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ACCELERATING WHOLE-CELL BIOCATALYSIS BY ENHANCING OUTER MEMBRANE PERMEABILITY

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A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Abstract

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Whole-cell biocatalysts are preferred in many biocatalysis applications. However, cell envelope often represents a formidable permeability barrier. As a result, reactions catalyzed by whole-cells are reportedly orders of magnitude slower than those of by their free enzyme counterparts. The present research addresses this critical issue by using membrane engineering approaches. Two *E. coli* strains with genetically altered outer membrane structures were used in the study, a lipopolysaccarides (LPS) mutant SM101 and a Braun's lipoprotein mutant E609L. The effects of outer membrane mutation on the permeability of substrates differing substantially in size and hydrophobicity were investigated by combining the mutant cells with model enzymes. The reduction of the outer membrane permeability barrier by these mutations led to significant accelerations

(2– to 14–fold) in reaction rates of all whole-cell catalyzed reactions investigated. In the case of tetrapeptide, LPS mutation of the outer membrane can render the outer membrane completely permeable to substrate, a barrier-less condition that maximizes the reaction rate. For reaction rates of toluene dioxygenase (TDO)-catalyzed reactions, a dramatic increase of up to six fold was observed with the lipoprotein mutant for each of the three small, hydrophobic substrates tested. Mutations in either the LPS or in the Braun's lipoprotein are effective for accelerating reactions with UDP-glucose, resulting in a striking acceleration (up to 14–fold) of reaction rate. The magnitude of reaction rate acceleration was found to be dependent upon the substrate concentrations, the enzyme expression level, and on the nature of the mutations and substrates. In addition, the mutations have been demonstrated to be far more superior to common permeabilization procedures like freeze-thaw (FT) or treatment with the chelating agent EDTA (ethylene diamine tetraacetic acid). Importantly, lipoprotein mutant E609L exhibited a normal growth rate and expressed the recombinant multi-component enzyme as well as the isogenic parent. The exact nature of *lpp* lipoprotein mutation in E609L was further studied and deletion of *lpp* was successfully introduced into *E. coli* strain with different genetic background for whole-cell biocatalysis applications. An example was provided by introducing an *lpp* deletion into an *E. coli* O44K74 strain to achieve a higher yield for L-carnitine production. This research and the results outlined in this dissertation demonstrate a valid strategy for addressing permeability issues in whole-cell biocatalysis. The work also highlights a need for accessing substrate permeabilities in biocatalysis research and development.

CHAPTER 1 Introduction

1.1 Background

Over the last two decades, the rising economic and medical importance of natural products and an ever-increasing pressure to mitigate the adverse impact of synthetic production practices have stimulated interest in the use of biocatalysis as a production tool. According to estimatesT[1], by 2010, the share of biocatalysis processes in the production of various chemical products will increase from 5% to about 20%, about $280 billion increase in the market value of products produced using biocatalysis. Another estimation[2] showed that 30–60% of all fine chemicals production is anticipated to involve a biocatalytic process step in 5 years.

Compared to isolated enzymes, whole-cell biocatalysis has a number of attributes that are particularly attractive for large-scale applications. First, the use of whole cells instead of free enzymes is more economical as the use of whole cell eliminates the need for tedious, expensive protein isolation and purification. Also, whole cell biocatalysis allows cascades of enzymatic reactions that involve multiple enzymes, cofactors, and substrates while the same reaction might be too complicated to perform *in vitro*. Moreover enzymes are generally more stable when protected by cell envelopes[3, 4].

In general, the reactions catalyzed by whole cells are 10– to 100–fold slower than rates observed for free enzyme reactions. The difference is usually attributed to the mass transport barrier imposed by cellular envelopes. For example, isonovalal synthesis from α-pinene oxide catalyzed by *Pseudomonas rhodesiae* whole cells was increased by 60–

fold after permeabilizing the cells[5]. In another recent study, mass transfer across a recombinant *E. coli* cell membrane caused a reduction in the Baeyer-Villiger oxidations of biocyclo[3.2.0] by almost ten fold[6]. In a more dramatic case, intact *Pseudomonas pseudoalcaligenes* cells showed no activity to maleate (substrate) in the process of D-malate production; the cell membrane completely blocked the entry of the substrate[7]. Other studies documenting the mass transfer limitations imposed by cell membranes during whole-cell biocatalysis have also been reported[8, 9, 10, 11, 12].

This often-encountered mass transport problem has thus far limited the commercial application of whole-cell biocatalysis. Current permeabilizing methods involve chemical treatments with detergents, solvents, chelators, or physical treatments such as extreme temperature fluctuations. All these methods are highly empirical, and are generally undesirable for large scale applications. Permeabilization treatments inevitably create additional steps in manufacturing processes, complicate downstream processing, and incur unintended damage to cells. Cell damage is particularly problematic as it disrupts the normal membrane functions and intracellular metabolism needed for efficient cofactor regeneration.

1.2 Objectives

The goal of this research is to accelerate the whole-cell biocatalysis reactions via genetic alteration of a cell membrane to mitigate or eliminate mass transport resistance to substrates. Eventually, we hope to be able to rationally engineer the cell membrane to enhance the permeability of a chosen substrate. The specific objectives of the study are:

1) To illustrate the use of two specific *E. coli* outer mutations (Lipid A and Braun's lipoprotein) to increase the permeability of substrates with different size and hydrophobicity.
2) To establish the correlations between the effects of the mutations on mass transport rates to the molecular properties of the substrates, and a generally applicable membrane modification method to accelerate the whole-cell biocatalysis reaction rate.

1.3 Rationale and Approaches

The outer membrane of Gram-negative bacteria cell envelope is a distinct bilayered structure. It is chemically different from usual biological membranes and has the ability to resist damaging chemicals. The outer leaflet of the outer membrane, lipopolysaccharide (LPS), is a highly ordered quasi-crystalline structure with very low fluidity and very low permeability to especially hydrophobic molecules. The LPS consists of lipophilic Lipid A, an oligosaccharide core, and a long polysaccharide chain comprising up to 40 sugars (called an O-specific chain). The hydrophilic polysaccharide component is responsible for the exclusion of hydrophobic molecules and the hydrophobic Lipid A of LPS limits the entry of hydrophilic compounds. The outer membrane of Gram-negative bacteria excludes even small hydrophobic compounds, and requires dedicated protein-based mechanisms for the entry of some larger hydrophilic molecules smaller than 600–700 Daltons. Passive diffusion through porin channels of the outer membrane is the mass transport mechanism for small hydrophilic compounds. Even with porins, diffusion may be slower than desired[10].

The rate of entry of molecules is often the rate-limiting step of a biocatalysis reaction. Since many biocatalysis substrates are synthetic hydrophobic and/or relatively big molecules and lack a natural uptake system, obtaining rapid substrate permeation through outer membranes is a common and formidable obstacle in whole-cell biocatalysis. In this study, we accelerate whole-cell biocatalysis by genetic modification of the outer membrane of a host cell to increase the permeability of the cell membrane to specific substrates. Two outer membrane mutants with genetic modifications at different

sites of the outer membrane were studied. The first mutant has a mutation inactivated the first enzyme in the LPS synthesis pathway, UDP-*N*-acetylglucosamine acyltransferase, resulting in an approximately 30% reduction in LPS synthesis. Another modification targeted a Braun's lipoprotein, a structural protein that connects the peptidoglycan with the inner leaflet of the outer membrane. This protein is the most abundant protein in a numerical sense in *E. coli*. About one-third of the lipoprotein molecules are covalently linked to murein and help hold the two structures together. Mutants defective in the structural gene for this protein are fully viable, indicating the gene is nonessential. However, the disruption of the lipoprotein likely affects the global membrane integrity and thus may cause some changes in substrate permeability. Our approach is first to interrogate the existing outer membrane mutants with different substrates and then rationally engineer the cell membrane for the whole-cell biocatalysis application. The approach is illustrated in Figure 1–1.

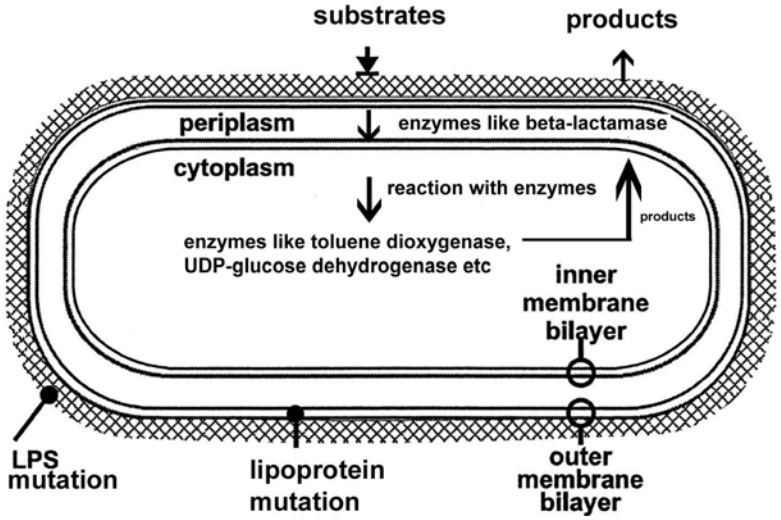


Figure 1–1. Illustration of the mechanism and permeability assay of *E. coli* whole-cell catalysis investigated in this study.

1.4 Organization of the Dissertation

This dissertation comprises 6 chapters as follows.

Chapter 1. Introduction

Chapter 2. Markedly Enhanced Whole-Cell Catalyzed Reaction Rates by Genetic Modification of Outer Membranes

Chapter 3. Acceleration of a Permeability Limited Toluene Dioxygenase–Catalyzed Reaction via Lipoprotein Mutation

Chapter 4. The Effect of Outer Membrane Mutations on UDP–Glucose Permeability and Whole-Cell Catalysis Rates

Chapter 5. The Significance and Application of *lpp* Lipoprotein Mutation in Whole-Cell Biocatalysis

Chapter 6. Summary

The experimental chapters contain the data and analyses used to prepare the three publications from these studies. Thus, the dissertation is organized according to the chronology of the work carried out. Chapter 2–4 therefore cover different aspects of genetic modification of cell membrane to enable improved permeability of interrogating substrates. In all, we studied the effect of two membrane mutations on the permeability of six substrates differing substantially in hydrophobicity and size.

In chapter 2, the effects of two outer membrane mutations on permeability of two substrates with different hydrophobicity are reported. By combining these two mutants with two model enzymes (β-lactamase and subtilisin) expressed in periplasmic space, we

were able to quantify the effects of the mutations on reaction rates with their respective substrates, nitrocefin (hydrophilic) and a tetrapeptide (hydrophobic).

In the work described in Chapter 3, the effect of lipoprotein mutation on the permeability of three small, hydrophobic substrates was evaluated with another model enzyme, toluene dioxygenase.

Chapter 4 reports the results of a study on Lipid A and lipoprotein mutations that were interrogated using a relatively large and hydrophilic molecule, UDP-glucose. This study considered two UDP-glucose utilizing reactions catalyzed by UDP-glucose dehydrogenase and UDP-galactose 4'-epimerase/β-1,4-galactosyltransferase fusion protein. The application of these mutants for whole-cell biocatalysis was illustrated by synthesizing a disaccharide, *N*-acetyllactosamine.

Mutation of *lpp* lipoprotein had a significant effect on the permeability of all six substrates tested. We therefore studied the genetic sequence of *lpp* lipoprotein mutation and introduced this mutation into *E. coli* strains with different genetic backgrounds. Chapter 5 discusses the effectiveness of this mutation on the production of L-carnitine by *E. coli* O44K74 strain with *lpp* lipoprotein to achieve a higher yield of production.

The significance and the potential impact of this research are summarized in Chapter 6. This final chapter also reiterates the major finding of these studies and offers recommendations for the future work.

CHAPTER 2

Markedly Enhanced Whole-Cell Catalyzed Reaction Rates by Genetic Modification of Outer Membranes

Whole-cell biocatalysts are preferred in many biocatalysis applications[3,4]. However, due to the permeability barriers imposed by cell envelopes, whole-cell catalyzed reactions are often 10–to–100 times slower than reactions catalyzed by free enzymes. In this study, we reduced the membrane permeability barrier of *E. coli* strains by genetically altering the membrane structure. A lipopolysaccarides *E. coli* mutant (SM101) and a Braun's lipoprotein mutant (E609L) were used in this study. Two model substrates that differ substantially in size and hydrophobicity, nitrocefin and the tetrapeptide N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, were employed to interrogate the outer membrane of the cells. The reduction of the outer membrane permeability by genetic methods led to significant increases (up to 380%) in reaction rates of whole-cell catalyzed reactions. The magnitude of the increase in biocatalysis rates was dependent on the substrates and on the nature of the mutations introduced in the outer membrane structure. Notably, some outer membrane mutations can render the outer membrane completely permeable to one substrate, a barrier-less condition that maximizes the reaction rate. The impact of the mutations introduced on the permeability barrier of the membranes was compared to the impact of polymixin B nonapeptide, a known potent permeabilizer acting on lipopolysaccharides. Based on our findings, genetic modifications to enhance the permeability of hydrophilic molecules should target the Lipid A region. However, molecular engineering strategies other than reduction of Lipid

A synthesis should be considered. As we have demonstrated with tetrapeptide, membrane engineering can be much more effective than exogenous permeabilizers in reducing the permeability barrier imposed by cell walls. To our knowledge, this study is the first use of molecular membrane engineering techniques to address the substrate permeability limitations encountered in biocatalysis applications.

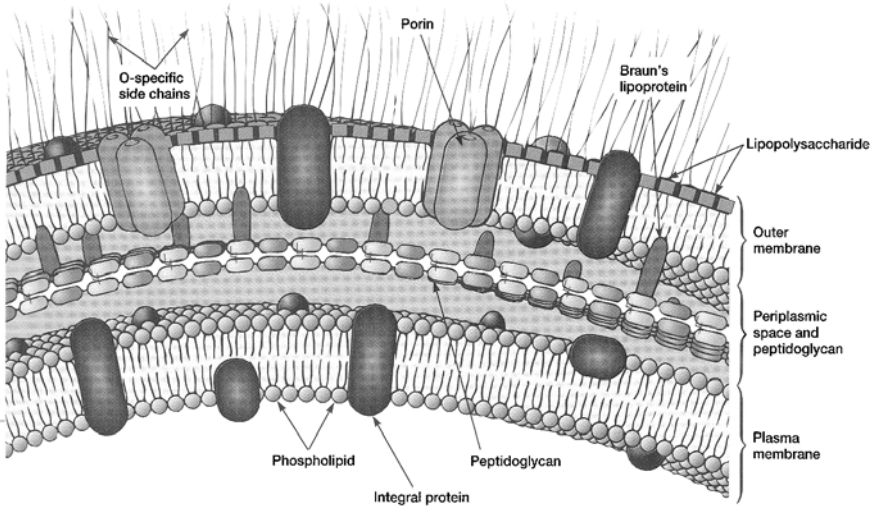
2.1 Introduction

Whole cell biocatalysis are preferred to purified enzymes especially for large-scale applications because it is usually more economical, easier and stable to use, particularly for reactions involving multiple enzymes or cofactor regenerations[9]. However, whole cell biocatalysis generally exhibits much slower reaction rates than does the use of free enzymes due to the mass transfer limitations imposed by low permeability cellular membranes. This often-encountered difficulty limits the application of whole-cell biocatalysis. It's been recently reported that the membrane permeability barrier cause a reduction in the reaction rate for about 60–fold in a study of whole-cell catalyzed isonovalal synthesis from α-pinene oxide, almost ten fold for a Baeyer-Villiger oxidations with recombinant *E. coli* cells. And more dramatically, the intact *Pseudomonas pseudoalcaligenes* cells showed no activity to maleate (substrate) in D-malate production.

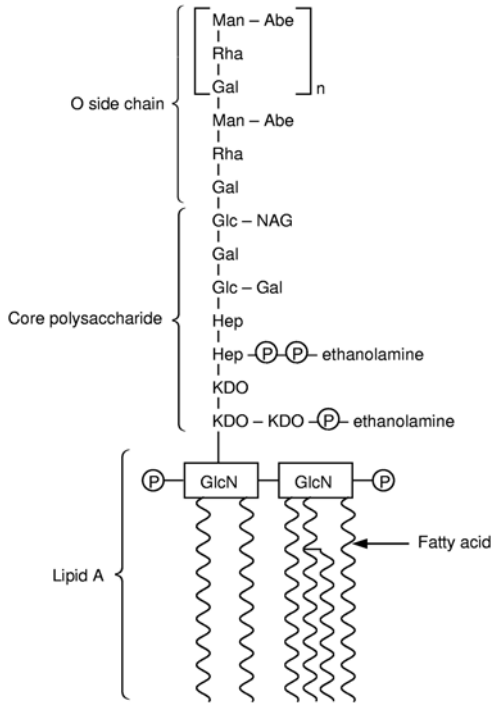
Current permeabilizing methods involve physical and/or chemical treatment of host cells. These methods are generally developed by trial-and-error and inevitably add several steps to a bioprocess synthesis. For example, permeabilizing cells with 1 % toluene, a commonly used procedure, requires five steps[11]. In addition, permeabilizer residues complicate downstream purification processing, especially when a surfactant is used to permeabilize the cell. Furthermore, excessive membrane damage and cell lysis are often observed when chemical permeabilizing agents are employed. Such damage precludes efficient cofactor regeneration since the regeneration process requires normal membrane function and intracellular metabolism.

The molecular basis of permeability limitations is well-known. The outer membrane of Gram-negative bacteria[12] (Figure 2–1A) excludes even small hydrophobic compounds, and allows the entry of some larger hydrophilic molecules (<700 Daltons) through dedicated protein-based structures, aptly called porins. Passive diffusion through porin channels (Figure 2–1A) of the outer membrane is the mass transport mechanism for small hydrophilic compounds. Even with porins, diffusion may be slower than desired. The lipopolysaccharide (LPS) (Figure 2–1A and Figure 2–1B) monolayer is a highly ordered, quasi-crystalline structure with very low fluidity, and the layer does not allow rapid diffusion of molecules. The LPS (Figure 2–1B) comprises lipophilic Lipid A, an oligosaccharide core, and a long polysaccharide chain containing up to 40 sugars (indicated in Figure 2-1B as the O-specific side chain). The hydrophilic polysaccharide component is responsible for the exclusion of hydrophobic molecules and the hydrophobic Lipid A of LPS limits the entry of hydrophilic compounds.

The molecular size and/or hydrophobicity of most biocatalysis substrates of commercial interest limit or preclude their entry into host cells by naturally established transport mechanisms. The concomitant limitation of biocatalysis reaction rates is therefore a commonly encountered and formidable obstacle to the commercialization of whole-cell biocatalysis synthesis.



(A)



(B)

Figure 2–1. A: Gram-negative envelope. B: Lipopolysaccharide structure. (adopted from Prescott et al., 2002)

In order to mitigate the mass transport limitations in whole-cell biocatalysis systems, we are genetically altering the barrier membrane to increase the transport rates of chosen substrates through the membrane. In this study, the effects of genetic modifications at two sites of the outer membrane were investigated. One modification affects the LPS synthesis pathway. An LPS mutant with approximately 30% reduction in LPS synthesis was used to probe the changes in biocatalysis reaction rates caused by the mutation. Another membrane modification targeted a Braun's lipoprotein (Figure 2–1A), a structural protein that connects the peptidoglycan with the inner leaflet of the outer membrane. In order to isolate the outer membrane for permeation studies, the model enzymes we used (β-lactamase and subtilisin) were expressed in periplasmic space. Their respective substrates, nitrocefin and a tetrapeptide, differ substantially in size and hydrophobicity, and were used as model substrates to probe the mutation effect on reaction rates.

2.2 Experimental

2.2.1 Chemicals

All chemicals were reagent grade and used as received. Nitrocefin was obtained from Oxoid Ltd. (Basingstoke, Hampshire, United Kingdom). Tetrapeptide, N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF-pNA) and polymyxin B nonapeptide (PMBN) were obtained from Sigma (St. Louis, Mo).

2.2.2 Sterile control

All vessels and media used for cell cultures were autoclaved at 121^{o}C for 15 minutes. All tips, pipettes and tubes were purchased in disposable, sterile form. All cell culture operations were performed in a class II biological safety cabinet to avoid air-borne contamination.

2.2.3 *Escherichia coli* strains and plasmids

E. coli strains and plasmids used in this study are shown in Table 2–1. pBR322 is a commonly used cloning vector in *E. coli* with a low copy number of about 20 per cell. pBR322 contains *bla* gene, coding for β-lactamase that confers resistance to ampicillin. The expression of β-lactamase is constitutively driven by P1 and P3 promoters (no inducer required), and the expression level is comparable between strains[13, 14]. The signal sequence in *bla* gene allows translocation of β-lactamase to periplasmic space[15].

Plasmid pGES201 was graciously supplied by Dr. Dordick (RPI). This plasmid was constructed by inserting WT-*sprE* (a wild-type gene for subtilisin E) into a commercial pBAD/gIII plasmid (Invitrogen) for periplasmic expression of subtilisin E. The signal peptide from the bacteriophage *fd* gene III (gIII) permits secretion of subtilisin E into periplasmic space. araBAD promoter (pBAD) provides tight (low basal expression level without inducer L-arabinose), dose-dependent regulation of WT-*sprE* gene expression using arabinose as an inducer[16].

