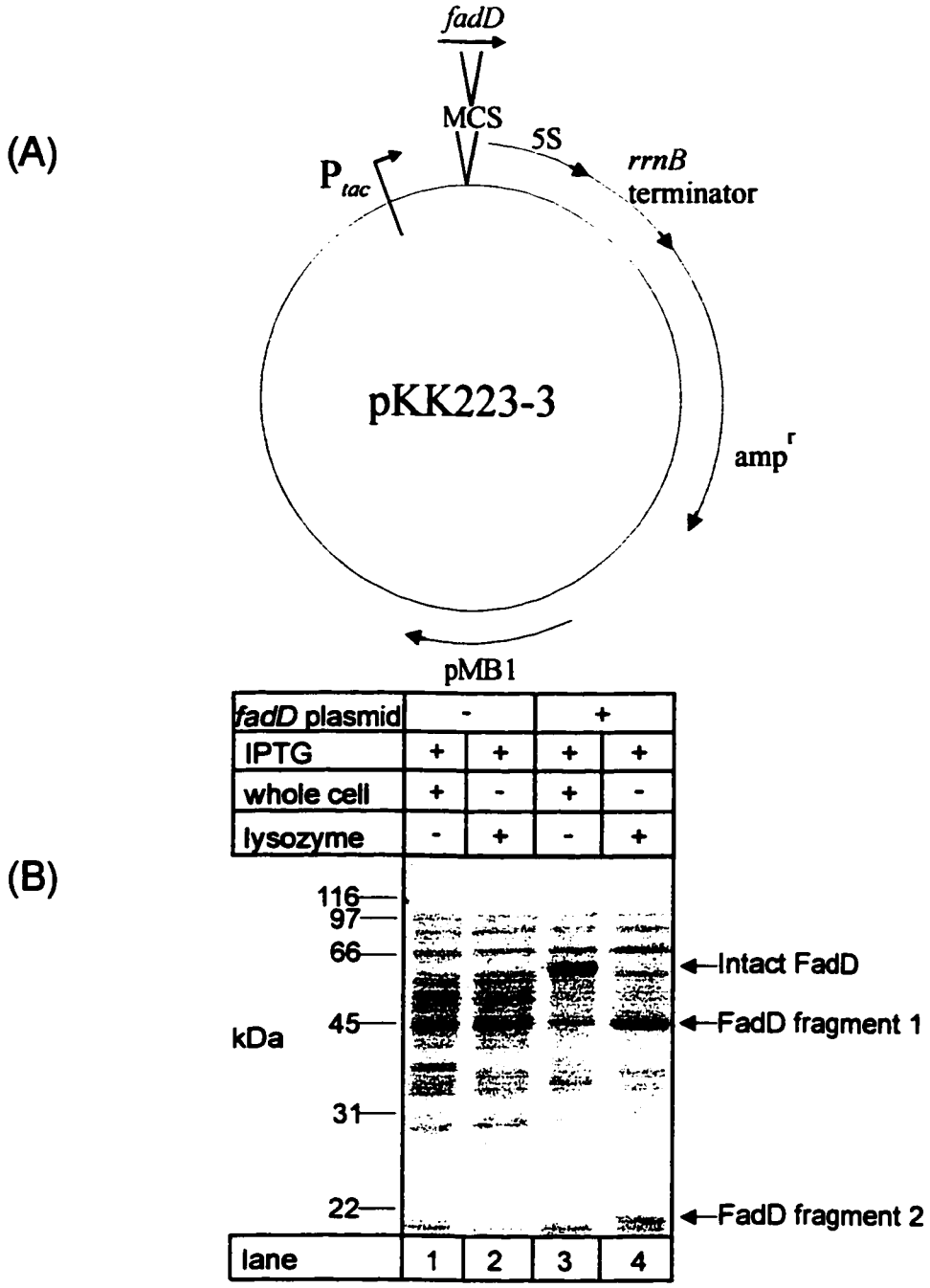


Figure 27. Overexpression of FadD using the IPTG inducible expression vector pKK223-3. **(A)** Schematic of pKK223-3 plasmid, with the *fadD* gene inserted into the multiple cloning site (MCS). **(B)** SDS-PAGE analysis of JM105 *E. coli* transformed with the pKK223-3** plasmid containing the *fadD* gene (+, lanes 3,4) or the plasmid alone (-, lanes 1,2). These cells were grown and induced with 1 mM IPTG before being boiled in SDS-PAGE sample buffer (lanes 1,3) or boiled in SDS-PAGE sample buffer after lysis by EDTA/lysozyme (lanes 2,4). The gel was visualized by Coomassie Blue staining.

protein, resulting in the formation of the 45- and 19-kDa fragments. The molecular weight of fragment 1 was similar to the previously described weight for purified native FadD. Although the relevance of cleaved FadD was unclear, the data showed for the first time that two forms of FadD actually existed. To determine the effect of cleavage on FadD LCFA binding activity, we performed photoaffinity labeling experiments with cleaved FadD. *E. coli* cell lysates produced by Tris-EDTA/lysozyme treatment, which were incubated with photoreactive fatty acid, subjected to photolysis and analyzed by SDS-PAGE/fluorography. Intense labeling of 62-kDa and 45-kDa proteins was observed (Figure 28, lane 2) indicating that both intact and cleaved FadD displayed affinity for LCFAs.

The LCFA binding region of FadD has been partially characterized by (Black et al., 2000) using a different photoreactive fatty acid, which contained an azido group esterified to the carboxyl of a long chain fatty acid. Identification of the fatty acid attachment sites revealed that labeling mostly occurred at sites just outside the FACS signature motif, which is thought to bind the acyl end of LCFAs. Since the photoreactive fatty acid used in our work utilizes a diazirinophenoxy group at the omega carbon of the fatty acid (Leblanc et al., 1982), it is possible that our photoreactive fatty acid may derivatize amino acids forming the FACS signature motif.

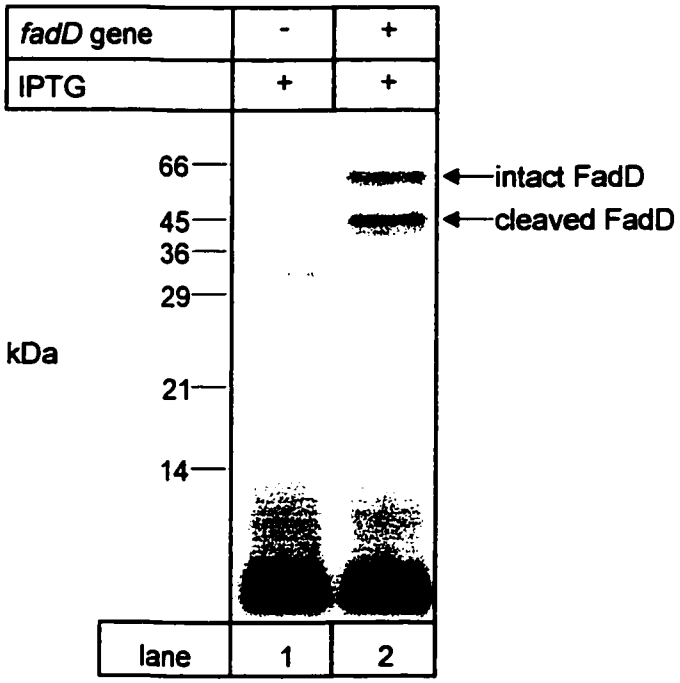


Figure 28. Photoaffinity labeling of intact and cleaved FadD with photoreactive fatty acid. SDS-PAGE/fluorograph of cell lysates prepared from JM105 *E. coli* transformed with pKK223-3 plasmid (lane 1) or pKK-223-3 with the *fadD* gene insert (lane 2) and incubated with 11-diazirinophenoxy-[11-^{3}H]-undecanoate and exposed to high intensity 365 nm radiation.