Two types of *E. coli* host strains were used in this study. The lipid A mutant SM101(*lpxA2*) is a thermo-sensitive, UDP-*N*-acetylglucosamine acyltransferase (*lpxA*) defective strain[17,] . Its isogenic parent strain, SM105, was used as a control. E609L is a periplasmic leaky *E. coli* host in which the gene encoding Braun's lipoprotein was disrupted by a Tn*10* insertion into the *lpp* gene[18]. E609 was its isogenic parent and was used as a control.

Strains SM101/pBR322, SM105/pBR322, E609L/pBR322 and E609/pBR322 for study with nitrocefin were constructed by transforming the four host strains (Table 2–1) with plasmid pBR322 using a Bio-Rad Micropulser following the standard procedures[19]. Similarly, strains SM101/pGES201, SM105/pGES201, E609L/pGES201 and E609/pGES201 were constructed by transformation of the host strains with plasmid pGES201 for study with tetrapeptide.

Figure 2–2 is a block diagram illustrating the experimental protocol used in this study. Detailed descriptions of the procedures referred to in the various blocks in Figure

2–2 follow below. Additionally, the terms used for various preparations (i.e., supernatant, cell pellet, etc.) are indicated in Figure 2–2 as a quick reference for the reader.

Table 2–1. *E. coli* strains and plasmids used in this study.

	Description	Source
Strains		
SM101	*lpxA2* mutant, thermosensitive, grows well at 28°C	M. Vaara (Martti.Vaara@Helsinki.fi)
SM105	*lpx A2*$^{+}$, isogenic parent of SM101	M. Vaara
E609L	*lpp*::Tn*10*; periplasmic leaky; Tc^{r}	K. W. Miller[, 25] (labcin@uwyo.edu)
E609	*HfrC pps*, isogenic parent of E609L	K. W. Miller [, 25]
Plasmids		
pBR322	periplasmic β-lactamase encoding plasmid	R. I. Lehrer (rlehrer@mednet.ucla.edu)
pGES201	subtilisin E (serine protease) gene WT-sprE in pBAD/gIII	J. Dordick (dordick@rpi.edu)

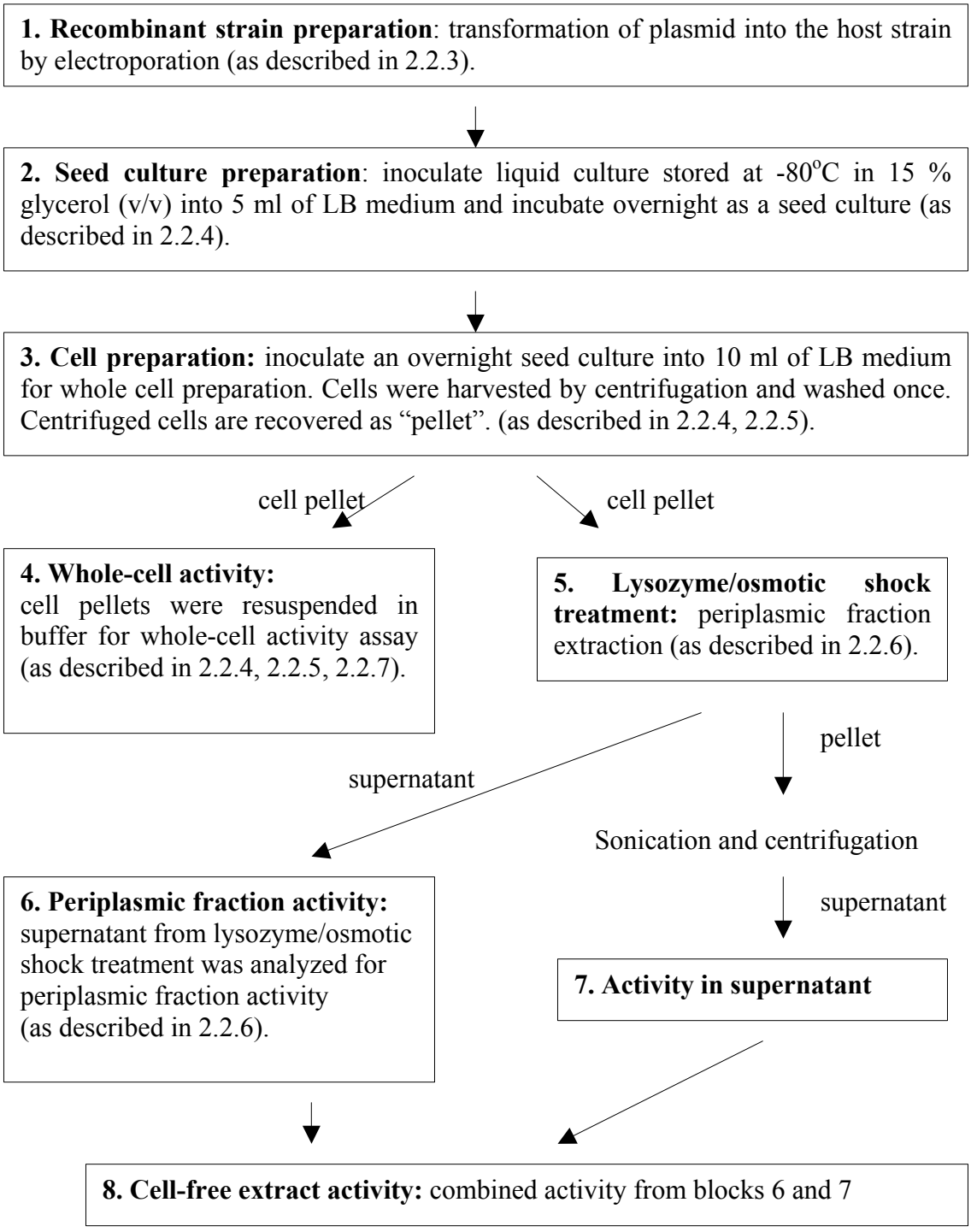


Figure 2–2. Block diagram illustrating the sequence of preparative steps and assays used for the measurements reported in this study.

2.2.4 Whole-cell β-lactamase activity assay with nitrocefin

All pBR322 transformants were grown in LB medium. The LB medium contained 10g/L tryptone, 5 g/L bacto-yeast extract, and 10g/L NaCl. SM101/pBR322, SM105/pBR322, E609L/pBR322 and E609/pBR322 were grown in 10 ml of LB medium containing 50 μg/ml ampicillin (to maintain selective pressure for ampicillin resistant gene carrying plasmid pBR322 in these strains) in a 50 ml flask and each inoculated with an overnight culture of (2% inocula). SM101/pBR322, which is thermo-sensitive, was grown at 28 ^{o}C; the others were grown at 37^{o}C. All cultures were continuously shaken at 250 rpm until OD_{600} (the optical density at 600 nm) reached around 0.5. Cells were harvested by centrifugation at 4000 × g for 10 minutes at 4^{o}C and washed once with 10 mM sodium phosphate buffer (pH 7.4) using a pipette. Cell pellets were then prepared by centrifugation. The pellets were isolated and then resuspended in 5 ml of sodium phosphate buffer (pH 7.4). Resuspension was accomplished by covering the cells with the buffer and then drawing the mixture into a pipette.

The nitrocefin reaction rates were measured by the Angus method with some modifications [20]. The reaction was carried out in a micro-cuvette. 100 μl of cell suspension prepared as above was diluted with 860 μl of 10 mM sodium phosphate buffer (pH7.4). The reaction was started by adding 40 μl of 0.5 mg/ml nitrocefin (20 μg/ml final concentration). The total assay volume was 1 ml. Initial velocities of nitrocefin cleavage (extinction coefficient ε_{500} = 15,000 M^{-1} cm^{-1}) were monitored by measuring the absorption at 500 nm at room temperature with a UV/VIS spectrophotometer (Lambda 40

Spectrometer, Perkin Elmer). Similar preparations without cell suspension or without nitrocefin addition were used as controls. One unit of β-lactamase activity was defined as the amount of enzyme required to hydrolyze 1 μmol of nitrocefin per minute at room temperature (typically 22°C ± 1°C). The typical range of cell density (absorption at 600 nm) for a reaction mixture was between 0.09 OD_{600} and 0.11 OD_{600} (corresponding to about 0.2 g wet cell/liter). The activities were normalized by OD_{600} to correct for small differences in cell density between the various preparations.

2.2.5 Whole-cell subtilisin activity assay with tetrapeptide

The expression of functional subtilisin E in *E. coli* strains was performed according to methods described in the literature. 10 ml of LB medium with 100 μg/ml ampicillin in a 50 ml flask was inoculated with an overnight culture of SM101/pGES201, SM105/pGES201, E609L/pGES201 and E609/pGES201 (2% inocula). The thermo-sensitive SM101/pGES201, which is thermo-sensitive, was grown at 28 ^{o}C; all others were grown at 37 ^{o}C. All cultures were continuously shaken at 250 rpm until OD_{600} reached around 0.5. Cells were harvested by centrifugation at 4000 × g for 10 minutes at 4^{o}C and resuspended in a complete minimal (CM, an enriched medium containing all of the growth requirements of a strain) of equal volume. CM Medium contained 10.50 g/L K_2HPO_4, 11.25 g/L KH_2PO_4, 1.00 g/L $(NH_4)_2SO_4$, 0.50 g/L sodium citrate·$2H_2O$, 0.25 g/L $MgSO_4 \cdot 7H_2O$, and 0.015 g/L $CaCl_2 \cdot 2H_2O$. Other supplements included all 20 amino acids at a final concentration of 40 μg/ml each, 5 mg/L vitamin B1, 0.20 % (v/v) glycerol, and 100 μg/ml ampicillin. Inducer arabinose was added to a final concentration of 0.10 %

(w/v). After induction, the incubation continued for 2–to–6 hours at 20°C with shaking to achieve comparable expression levels. 100 µl cells were harvested by centrifugation at 4000 × g for 10 minutes at 4 °C. Cell pellets were resuspended in 200 µl of 10 mM sodium phosphate buffer (pH 7.4) and AAPF-pNA was added to a final concentration of 300 µM. The reactions were carried out in 96-well microtiter plates for 120 minutes at 30 °C. Initial velocities of *p*-NA release (extinction coefficient $\varepsilon_{405} = 9.33 \times 10^{-3}$ μM^{-1} cm^{-1}) from AAPF-NA were measured spectrophotometrically at 405 nm in a Wallac 1420 Victor[3] Multi-label Counter (Perkin Elmer). One unit of subtilisin E activity was defined as the amount of enzyme required to hydrolyze 1 µmol of AAPF-pNA per minute at 30 °C. As with β-lactamase activities, subtilisin activities were normalized by absorption measured at 600 nm to correct for small differences in cell density between samples.

2.2.6 Preparation of periplasmic fractions

A combined lysozyme and osmotic shock treatment[21] was used to extract the periplasmic fraction. Cell pellets harvested from 4 ml of broth were resuspended in 2 ml of OSI (osmotic shock buffer I) containing 100 µg/ml lysozyme. The OSI contained 200 mM Tris-HCl (pH 7.8), 2.5 mM EDTA, 2 mM $CaCl_2$, and 20% sucrose. The suspension was incubated quiescently at room temperature for 15 minutes before an equal volume of ice-cold, deionized water was added. The suspension was incubated for another 15 minutes and then centrifuged at 13000 × g for 15 minutes at 4 °C. The supernatant was analyzed for either β-lactamase or subtilisin activities using the same method as described

in whole-cells (see Section 2.2.5). The activity measured was assigned as the periplasmic activity (Block 6, Figure 2–2).

2.2.7 Preparation of cell–free extract

The pellets from the last step of periplasmic fraction preparation (Block 6, Figure 2–2) were resuspended in 2 ml of 10 mM sodium phosphate buffer (pH 7.4) containing 5 mM DTT (dithiothreitol). This was followed by ultrasonication (70 Watts output, 15 s × 4 bursts with 45 s intervals). The supernatant was collected after centrifugation at 13000 × g for 15 minutes at 4^{o}C and was analyzed for either β-lactamase or protease activity as described in 2.2.4 and 2.2.5. The activity was added to the periplasmic activity to obtain the cell-free extract activity (Block 8, Figure 2–2).

2.2.8 Polymyxin B Nonapeptide Permeabilization

PMBN treatment was conducted by pre-incubating the cells with 50 μg/ml PMBN for 5 minutes at room temperature before reaction.

2.3 Results

Two outer membrane mutants with their respective isogenic parents were used in this study. One mutant, SM101, carries a mutation in the lipopolysaccharide (LPS) synthesis pathway (Table 2–1). In particular, the thermo-sensitive mutation occurs at *lpxA*, which encodes UDP-N-acetylglucosamine O-acyltransferase, the enzyme that catalyzes the first step of lipid A biosynthesis[22]. As a result of this mutation, both lipid A and lipopolysaccharide synthesis were significantly reduced. At a permissive temperature (below 37 ^{o}C), the mutant produces 30% less lipid A than the control strain[,,23]. The reduced LPS synthesis is thought to be compensated by glycerophospholipid, resulting in a hybrid outer leaflet of the outer membrane. Previously, the *E. coli* strain carrying this mutation was found to be particularly susceptible to hydrophobic antibiotics. The MICs (minimal inhibitory concentration) of a large number of antibiotics for the LPS mutant are 30– to–1000–fold lower than for its parent[24].

The other outer membrane mutant (E609L) used in this study carries a transposon mutation in a structural protein called Braun's lipoprotein. The function of this lipoprotein is to maintain rigidness and integrity of the outer membrane by linking the inner leaflet of the outer membrane with the peptidoglycan (Figure 2–1A). As a result of the mutation, the cells were susceptible to chelating agents (such as ethylene diamine tetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA)), as manifested by a decrease in cell density OD_{600}. Some leakage of proteins was also observed[,25].

Table 2–1 shows the four strains and two plasmids used in this study. In order to focus our study on outer membrane permeability, we used two plasmids that directed the

expression of two enzymes of interest into the periplasmic space. Plasmid pBR322 carries a gene coding for β-lactamase along with its signal sequence that allows secretion of the expressed enzyme to the periplasmic space. Plasmid pGES201 allows periplasmic expression of subtilisin, a serine protease.

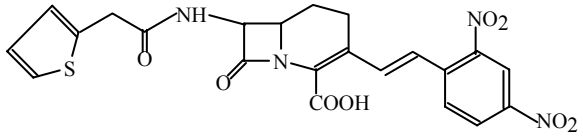
Mutant strains SM101, E609L and their respective isogenic parents were transformed with either pBR322 (β-lactamase) or pGES201 (subtilisin). No appreciable differences in growth behavior were observed between E609L and E609. SM101 grew slightly slower than SM105 (according cell density OD_{600} measurement), with a doubling time about 20% longer than the wild type grown at 28^{o}C in LB medium. Whole cells of these transformants were prepared as described in the Experimental section. Enzyme activities were measured and were compared to the activities of periplasmic and cell extracts.

The permeabilizing effect of polymixin B nonapeptide (PMBN) on whole cell reactions with nitrocefin and tetrapeptide was also studied. PMBN is a proteolytic product from the natural antibacterial peptide Polymixin B (PMB). PMBN, an extremely poor antimicrobial agent, is about 100–fold less toxic to *E. coli* cells than PMB, but is structurally similar to its parental compound and is still capable of binding to Lipid A. Consequently, PMBN is a good outer membrane permeabilizer for gram-negative bacteria[26, 27].

2.3.1 Reaction with nitrocefin substrate

Nitrocefin (MW = 516.5, Figure 2–3A) is a substrate for β-lactamase. Nitrocefin is a relatively hydrophilic molecule compared to the other substrate used in this study, the tetrapeptide (MW = 624.6, Figure 2–3B). The penetration of nitrocefin into the host cells is hindered by the presence of the outer membrane[28]. β-lactamase activities measured with nitrocefin as the substrate are tabulated in Table 2–2A. The activities of cell-free extract for all four constructs were similar, indicating a similar level of the β-lactamase expressions by each strain. Therefore, neither mutation in the outer membrane structure of the host cells significantly affects the ability of the cells to synthesize the recombinant enzymes. As well, the mutant cells maintained the ability to recognize and cleave the signal peptide sequence, and folded β-lactamase in the periplasm to its correct conformation.

A. Nitrocefin



B. N-succinyl-Ala-la-Pro-Phe-*p*-nitroanilide

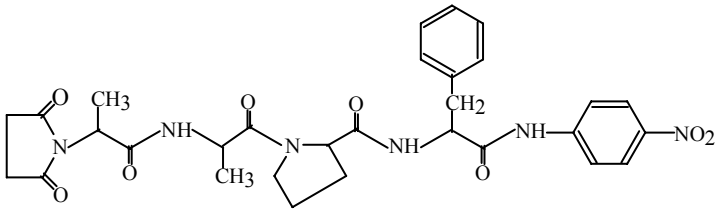


Figure 2–3. A: Chemical structure of nitrocefin. B: Chemical structure of N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide.

Notably, whole-cell activities are significantly lower than the activities of either the periplasmic fraction or the cell extract. The activities expressed as a percentage of the respective cell-free extract activities are tabulated in Table 2–2B. Whole cells had only 5%–to–20% of the cell-free extract activity, indicating the rate-limiting effect of the cell membranes. Since the enzyme is expressed in the periplasmic space, the main penetration barrier is the outer membrane (Table 2–2A and 2–2B). LPS mutation increased the activity of the whole cells from 0.088 to 0.13 Units/OD_{600} Cell (Table 2–2A), or percentage activity increase from 13%–to–20%, (50% increase, Table 2–2B). An increase in the percentage activity from 5%–to–18% after lipoprotein mutation was also observed (a 3.8–fold increase). Although a measurable increase in the reaction rate accompanied each mutation, the permeability resistance of the outer membrane to nitrocefin still existed.

Table 2–2A. β-lactamase activity.

Strains	Activity (Units/OD_{600} cell)[a]			
	Whole-cell	Periplasmic fraction	Cell-free extract	PMBN treated cells
SM101/pBR322	0.13 ± 0.03	0.61 ± 0.06	0.63 ± 0.09	0.25 ± 0.03
SM105/pBR322	0.09 ± 0.02	0.67 ± 0.12	0.68 ± 0.10	0.49 ± 0.06
E609L/pBR322	0.12 ± 0.01	0.63 ± 0.09	0.63 ± 0.07	0.33 ± 0.07
E609/pBR322	0.03 ± 0.01	0.69 ± 0.11	0.71 ± 0.11	0.59 ± 0.13

[a] All results are averages of at least three separate experiments and are represented as means ± 1 standard deviation.

Table 2–2B. Percentage β-lactamase activity.

Strains	Percentage Activity (with respect to cell-free extract)	
	Whole-cell [a]	PMBN treated cells[b]
SM101/pBR322	20	39
SM105/pBR322	13	71
E609L/pBR322	18	52
E609/pBR322	4.8	83

[a] $\frac{\text{Activity of whole - cell (Table 2a)}}{\text{Activity of cell - free extract (Table 2a)}} \times 100$

[b] $\frac{\text{Activity of PMBN - treated cells (Table 2a)}}{\text{Activity of cell - free extract (Table 2a)}} \times 100$

Higher rates were observed when a peptide permeabilizer, PMBN, was employed. For PMBN-treated cells, percentage activities reached 40%–to–80% of the respective cell-free extract levels (Table 2–2B), indicating the importance of the Lipid A region as a permeability barrier. A comparison of whole cell activities with and without PMBN treatment is shown in Figure 2–4, a plot of activity ratios for treated and untreated cells. The effect of permeabilizing with PMBN is more pronounced for wild-type cells than for mutant strains. This is not surprising for the LPS mutant, as less Lipid A was synthesized in the mutant and, consequently fewer PMBN binding sites were available in the membrane. The reason for a greater effect on permeation of PMBN toward E609 compared to E609L is not yet understood.

Interestingly, the two wild-type strains (SM105 and E609) exhibited significant differences in whole-cell activities. E609 had an activity of 0.034 units, a result 2.6–fold lower than the activity of 0.088 units measured for SM105. This difference is not due to

the expression level of the enzymes, as both strains have a similar level of expression as judged by the activities in the cell-free extract. Rather, the difference may be attributed to the different outer membrane structures that result from their different genetic background. Particularly, if the Lipid A concentration in the outer membrane outer leaflet was significantly higher in E609 than SM105, one would expect to see lower whole-cell activities but more pronounced PMBN effects on permeability in E609. The observed activities are consistent with this hypothesis (Table 2–2 and Figure 2–4) but further confirmation of this explanation is desirable and should be addressed in future studies.

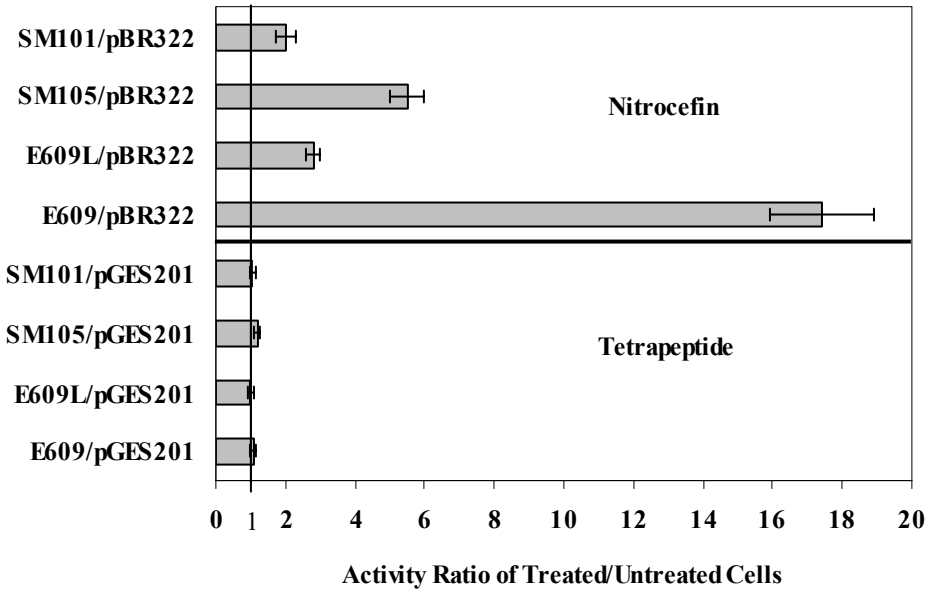


Figure 2–4. PMBN permeabilizing effect. Error bars represent standard deviations from the mean. All activities are averages of the results from at least three separate experiments.

The Lipid A region is very hydrophobic and therefore suspect as the barrier for hydrophilic molecules such as nitrocefin. Accordingly, PMBN is highly effective in

increasing the outer membrane permeability to this substrate. Yet, reduction of Lipid A synthesis by LPS mutation is less effective for this purpose. This may be due to the hybrid outer membrane structure that results from LPS mutation. The reduced Lipid A may have been replaced with other hydrophobic lipid molecules by a compensatory mechanism, effectively diminishing the effect of reduced Lipid A in increasing permeability. Genetic modifications of the fatty-acid moiety of Lipid A may be more effective in reducing the resistance of the outer cell membrane to hydrophilic molecules (Figure 2–1B).

2.3.2 Reaction with a tetrapeptide substrate

We have also investigated the transport of the tetrapeptide N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF-pNA, Figure 2–3B) across the cell walls of our *E. coli* strains. To our knowledge, the outer membrane permeability to this substrate has not been investigated. In a previous study, whole cells with periplasmically-expressed subtilisin exhibited some activity, indicating some permeability through the outer membrane. However, the authors did not identify low cell-wall permeability as the rate-limiting step in the synthesis.

The effect of mutations on the reaction rate of the tetrapeptide was investigated in the present study. Four strains (Table 2–1) were transformed with plasmid pGES 201, which carries a gene coding for subtilisin E. The expression of the enzyme is driven by an arabinose-inducible promoter. The majority of the enzyme activity was found in periplasmic space (Table 2–3A). Judging by the activity levels in cell-free extract, both

mutants had lower expression of the enzyme than the wild types. SM101 had an expression level about 15% lower than the parental strain and the expression level in E609L was about 25% lower than that in the wild type. Despite the lower expressions, mutant whole-cells exhibited activities either similar to (E609 pair) or significantly higher (SM pair) than the activities obtained by their respective parental stains (Table 2–3A, column 1), indicating increased permeability by either mutation.

When whole-cell activities are compared with their respective cell-free extract activities, wild-type cells SM105/pGES201 and E609/pGES201 have about 46% and 60%, respectively, of their associated cell-free extract activities. The existence of a permeability barrier to AAPF-pNA in each cell type is indicated. Mutation of LPS increased the whole-cell activity to 95% of the cell-free extract level, or 105% of the periplasmic level. This result is particularly striking as it implies that the mutation rendered the whole-cells completely permeable to the tetrapeptide and eliminated the mass transport resistance. Whole-cell catalysts reach a membrane-less or barrier-less condition that maximizes reaction rates. Lipoprotein mutation was also effective in increasing the permeability of the substrate. The reaction rates for whole-cells reached about 80% of their cell-free extract level or 94% of their periplasmic level. Thus, for this mutant, the mass transport limitation imposed by the outer membrane was almost totally eliminated.

Table 2–3A. Subtilisin activity.

Strains	Activity (mUnits/OD_{600} cell)[a]			
	Whole-cell	Periplasmic fraction	Cell-free extract	PMBN treated cells
SM101/pGES201	3.9 ± 0.5	3.7 ± 0.3	4.1 ± 0.2	4.1 ± 0.4
SM105/pGES201	2.2 ± 0.4	4.6 ± 0.4	4.8 ± 0.4	2.6 ± 0.3
E609L/pGES201	6.7 ± 0.8	7.1 ± 0.8	8.1 ± 0.9	6.7 ± 0.7
E609/pGES201	6.4 ± 0.7	10 ± 1.1	11 ± 1.2	6.9 ± 0.7

[a] All results are averages of at least three separate experiments and represented as means ± 1 standard deviation. Activities are in milli-units.

Table 2–3B. Percentage subtilisin activity.

Strains	Percentage Activity (with respect to cell-free extract)		Percentage Activity (with respect to periplasmic fraction)
	Whole-cell[a]	PMBN treated cells[b]	Whole-cell[c]
SM101/pGES201	95	100	105
SM105/pGES201	46	54	48
E609L/pGES201	82	82	94
E609/pGES201	59	64	66

[a] $\frac{\text{Activity of whole - cell (Table 3a)}}{\text{Activity of cell - free extract (Table 3a)}} \times 100$

[b] $\frac{\text{Activity of PMBN - treated cells (Table 3a)}}{\text{Activity of cell - free extract (Table 3a)}} \times 100$

[c] $\frac{\text{Activity of whole - cell (Table 3a)}}{\text{Activity of Periplsmic fraction (Table 3a)}} \times 100$

The PMBN permeabilizing effect was also investigated with tetrapeptide. The ratios of activities of treated cells to the activities of untreated cells are plotted in Figure 2–4. The activity ratios for PMBN-treated cells relative to untreated cells are in the range of 1.0 to 1.2, indicating only a small reduction in the barrier to permeation for the tetrapeptide. This is expected for mutant strains, as they no longer experience any

hindrance to permeation through the outer membrane. However, the minimal effect of PMBN on wild-type strains is in sharp contrast with the results from studies with nitrocefin, for which the ratios fell in the range of 2–to–18 (Figure 2–4) and for which the more pronounced effect was observed for the wild type strains. The difference in hydrophobicity of the two substrates may be responsible for this observation. PMBN acts by binding to Lipid A of the LPS, the highly hydrophobic region of the outer membrane. Based on the solubility of nitrocefin in an aqueous solution that did not dissolve the tetrapeptide, we consider the tetrapeptide to be more hydrophobic than nitrocefin. Therefore, the tetrapeptide can be expected to permeate through the Lipid A region more quickly than does the nitrocefin.

The LPS mutation and lipoprotein mutation do not cause detectable leakage of the β-lactamase or subtilisin under the conditions used for whole-cell activity assay. This was ascertained by measuring the respective activities of the supernatant samples taken during whole-cell reactions under identical experimental conditions. The supernatant showed negative β-lactamase or protease activity. Therefore, the increased whole-cell activity is attributable to increased permeability via mutations rather than to leakage of the enzymes.

2.4 Discussion

The substrate permeation barrier presented by cell envelopes is the underlying cause of low whole-cell biocatalysis rates. In this study, significant acceleration of whole-cell biocatalysis was achieved by reducing the barrier properties of the outer membrane via genetic modifications of the outer membrane structure. In the case of the tetrapeptide, mutation of the outer membrane accelerated the biocatalytic reaction to its maximum since the cellular membrane no longer impeded mass transport of the enzyme reactants. By using a single mutation, whole-cell biocatalysis exhibited the same rates as for free enzymes, yet all the advantages of whole-cell catalysis were preserved.

For the nitrocefin reaction, however, the same mutations had less impact on increasing the whole-cell reaction rates and only about 20% of the achievable cell-free extract level was measured. The gains to be realized by mutating the outer membrane are therefore dependent on the nature of the substrate. The results of experiments using PMBN to increase substrate permeation rates indicate the importance of the relative hydrophobicities of the substrates. This explanation is consistent with the earlier studies, which showed that *E. coli* cells with an LPS mutation were particularly susceptible to hydrophobic antibiotics due to increased permeability. Other differences, such as the molecular size of substrates and their ability to carry an ionic charge, certainly contribute to the differences in mass transport rates across the genetic altered cell walls.

To further probe the relationship of membrane permeability to the substrate molecular structure, the *E. coli* mutants could be interrogated with substrates of systematically varied sizes, hydrophobicities, and ionic charges. Other mutants,

engineered to allow passage of particular molecular structures, could also be developed. Understanding these relationships is important for predicting the effects of genetic modifications on catalysis rates and can ultimately lead to other rational approaches for modifying cellular membranes. This study was one step toward that goal.

In the case of tetrapeptide, mutations of the outer membrane structure resulted in a significant increase in permeability, whereas treatment with PMBN had little effect. Modifying the membrane structure genetically was more effective in increasing the permeation rate of tetrapeptide, than was treatment with the permeabilizer, strongly supporting our rationale for pursuing genetic alterations as a means of improving whole-cell biocatalysis rates. The PMBN treatments, however, identified the Lipid A region of *E. coli* as a potentially useful site for genetic modifications targeting increased permeability to hydrophilic molecules like nitrocefin. More importantly, the results presented here suggest that the genetic modification of Lipid A might be a better strategy than reducing the Lipid A content of the cell membrane.

CHAPTER 3

Lipoprotein Mutation Accelerates Substrate-Permeability Limited Toluene Dioxygenase–Catalyzed Reaction

One of the major problems in whole-cell biocatalysis is its low reaction rate. The underlying cause is the substrate permeation barrier presented by cell envelopes. The present research investigates mutation effects of the Braun's lipoprotein, the most abundant outer membrane structural protein in *E. coli*, on toluene dioxyengase (TDO)-catalyzed reaction. Dramatic enhancement of the reaction rate, an increase of up to six fold, was observed with the mutant for all three small, hydrophobic substrates tested (toluene, ethylbenzene and 2-indanone). The increase was observed over a wide range of substrate concentrations (0.1–5 mM). The mutant exhibited the same growth rate and comparable recombinant multi-component enzyme expression level as the isogenic parent strain. Taken together, the lipoprotein mutant carrying a plasmid encoding TDO is a much better whole-cell catalyst for the oxidation reaction. The beneficial effect of the lipoprotein mutation may be general for a broad range of substrates and enzyme systems as the mutation affects the global integrity of the cell membrane. A comparison of the mutation effect with a common permeabilizing procedure, the EDTA treatment, further illustrated the clear advantages of using genetic modification in cellular membrane engineering for improved whole-cell catalysts.

3.1 Introduction

Toluene dioxygenase (TDO) is a multi-component enzyme system that catalyzes the dioxygenation of toluene to toluene *cis*-dihydrodiol. In addition to toluene, it accepts other aromatic compounds, non-aromatic nuclei and aliphatic olefins as substrates. It catalyzes not only dioxygenation, but also monooxygenation, desaturation, O dealkylation, N dealkylation, sulfoxidation, and oxidative dehalogenation reactions[29, 30]. Its remarkable versatility has stimulated numerous studies in recent years for potential applications in bioremediations of recalcitrant aromatic hydrocarbons such as toluene and related compounds, and for chiral synthon synthesis in green chemistry applications[31, 32, 33, 34, 35, 36, 37].

So far, the studies of application of TDO have been exclusively with whole-cell catalysts. This is not surprising as the oxidation involves multiple enzyme components reductase, ferredoxin and terminal dioxygenase, and requires cofactor NADH. It would be too complicated if each component of the system were to be isolated and reconstituted *in vitro*. However, one obstacle in applying such whole-cell biocatalysts is the limited substrate permeability imposed by the cell envelopes, which could result in reduced reaction rate and low productivity

Toluene is a small hydrophobic molecule. In general, the outer membrane of gram-negative cells such as *E. coli,* especially the outer leaflet that consists of lipopolysaccharides (LPS), is a formidable barrier for hydrophobic molecules. The LPS monolayer is a highly ordered quasi-crystalline structure with very low fluidity, which does not allow rapid diffusion of molecules. Additional rigidity of the outer membrane

can be attributed to its interaction with the murein layer involving an outer membrane lipoprotein, called Braun's lipoprotein. This protein is present in some 700,000 copies per cell, which makes it the most abundant protein in numerical sense in *E. coli*. About one-third of the lipoprotein molecules are covalently linked to murein and help hold the two structures together. Mutants defective in the structural gene for this protein are fully viable, indicating it is nonessential. However, it is possible the disruption of lipoprotein will affect the global membrane integrity and thus may effect some changes in substrate permeability. Indeed, our earlier studies have found that the permeability for nitrocefin and a tetrapeptide, substrates for a β-lactamase and a protease, respectively, through the outer membrane were increased in a mutant which was absent of a major lipoprotein. Consequently, reaction rates catalyzed by the respective enzymes were dramatically increased with the mutant cells[38]. It was not clear, though, how the mutant would fare with smaller but very hydrophobic molecules. In this study, we examined the mutation effect of lipoprotein on reaction rates catalyzed by TDO. Exploiting the relaxed substrate specificity of this enzyme system, we investigated TDO catalyzed reactions with three different substrates, toluene, ethylbenzene, and 2-indanone. To fully assess the suitability of the mutant as a whole-cell catalyst, a comparison study of cell growth and level of recombinant protein production was also carried out.

3.2 Experimental

3.2.1 Reagents, bacterial strains and plasmids

All chemicals were reagent grade and used as received. Toluene, o-cresol, ethylbenzene, 2-ethylphenol, 2-indanone, ethyl acetate, and butyl acetate were purchased from Aldrich (St. Louis, Mo). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from BioVectra (Canada). *cis*-(1*S*,2*R*)-Dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) was purchased from CSS (Northern Ireland). Ready Tris-HCl gel, Laemmli sample buffer, 2-mercaptoethanol, SDS-PAGE Low Range Molecular Weight Standards and Coomassie Brilliant Blue R-250 stain and destain solution were purchased from Bio-Rad (Hercules, CA).

Plasmid pDTG601, which was constructed by inserting gene *todC1C2BA* into pKK223-3 for TDO expression under the control of the *tac* promoter, was a gift from Dr. D. T. Gibson[39]. Two *E. coli* host strains were used in this study. E609L, in which the gene *lpp* encoding Braun's lipoprotein was disrupted by a Tn*10* insertion, is designated as the mutant. E609 was its isogenic parent and was used as the control. Strains E609L/pDTG601 and E609/pDTG601 were constructed by transforming the host strains with plasmid pDTG601.

3.2.2 Cell growth curve and protein expression

LB medium supplemented with appropriate antibiotics (as described below) was used in all cultivations. The medium for plasmid-carrying strains was supplemented with

100 μg/ml ampicillin and the medium for the mutant was additionally supplemented with 12.5 μg/ml tetracycline. For cell growth study, 100 ml of medium in a 500 ml flask was inoculated with an overnight culture of E609L, E609, E609L/pDTG601, and E609/pDTG601 (2% inocula). The flasks were shaken in a rotary shaker at 250 rpm at 37 ^{o}C. Samples were taken from each culture every hour to monitor the growth pattern by measuring optical density at 600 nm with a UV/VIS spectrophotometer (Lambda 40 Spectrometer, Perkin Elmer). For cell growth study with induction, the same medium as described above was used and initial cultivation was carried out at 37^{o}C until OD_{600} reached 0.5–0.7, when IPTG was added to a final concentration of 1 mM and the cultivation temperature was shifted to 30 ^{o}C.

To evaluate the TDO expression levels by SDS-PAGE analysis, samples were taken every hour for four hours after induction. Whole-cells pellets from 1 ml culture were suspended in 100 μl 1 × SDS-PAGE sample buffer containing 2.5% 2-mercaptoethanol. Samples were boiled for 5 minutes and centrifuged briefly. 5 μl of samples and a marker were loaded into a Ready 10 % Tris-HCl gel (6.8 cm × 8.6 cm, 1.0 mm thickness). The gel was stained using Coomassie Brilliant Blue R-250 stain solution.

3.2.3 Initial velocity measurement

Ten ml of LB medium supplemented with 100 μg/ml ampicillin in a 50 ml flask was inoculated with an overnight culture of E609L/pDTG601 or E609/pDTG601 (2% inocula). Other cultivation and induction conditions were as described in the cell growth studies.

Two hours after induction, 1 ml of culture was collected in a 1.5 ml Eppendorf tube. Cells were harvested by centrifugation at 4,000 × g for 10 minutes at 4°C and washed once with 10 mM sodium phosphate buffer (pH 7.4). Cell pellets were collected by centrifugation and resuspended into 0.5 ml of same sodium phosphate buffer containing 0.4% glucose. The reaction was started by addition of substrate to a desired final concentration. The reaction mixture in the Eppendorf tube was then sealed with parafilm and shaken at 250 rpm at 37°C for 15 minutes for initial rate measurement. The product of the oxidation reaction was extracted by ethyl acetate. In the case of reactions with toluene and ethylbenzene, before subject to GC analysis, the products were dehydrated to their respective dihydrodiol products by an acid treatment (25 μl of 2M HCl) (Figure 3–1)[40]. The extract was analyzed by a Hewlett-Packard GC/MS system. All activities were normalized by A_{600} to correct for small differences in cell density between preparations.

3.2.4 Time course

The time course of the whole cell catalyzed reactions was conducted under the same conditions as described above, except that the reaction was allowed to proceed to a longer period of time and samples were taken from time to time to monitor the progress of the reactions.

3.2.5 Analytical methods

Samples as prepared above were analyzed using a Hewlett-Packard 6890 (Palo Alto, CA) Gas Chromatograph equipped with an MSD, an autosampler, and an injector. A HP-5MS methylpolysiloxane phase column (30 m × 0.25 mm × 0.25 μm) was used. The temperature for the injector and the MSD was 280 ^{o}C. Column temperature profile was according to a pre-determined program (40^{o}C for 1 minute, first temperature gradient to 180^{o}C at 10 ^{o}C/min; second temperature gradient to 300^{o}C at 25 ^{o}C/min; and holding the final temperature for 10 minutes). The flow rate of the carrier (He) was 1 ml/min. Under these conditions, the retention times for butyl acetate (internal standard), toluene, o-cresol, ethylbenzene, 2-ethylphenol, 2-indanone and (*S*)-2-hydroxy-1-indanone were 4.46, 3.99, 7.90, 5.17, 9.17, 7.10 and 12.55 min, respectively.

3.2.6 EDTA treatment

The effect of EDTA on whole-cell catalyzed-reaction rate was studied. EDTA was added to a cell suspension to a final concentration in the range of 0.1 to 1.0 mM. The mixture was incubated quiescently at room temperature for 15 minutes before 2 mM toluene was added. The reaction was carried out under the conditions as described above.

3.3 Results

In order to evaluate the mutation effect of lipoprotein on TDO catalyzed reactions and the suitability of lipoprotein mutant as whole-cell catalyst in bioremediation and green chemistry applications, we used three different TDO substrates in this study, toluene, ethylbenzene, and 2-indanone. The respective reactions and products are illustrated in Figure 3–1. *E. coli* strain E609L carries a transposon mutation in an outer membrane structural protein called Braun's lipoprotein, and E609 is its isogenic parent and is used here as the control. The two strains were transformed with a plasmid pDTG601, which harbors the genes of TDO from *Pseudomonas putida* F1, a multi-component system.

3.3.1 Cell growth and recombinant protein expression

One essential attribute for microbial whole-cell biocatalysts is their ability to grow and attain high cell density. In the process of engineering a whole-cell biocatalyst, one important consideration is to balance the need for increased permeability of substrate with the need for the cell to maintain sufficient membrane functions and to retain the ability for growth as much as possible. Since the membrane function is critical to cell growth, any mutation aimed to increase the substrate permeability should be evaluated with respect to the effect on cell growth. We therefore carried out a comparison study to evaluate the lipoprotein mutation on cell growth.