C.3.2. Role of OmpT protease in cleavage of FadD *in vitro*

It was unknown if cleavage of FadD represented a physiologically relevant event. To determine if processing of FadD occurred *in vivo*, we overexpressed FadD under various inducing and non-inducing conditions (growth in oleate and glucose minimal media respectively) as well as switching carbon sources and brief incubation without any carbon sources (starvation). Under all these conditions however, cleavage of the 62-kDa protein to the cleaved form could not be observed by SDS-PAGE (data not shown). Tsp protease had been implicated in *E. coli* LCFA uptake but its role was unclear (Azizan and Black, 1994). To determine if Tsp was responsible for cleavage of FadD, we overexpressed the FACS in the Tsp deficient strain KS1000 and the isogenic wild type strain, X90. Lysates were prepared from these cells using Tris-EDTA/lysozyme and then analyzed by SDS-PAGE. Cleaved FadD was observed in both the Tsp-deficient as well as wild type cells, indicating that Tsp protease is not required for FadD cleavage (see Figure 29(B)). The identity of the protease responsible for FadD cleavage remained unknown.

Oscar Cheng carried out purification of intact and cleaved FadD enzyme (Cheng, 1998). During development of the purification scheme, it was found that Triton X-100 detergent increased the efficiency of FadD cleavage while oleate inhibited proteolysis *in vitro*. These observations were subsequently used for purifying both forms of the enzyme, although purification of intact FadD without any cleaved enzyme was difficult. The effects of cleavage on FadD function were also investigated, which revealed that cleaved FadD exhibited over two-fold higher V_{max} and apparent K_m values

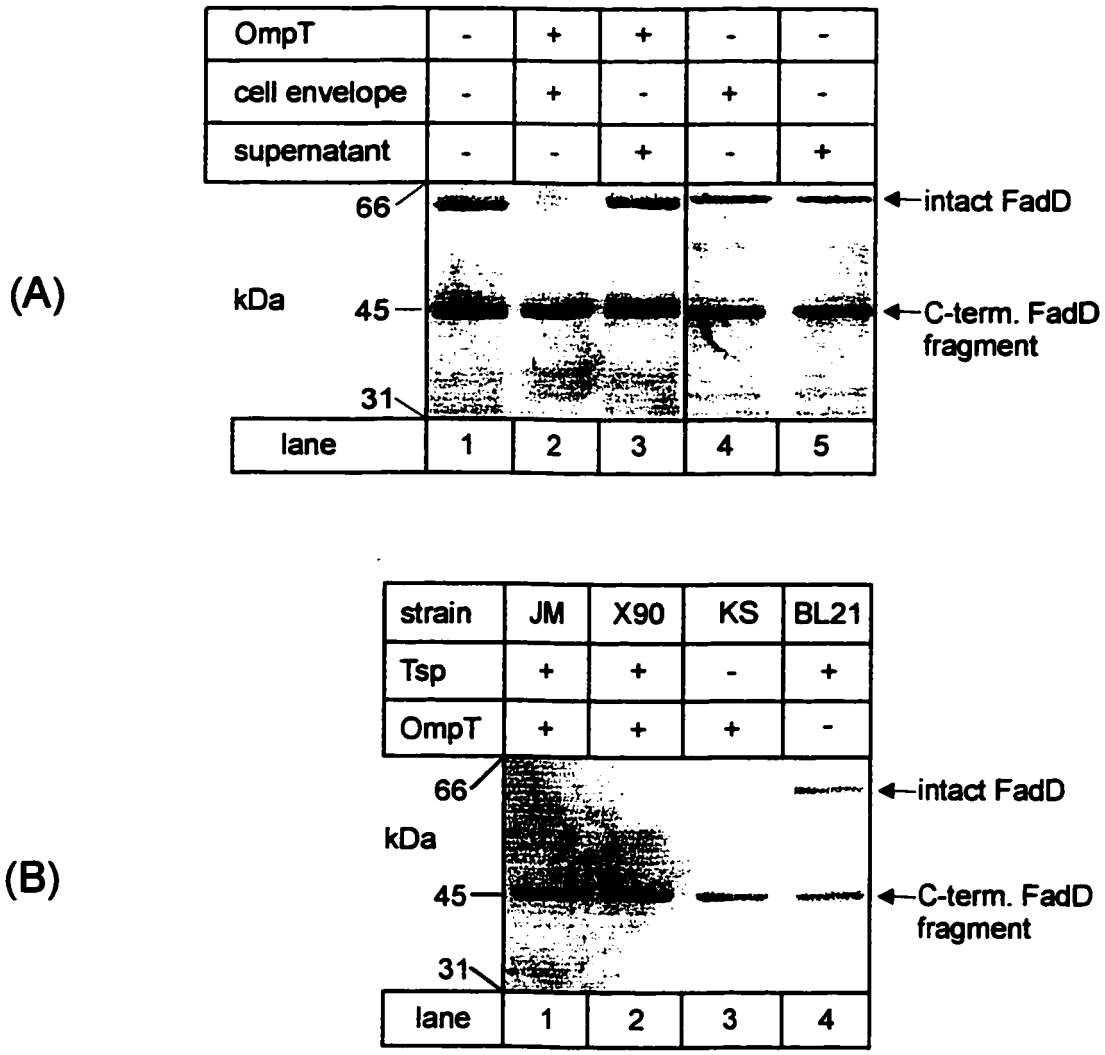


Figure 29. Incubation of partially purified FadD with *E. coli* cell envelope fractions or whole cells. **(A)** SDS-PAGE analysis of partially purified FadD (lane 1) incubated with cell envelope or supernatant fractions from wild type JM105 (lanes 2 and 3) or *ompT* deficient BL21(DE3) (lanes 4 and 5) *E. coli*, visualized by Coomassie staining. **(B)** SDS-PAGE analysis and Coomassie staining of partially purified FadD incubated with various intact *E. coli* cells. The following cell lines were used JM-(JM105), X90-(X90), KS-(KS1000), BL21-(BL21(DE3).

than intact FadD. The k_{cat}/K_m values for the two forms were similar though, indicating that the catalytic efficiency was unaffected by cleavage. At this point, there were no obvious functional differences between cleaved and intact FadD *in vitro*. Purified cleaved FadD fragments were analyzed by protein microsequencing to determine the N-terminal amino acid sequence for each fragment. From these results, it was determined that FadD cleavage occurred between amino acid residues lysine 172 and arginine 173. Cleavage at this site predicted the formation of a 43.0 kDa C-terminal and a 19.4 kDa N-terminal fragment. Although the C-terminal fragment appeared to migrate as a 45-kDa polypeptide on a SDS-polyacrylamide gel, mass spectrometry performed on this fragment confirmed that it was 43.0 kDa (O. Cheng, personal communication). The data thus far suggested that the protease(s) responsible for cleaving FadD displayed a preference for cleavage sites containing basic residues. As shown in figure 27, cleavage of FadD only occurred after cell lysis suggesting that FadD and the unidentified protease were located in different subcellular compartments. Since FadD is a cytoplasmic enzyme, it suggested that the unidentified protease might be located in the cell envelope. To test this, *in vitro* cleavage assays were performed by incubating partially purified FadD with *E. coli* cell envelope or supernatant fractions. The partially purified FadD substrate was prepared using a modified purification protocol by (Cheng, 1998) (see materials and methods, section B.2.12).