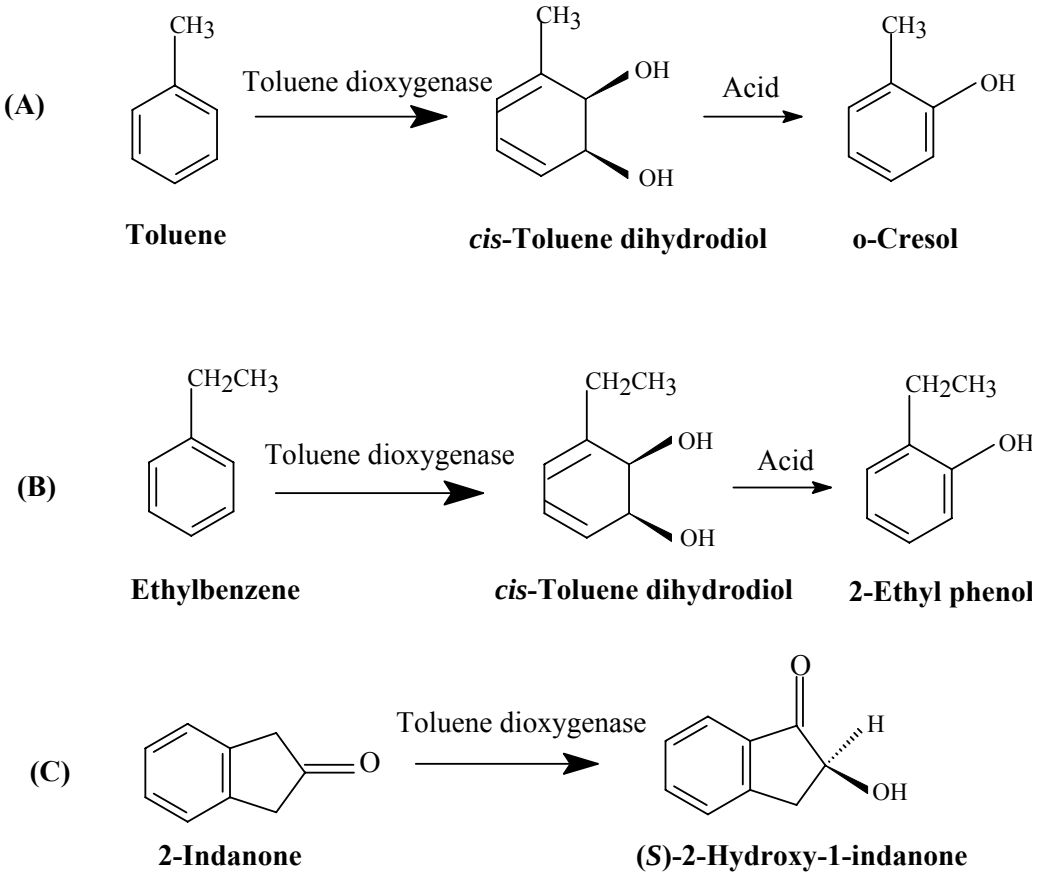


Figure 3–1. Oxidation reactions catalyzed by toluene dioxygenase. (A) toluene, (B) ethylbenzene, (C) 2-indanone.

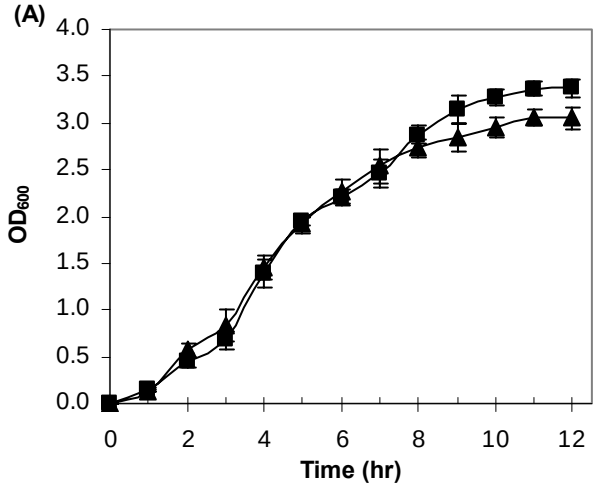
The mutant and control were grown in shaker flasks under identical conditions except that for the mutant, the LB medium was supplemented with tetracycline in order to apply a selective pressure for the transposon insert. As shown in Figure 3–2A, the two strains exhibited, in the first seven hours of cultivation, an almost identical growth rate with doubling time of 0.96 hour. After seven hours, the growth rates were reduced, but more so with the mutant strain, resulting in a difference in cell concentrations about 10%.

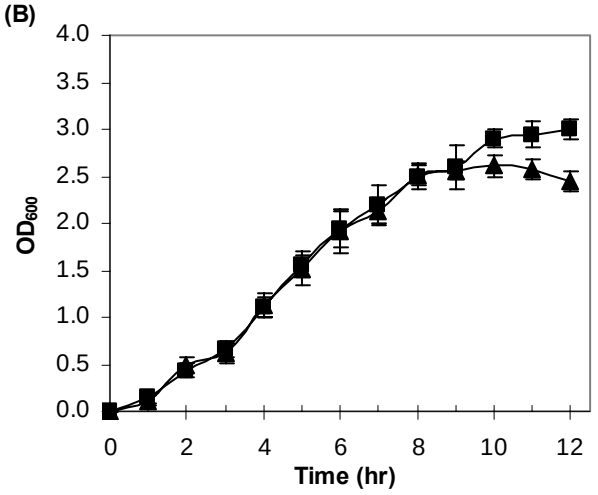
The highest cell concentrations reached were 3.1 and 3.4 OD_{600} for the mutant and the control, respectively.

Similar growth behaviors were observed when cell growth studies were carried out with the two strains carrying plasmid pDTG601 but without induction (Figure 3–2B). The only difference observed in growth was in the last few hours of cultivation. The highest cell concentrations reached were 2.6 and 3.0 OD_{600} for the mutant and the control, respectively, both lower than what were obtained without the plasmid, reflecting the extra burden of maintaining an 8.6 kb plasmid. The difference in cell density between the two strains was similar with or without plasmid (0.4 versus 0.3). Therefore, the mutation does not dramatically affect the cell's growth yield when carrying the plasmid. However, the mutant did not seem to be able to maintain the highest cell density obtained and there was a decrease of OD as soon as the highest density was reached, unlike the case without the plasmid and with the control (Figure 3–2A), suggesting that the presence of the plasmid made the mutant cells somehow vulnerable and the cells were on the verge of death (or lysis) at the end of the growth period.

Growth comparison was also conducted for the two strains carrying pDTG601 with inductions. As shown in Figure 3–2C, for many hours after induction, mutant cells were able to grow at the same rate as the parent strain. This is consistent with the results from Figure 3–2B without induction, but surprising considering that as soon as the growth stopped, the cell death (or lysis) ensued as manifested by the decrease of the OD. The parent strain, however, maintained the highest cell density in the stationary phase.

Consequently, after the cessation of the growth, the difference in cell concentrations was widened from about 0.4 at 9th hour to about 0.6 at 12th hour.





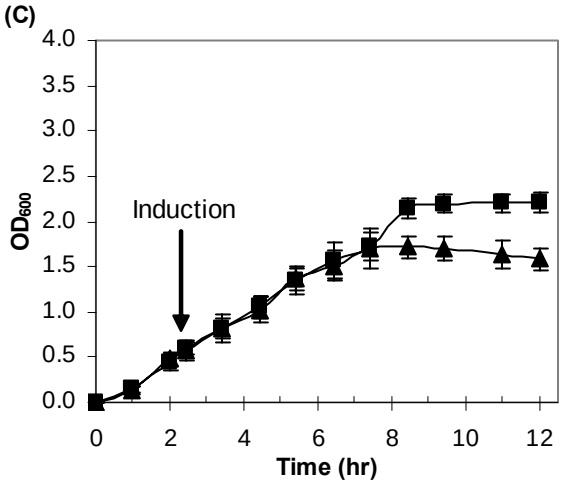


Figure 3–2. Growth pattern of (A) E609L (▲) and E609 (■); (B) E609L/pDTG601 (▲, without induction) and E609/pDTG601 (■, without induction); (C) E609L/pDTG601 (▲, IPTG induction) and E609/pDTG601 (■, IPTG induction). Error bars represent standard deviations from the mean. All data are measured in at least three separate experiments.

Taken together, the lipoprotein mutation does not seem to affect the growth rate even when cells are carrying a plasmid and are producing recombinant proteins. However, the mutation apparently sensitizes the cell to conditions that are typically found at the end of growth, or in stationary phase. We speculate that limitations of nutrients and accumulation of waste products such as acetate may be responsible for the difference observed at the end of the cultivation. Further study is needed to fully understand the underlying reasons for the phenomenon observed.

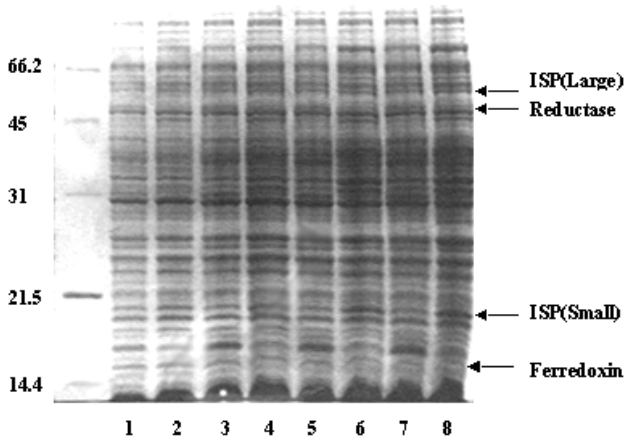


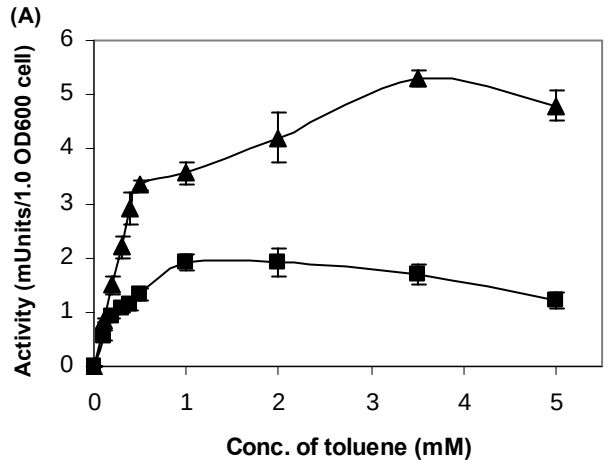
Figure 3–3. SDS-PAGE comparison of the toluene dioxygenase expression in *E. coli* E609L/pDTG601 and E609/pDTG601. The cellular fractions were loaded on the gel as follows: 1 hr induced E609L/pDTG601 (lane 1), 1 hr induced E609/pDTG601 (lane 2), 2 hr induced E609L/pDTG601 (lane 3), 2 hr induced E609/pDTG601 (lane 4), 3 hr induced E609L/pDTG601 (lane 5), 3 hr induced E609/pDTG601 (lane 6), 4 hr induced E609L/pDTG601 (lane 7), 4 hr induced E609/pDTG601 (lane 8).

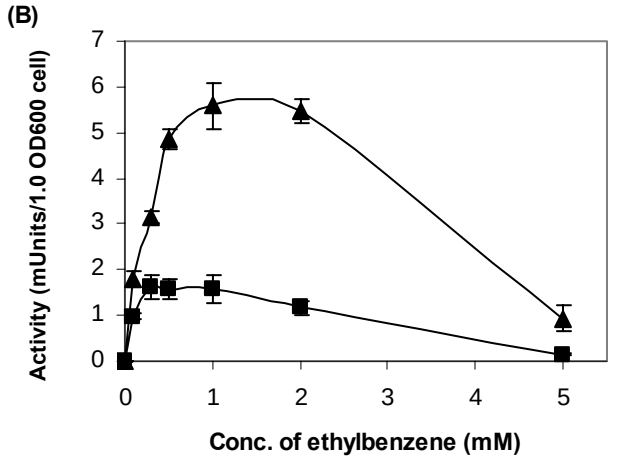
Since engineered whole-cell catalysts with overexpression of one or more recombinant enzymes are common in biocatalysis, another essential attribute of the whole-cell catalyst is the ability for high level expression of recombinant proteins. For this reason, we carried out studies to compare the expression levels of TDO for the two strains. Samples were taken every hour after induction for four hours, and expression levels were evaluated using SDS-page gel and Coomassie Blue stains. Figure 3–3 shows that there is essentially no detectable difference in expression levels of TDO between the two strains for all the samples (1–4 hours after induction) analyzed and for all components of the TDO system. Therefore, the mutation in lipoprotein does not affect the

expression of the multi-component TDO system. Samples taken after 4 hours exhibited lower activities for reasons not clear to us, but this is the case for both strains and therefore is not an effect associated with the mutation. In subsequent catalysis studies, conditions (2 hrs after induction) that gave rise to comparable expression levels of TDO in two strains were used.

3.3.2 Lipoprotein mutation on initial velocity

The initial velocities for the oxidation reactions catalyzed by the two recombinant strains were measured for substrate concentrations over the range of 0.1-5 mM. As shown in Figure 3–4A, B, and C, the mutant strain exhibited much higher initial activities for each of three substrates and at all substrate concentrations tested, suggesting a positive effect of mutation on increasing reaction rate. The difference in the initial velocity was not due to the difference in expression as comparable level of expression was achieved (as discussed earlier). Although some of the components of TDO are inner membrane bound and some reside intracellularly, toluene, ethylbenzene, and 2-indanone are sufficiently hydrophobic and their passage through inner membrane is not expected to be rate-limiting. The increased initial reaction rate is therefore likely through improved outer membrane permeability to substrates due to the mutation.


(A)
Activity (mUnits/1.0 OD600 cell)
0
1
2
3
4
5
6
0
1
2
3
4
5
Conc. of toluene (mM)


(B)
Activity (mUnits/1.0 OD600 cell)
0
1
2
3
4
5
6
7
0
1
2
3
4
5
Conc. of ethylbenzene (mM)

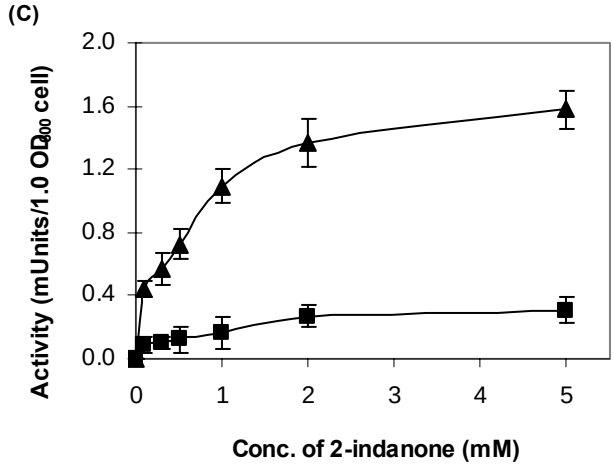

(C)
Activity (mUnits/1.0 OD600 cell)
0.0
0.4
0.8
1.2
1.6
2.0
0
1
2
3
4
5
Conc. of 2-indanone (mM)

Figure 3–4. Effect of substrate concentration on the initial degradation rate by whole-cell *E. coli* E609L/pDTG601 (▲) and E609/pDTG601 (■). (A) toluene, (B) ethylbenzene, (C) 2-indanone. Error bars represent standard deviations from the mean. All data are measured in at least three separate experiments.

Over the range of toluene concentration tested, the initial rate ratios of mutant to parent strains varied over a range of 1.5 (at 0.1 mM) to 3.9–fold (at 5 mM). The initial velocity profile was complicated by the presence of possible substrate and/or product inhibitions, evident from the decline of the reaction rate with the increase of the toluene substrate concentrations (1-5 mM) for the parent strain. The mutant strain seemed to fare better in terms of inhibition and only a slight decrease of reaction above substrate concentration of 4 mM was evident (Figure 3–4A).

For the second substrate, ethylbenzene, the initial velocity-substrate concentration profile was similar to that of toluene except that there was a sharp decline of enzyme activities at high substrate concentrations for both strains. The ratio of reaction rates of the two strains fall in the range of 1.8 (at 0.1 mM) and 6.3 (at 5 mM). The reasons for the difference between the two substrate, with respect to possible substrate or/and product inhibitions, were not clear, and were not investigated further as it was deemed to be beyond the scope of this work.

For 2-indanone (Figure 3–4C), the mutant strain had a substantially higher reaction rate than that of the parent over the entire range of concentrations examined. For example, at substrate concentration of 5 mM, the initial reaction rate of the mutant strain

is about 5 times of that of the parent strain. There was apparently no inhibition for this substrate.

A departure of the enzyme kinetics from the Michaelis-Menten model was evident from inspection of Figure 3–4 and Eadie-Hofstee plots (Figure 3–5, shown only for toluene). This may be due to the multi-component nature of the system and the presence of inhibitions in the case of toluene and ethylbenzene.

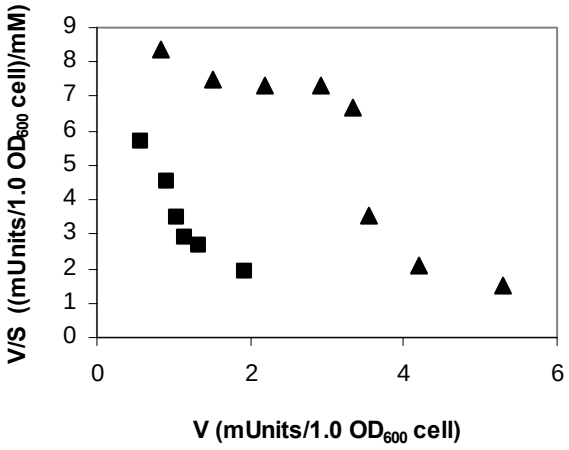


Figure 3–5. Eadie-Hofstee plots of whole-cell enzyme kinetic properties of E609L/pDTG601 (▲) and E609/pDTG601 (■).

3.3.3 Time courses of TDO catalyzed reactions

Additional experiments were carried out to further evaluate the potential of the lipoprotein mutant as whole-cell biocatalyst in bioremediation and green chemistry applications. Time course of product formation from toluene degradation for initial toluene concentration of 2 mM is shown in Figure 3–6. Within 2 hours, the mutant converted 2 mM toluene to 1.5 mM product *cis*-dihydrodiol, which subsequently

chemically converted to o-cresol under acidic conditions, a conversion of approximately 75%, whereas the parent strain had a conversion only about 30% and the product concentration reached was about 0.6 mM. For reasons not clear to us, the reactions for both strains completed within about two hours and there was no further reaction beyond that. The observed difference in conversion and product concentration was a result of substantial difference in the reaction rate between the two strains, reflected by the difference in the slopes of the time course (Figure 3–6), a result consistent with the observed faster initial velocity (Figure 3–4A) for the mutant. Substantial differences in conversion and product concentration were also observed when different initial toluene concentrations were used. In all concentrations tested, the mutant degraded more toluene and achieved higher product concentrations. At an initial concentration of 1 mM toluene, for example, nearly 100% toluene was degraded by the mutant while the parent could only degrade about 50% (data not shown).

Similar results were obtained with other two substrates (data not shown). The product concentration formed with the mutant cells was 2-3 times higher than that obtained with the parent strains, a result that correlated well with the initial velocity differences between the two strains (Figure 3–4).

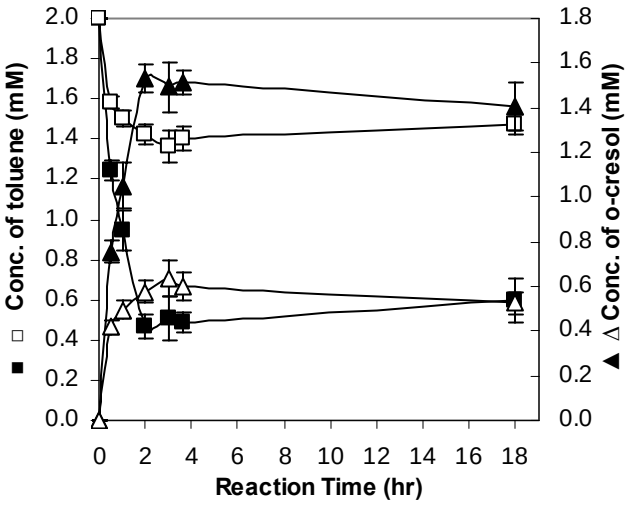


Figure 3–6. Time course of toluene degradation by whole-cell *E. coli* E609L/pDTG601 (solid) and E609/pDTG601 (open). Initial toluene concentration was 2 mM. Error bars represent standard deviations from the mean. All data are measured in at least three separate experiments.

3.3.4 EDTA Treatment versus Lipoprotein Mutation

EDTA is an effective membrane permeabilizer. It has long been used to treat bacterial cell membranes to increase permeability[41]. It functions through chelation of metal ions such as Ca^{++} and Mg^{++}, which cross-link lipopolysaccharides, consequently adding to the rigidity of the outer membrane. Since the LPS layer is suspected to be the main permeability barrier to hydrophobic molecules such as toluene, removal of these metal ions is expected to weaken the intra-molecular interaction of LPO and loosen up the outer membrane layer, thus allowing passage of hydrophobic molecules through the outer membrane layer at a higher rate.

We carefully studied conditions of treatment, and under optimal conditions (0.5 mM EDTA and a treatment time of 15 minutes), pretreatment with EDTA increased reaction rate with toluene about 23% (standard deviation 5%) for the parent strain E609 (Figure 3–7). This compares with the lipoprotein mutation that increased the reaction rate more than two fold (Figure 3–7, Figure 3–4A), indicating the mutation in envelope structure could be far superior to chemical treatment. Increasing EDTA concentration higher than 0.5 mM caused a decrease of TDO activities, probably due to the removal of the required metal cofactors for TDO (Fe^{++}) and the destabilizing of the inner membrane. This result further illustrates that the chemical treatment causes undesired, generalized damaging effects that go beyond permeabilizing.

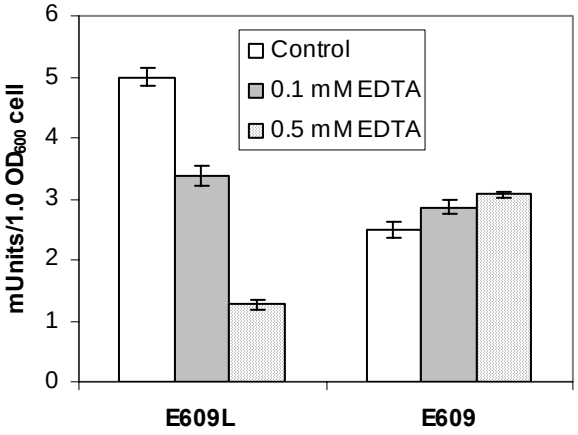


Figure 3–7. Effect of EDTA on the permeability of E609L/pDTG601 and E609/pDTG601. Error bars represent standard deviations from the means. All data are measured in at least three separate experiments.

Interestingly, no increase in reaction rate by EDTA was observed for the mutant strain. The treatment, in fact, caused a sharp decrease of TDO activities (Figure 3–7).

This is probably due to the defect in membrane structure caused by the lipoprotein mutation that sensitizes the cells for EDTA treatment.

3.4 Discussion

We have demonstrated that mutation in an outer membrane structural lipoprotein resulted in a several fold increase in the TDO-catalyzed reaction rate with the three small hydrophobic substrates investigated. The substantial increase in reaction rate indicates that modification of cellular membrane composition and mass transfer barrier properties through genetic engineering could be an effective method of accelerating whole-cell biocatalysis.

The significant acceleration of the oxidation reaction implies that the defect in membrane structures caused by the lipoprotein mutation may be extensive. Yet remarkably, the mutant cells grow as fast as the parent strain, even under stressful conditions such as carrying a medium-sized plasmid and overproducing a four-component recombinant enzyme system. It seems that the mutant cells were able to compensate the defect in membrane by some unknown mechanisms. The defect may affect the energetic state of the membrane, the proton gradient, for example. If this is true, it is conceivable that the mutant cells can compensate the loss of proton gradient by metabolizing more of an energy source, which would lead to a lower growth yield. Consistent with this explanation, we observed a slightly lower growth yield for the mutant in all three cases investigated. The mutant cells appear to be sensitive towards the end of the growth period, indicated by the loss of its ability to maintain cell biomass and an immediate transition to cell death phase when carrying a plasmid. The exhaustion of an energy source, which mutant cells are relying on to maintain the membrane energetic state and compensate the defect, could be responsible for the observed behavior at the end

of the cultivation. If this proves to be true, some additional carbon source would be sufficient to curb the loss of the biomass. Thus the difference in growth behavior caused by the mutation may not be a serious obstacle in biocatalysis applications, especially considering the difference is rather small and the gain in rate increase is substantial.

We have shown in this study that even with small molecules (molecular weight less than 100 Daltons), the substrate permeability can severely limit the reaction rate. A full evaluation of the permeability barrier was not possible as attempts to remove the cell membrane barrier and reconstitution of TDO components *in vitro* failed to recover a majority of the enzyme activity. However, from the dramatic acceleration by disruption of a structural protein, the severe limitation in substrate transfer across cell membrane can be appreciated. This common problem in whole-cell biocatalysis has been under-addressed, and we hope our research and the encouraging results we presented here will stimulate interest in this important area.

The genetic modifications on membrane structure with lipoprotein mutation, as shown here and in an earlier work, brought about significant increases of several fold in the reaction rate with three enzyme systems (including simple hydrolases and a multi-component redox enzyme system), and five substrates encompassing different sizes and different hydrophobicities. It appears that the beneficial effect on the whole-cell catalysis rate of the particular mutation is general.

CHAPTER 4

Outer Membrane Mutation Effects on UDP–Glucose Permeability and Whole-Cell Catalysis Rate

In whole-cell biocatalysis, cell envelopes represent a formidable barrier for substrates to permeate. As a result, the reaction catalyzed by the whole-cells is often orders of magnitude slower than that of their isolated enzyme counterparts. The present research addresses this critical issue by investigating the effects of outer membrane mutation on UDP-glucose utilizing enzymes in whole-cell systems. Owing to the severe limitation in substrate permeability, the wild type cells only exhibited as low as 4% of available enzyme activities. Reducing the barriers of the outer membrane permeability (by mutations in its structure) led to a striking acceleration (up to 14–fold) of reaction rate in cells expressing UDP-glucose dehydrogenase. Mutations in the lipopolysaccharide synthesis pathway or Braun's lipoprotein are both effective. The acceleration was dependent upon the substrate concentrations as well as the enzyme expression level. In addition, the mutation has been demonstrated to be much more effective than the freeze-thaw (FT) permeabilizing method. An application of outer membrane mutants was illustrated with the synthesis of a disaccharide (*N*-acetyllactosamine) from UDP-glucose. Both reaction rate and product yield were enhanced significantly (more than two fold) in the lipoprotein mutant, demonstrating the importance of the outer membrane permeability barrier and the advantages of using outer membrane mutants in synthesis.

4.1 Introduction

Whole-cell biocatalysis allows more complicated biotransformation that requires multi-enzymes or even multi-pathways. Enzymes enclosed inside and protected by the cell envelopes are often more stable than their isolated counterparts. Moreover, whole-cells provide what is often the only economically feasible way to regenerate expensive cofactors. However, whole-cell catalysts suffer a major problem. The cell envelopes represent a formidable barrier for substrates to permeate. As a result, the reaction catalyzed by the whole-cells is often orders of magnitude slower than that of the isolated enzymes.

Until recently, the only effective way to address this was to permeabilize cells by chemical (such as detergent, solvent, and chelators) or physical (such as extreme temperature fluctuations) treatment. These methods are not desirable, especially for large scale applications, since they create additional steps in manufacturing processes, complicate downstream processing, and incur unintended damage to cells. Previously, we demonstrated that modifications in cellular membrane structure through a molecular engineering approach could be very effective in reducing the permeability barriers[, 42]. Some benefits of this approach are that the modification to the cell membrane structure is localized and effects on cell growth are minimal.

In this chapter, we report a permeability study of a relatively large and hydrophilic molecule, UDP-glucose, using *E. coli* cells that express recombinant UDP-glucose utilizing enzymes. The results reveal a severe limitation in permeability for this molecule and a striking acceleration of reactions by single mutations in membrane

structure. Also, the application of these mutants is illustrated with the synthesis of a important disaccharide, *N*-acetyllactosamine (LacNAc). LacNAc is one of the most common core structures in oligosaccharides of glycoproteins, some of which are significantly elevated in various types of cancer patients[43,44]. LacNAc also play an important role in the initial event in many inflammatory responses[45]. The widespread biological role of LacNAc-based oligosaccharides has led to an increased demand for these structures for biomedical studies, and therefore, simple and efficient synthetic routes to these compounds are desirable. Compared to tedious chemical approaches (involving multiple protection and deprotection steps)[46,47], the enzymatic preparation of these structures using glycosyltransferases is highly efficient, and has been applied in gram-scale production[48]. In this study, the product yield of LacNAc was increased using membrane mutant strains. Also, important implications of the permeability issues in whole-cell biocatalysis research are discussed.

4.2 Experimental

4.2.1 Chemicals and reagents

All chemicals were reagent grade and used as received. UDP-glucose (UDP-Glc), NAD, UDP-glucuronic acid (UDP-GA), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-lactosamine (LacNAc), isopropyl-β-D-thiogalactopyranoside (IPTG), and dithiothreitol were purchased from Sigma (St. Louis, Mo). Primers were from Invitrogen (Carlsbad, CA). Ready Tris-HCl gel, Laemmli sample buffer, 2-mercaptoethanol, SDS-PAGE low range molecular weight standards, Coomassie Brilliant Blue R-250 stain, and destain solution were purchased from Bio-Rad (Hercules, CA).

4.2.2 Bacterial strains and plasmids

The *E. coli* strains and the plasmids used in this study are listed in Table 4–1. Two types of *E. coli* membrane mutant strains were used in this study. The lipid A mutant SM101(*lpxA2*) is a thermo-sensitive, UDP-N-acetylglucosamine acyltransferase (*lpxA*) defective strain[49]. SM105 is its isogenic parent strain. E609L carries a Tn*10* insertion in *lpp* coding for Braun's lipoprotein. E609 is its isogenic parent.

Table 4–1. *E. coli* strains and plasmids used in this study.

Strain or Plasmid	Description	Reference
E. coli strains		
SM101	*lpxA2* mutant, thermosensitive, grows well at 28°C	,
SM105	*lpx A2*$^{+}$, isogenic parent of SM101	49,
E609L	*lpp*::Tn*10*; periplasmic leaky; Tc^{r}	,
E609	*HfrC pps*, isogenic parent of E609L	18,
E609L(*ΔlacZ*)	E609L with deleted *lacZ* gene on chromosome	This study
E609(*ΔlacZ*)	E609 with deleted *lacZ* gene on chromosome	This study
E.coli O12:K5(L)	ATCC 23508	ATCC
Plasmids		
pCW-lgtB	amp^{r}, containing β-1,4-galactosyltransferase gene	50
pLysTE7	amp^{r}, containing *E. coli* UDP–galactose 4-epimerase.	51
pGEM-T-Easy vector	amp^{r}	Promega
pQE80L	commercial expression vector from Qiagen containing T5 promoter and *lacI*^{q}	Qiagen
pBBR122	gram negative broad host vector	MoBiTech
pT-GalE	containing *galE* gene with Bgl II, Bam H I site	This study
pT-LgtB	*lgtB* gene containing Bam H I and XhoI sites	This study
pT-EL	amp^{r} containing *galE*:*lgtB* with Bgl II XhoI sites	This study
pQEL	amp^{r} with T5 drive *galE*:*lgtB* fusion expression	This study
pT-KfiD	amp^{r}, containing *KfiD* gene	This study
pQEkfiD	UDP-glucose dehydrogenase gene *kfiD* in pQE80L	This study
pCEL	T5 directed β-1,4-galactosyltransferase (*lgtB*)/UDP-galactose 4-epimerase (*galE*) fusion protein encoding plasmid	This study
pKD13	template plasmid	52
pKD46	phage λ Red recombinase helper plasmid	52
pCP20	FLP recombinase elimination helper plasmid	52

4.2.3 Construction of lacZ knockout strains

The strains E609L(*ΔlacZ*) and E609(*ΔlacZ*) were constructed for LacNAc production using the gene inactivation method developed by Wanner and coworkers52. The FRT-flanked kanamycin resistance gene was PCR amplified along with a 50–nucleotide extension at each end, which is homologous to the adjacent regions of the genomic *lac*Z gene. The PCR product was generated using plasmid pKD13 as the template and the following primers: the 5' primer, 5'-GCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCGTGTAGGCTGGAGCTGCTTC-3') and the 3' primer, 5'-TTAAATAGTACATAATGGATTTCCTTACGCGAAATACGGGCAGACATGGCATTCCGGGGATCCGTCGACC-3' (homologous regions underlined). Subsequently, the purified PCR product was introduced by electroporation into the target strains E609L and E609, which were carrying Red helper plasmid pKD46. Homologous recombination mediated by the plasmid-borne (pKD46) phage λ Red recombinase resulted in kanamycin resistant transformants. The kanamycin gene was then excised by introducing the FLP helper plasmid, pCP20, harboring the gene for FLP recombinase. Finally, both the Red helper plasmid and the FLP helper plasmid were cured by growth at above 37°C as they contain temperature-sensitive replicons.

4.2.4 Construction of pQEkfiD, a T5 driven KfiD expression vector

The UDP-glucose dehydrogenase gene, *kfiD*[53], was amplified from the genomic DNA of *E. coli* O12:K5(L) (ATCC 23508) with the primers designed according to the

*kfi*D sequence (GenBank accession #: X77617). The 5' primer (5'-TGG***AGATCT*****ATG**TTCGGAACACTAAAAATAACTG-3') is a 34-mer that includes a *Bgl* II site (bold italics) and ATG start codon (bold underlined). The 3' primer (5'-TT***CTCGAG***TTAGTCACATTTAAACAAATCGCGAC-3') is also a 34-mer with a *Xho* I site (bold italics) and stop codon TAA (shown its reverse complementary sequence, underlined). The 1.1 kb fragment was first inserted into the pGEM T-easy vector (Promega) to give pT-kfiD. Then the final expression vector pQEkfiD was constructed by inserting the *kfi*D gene, obtained from pT-kfiD by the double digestion by *Bgl* II and *Xho* I, into the *Bam*HI and *Sal*I site of pQE80L (Qiagen). The organization of the plasmid pQEkfiD is shown in Figure 3–1A.

4.2.5 Construction of pCEL, a T5 driven galE: lgtB fusion expression vector

The *E. coli* UDP-galactose 4'-epimerase gene was amplified from pLysPTE7 using the following primers. The 5' primer, 5'-TGG***AGATCT***ATGAGAGTTCTGGTTACCGGTGGT-3', contains a *Bgl* II site (in bold italics) and start codon, ATG (underlined); the 3' primer, (5'-TA***GGATCC***GCCAGCGC*TGA*ATCGGGATATCCCTGTGGATG-3') contains a *Bam*HI site (shown in bold italics) with the original stop codon, TAA, mutated to Ser codon TCA, (reverse complementary sequence underlined) and a mini-linker sequence, coding for a linker peptide, Ala-Ala-Gly-Gly-Ser. The PCR fragment, about 1 kb, was ligated into the pGEM T-easy vector to give the plasmid pT-GalE. The gene for β-1,4-galactosyltransferase from *Neisseria meningitidis*, was cloned from a plasmid pCW-lgtB

with the following primers. The 5' primer, (5'-TA***GGATCC***ATGCAAAACCACGTTATCAGCTTAG-3'), contains a *Bam*HI site (bold italics) and start codon ATG (underlined); the 3' primer, (5'-TA***CTCGAG***TTATTGGAAAGGCACAATGAACTG-3'), contains an *Xho* I site (bold italics). The amplified PCR fragment (about 0.8 kb) was inserted into the pGEM T-easy vector to give the plasmid pT-lgtB. The lgtB gene fragment, obtained from pT-LgtB by the double digestion by *Bam*HI and *Sac*II, was inserted into the same sites in pT-GalE downstream from *galE* to give pT-EL. The fusion gene fragment obtained by *Bgl* II and *Xho* I digestion was fused into the *Bam*HI and *Sal*I sites of pQE80L to give pQEL. The plasmid pCEL was obtained by inserting the entire *Xho*I-hydrolyzed broad range host plasmid pBBR122 into the *Xho*I site of the pQEL. The organization of the plasmid pCEL is shown in Figure 3–1B.

4.2.6 Expression of UDP-glucose dehydrogenase and GalE-LgtB fusion protein in *E. coli*

The LB medium supplemented with appropriate antibiotics (as described below) was used in all cultivations. The medium for pQEkfiD carrying strains was supplemented with 100 μg/ml ampicillin and the medium for pCEL carrying strains was supplemented with 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. All medium for the E609L and E609L(*ΔlacZ*) strains were additionally supplemented with 12.5 μg/ml tetracycline, due to the Tn*10* insertional mutation of the *lpp* gene.

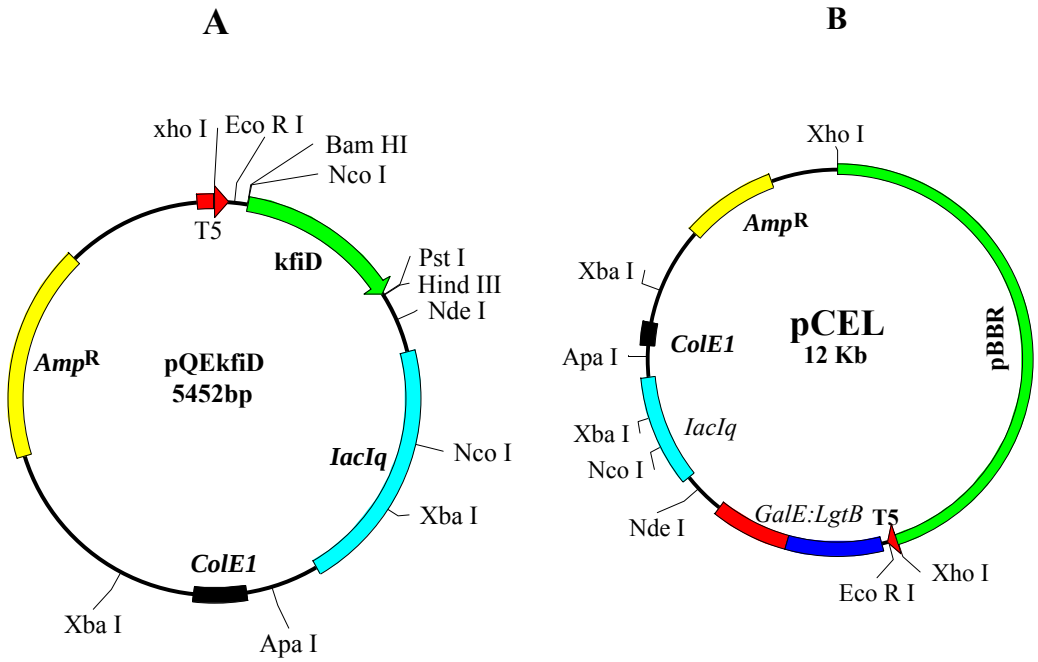


Figure 4-1. Plasmid map of pQEkfiD(A) and pCEL (B). Abbreviations are as follows: *Amp*^{R}: ampicillin resistance gene; *ColE1*: *colE*1 origin of replication; *gal*E:*lgt*B: fusion gene of UDP-galactose-4-epimerase and β-1,4-galactasyltransferase; *kfiD*: UDP-glucose dehydrogenase gene, *lacIq*: *LacIq* repressor; T5: phage T_5 promoter; pBRR: Broad-Host-Range Vectors pBBR122 (MoBiTec).

For the expression of UDP-glucose dehydrogenase, 10 ml of LB medium in a 50 ml flask was inoculated with an overnight culture of SM101/pQEkfiD, SM105/pQEkfiD, E609L/pQEkfiD, and E609/pQEkfiD (2% inocula). Cells were grown at 28^{o}C with shaking at 250 rpm. IPTG was added to a final concentration of 1 mM when OD_{600} reached 0.5~0.7 and the cells were incubated for additional four hours at 28 ^{o}C. Cells were then collected for further analysis. For the study with different IPTG induction

concentrations, the cultivation was conducted under the same conditions as described above, except that different IPTG concentrations were added.

For the expression of GalE-LgtB fusion protein, 100 ml of LB medium in a 500 ml flask was inoculated with an overnight culture of E609L(*ΔlacZ*)/pCEL and E609(*ΔlacZ*)/pCEL and incubated at 30^{o}C until OD_{600} reached 0.2~0.3, upon which IPTG was added to a final concentration of 1 mM. After induction, the cultivation was continued for another four hours at a lower temperature (25 ^{o}C).

4.2.7 UDP-glucose dehydrogenase activity assay

4.2.7.1 Whole cell activity assay

Cultivation and induction conditions were as described in section 4.2.6. 0.5 ml of induced culture was collected in a 1.5 ml Eppendorf tube. Cells were harvested by centrifugation at 4,000 × g for 10 minutes at 4^{o}C and washed once with 100 mM Tris-HCl buffer (pH 9.0). Cell pellets were collected by centrifugation and resuspended into 0.5 ml of same Tris-HCl buffer. The reaction was started by the addition of substrates, UDP-Glc and NAD^{+}, to desired final concentrations. Samples were taken during the first 30 minutes of the reaction at 37^{o}C to measure the initial rate. The reaction was terminated by centrifugation at 13,000g for 5 minutes and the supernatant was subjected to an HPLC analysis (Hewlett-Packard 1100 Series). All activities were normalized by A_{600} to correct for small differences in cell density between preparations.

4.2.7.2 Cell extract activity assay

The cell extract was prepared by removing cell envelopes using Bugbuster (Novagen, San Diego, CA) and lysozyme. Cell pellets were suspended in Bugbuster with 20 μg/ml lysozyme, upon which they were incubated at 10^{o}C and 250 rpm for 60 minutes, followed by centrifugation at 13000 × g for 15 minutes at 4 ^{o}C. The supernatant was then analyzed for enzyme activity. The UDP-glucose dehydrogenase activity of cell extract was assayed spectrophotometrically by following the reduction of NAD^+ at 340 nm, using a UV/VIS spectrophotometer (Lambda 40 Spectrometer, Perkin Elmer). The reaction mixture contained 1 mM UDP-Glc, 2 mM NAD^+, 2 mM dithiothreitol, 50 μl of cell extract, and 100 mM Tris-HCl buffer (pH 9.0) for a total volume of 1 ml. The reaction was initiated by the addition of the cell extract. The initial velocity of the NAD^+ reduction (ε_{340} = 6,220 $M^{-1}cm^{-1}$) was measured during the first minute. The whole-cell activity assay and cell extract assay were compared and found to be consistent with each other.