Lysis of *E. coli* cells and separation by centrifugation results in two fractions. The soluble fraction contains proteins found in the cytoplasm and periplasm while the pellet fraction contains membrane and cell wall associated proteins. As shown in figure

29 (A), cleavage of FadD only occurred with cell envelope fractions (compare lanes 2 and 3) suggesting that the protease(s) responsible for cleavage was associated with the cell envelope. It should be noted that there was a significant amount of cleaved 43-kDa FadD fragment already present in the partially purified preparation (Figure 29 (A), bottom arrow). This was the result of cleavage that occurred during purification of partially purified FadD since oleate (which inhibits lysis, see previous page) was not added to avoid potential inhibition of cleavage during subsequent *in vitro* proteolysis assays.

Investigation of known membrane associated proteases suggested OmpT, a serine type protease with specificity for di-basic residues (Grodberg and Dunn, 1988), might be responsible for FadD cleavage (Sedgwick, 1989; Baneyx and Georgiou, 1990). To test this hypothesis, cell envelope and supernatant fractions from the OmpT-deficient cell line BL21(DE3) were incubated with partially purified FadD. Cell envelope fractions from the OmpT-negative cells did not appear to cleave intact FadD (Figure 29 (A), compare lanes 2 and 4), suggesting that OmpT was required for cleavage. The active site of OmpT is thought to reside on the cell surface (Grodberg and Dunn, 1988). Consistent with this, we found that FadD was cleaved when incubated with intact *E. coli* cells expressing OmpT (Figure 29 (B), lanes 1-3) but not with intact OmpT-deficient cells (BL21(DE3)) (lane 4). Due to the presence of cleaved FadD in the partially purified substrate, we could not absolutely rule out the possibility that some of the intact FadD was cleaved during incubation with cell envelope fractions or intact *E. coli* cells deficient in OmpT. These results strongly suggested that OmpT cleaved the 62-kDa FadD enzyme

to produce an enzymatically active complex composed of the 19-kDa N-terminal and the 43-kDa C-terminal FadD fragments. In addition, localization of OmpT activity to the cell surface further confirmed that FadD cleavage did not represent processing *in vivo*. In fact, OmpT deficient *E. coli* grew on oleate minimal media, indicating that the protease was not required for growth on fatty acids (data not shown).

Subsequent to these findings, O Cheng generated antibodies against the 45-kDa FadD fragment which recognized both intact 62-kDa FadD and the 45-kDa fragment. These were used to show that only intact FadD could be detected *in vivo,* in *E. coli* grown on oleate minimal media. This finding was consistent with the above results and again indicated that cleaved FadD was a product of degradation due to cell lysis.

The native substrate(s) for OmpT is unknown but is responsible for proteolysis of many proteins *in vitro* (Grodberg and Dunn, 1988; Hatzfeld et al., 1992; Henderson et al., 1994; Kycia et al., 1995) and *in vivo* (Baneyx and Georgiou, 1990; Skare et al., 1993), which have led to artefactual observations. An implication of the above findings is that some studies may have purified and characterized cleaved FadD enzyme and future studies should therefore be cautious of *in-vitro* proteolysis.

FadD cleavage appears to explain the differences in molecular weight of purified FadD from endogenous and plasmid encoded sources since some groups had used the OmpT deficient cell line BL21(DE3) to express the cloned *fadD* gene (Black et al., 1992). In addition, proteolysis may also explain the reported isolation of different substrate specific and charge isoforms (Samuel et al., 1970; Kameda and Imai, 1985) of *E. coli* FACS.

C.3.3. Overexpression and purification of MBP-FadD fusion protein

Since FadD was cleaved by OmpT protease, it prompted us to attempt to overexpress FadD in the OmpT-deficient cell line BL21(DE3), as a means of 1) confirming that OmpT was responsible for FadD cleavage and 2) purifying intact FadD free of the cleaved form. However, attempts at transforming the OmpT-deficient cell line with the expression vector were unsuccessful. We therefore constructed a FadD fusion protein expression system to facilitate the purification of FadD in an OmpT deficient cell line.

The FadD fusion protein expression plasmid was constructed by inserting the *fadD* gene into the pMAL-p2 plasmid (see figure 15(B)) which fused it to the C-terminus of maltose binding protein (MBP). This plasmid was transformed into the OmpT deficient cell line BL21(DE3) and induced to express by IPTG. Figure 30 shows the overexpression of a protein between the 97.4-kDa and 116-kDa protein markers, close to the expected combined weight of MBP (43-kDa)(Duplay et al., 1984) and FadD (62-kDa)(Black et al., 1992).

The MBP-FadD fusion protein remained uncleaved after cell lysis with Tris-EDTA/lysozyme and during the purification process. Cell lysates containing the fusion protein also displayed high levels of long-chain fatty acyl-CoA synthetase activity (Table 2). SDS-PAGE analysis of the affinity purified MBP-FadD fusion protein revealed that it

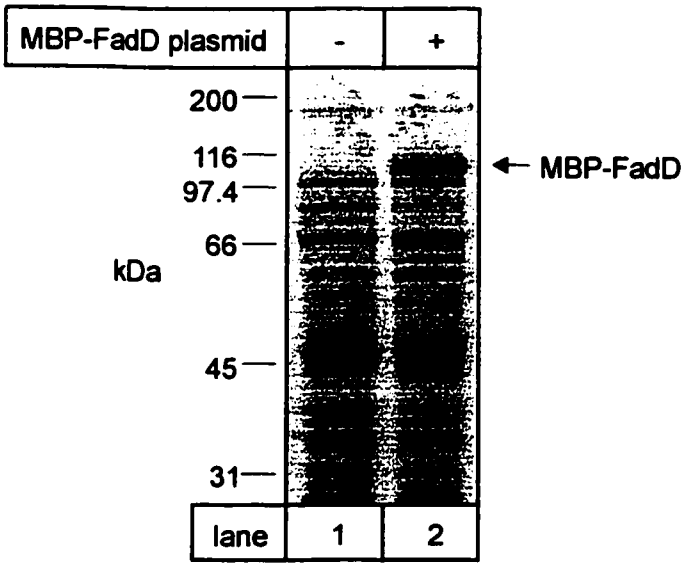


Figure 30. Overexpression of MBP-FadD fusion protein. BL21 (DE3) *E. coli* were transformed with the pMAL-p2 plasmid containing the *fadD* gene (+, lanes 2) or the plasmid alone (-, lanes 1). These cells were grown and induced with 1 mM IPTG before being boiled in SDS-PAGE sample buffer and analyzed by SDS-PAGE, followed by Coomassie Blue staining.

Table 2. Purification table for MBP-FadD fusion protein.

Procedure	Total protein	Total Recovery	Total Activity	Recovery of activity	Specific activity
	(mg)	(%)	(U[a])	(%)	(U/mg)
clear lysate	239.4	100	595, 333	100	2486
solubilized membrane	40.6	16.9	99, 190	16.7	2446
detergent soluble	3.7	1.5	43, 366	7	11, 721
maltose eluted	0.24	0.1	591	0.01	2463

a- U refers to units which is defined as pmole of product formed/min

consisted of a major band of approximately 105-kDa and a minor amount of a 62-kDa protein (Figure 31 (A), lane 6). Both proteins were detected with the anti-C-terminal FadD antibody, confirming that the 105-kDa protein was the FadD fusion protein (Figure 32 (A), lane 3). The 62-kDa protein appeared to co-elute with the FadD fusion protein and represent FadD generated by non-specific cleavage. Table 2 represents a purification table for the FadD fusion protein with a large amount of activity apparently lost during the final step of elution with maltose.

The region of the fusion protein connecting MBP to FadD encodes a Factor Xa cleavage site. To ensure that FadD could be separated from the MBP moiety, we cleaved the fusion protein with Factor Xa. Treatment of the fusion protein with the protease produced two major fragments, a 45- and 62- kDa species (data not shown) with the 62-kDa band likely representing FadD, while the 45-kDa protein corresponded to MBP.

C.3.4. Effect of FadD ligands on OmpT proteolysis

As mentioned in section C.3.3, proteolysis of FadD by OmpT was sensitive to the presence of detergents and fatty acids. While fatty acids are a substrate for FadD, detergents are thought to represent a ligand since FadD requires a lipid interface for catalysis (Mangroo and Gerber, 1993; DiRusso et al., 1999). Considering that proteolysis of FadD was affected by the presence of a substrate and a likely ligand, we decided to pursue structure-function analysis using limited proteolysis (Wilson, 1991) with OmpT protease.

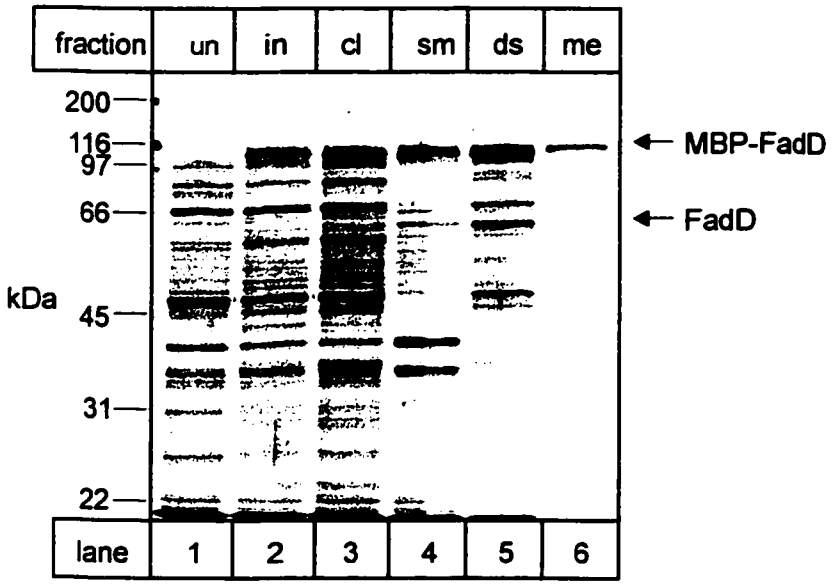


Figure 31. Purification of the MBP-FadD fusion protein. BL21(DE3) cells were transformed with the MBP-FadD expression plasmid and grown to mid-log in LB media (lane 1) before being treated with IPTG for 90 minutes (lane 2). The cells were harvested and washed before passage through a french press, three times. The resulting lysate was spun at 15,000 *g* for 15 min. and the supernatant containing cleared lysate (**cl**, lane 3) recovered. The cleared lysate was then spun at 100,000 *g* for 60 min and the resulting supernatant discarded. The total membrane pellet was then resuspended with buffer A (25 mM Tris pH 7.5, 25 mM NaCl, 0.25% Triton X-100), to obtain solublized membrane sample (**sm**, lane 4). This suspension was then spun at 100,000 *g* for 60 minutes and the supernatant recovered (detergent soluble fraction, **ds**, lane 5). This fraction was then incubated with amylose resin (New Eng. Biolab.) and washed with buffer A containing 1mM β-mercaptoethanol. The loaded resin was then resuspended with buffer A containing 10 mM maltose and the supernatant saved (maltose elutant, **me**, lane 6). Samples from each step were analyzed by SDS-PAGE and Coomassie Blue staining.

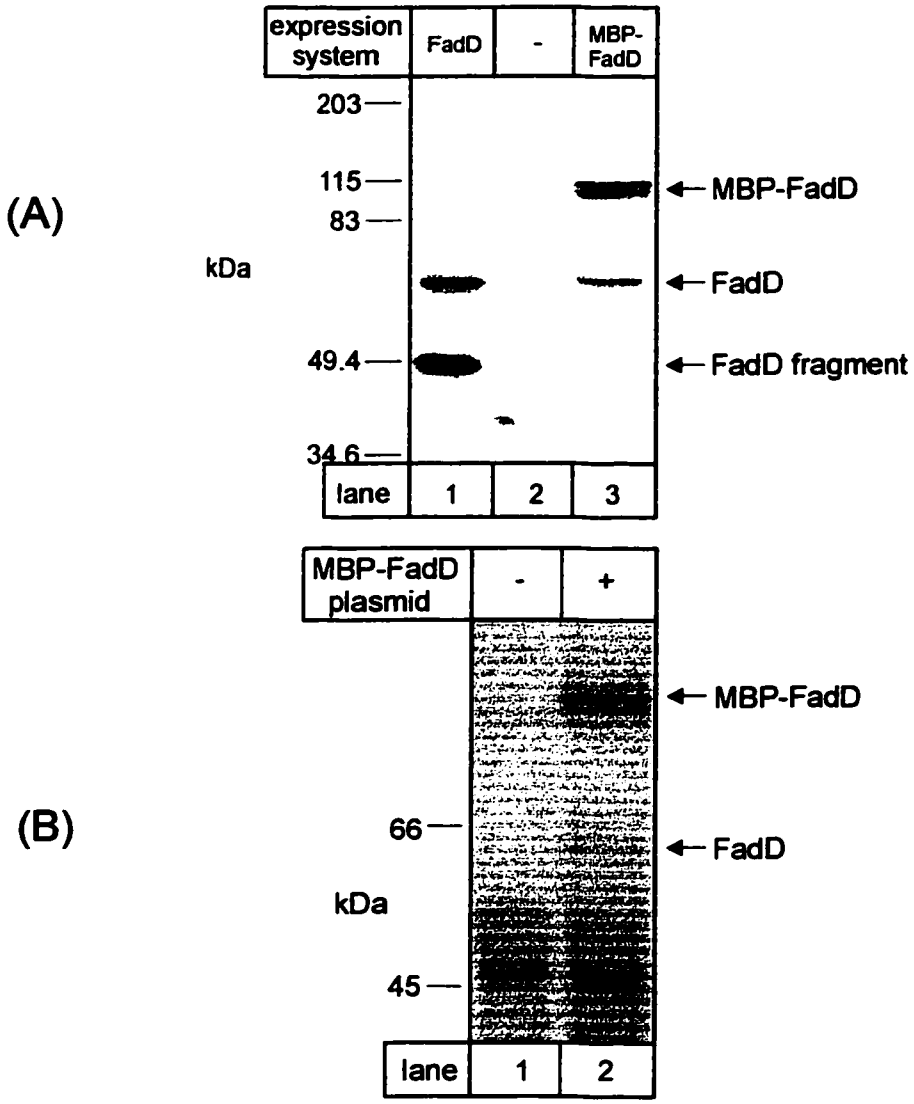


Figure 32. Characterization of the MBP-FadD fusion protein **(A)** Immunoblot analysis with anti-C-terminal FadD antibody of JM105 cells transformed with the FadD overexpression plasmids, pJ8 (pKK223-3** based) and pMD62 (pMAL-p2 based). *Lane 1* consists of lysates prepared from JM105 transformed with the FadD expression vector with the top band representing intact FadD (62 kda) and the bottom band representing the C-terminal FadD fragment (45 kDa). *Lane 2* samples were prepared by harvesting and boiling JM105 transformed with pMAL-p2 with SDS-PAGE sample buffer. *Lane 3* samples were prepared in a manner similar to lane 2 samples, except JM105 cells were transformed with MBP-FadD expression vector. **(B)** Photolabeling with the photoreactive fatty acid was performed on lysates from JM105 cells transformed with pMAL-p2 (lane 1) or pMal-p2 + *fadD* gene (lane 2). Lysates were prepared by taking IPTG induced cells and treating them with Tris-EDTA/lysozyme. These lysates were then incubated with photoreactive fatty acid and photoactivated. These samples were then analyzed by SDS-PAGE and fluorography.