4.2.8 Freeze-thaw treatment

The effect of freeze-thaw on the whole-cell catalyzed-reaction rate was also studied. Cell pellets were frozen at -80^{o}C for 30 minutes and thawed at room temperature. The reaction was then carried out under the conditions as described above.

4.2.9 Cell extract activity assay for GalE-LgtB fusion enzyme

The GalE-LgtB fusion protein activity assay was conducted at 37^{o}C in 100 mM pH 7.5 Tris-HCl buffer (0.5 ml final volume), which contained 5 mM UDP-Glc, 10 mM GlcNAc, 10 mM $MnCl_2$, 1% bovine serum albumin and 0.44 ml cell extract. Cell extract was prepared as described in section 4.2.7.2 for UDP-glucose dehydrogenase. The reaction was terminated after 15 minutes by boiling the reaction mixture for 5 minutes. The precipitates were then removed by centrifugation at 13,000g for 5 minutes. The product of the reaction, LacNAc, in the supernatant, was analyzed by a Dionex BioLC system. One unit of GalE-LgtB fusion protein activity is defined as the amount of enzyme required to produce 1 μmol of LacNAc per minute. All activities were normalized by A_{600} to correct for small differences in cell density between preparations.

4.2.10 Time course of LacNAc production

Cells from 50 ml of culture were harvested by centrifugation at 4,000 × g for 10 minutes at 4^{o}C and washed once with 100 mM Tris-HCl buffer (pH 7.5) with 10 mM $MnCl_2$. Cell pellets were collected by centrifugation and resuspended into 0.5 ml of the same buffer. The reaction was started by the addition of 10 mM UDP-Glc and 40 mM GlcNAc. The reaction mixture was shaken at 250 rpm and 37 ^{o}C, and samples were taken at regular intervals to monitor the progress of the reaction. Samples were centrifuged at 13,000 × g for 5 minutes and the supernatant was analyzed by a Dionex BioLC system.

4.2.11 SDS-PAGE analysis

The expression levels of UDP-glucose dehydrogenase and GalE-LgtB Fusion Protein were evaluated by SDS-PAGE analysis. Cell pellets were suspended in 100 μl 1 × SDS-PAGE sample buffer containing 2.5% 2-mercaptoethanol. The samples were boiled for 5 minutes and centrifuged briefly. 10 μl of samples and the marker were then loaded into a Ready 10 % Tris-HCl gel. The gel was stained using Coomassie Brilliant Blue R-250 stain solution.

4.2.12 Analytical Methods

4.2.12.1 UDP-glucuronic acid assay

The samples were analyzed using a Hewlett-Packard 1100 Series HPLC system (Palo Alto, CA) equipped with a quaternary pump and diode-array detector. A YMC reversed-phase J'Sphere ODS column, 150 m × 4.6 mm I.D., 4 μm particle size (Waters, Milford, MA) was used. The ion-pairing method used was described previously[54].

4.2.12.2 LacNAc assay

A Dionex BioLC HPLC system (Sunnyvale, CA) equipped with a GS50 gradient pump, ED50 electrochemical detector, and Dionex CarboPac PA10 column (4 × 250 mm) was used. Two solvents were used as eluents: 200 mM NaOH and H_2O. The HPLC method consists of 3 steps: elution, regeneration, and equilibration. It was performed as follows: elution: 18% 200 mM NaOH and 82% H_2O for 23 minutes; regeneration: 50% 200 mM NaOH and 50% H_2O for 15 minutes; equilibration: 18% 200 mM NaOH and 82% H_2O for 14 minutes.

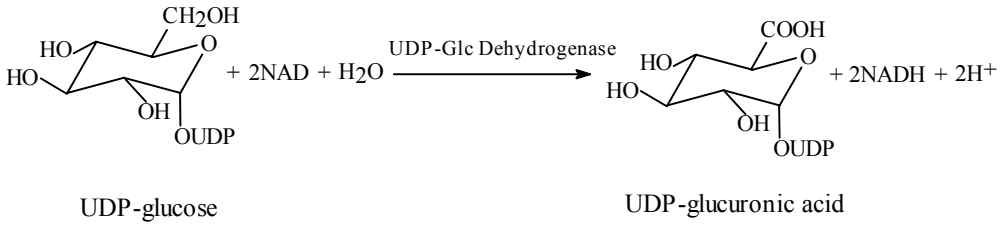
4.3 Results

4.3.1 Mutation effects on UDP-glucose dehydrogenase activity

UDP-glucose is a relatively large molecule (molecular weight 610), close to the maximum size allowable through porins. In addition, since it is hydrophilic, there could be considerable mass transfer resistance in the highly hydrophobic inner leaflet of the outer membrane, which is made of phospholipids. Mutations at the outer membrane structures could lead to increased permeability. Two types of outer membrane mutants were used in this study. SM101 carries a mutation in the first enzyme of the Lipid A synthesis pathway, resulting in a reduced synthesis of LPS. E609L carries an insertional mutation in a structural protein, Braun's lipoprotein, which provides a covalent link between the outer membrane and the peptidoglycans. The permeability of the membranes was evaluated by measuring the reaction rate catalyzed by an overexpressed enzyme, UDP-glucose dehydrogenase (Figure 4–2A). The respective parental strains were used as controls. Figure 4–3 shows a comparison of whole-cell activities between the mutants and the respective controls. Whole-cells carrying either type of mutation exhibited a much higher activity than their respective parental strains, increasing reaction rate by a factor of 2.0–to–14, depending on the substrate concentrations used. Under all conditions, there were no significant differences in the expression levels of the enzyme between the mutant and the control. A typical SDS-PAGE gel is shown in Figure 4–4. This was further supported by the activity measurement in the cell extracts (prepared by removing cell

membranes) (Table 4–2). Therefore, the differences observed in the reaction rate can be attributed to the altered rate of substrate uptake due to the mutations.

(A)



(B)

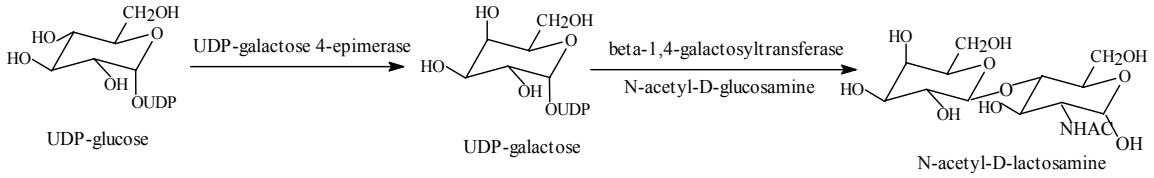


Figure 4–2. Reactions catalyzed by (A) UDP-glucose dehydrogenase and (B) GalE-LgtB fusion protein.

When whole-cell activities are compared to their cell extract activities, striking differences are evident (Table 4–2). At a relatively low substrate concentration (5 mM), the whole-cell activities for the parental strains only represent a small fraction of total activity inside the cells, about 4% for both E609 and for SM105, suggesting that severe permeability limitations imposed by the cell membranes. The mutations at the outer membrane structure dramatically increased the whole-cell activity, 9–fold for SM101 and 14–fold for E609L, bringing it to about 34% of the cell extract level for SM101 and 60% for E609L. At a higher substrate concentration (20 mM), the LPS mutant recovered about 73% of the activity, compared to about 19% with the parental strain. The lipoprotein

mutant recovered nearly 100% of the cell extract activity whereas only about 10 % activity was exhibited by the control strain. The results indicate that mutations at the outer membrane structures were effective in reducing the substrate permeability barrier in whole-cell catalysts. The lipoprotein mutation seemed to be particularly effective, capable of eliminating the permeability barrier under certain conditions.

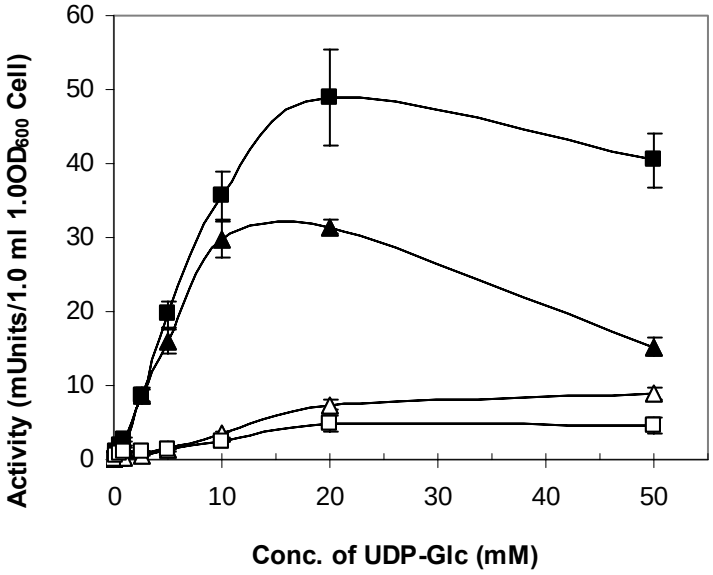


Figure 4–3. The effect of substrate concentrations on the initial rate of whole-cell *E. coli* SM101/pQEkfiD (▲), SM105/pQEkfiD (△), E609L/pQEkfiD (■) and E609/pQEkfiD (□). 2 mM NAD^+ was used in all reactions. Error bars represent standard deviations from the mean. All data are measured in at least three separate experiments.

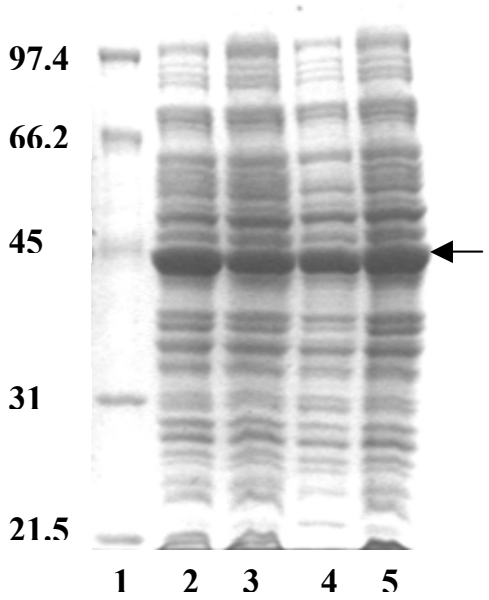


Figure 4–4. SDS-PAGE analysis of the UDP-glucose dehydrogenase expression in *E. coli* strains*:* SM101/pQEkfiD, SM105/pQEkfiD, E609L/pQEkfiD and E609/pQEkfiD. The cellular fractions were loaded on the gel as follows: marker (lane 1), 4 hr induced SM101/pQEkfiD (lane 2), 4 hr induced SM105/pQEkfiD (lane 3), 4 hr induced E609L/pQEkfiD (lane 4), 4 hr induced E609/pQEkfiD (lane 5).

Table 4–2. A comparison of UDP-glucose dehydrogenase activity in whole-cell and cell extract of SM101/pQEkfiD, SM105/pQEkfiD, E609L/pQEkfiD and E609/pQEkfiD

	Activity[a] (mUnits/1 ml 1.0OD_{600} cell)			
	SM101	SM105	E609L	E609
Cell extract	43.0 ± 2.2	39.9 ± 0.4	49.5 ± 1.6	51.7 ± 5.7
	At 5 mM Substrate Concentration			
Whole cell	14.7 ± 1.7	1.6 ± 0.1	29.5 ± 2.6	2.1 ± 0.2
Whole cell/Cell extract (%)	34.2	4.0	59.6	4.1
	At 20 mM Substrate Concentration			
Whole cell	31.4 ± 0.9	7.4 ± 0.7	48.8 ± 6.5	4.9 ± 1.1
Whole cell/Cell extract (%)	73.0	18.5	98.6	9.5

[a]All activities are measured in at least three separate experiments and expressed as means ± 1 standard deviation.

4.3.2 Enzyme expression levels on whole cell activities

It is nowadays a common practice to use whole cells, overexpressing one or more enzymes, as catalysts. Often in these systems, the inducer concentration is used to modulate their activities. Higher activities usually correlate with higher expression levels. Although this is true in most cases with activities measured in cell extracts, in whole-cell system, if substrate permeability is a problem, the relation between the inducer concentration, the expression level, and the enzyme activity can be more complicated. Table 4–3 illustrates how the enzyme activity responds to the inducer concentrations. For E609, in the cell extract, the activity increased from about 2 mU to about 50 mU, a 25–fold difference, when IPTG concentrations jumped from 0.01 mM to 1 mM. However, the whole-cell activity did not respond to the increase of IPTG nearly as dramatically, increasing the activity only 7 times for the same inducer concentration change. Clearly, the substrate permeability limitation diminished the cell's gain from increased inducer concentrations. The mutant strain, E609L, which allowed the substrate greater permeability, was more sensitive to IPTG concentration change. The enzyme activity jumped about 11 times (Table 4–3). This result indicates that substrate permeability is an important (yet often overlooked) factor that needs to be taken into account in the construction of recombinant whole-cell catalysts.

Table 4–3. UDP-glucose dehydrogenase activity of whole-cell and cell extract of E609L/pQEkfiD and E609/pQEkfiD at different IPTG concentrations.

	Whole Cell Activity (mUnits)/1 ml 1.0 OD_{600} Cell[a]			
Conc. of IPTG (mM)	0.01	0.1	0.5	1
E609L	2.6 ± 0.4	20.5 ± 3.5	27.8 ± 1.7	29.5 ± 2.6
E609	0.3 ± 0	1.8 ± 0.3	2.3 ± 0.1	2.1 ± 0.2

	Cell Extract Activity (mUnits)/1 ml 1.0 OD_{600} Cell			
Conc. of IPTG (mM)	0.01	0.1	0.5	1
E609L	1.6 ± 0.3	32.6 ± 0.3	44.2 ± 1.6	49.5 ± 1.6
E609	1.9 ± 0.1	33.2 ± 5.0	46.5 ± 1.4	51.7 ± 5.7

[a]The whole cell activity was measured with 5 mM UDP-Glc and 5 mM NAD+ as described in Materials and Methods. All activities are measured in at least three separate experiments and expressed as means ± 1 standard deviation.

Interestingly, as the mutant E609L is more responsive to IPTG concentration than E609, the activity ratio of E609L to E609 increased with the increase of the inducer concentration (Figure 4–5), with more pronounced effects observed at lower IPTG concentrations (0.01–0.1 mM). The beneficial effects of outer membrane mutation, therefore, depend on the expression levels.

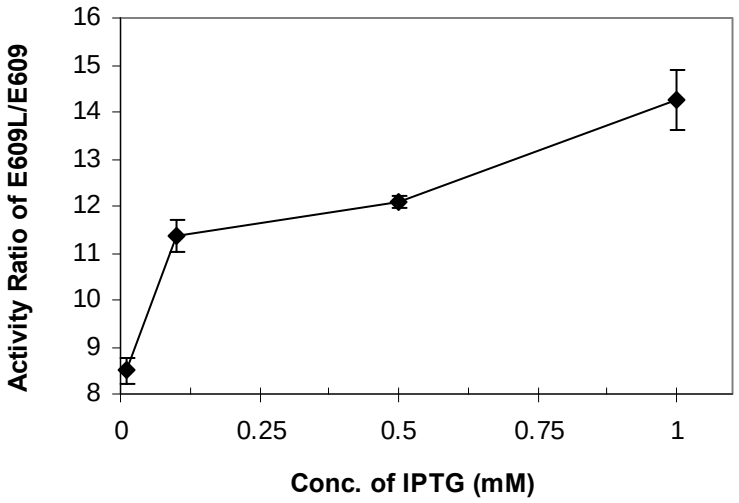


Figure 4–5. The effect of different IPTG concentrations on the whole-cell activity ratio of E609L/pQEkfiD to E609/pQEkfiD. 5 mM UDP-Glc and 5 mM NAD^+ were used in all reactions. Error bars represent standard deviations from the mean. All activities are measured in at least three separate experiments.

4.3.3 Mutation versus freeze-thaw

The effectiveness of outer membrane mutation in reducing substrate permeability limitations was compared to a common permeabilization procedure, freeze-thaw (FT), known to be effective in permeabilizing cell membranes for UDP-glucose[55]. Figure 4–6 shows that mutation of the LPS synthesis pathway is much more effective, increasing the whole-cell activity by about 9 times versus 2 times with FT. Similarly, the lipoprotein mutation led to an increase of nearly 14–fold, compared to about 5–fold with FT. Besides being more effective, the genetic engineering approach eliminates the need for additional processing steps, time, and equipment. Therefore, modification of the outer membrane is far superior to FT treatment.

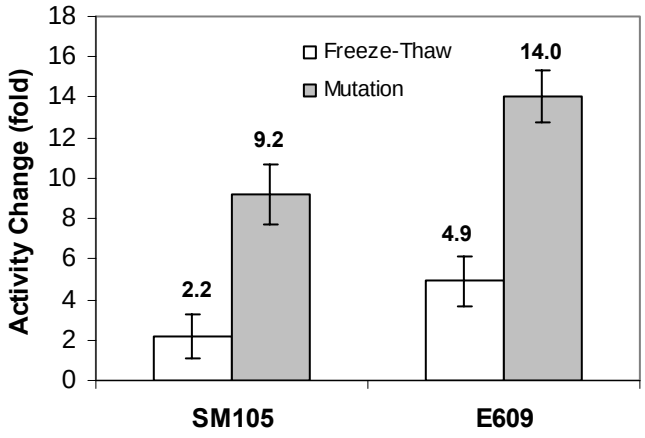


Figure 4–6. A comparison of membrane mutation and freeze-thaw method. 5 mM UDP-Glc and 5 mM NAD^+ were used in all reactions. Error bars represent standard deviations from the mean. All data are measured in at least three separate experiments.

4.3.4 LacNAc synthesis from UDP-glucose

As an example of the application of outer membrane mutants in synthesis, we studied the LacNAc synthesis from UDP-glucose. LacNAc is one of the most common core structures in glycans, some of which are significantly elevated in various types of cancer patients[56]. Its synthesis from UDP-glucose is of particular interest as UDP-glucose is 100 times less expensive than UDP-galactose[7]. To achieve high synthesis with whole-cell catalysts, host modification is necessary to remove the β-galactosidase activity, which hydrolyzes the product. This was done by knocking out the β-galactosidase encoding gene (*lacZ*) from the chromosome of E609 and E609L. A PCR-based gene-

knockout method was used. The resulting knockouts were verified by gene sequencing and activity assay (data not shown). The two required enzymes, UDP-galactose 4'-epimerase (*galE*, catalyzing the reversible conversion between UDP-glucose to UDP-galactose) and a β-1, 4-galactosyltransferase (*lgtB*, catalyzing the addition of a galactose from UDP-galactose in beta 1 to 4 linkage to *N*-acetylglucosamine to yield LacNAc), were introduced as a fusion (Figure 4–1B; Detailed construction of the plasmid is given in the section 4.2.5). The reaction yielding LacNAc is shown in Figure 4–2B.

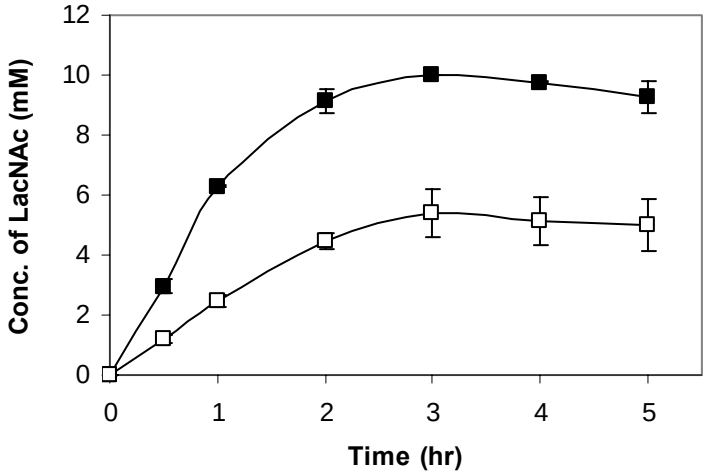


Figure 4–7. The time course of LacNAc production by whole-cell E609L(*ΔlacZ*)/pCEL (■) and E609(*ΔlacZ*)/pCEL (□). 10 mM UDP-Glc and 40 mM GlcNAc were used in the reaction. Error bars represent standard deviations from the mean. All data are measured in at least three separate experiments.

Figure 4–7 is a comparison between E609L(*ΔlacZ*)/pCEL and E609(*ΔlacZ*)/pCEL in the synthesis of LacNAc. The initial rate of synthesis was about 2.5 times higher for the lipoprotein mutant. In addition, there were substantial differences in product yields. Under the same conditions, the mutant gave a product yield of 100% (based on the input

of UDP-glucose), whereas the parental strain gave only 50% yield. This difference may be due to the product inhibition by UDP, which was found to have accumulated in the parental strain (data not shown). The accelerated reaction rate can be attributed to the increased permeability resulting from the lipoprotein mutation, as there was no significance difference in the expression levels of the two strains (Figure 4–8).