Purification of intact MBP-FadD fusion protein allowed us to test the effects of Triton X-100, oleate, ATP and CoA on cleavage of FadD *in vitro*. Like intact native FadD, purified MBP-FadD was cleaved after incubation with intact *E. coli* cells expressing OmpT, yielding two protein fragments, 45-kDa and 60-kDa in size (Figure 33 (A), lane 2). The 45-kDa band represented the C-terminal FadD fragment since it was detected with the anti-FadD antibody specifically raised against that fragment (Figure 33 (B), lane 1). The 60-kDa band could not be detected with the FadD antibody indicating that it represented MBP (43-kDa) fused to the 19-kDa N-terminal FadD fragment. Smaller, faint bands below the 45-kDa fragment could also be seen by Coomassie staining and immunoblotting, indicating that they represented minor degradation products of the C-terminal FadD fragment. Cleavage of MBP-FadD did not occur when the fusion protein was incubated with OmpT deficient *E. coli* cells (data not shown) confirming again that OmpT cleaves FadD.

The various ligands and substrates for FadD were then added separately to see what effect they had on proteolysis of MBP-FadD by OmpT. Addition of Triton X-100 detergent to the digestion reactions resulted in more efficient cleavage of MBP-FadD (Figure 33 (A), compare lane 2 with 3) Conversely, the addition of oleate, prevented proteolysis (figure 33 (A), lane 4). Addition of ATP enhanced proteolysis of the fusion protein and even blocked the effects of oleate (Figure33 (B), compare lane 1 with lanes 3 and 5). Addition of CoA, another substrate for FadD, had no effect on MBP-FadD cleavage (Figure 33 (B), lane 4), which was expected since CoA is bound after ATP and the LCFA (see pg. 33, equation (1)).

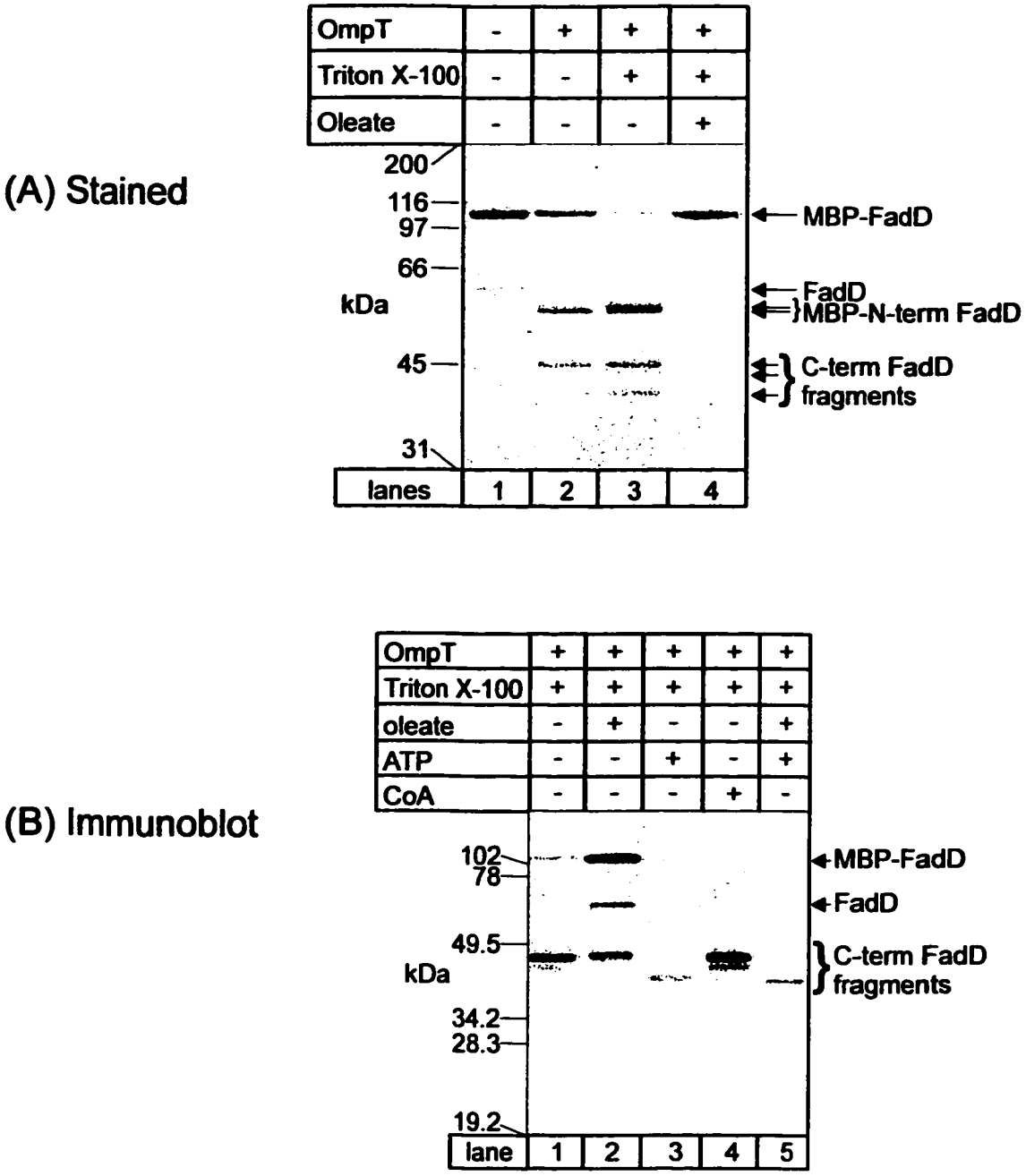


Figure 33. Effect of detergents and FadD substrates on the proteolysis of maltose binding protein-FadD (MBP-FadD) fusion protein. Purified MBP-FadD fusion protein was incubated with various ligands and Triton X-100 before incubation with intact JM105 *E. coli* cells. The reactions were subjected to centrifugation and the supernatants analyzed by SDS-PAGE/Coomassie staining (A) or immunoblotting with antibodies raised against the C-terminal FadD fragment (B).

To ensure that the FadD substrates and ligands themselves were not affecting the enzymatic activity of OmpT protease, the proteolysis experiments were repeated with another OmpT substrate, T7 RNA polymerase (Grodberg and Dunn, 1988). As figure 34 shows, Triton X-100, oleate and ATP did not affect the ability of OmpT-expressing cells to cleave T7 RNA polymerase, indicating that the effects of these molecules on FadD cleavage were specific.

These results imply that interaction of FadD with detergents or its substrates results in the exposure or protection of OmpT cleavage sites caused by conformational changes in FadD. These observations also show that OmpT is a useful probe for detecting changes in FadD conformation.

C.3.5. Investigation of FadD structure-function relationships

OmpT-mediated cleavage of FadD enzyme results in the formation of an enzymatically active complex comprised of a 19-kDa N-terminal and a 43-kDa C-terminal fragment that remain associated, which also indicated that the OmpT cleavage site was not essential for enzyme activity. Previous work from our laboratory had shown that FadD is activated in the presence of Triton X-100 and bacterial membranes (Mangroo and Gerber, 1993). *E. coli* pyruvate oxidase is a lipid-surface activated enzyme that is sensitive to limited proteolysis by chymotrypsin. Proteolysis of pyruvate oxidase by chymotrypsin causes the enzyme to be active in a lipid-independent manner (Hamilton et al., 1986; Grabau and Cronan Jr, 1986; Grabau et al., 1989). Cleavage of FadD by

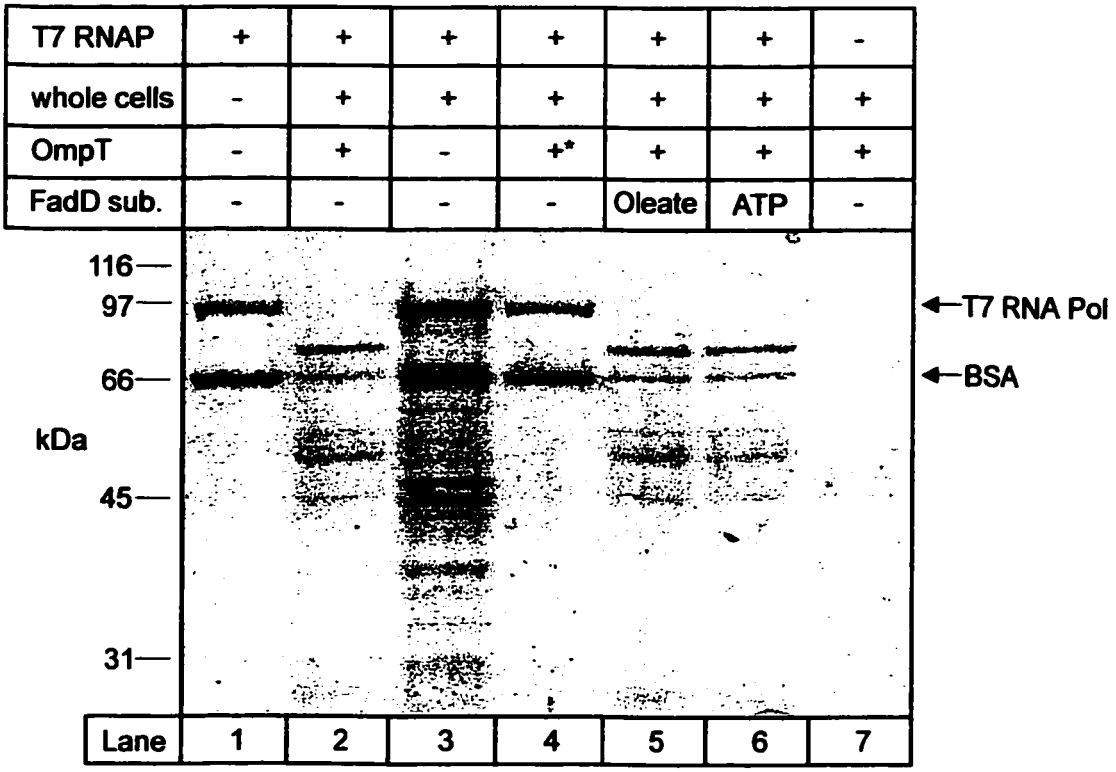


Figure 34. Effect of FadD substrates on OmpT-mediated proteolysis of T_7 RNA polymerase. SDS-PAGE/Coomassie Blue staining of the proteolysis assay performed as described in figure 29 (B), except using commercially available T_7 RNA Polymerase (Fermentas) as the substrate. *- represents a 0 time incubation.

OmpT did not have a similar effect, although Triton X-100 increased the efficiency of FadD cleavage, which may be due to interaction of the enzyme with detergent. As previously shown, proteolysis of FadD could be inhibited by fatty acids which could then be blocked by the presence of ATP. These observations suggested that the OmpT cleavage site is located within a solvent accessible region of FadD.

This region appears to undergo conformational changes upon binding LCFAs such that the OmpT cleavage site is no longer accessible and/or more structurally rigid (Figure 33 (A), (B)). ATP also altered the proteolytic pattern of FadD, implying that ATP binding also induced conformational changes in this area but in a manner different from oleate. It appears that upon ATP binding, the region surrounding the OmpT cleavage site becomes less structured and/or more accessible to the protease. Potential cleavage sites are most susceptible when the surrounding region is unstructured and highly mobile (Fontana et al., 1986; Wilson, 1991). Due to their mobility and solvent accessibility, hinge or linker regions of proteins containing consensus cleavage sites, are highly sensitive to proteases including OmpT (Fontana et al., 1986; Wilson, 1991; Zhao and Somerville, 1993; Kycia et al., 1995). It is tempting to speculate from our results that the region in FadD surrounding the OmpT cleavage site also represents a flexible hinge region or domain linking the 19-kDa amino-terminal and the 43-kDa carboxyl- terminal FadD fragments.

Photoaffinity labeling of cleaved FadD also revealed that the hydrocarbon chain of fatty acids interacts with the 43-kDa FadD fragment, suggesting that a fatty acid binding site resides in this portion of the enzyme. Black et al, using a different

photoreactive fatty acid, reported a similar result (Black et al., 2000). They determined that the probe mostly cross-linked to a region just adjacent to the FACS signature motif, located near the carboxyl terminus of FadD (figure 37). As mentioned previously, the FACS signature motif is thought to be involved in binding fatty acids and determining substrate specificity (Black et al., 2000). Using computer modeling, Black and colleagues also proposed a partial structural model for FadD (starting at Thr^{213}) in which the ATP/AMP and fatty acid binding domains reside within the 43-kDa C-terminal FadD fragment (DiRusso et al., 1999; Black et al., 2000). This model also appears to place the OmpT cleavage site at a boundary between the N-terminal and C-terminal FadD fragment. To determine if FadD cleavage was occurring at an inter-domain site, we wanted to isolate the individual FadD fragments and test if the 43-kDa C-terminal fragment alone possessed enzymatic activity or if combining the C-terminal and N-terminal fragments of FadD together could restore activity.