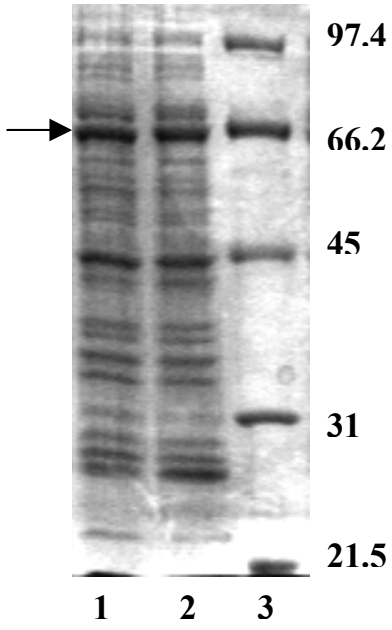


Figure 4–8. SDS-PAGE analysis of the GalE-LgtB fusion protein expression in *E. coli* E609L(*ΔlacZ*)/pCEL and E609(*ΔlacZ*)/pCEL. The cellular fractions were loaded on the gel as follows: 4 hr induced E609L(*ΔlacZ*)/pCEL (lane 1), 4 hr induced E609(*ΔlacZ*)/pCEL (lane 2), marker (lane 3).

4.4 Discussion

This study demonstrates that UDP-glucose, as a large hydrophilic molecule, has severe limitations of permeability in the outer membrane. At 5 mM substrate concentration, only a small percentage (4%) of the possible enzyme activities was exhibited in a whole-cell system. Modifications of the outer membrane structure are effective in reducing these limitations as shown by the dramatically increased rate of reactions (up to 14–fold) in the mutants. In particular, nearly 100 % of the available enzyme activity (in cell extracts) was recovered in cells with the lipoprotein mutation at 20 mM substrate concentration. The gains of the mutation in reaction rate are the result of increased intracellular substrate concentrations, which can be illustrated with the following analysis. We assume that the simple Michaelis-Menten kinetics applies to the UDP-glucose dehydrogenase, and K_m value for UDP-glucose is 0.015 mM[57], and V_{max} measured in this study are 41.5 mU for SM105 and SM101, and 50 mU for E609 and E609L. The intracellular concentrations of UDP-glucose in the whole cells were estimated using this model. They are tabulated in Table 4–4. The intracellular substrate concentrations for wild-type cells are 3–4 orders of magnitude lower than the extracellular concentration. This is consistent with the general observation that the whole-cell catalyzed rate is usually orders of magnitude lower than of the isolated enzyme counterpart. Mutations at the outer membrane increase the intracellular concentration by 10–to–380–fold (Table 4-4). The mutations coupled with increased extracellular substrate concentration raised the level of intracellular substrate concentration 3–to–40–fold above the K_m value; consequently, whole cells exhibited almost the maximum activity. It is

important to note that a substrate concentration of 5 mM is 330–fold higher than the K_m value. Supplying a substrate at this high concentration allows the reaction to proceed at approximately two orders of magnitude (instead of four orders of magnitude as predicted from the intracellular concentration) lower than the isolated enzyme counterpart. If the substrate concentrations was not supplied that high, the whole cell activity could well fall below the detection level. This could lead to erroneous conclusions in biocatalyst screening and development. This model analysis depicts a drastically different environment that an enzyme experiences intracellularly and illustrates, once again, the need and the importance to evaluate substrate permeability in whole-cell biocatalysis research.

Table 4–4. Intracellular UDP-glucose concentration estimated using the Michaelis-Menten Model.

	Modeled Intracellular Concentration (μM)			
	SM101	SM105	E609L	E609
	At 5 mM Extracellular Concentration			
Intracellular Concentration	8.23	0.60	21.59	0.66
Ratio: Extracellular to Intracellular	608	8313	232	7603
Ratio: Mutant to Parent	13.68		30.82	
Ratio: Concentration to K_m	0.55	0.04	1.44	0.04
	At 20 mM Extracellular Concentration			
Intracellular Concentration	46.63	3.26	610.00	1.63
Ratio: Extracellular to Intracellular	429	6144	33	12272
Ratio: Mutant to Parent	14.33		374.30	
Ratio: Concentration to K_m	3.11	0.22	40.67	0.11

The enzymes investigated, UDP-glucose dehydrogenase and the epimerase-galactosyltransferase fusion, were both over-expressed in the cytoplasm. As the inner

membrane is highly hydrophobic, it is expected that the molecule also experiences limitations in the penetration of the inner membrane. This may be reflected by the fact that even with substantial outer membrane permeabilizing, only up to 60% of the activity was recovered at 5 mM substrate concentration. Only at a much higher substrate concentration (20 mM) was 100% activity recovery achieved. Our current system does not allow an assessment of the inner membrane permeability. As a matter of fact, our initial efforts in isolating the outer membrane permeability from inner membrane permeability (by expressing these enzymes in periplasmic space) resulted in either inactive enzymes or very low level expression, not conducive to the permeability study. Nevertheless, the significant rate acceleration achieved by mutations on the outer membrane structure suggests that the outer membrane permeability barrier for this molecule is significant and substantial reduction could be achieved through membrane engineering. If inner membrane permeability can be addressed, the maximum activity could be reached at an external substrate concentration lower than 20 mM.

This, along with our earlier work[7], indicates that the beneficial effects of outer membrane mutation seem to be universal to all six molecules investigated. However, the gains in rate acceleration due to mutations varies from a mere 120%–to–1400%, and appears to be dependent, in a complicated manner, on the molecular structures of the substrate molecules (such as size and hydrophobicity), and the exact nature and locations of the mutation. This warrants further investigation. Finding a correlation of mutational effects to the molecular features of substrates will allow membrane structures to be tailored for specific process needs. The gains, as illustrated in this work, are also

dependent on the enzyme expression levels and substrate concentrations. Other conditions that influence enzyme reaction rates such as pH and temperature could also affect the rate accelerations, which await further studies.

The severe limitations of substrate permeability on whole-cell reaction rates, as revealed in this paper, as well as our earlier work and that of others[–7, , , , 58, 59], clearly demonstrates a need for an assessment of membrane permeability in biocatalysis research and development. The dramatic improvement in the reaction rate with outer membrane mutants testifies to the effectiveness of using a molecular engineering approach to address this permeability problem. Although so far our work has been done exclusively with substrates, permeability problems with products produced and accumulated intracellularly could conceivably be addressed using the same membrane engineering strategies as illustrated in this work.

CHAPTER 5

Significance and Application of *lpp* Lipoprotein Mutation in Whole-cell Biocatalysis

Our previous studies with a Braun's lipoprotein mutant E609L demonstrated that lipoprotein mutation in outer membrane could be an effective method to increase permeability of a variety of substrates[38, , 60]. The results showed that reactions with all six substrates were dramatically accelerated by this lipoprotein mutation (up to 14–fold increase in reaction rate). In order to establish this as a generally applicable methodology for accelerating whole-cell biocatalysis, we decided to investigate an alternative genetic modification to obtain the same phenotype. The *lpp* gene deletion was successfully introduced into *E. coli* strains from different genetic background and the resulted strains were characterized. It showed that the deletion had the same effect on substrate permeability as the original transposon mutation. Also, the *lpp* deletion was found to have no significant effect on cell growth, carbon metabolism, and lipid compositions. An example was illustrated with L-carnitine production by introducing *lpp* deletion into the *E. coli* O44K74 strain to achieve a higher product yield. This method might be generally applicable to biocatalysis processes as the permeability change effected by the mutation is of global nature. These results demonstrate that a useful phenotype can now be transferred to any *E. coli* strain with a straightforward genetic manipulation to effectively enhance the rate of a whole-cell biocatalysis reaction.

5.1 Introduction

The increasing environmental and economic pressure in past decade has stimulated tremendous progress in the use of enzymes and microorganisms for the production of fine chemicals, pharmaceuticals as well as food ingredient. Biocatalysis has developed from a niche technology to a widely used manufacturing method. In many cases, especially in large scale applications, whole-cell biocatalysts are preferred to free enzymes. First, the isolation and purification of enzymes not only significantly increase the cost but also might render full or partial loss of activity, while the use of whole cells don't have these problems. Also, the whole-cell system allows the cascades of enzyme reactions involve multiple enzymes, cofactors and substrates[,] . However a major obstacle in the application of whole-cell biocatalysts is the limited reaction rate and yield due to the formidable permeability barrier imposed by cell envelope.

Our previous studies with a Braun's lipoprotein mutant E609L demonstrated that genetic modification in outer membrane could be an effective method to increase the reaction rate by reducing the substrate permeability barrier. It showed that reactions with all six substrates were dramatically accelerated by this lipoprotein mutation (up to 14–fold increase in reaction rate). The lipoprotein mutation was proven to be effective towards substrates with various size and hydrophobicity. Additionally, the lipoprotein mutation has essentially no effect on its growth and high level expression of recombinant protein[38, ,] . The extraordinary performance of the lipoprotein mutant E609L suggests that it would be of great interest to apply this as a generally methodology for accelerating whole-cell biocatalysis. The sequence result of E609L suggests that the *lpp* gene is

completely inactivated by an transposonal insertion containing multiple stop codon. Consequently, the same phenotype could be obtained by introduction of *lpp* gene deletion into strains with different genetic background. The resulted *lpp* deletion strain showed the same effect on substrate permeability as the original transposon mutant E609L. Further, the *lpp* deletion had no significant effect on cell growth, carbon metabolism and lipid compositions. The result indicates that the *lpp* deletion could be established as a general method for accelerating whole-cell biocatalysis.

An application was illustrated with L-carnitine production. L-carnitine [R-(-)-3-hydroxy-4-trimethylaminobutyrate] is essential for the transport of long-chain fatty acids through the inner mitochondrial membrane in eukaryotes. With the identification of its clinical and biotechnological importance, the demand for L-carnitine has increased worldwide. Several chemical and biological processes have been developed for its synthesis [61]. Compared to the racemic carnitine product from chemical process, biotransformation converts achiral precursors, such as crotonobetaine to optical purity L-carnitine, which is safe to be administered to the patients. *E. coli* O44K74 has a significantly higher carnitine dehydratase than other *E. coli* strains and has been employed in the production of L-carnitine[, ,]. However, the biotransformation process using whole cells resulted in a maximum of only 30–43% conversion ratio due to unfavorable reaction equilibrium, limitations in transport system and side product formation[62, 63] (as depicted in Figure 5–1). Permeabilization by either detergents or polyethylenimine to achieve higher yield of L-carnitine was reported[64]. But the chemical treatment is far from being desirable, especially for large scale applications, since they

are usually non-specific and incur unintended damage to cell (even lysis), and also complicate the downstream process. In this study, the lipoprotein mutation was successfully introduced into L-carnitine production strain *E. coli* O44K74 by deleting the lipoprotein encoding gene *lpp* using a straightforward and efficient PCR-based method. The results showed that higher product yield of L-carnitine synthesis was achieved by *lpp* deletion strain due to its increased outer membrane permeability. The faster export of L-carnitine by the mutant cells allowed the reversible reaction catalyzed by carnitine dehydratase to continue in a favorable direction and the product to escape from the degradation by side reactions (Figure 5–1). These results demonstrate that a useful phenotype can now be transferred into any *E. coli* strain to effectively enhance the rate of a whole-cell biocatalysis reaction.

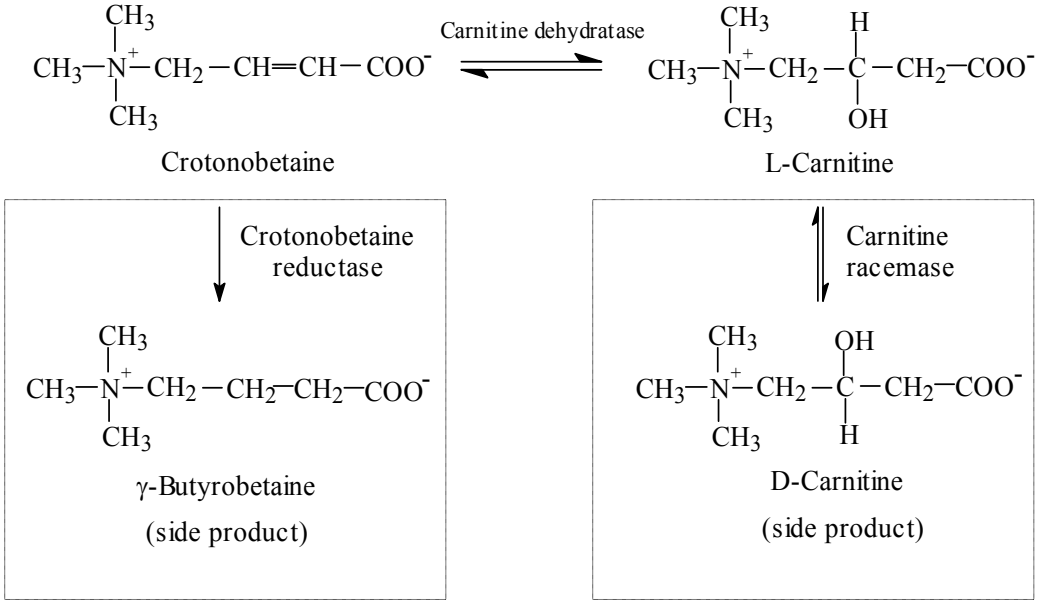


Figure 5–1. Biotransformation of crotonobetaine to L-carnitine catalyzed by carnitine dehydratase and the related side reactions (in squares) in *E. coli* whole cells.

5.2 Experimental

5.2.1 Chemicals and reagents

All chemicals were reagent grade and used as received. Methyl esters of myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1) and cis-vaccenic acid (C18:1), L-carnitine, Acetyl-CoA, dithiobisnitrobenzoic acid (DTNB), carnitine acetyltransferase (CAT), and N-phenyl-1-naphthylamine (NPN) were purchased from Sigma Chemical Co. (St. Louis, MO). Crotonobetaine was graciously provided by Dr. Manuel Canovas' . All primers used in this study were from Invitrogen (Carlsbad, CA) and listed in Table 5–1.

Table 5–1. List of oligonucleotide primers used in this study.

Primer name	Primer sequence[a]
lpp-f	5' -CTTTGTGTAATACTTGTAACGCTACATGGAGATTAACTCAATCTAGAGGGGTGTAGGCTGGAGCTGCTTC- 3'
lpp-r	5' -CGTGACGCAGTAGCGGTAAACGGCAGACAAAAAAAATGGCGCACAATGTGATTCCGGGGATCCGTCGACC- 3'
lpp-up	5' -CACTACTAACACCGCATCTG- 3'
lpp-dn	5' -GCCATACACACTGCCAGCAG- 3'

[a] Homologous regions are underlined

5.2.2 *E. coli* Strains

E. coli strains used in this study are shown in Table 5–2. E609L carries a Tn*10* insertion in *lpp* coding for Braun's lipoprotein. E609Y is the *lpp* knockout strain

constructed in this study. E609 is the isogenic parent of E609L and E609Y. *E. coli* O44K74, a gift from Dr. Marie-Andree Mandrand, has a significant higher carnitine dehydratase activity and was used for L-carnitine synthesis[65]. *E. coli* O44K74Y is the *lpp* knockout strain constructed using *E. coli* O44K74 as the parent strain.

Table 5–2. *E. coli* strains used in this study.

	Description	Source
E609L	*lpp*::Tn*10*; periplasmic leaky; Tc^r	K. W. Miller, (labcin@uwyo.edu)
E609Y	*lpp* deletion strain	this study
E609	*HfrC pps*, isogenic parent of E609L and E609Y	K. W. Miller,
O44K74Y	*lpp* deletion strain	this study
O44K74	Isolated from the intestine of a rat for its higher carnitine dehydratase activity. Isogenic parent of O44K74Y	M-A Mandrand (marie-andree.mandrand@insa-lyon.fr)

5.2.3 Construction of *lpp* knockout strains

The *lpp* knockout strain was constructed using the gene inactivation method developed by Wanner and coworkers. Briefly, the FRT–flanked kanamycin resistance gene was PCR amplified using primers (lpp-f and lpp-r, as shown in Table 5–1) with 50–nucleotide extensions that are homologous to the adjacent regions of the genomic *lpp* gene. Homologous recombination was mediated by the plasmid–borne phage λ Red recombinase. The kanamycin gene was then excised by helper plasmid encoding the FLP

recombinase. Finally, both plasmids were easily cured by growth at above 37°C as they contain temperature-sensitive replicons.

5.2.4 Characterization of lipoprotein mutant E609Y

5.2.4.1 Growth curve

LB and M9 minimal medium were used in cultivations. M9 minimal medium contained per liter: 6.8 g Na_2HPO_4; 3.0 g KH_2PO_4; 0.5 g NaCl; 1.0 g NH_4Cl; 3.6 g glucose; 0.50 g $MgSO_4 \cdot 7H_2O$, 0.015 g $CaCl_2 \cdot 2H_2O$. Other supplements included 20 amino acids at a final concentration of 40 μg/ml each. The medium for E609L was additionally supplemented with 12.5 μg/ml tetracycline. For cell growth study, 100 ml of medium in a 500 ml flask was inoculated with an overnight culture of E609Y, E609L and E609 (2% inocula). The flasks were shaken in a rotary shaker at 250 rpm at 37 ^oC. Samples were taken from each culture at regular intervals to monitor the growth pattern by measuring optical density at 600 nm with a UV/VIS spectrophotometer (Lambda 40 Spectrometer, Perkin Elmer).

5.2.4.2 Lipid extraction, fractionation and analysis

Phospholipids were extracted by the method of Bligh and Dyer, as modified by Ames[66]. Briefly, stationary phase cells (500 mg wet weight) were washed with 10 mM, pH7.4 sodium phosphate buffer and resuspended in 1.6ml of same buffer, after which 4 ml of methanol and 2 ml of chloroform were added with thorough mixing (water, methanol and chloroform are in the proportion of 0.8:2:1, v/v). The suspension was incubated in room temp for 2 h with periodic vortexing. The extract mixture was diluted

with 2 ml of chloroform followed by 2 ml of same buffer, whereupon the phase was thoroughly mixed. After brief centrifugation, the lower chloroform phase (containing lipids) was removed and subjected to further esterification. The lipid extracts were esterified with 5% H_2SO_4 in methanol at 70^{o}C for 2 hours. The fatty acid methyl esters (FAMEs) were extracted with *n*-pentane. The pentane was removed with air stream, and the FAMEs were dissolved in 1 ml of *n*-heptane for further gas chromatography-mass spectrometry (GC/MS) analysis.

The fatty acid profile was determined with Hewlett-Packard 6890 GC coupled to a HP5973 MSD (Palo Alto, CA). The temperature for the injector and the MSD was 280 ^{o}C. The GC inlet was operated in the splitless mode at 230 ^{o}C. One microliter was injected from a 10-μl syringe by an HP7683 autosampler. An HP-5MS methylpolysiloxane phase column (30 m × 0.25 mm × 0.25 μm) was used, with helium as carrier gas at 1 ml/min. The GC oven heated the column with a program started at 60^{o}C for 2 minutes, ramped at 10 ^{o}C/min to 150 ^{o}C, then ramped at 3 ^{o}C/min to 250 ^{o}C, finally ramped at 10 ^{o}C/min to 300 ^{o}C, held for 5 minutes. Authentic FAME samples and the MS spectrum were used to determine the retention times and identify individual fatty acids derived from the strains. Retention times of identified FAMEs are shown in Table 5–3.

5.2.5 Application of lipoprotein mutant for L-carnitine production

5.2.5.1 Cell preparation

E. coli strains O44K74 and O44K74Y (Table 5–2) were aerobically grown in LB medium at 30^{o}C with 250 rpm 15 hrs to obtain the seed culture. Cells were cultivated

in complete medium inoculated with 2 % (v/v) of overnight seed culture and incubated for 24 hrs under anaerobic conditions at 37^{o}C in completely filled, air-tight flasks without stirring. The complete medium contained per liter: 20 g peptone, 12.6 g glycerol, 5 g NaCl, 2 g fumarate, supplemented with 50 mM crotonobetaine to induce the expression of the enzymes involved in carnitine metabolism.

5.2.5.2 Biotransformation using resting cells

Cells were harvested after overnight growth by centrifugation at 4,000 g for 15 minutes, washed twice with 35 mM potassium phosphate buffer (pH 7.5), and resuspended to around 10 OD_{600} (~20 g wet cell/liter) in 60 mM potassium phosphate buffer (pH 7.5) with 50 mM crotonobetaine. Biotransformation was carried out aerobically at 37^{o}C and 250 rpm shaking. Samples were taken every hour to monitor the progress of the biotransformation.

5.2.5.3 L-carnitine analysis

L-carnitine concentration was determined enzymatically with the carnitine acetyl transferase methods, based on a colorimetric reaction. Reaction mix included 0.3 mM DTNB, 0.06 mM Acetyl-CoA, 2.5 mM EDTA, 0.25 U CAT, and 100 μl of appropriately diluted sample in 50 mM phosphate buffer (pH 7.5). Absorbance at 412 nm was monitored using a UV/VIS spectrophotometer (Lambda 40 Spectrometer, Perkin Elmer) after the reaction mixture was incubated at 30^{o}C for 30 min. L-carnitine production yield was determined as the percentage of L-carnitine produced over the crotonobetaine supplied in biotransformation.