We first attempted to express the two fragments separately. Expression systems for truncated FadD protein with 172 amino acids deleted from the N-terminus (ΔN172FadD) was constructed. The truncated FadD protein was expressed alone (Figure 35(A)) and fused to MBP (Figure 35(B)). As evident from the immunoblots, expression of the C-terminal FadD fragment was very low when expressed alone or as a fusion protein. This suggested that the C-terminal FadD fragment is unstable and specifically requires the N-terminal FadD fragment for structural stability. This is based on two observations, the first being the tight association seen between the two FadD fragments

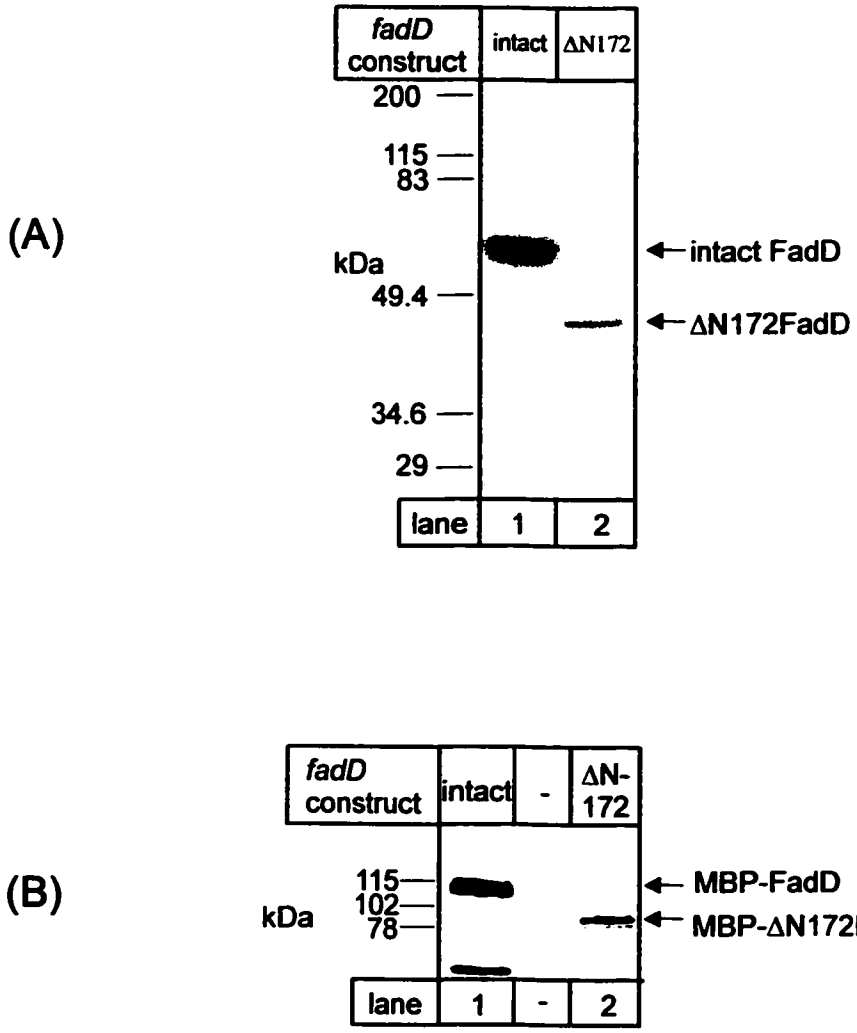


Figure 35. Expression of 43-kDa FadD C-terminal fragment alone or fused to MBP. **(A)** Immunoblots with anti-FadD serum of *E. coli* cell lysates expressing FadD protein with 172 amino acids deleted from the amino terminus. Using PCR, we constructed a *fadD* gene such that only the last 386 carboxyl terminal residues of FadD would be expressed. Lane 1 contained full length FadD protein, while lane 2 contained the deleted FadD protein. **(B)** The amino truncated FadD protein was also expressed as a MBP fusion protein. Lane 1 contains the MBP-FadD fusion protein while lane 2 contains the N-terminal truncated FadD fused to MBP.

from our previous results and the second was the instability of the truncated FadD protein even when it is fused to a complete MBP protein. In spite of the low expression, photoaffinity labeling and ACS activity assays were performed on cell lysates containing either truncated FadD protein but no activity could be detected. Another approach that was used to obtain the 45- and 19-kDa FadD fragments separately involved using the MBP-FadD fusion protein. This involved incubating cell lysate containing MBP-FadD with whole *E. coli* cells expressing OmpT protease. The digestion reaction containing nicked FadD was then incubated with amylose resin and washed several times with buffer. It was anticipated that the MBP-19-kDa FadD fragment would bind to the amylose resin, while the 45-kDa C-terminal fragment would remain in solution. After very extensive washing of the loaded amylose resin with buffer containing detergent, we obtained a single 60-kDa protein, close to the expected combined weight of the N-terminal FadD fragment (19-kDa) fused to MBP (42.7-kDa) (Figure 36). Eventually, this fragment would be incubated with the ΔN172 FadD fragment and tested for FACS activity. Observation of activity would then confirm that the OmpT cleavage site is located in a linker domain and both fragments are required for enzyme activity.

C.3.6. Conclusions

We report that FadD is a 62-kDa protein *in vivo* that becomes cleaved upon cell lysis. Cleavage of FadD produces 43-kDa and 19-kDa fragments that remain tightly

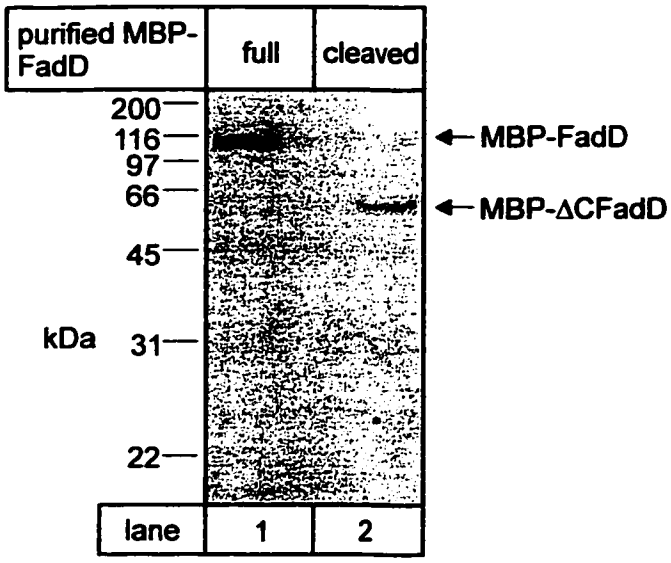


Figure 36. Isolation of MBP fused to 172 amino acid N-terminus of FadD. The soluble fraction of Triton X-100 solubilized total membranes from IPTG induced BL21(DE3)(pMD62) were prepared, as described in figure 31. The detergent soluble fraction was then incubated with harvested/washed JM105 cells for 30 min. The proteolysis reaction was stopped by spinning the reaction and isolating the supernatant. The resulting supernatant was then incubated with amylose resin as described in figure 31. The loaded resin was then washed several times with 25 mM Tris-Cl pH 7.5, 25 mM NaCl, 0.25% Triton X-100, 1 mM β-mercaptoethanol and eluted with 10 mM maltose (lane 2, cleaved FadD fragment). The samples were analyzed by SDS-PAGE and visualized by Coomassie Blue staining.

associated. Photoaffinity labeling experiments revealed that both intact FadD and the 43-kDa C-terminal fragment are labeled by photoreactive fatty acid, indicating that the C-terminal fragment is involved in binding the acyl end of LCFAs (Figure 28). Our data shows that the periplasmic protease Tsp is not involved in the cleavage of FadD, and instead implicate the outer membrane protease, OmpT. We made use of FadD sensitivity to OmpT protease to demonstrate that FadD undergoes ligand-induced conformational changes. Limited proteolysis studies revealed that the FadD substrates, oleate and ATP, altered the proteolytic patterns of FadD. This suggested that the region surrounding the cleavage site is mobile and solvent accessible, common characteristics of linker domains. This proposed linker domain appears to connect the C-terminal end of FadD containing the ATP/AMP and fatty acid binding domains to the 19-kDa N-terminal region of unknown function (Figure 37).

C.3.7. Future investigations

Further studies should be performed to determine if the region surrounding the OmpT cleavage site represents an intradomain region. This proposal would be strengthened if a specific function could be demonstrated for each fragment and FadD activity could be reconstituted by combining the isolated FadD fragments. Future work should also be focused towards determining what functions are contained within the N-terminal fragment and its functional relationship with the C-terminal fragment of FadD.

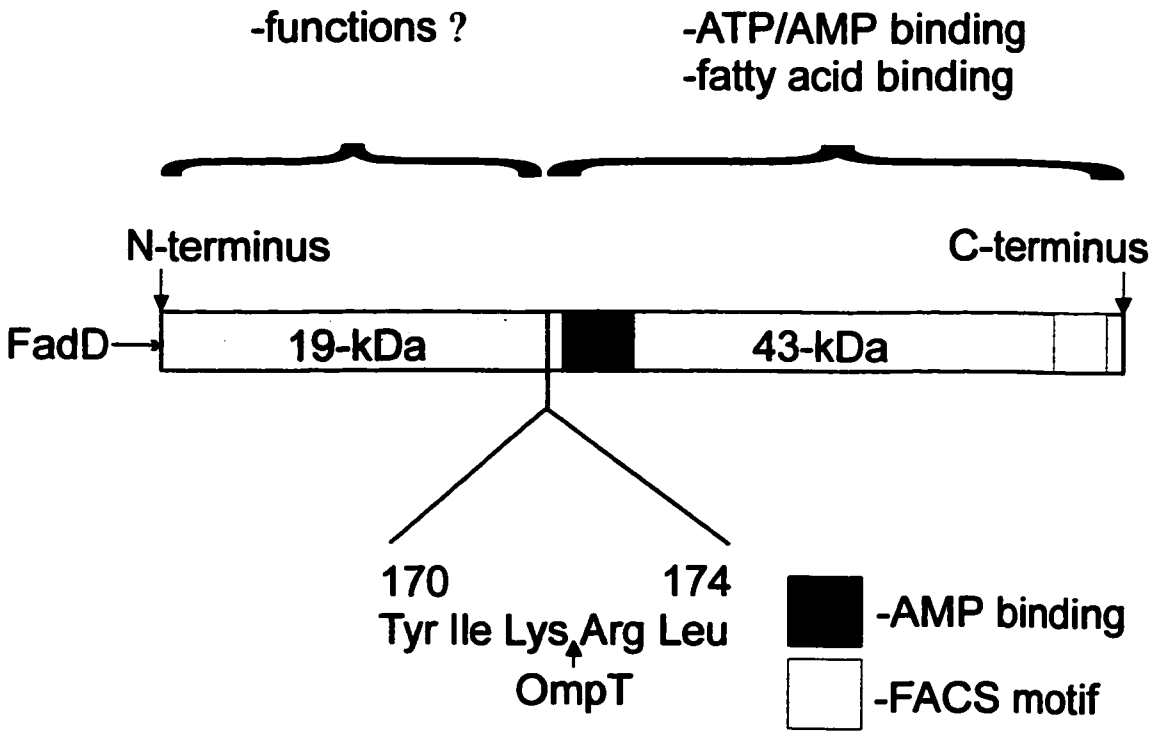


Figure 37. Domain structure diagram of FadD. Diagram depicting the OmpT cleavage site and proposed functions contained within the two fragments of cleaved FadD. FadD contains regions of homology found in other AMP-binding proteins (amino acids 200-273) (Black *et al.* 1992, Fulda *et al.* 1994) and other fatty acyl-CoA synthetases (FACS) (amino acids 431-455) (Black *et al.* 1998). The AMP binding sequence is thought to be involved in binding nucleotides, while the FACS motif is involved in forming a binding pocket for long-chain fatty acids (Black *et al.* 2000). Our data suggest that the region surrounding OmpT cleavage site is a linker domain.

What are also not understood are the implications of FadD conformational changes on its function/activity. Mammalian cells express an integral membrane form of a long-chain FACS, which appears to facilitate LCFA movement across the plasma membrane (Gargiulo et al., 1999). It is possible then, that binding of either ATP or fatty acid or both, leads to conformational changes in FadD that facilitate recruitment to the membrane. Recruitment of the enzyme to the plasma membrane may be essential for its activity since it is thought that membrane dissolved fatty acids represent the prime source of substrate for FadD (Mangroo and Gerber, 1993). To investigate this, observations should be made concerning the effects of FadD ligands on its ability to bind to membranes. In this model, FadD would appear to play a direct role in LCFA transport and not act as a simple "sink" for fatty acids (Higgins, 1994).

D. SUMMARY AND CONCLUSIONS

Growth of *E. coli* on LCFAs requires both FadL and FadD. Both proteins, including the periplasmic protease Tsp, are thought to be involved in LCFA transport. The focus of our research was to investigate the functions of these proteins and understand the mechanism for transport of LCFAs across the *E. coli* cell envelope. Bacterial fatty acid metabolism represents a relevant subject for clinical and industrial research in fields such as bacterial pathogenesis and food preservation.

Unlike other carbon sources, transport of LCFAs is a paradox for *E. coli.* The lipid is utilized as a sole carbon source but can also be toxic at abnormally high levels in the periplasm. The toxic properties of LCFAs are especially evident when the OM is permeabilized with EDTA. Interestingly, overexpression of FadL did not sensitize *E. coli* to LCFAs. FadL appears to balance LCFA uptake concurrently with protecting cells from inhibiting levels of LCFA. To investigate the mechanism of FadL activity, an assay was developed to monitor LCFA permeation. Although FadL is thought to facilitate the permeation of LCFAs across the outer membrane, there has never been any direct demonstration of this activity. Using a photoreactive LCFA, a permeation assay was used to directly demonstrate FadL activity. In addition, observation of this activity represents the first direct demonstration for the role of a protein in fatty acid uptake across a biological membrane. Although a definite increase in LCFA permeation was evident when FadL was expressed, a sudden plateau followed permeation. The level of permeation was well below levels seen in permeabilized *E. coli,* which suggests that

FadL did not function as a simple channel for LCFAs. These results also suggest that FadL may exist in open and closed states, and demonstrates for the first time *in vivo*, regulation of a specific-type porin.

Investigation of the role of Tsp in LCFA uptake indicates that the protease is required for normal outer membrane function and appears to play an indirect role in LCFA uptake. It appears that the effects of the *tsp* mutation on LCFA uptake are related to changes in outer membrane permeability, although it is uncertain how Tsp is involved in OM integrity.

Results from the FadD studies reveal that the 62-kDa enzyme is cleaved by the outer membrane protease OmpT producing a 19- and 43-kDa fragment *in vitro*. Proteolysis of FadD likely accounts for discrepancies in the description of native FadD, by other groups. Cleavage did not significantly affect FadD activity, indicating that the region surrounding the cleavage site is not essential for enzyme function. OmpT-mediated proteolysis reveals that the fatty acyl-CoA synthetase undergoes conformational changes in the presence of ligand or substrates. These observations suggest that the OmpT cleavage site is located within a linker domain, connecting the 19-kDa N-terminal region of FadD to the 43-kDa carboxyl end of FadD, which contains the nucleotide and fatty acid binding sites. This data demonstrates that OmpT is a useful tool for monitoring FadD structure and suggests that changes in conformation due to substrate binding, may play a role in recruitment of the enzyme to the inner membrane.

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