5.3 Results

5.3.1 Sequence analysis of *lpp* in E609L

Braun's lipoprotein, the major outer membrane lipoprotein encoded by *lpp*, is present in some 700,000 copies per cell, which makes it the most abundant protein in numerical sense in *E. coli*. About one–third of the lipoprotein molecules are covalently linked to murein and help hold the two structures together. Therefore the rigidness of the outer membrane can be attributed to the interaction between the lipoprotein and the murein layer. Mutants defective in the structural gene for this protein are fully viable, indicating it is nonessential. However, it is possible the disruption of lipoprotein will affect the global membrane integrity and thus may effect some changes in substrate permeability. Our previous studies[,] with the mutant, showed dramatically increased permeability with various substrates, ranging from hydrophilic (e.g. nitrocefin) to hydrophobic (e.g. tetrapeptide), small (e.g. toluene) to big molecules (e.g. UDP-glucose). The acceleration in reaction rate due to the mutation was as high as 14 times, suggesting the important effect of lipoprotein on membrane permeability. To have an insight into the molecular basis behind this phenotype, and also to provide knowledge for future rationale design of membrane mutants, the *lpp* region in E609L were sequenced and compared to E609 (Fig. 5–2A). Primers lpp-up and lpp-dn (Table 5–1) were used for DNA sequencing. As expected, a 59-bp insertion (in grey) at 17th bp of *lpp* was found in E609L. Moreover, the insertion contains two double-stop codon (in bold), indicating *lpp* is not expressed at all. Therefore, the absence of this major outer membrane lipoprotein is

responsible for the strikingly increased permeability. This is the first time that the genetic sequence information of this lipoprotein mutation was revealed. And most importantly, it indicates that the same phenotype as transposonal mutation could be obtained by a simple *lpp* deletion.

```
E609          ACTCAATCTAGAGGGTATTAATAATGAAAGCTACTAAACT--------------------
E609L         ACTCAATCTAGAGGGTATTAATAATGAAAGCTACTAAACTCTGATGAATCCCCTAATGAT
              ************************************

E609          ------------------------------------GGTACTGGGCGCGGTAATCCT
E609L         TTTGATAAAAATCATTAGGGGATTCATCAGTACTAAACTGGTACTGGGCGCGGTAATCCT
                                                   ********************

E609          GGGTTCTACTCTGCTGGCAGGTTGCTCCAGCAACGCTAAAATCGATCAGCTGTCTTCTGA
E609L         GGGTTCTACTCTGCTGGCAGGTTGCTCCAGCAACGCTAAAATCGATCAGCTGTCTTCTGA
              ************************************************************

E609          CGTTCAGACTCTGAACGCTAAAGTTGACCAGCTGAGCAACGACGTGAACGCAATGCGTTC
E609L         CGTTCAGACTCTGAACGCTAAAGTTGACCAGCTGAGCAACGACGTGAACGCAATGCGTTC
              ************************************************************

E609          CGACGTTCAGGCTGCTAAAGATGACGCAGCTCGTGCTAACCAGCGTCTGGACAACATGGC
E609L         CGACGTTCAGGCTGCTAAAGATGACGCAGCTCGTGCTAACCAGCGTCTGGACAACATGGC
              ************************************************************

E609          TACTAAATACCGCAAGTAATAGTACCTGTGAAGTGAAAAATGGCGCACATTGTGCGCCAT
E609L         TACTAAATACCGCAAGTAATAGTACCTGTGAAGTGAAAAATGGCGCACATTGTGCGCCAT
              ************************************************************
```

Figure 5–2A. Comparison of nucleotide sequences of *lpp* from E609L and E609Y. Start and stop codon are in bold. The grey part represents the 59-bp insertion introduced by transposon.

5.3.2 Construction of *lpp* knockout strains

The significant effect of the lipoprotein mutation also suggests that it would be of interest to introduce *lpp* knockout into strains with different genetic backgrounds and establish this as a general method to accelerate reaction rate of whole-cell biocatalysis. The lipoprotein mutant E609L used in earlier studies[,] was generated by phage

transduction mediated transposon insertion, a time-consuming and labor-intensive method in which tedious selection steps are involved. Here, a straightforward and highly efficient, one-step PCR-based gene inactivation method was investigated to introduce *lpp* deletion. The method leaves no antibiotic resistance gene marker, which is another advantage over transposon insertion.

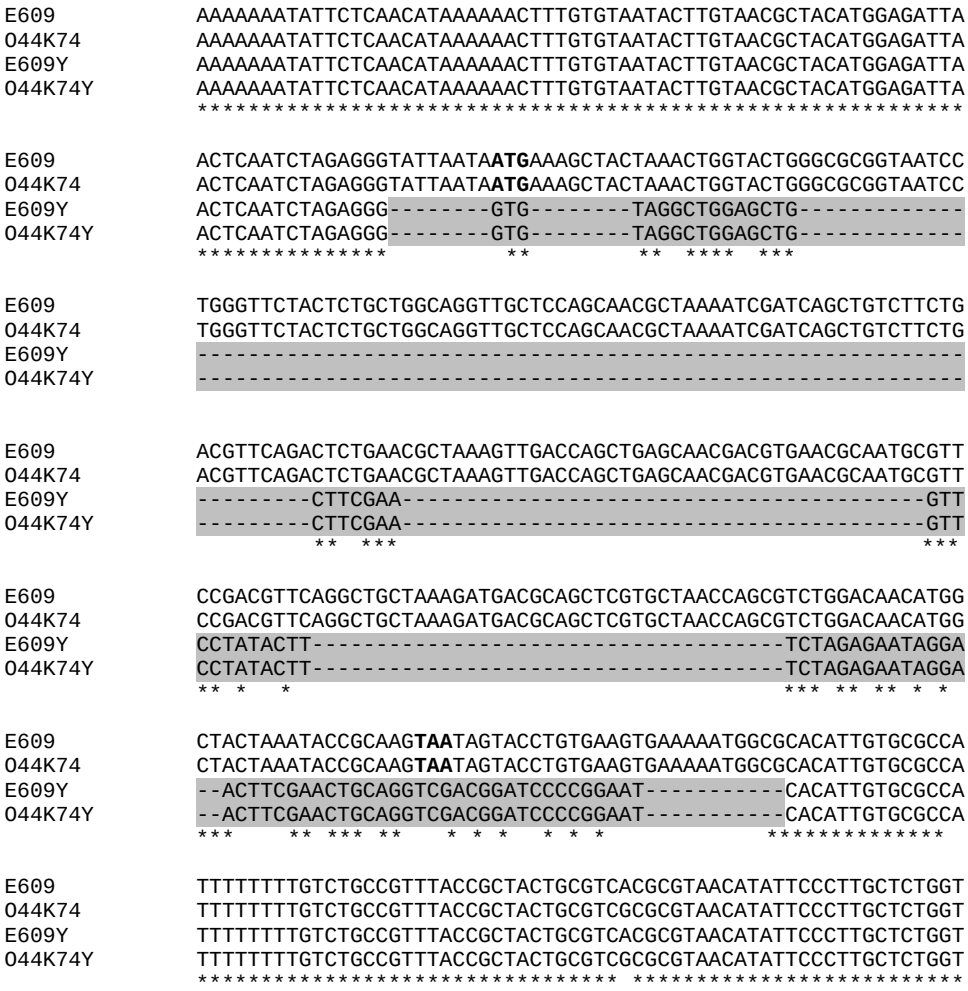


Figure 5–2B. Comparison of nucleotide sequences of *lpp* from *E. coli* E609, E609Y, O44K74 and O44K74Y. Start and stop codon are in bold. Grey part represents the 82-bp scar after the gene deletion.

As our first application of this *lpp* deletion (detailed in the Experimental section), lipoprotein mutant strains E609Y and O44K74Y were generated by deleting gene *lpp* from their chromosomal DNA of their respective parent strains, E609 and O44K74. The resulting *lpp* deletion strains were verified by PCR and sequencing. As shown in Fig. 5–2B, the gene deletion leaves behind an 82–bp scar (in grey) in place of *lpp*.

The effect of the *lpp* deletion on the membrane permeability was studied with UDP-glucose dehydrogenase catalyzed reaction utilizing UDP-glucose as substrate (as described in Chapter 4). The result showed that the *lpp* deletion had the same effect on the membrane permeability as transposon mutation in E609L, ~15–fold increase in the reaction rate was observed.

5.3.3 Characterization of the *lpp* knockout strain E609Y

5.3.3.1 Growth and metabolism

As one of the essential attributes of whole-cell biocatalysts, growth ability of *lpp* deletion strain E609Y was studied and compared to its parental strain E609 and the transposon mutant, E609L. Two different media, LB and M9 minimal were used to evaluate the growth of the mutant cells in both rich and minimal environment. As shown in Figure 5–3A, three strains exhibited the similar growth rate in LB media with a doubling time of about 0.7 hour. At the 8^{th} hour of the growth, the cell density of ~3.0 OD_{600} was reached for three strains with the highest of ~3.2 OD_{600} observed for E609.

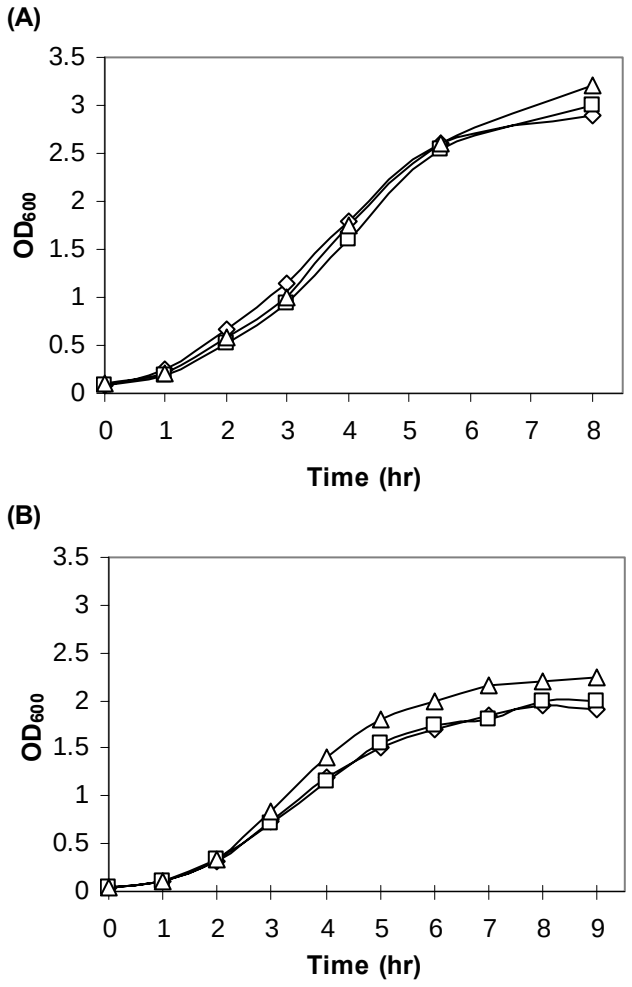


Figure 5–3. Comparison of growth curve of E609Y(□), E609L(◇) and E609(△). (A) LB medium; (B) M9 minimal medium.

In M9 minimal medium (Fig. 5–3B), two lipoprotein mutants, E609Y and E609L, exhibited the exactly same growth profile as expected. E609 showed higher growth rate than that of the mutants throughout the growth. The doubling time is 0.95 hour and 0.8 hour for the mutants and the parent strain, respectively. The final cell density in M9 is

~2.0 OD_{600}, one third lower than that of LB medium. Similarly, E609 ended up with a cell density bit higher (~0.2 OD_{600}) than that of the mutants.

Also, the glucose consumption was monitored when cells were grown in minimal medium under aerobic and anaerobic conditions. To our surprise, the result showed that the glucose consumption rate of the mutant cells was exactly the same as the parent strain, even under anaerobic condition (data not shown). It suggests that the mutant cells are able to maintain the normal cell growth and metabolism without consuming more carbon source. This is especially attractive in large-scale applications since no additional cost on medium material is required.

Taken together, the lipoprotein mutant E609Y cells fare quite well in both LB and minimal medium. The lipoprotein mutation doesn't significantly affect the cell growth and carbon metabolism. The slower growth rate of mutant is likely to be caused by the involvement of more complicated metabolism in minimal medium to compensate the membrane defect. Limitation of energy source and accumulation of waster products could be responsible for this observation.

5.3.3.2 Fatty acid profile

The lipids of *E. coli*, exclusive of lipid A of lipopolysaccharide, are entirely phospholipids, comprising 9.1% of total dry cell weight. The most common fatty acids found in phospholipids are palmitic acid (16:0); palmitoleic acid (16:1); *cis*-vaccenic acid (18:1). Modulation of fatty acid composition to ensure appropriate membrane fluidity and rigidity is an important bacterial strategy for growth in different environment. It reported[67, 68, 69, 70] that most bacteria can maintain membrane fluidity and integrity by

adaptation accomplished by changing membrane fatty acid chain length, branching pattern, and unsaturation degree. Proper membrane rigidity can be achieved by increasing longer-chain fatty acids and decreasing shorter-chain fatty acids. Cyclopropane fatty acid was also found to be important to the rigidity of the cell membrane and it showed that decrease of *cis*-9,10-methylene hexadecanoic acid (C17:cyc) resulted in increased membrane rigidity[71]. These studies suggest that there is a control mechanism in *E. coli* that regulates the fatty acid compositions to keep the variations in the physical properties of the membrane phospholipids to a minimum.

In this study, we speculate that lipoprotein mutant E609Y cells is probably subjected to the same control mechanism, that is, altering fatty acid composition to compensate the compromised membrane integrity and rigidity. We therefore carried out a comparison study to evaluate the effect of lipoprotein mutation on membrane fatty acid profiles in E609 and E609Y cells. The GC-MS analysis result showed that eight major fatty acids were found in membrane lipid extracts (Figure 5–4). In agreement with earlier studies, C16:1, C16:0 and C18:1, are 3 of the most abundant membrane fatty acids in both strains, constituting over 90% of total fatty acids. Other lipids including C14:0, C15:0, C17:cyc, C17:0, C18:0 make up the rest. The changes in fatty acid compositions of two strains were not dramatic, small but reproducible changes were noted. Compared to E609, the levels of short-chain fatty acids (C14:0, C15:0, C16:1 and C16:0) and cyclopropane fatty acid (C17:cyc) in E609Y were decreased for about 5% (Table 5–3). This was accompanied by an increase of long-chain fatty acids (C17:0, C18:1 and C18:0) (Table 5–3). Noticeably, one of the most abundant fatty acid C18:1 increased 4% in

E609Y. Therefore, the E609Y cells could manage to balance the increased membrane fluidity caused by lipoprotein deficiency by modulating proportions of membrane fatty acids without significant change in their compositions.

Table 5–3. Comparison of fatty acid compositions of E609Y, E609L and E609.

Major Fatty acids	Molecular weight	Retention Time (min)	Fatty acid (%)[a]		Difference between E609Y and E609
			E609Y	E609	
C14:0	242	18.3	3.7±0.8	5.0±0.2	-1.3
C15:0	256	20.8	2.7±0.8	2.8±1.0	-0.1
C16:1	268	23.2	23.7±1.4	23.9±2.8	-0.2
C16:0	270	23.8	38.3±1.8	40.3±1.1	-2.0
C17:cyc	282	26.0	6.7±1.8	7.6±2.1	-0.9
C17:0	284	26.4	2.0±0.8	1.3±0.3	0.7
C18:1	296	28.8	20.6±2.9	16.9±0.2	3.7
C18:0	298	29.2	2.4±0.4	2.1±0.4	0.3

[a] The values represent weight percentages of total fatty acids and are expressed as means ± 1 standard deviation for three independent experiments.

A

```
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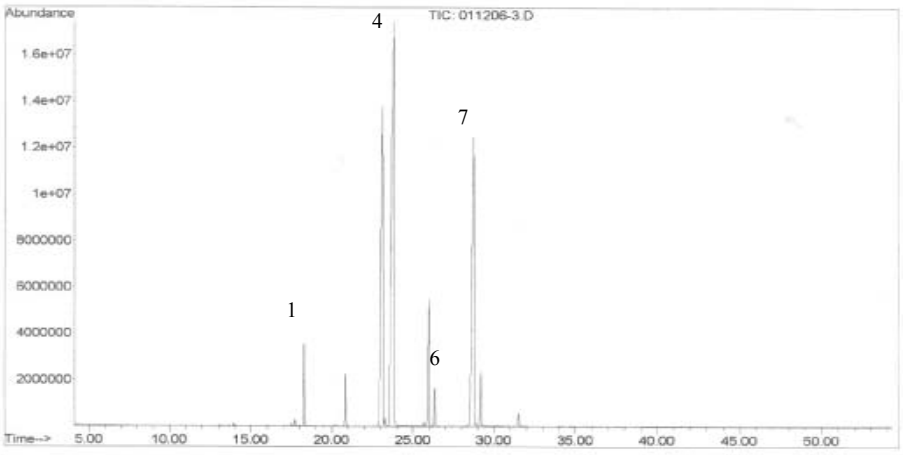

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Vial Number: 1
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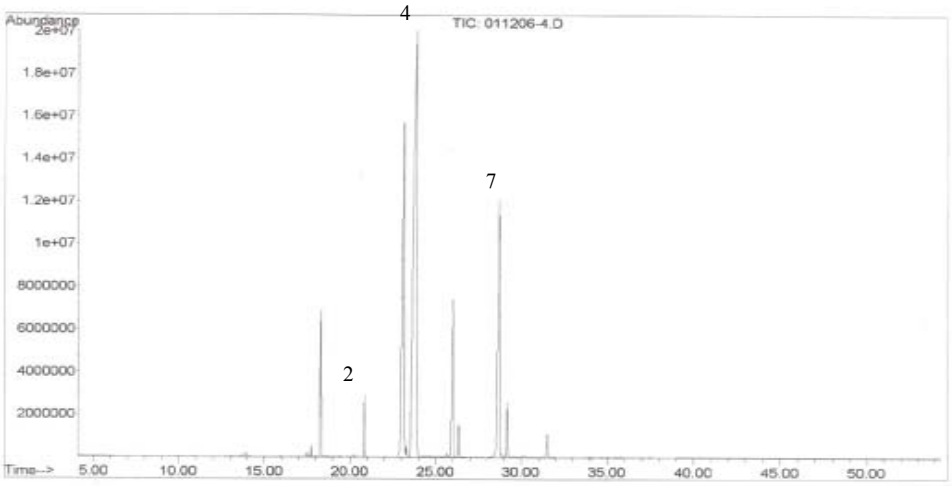

Figure 5–4. Gas chromatography of membrane fatty acids of E609Y(A) and E609(B). 1. myristic acid (C14:0); 2. pentadecanoic acid (C15:0); 3. palmitoleic acid (C16:1); 4. palmitic acid (C16:0); 5. *cis*-9,10-methylene hexadecanoic acid (C17:cyc); 6. heptadecanoic acid (C17:0); 7. *cis*-vaccenic acid (C18:1); 8. octadecanoic acid (C18:0).

5.3.4 Biotransformation using E. coli O44K74Y resting cells

It has been reported that the biotransformation processes for L-carnitine production (Figure 5-1) using *E. coli* O44K74 cells can only result in a maximum of 30–43% yield due to unfavorable reaction equilibrium, transport limitations and side product formation[64]. Both substrate (crotonobetaine) and product (L-carnitine) are hydrophilic with MW of around 200. Chemical permeabilizers such as organic solvents (ethanol, acetone and toluene), detergents (Tween 20 and Triton X-100) and polyethylenimine were used to treat the O44K74 cells to achieve a higher yield of biotransformation. However, chemical treatment of cells can often incur unintended damage to cells (even lysis), and generate serious downstream problem, especially when detergent is used. Our previous studies[,] demonstrated that *lpp* lipoprotein mutation was extremely effective in accelerating whole cell biocatalysis rates towards various substrates. Since the reaction rates were decided by measuring the product concentrations outside the cells, the increase in the reaction rates should also be attributed to the fast product export due to the mutation. In this study, an *lpp* knockout strain *E. coli* O44K74Y was generated using a previously reported gene inactivation method to evaluate the mutational effect on the L-carnitine export and product yield.

5.3.4.1 Time course of L-carnitine production

Cells of lipoprotein mutant *E. coli* O44K74Y and O44K74 were grown under the anaerobic conditions as described in 5.2.5.1. The biotransformation was performed aerobically using resting cells (~20 g wet cell/liter) suspended in 60 mM potassium phosphate supplied with 50 mM crotonobetaine. The time course of biotransformation

process was monitored by measuring the L-carnitine concentration as shown in Figure 5–5. The biotransformation started off quickly in the first two hours, with initial production rates of 3.0 g l^{-1} h^{-1} and 2.7 g l^{-1} h^{-1} for mutant and parent strain, respectively. The product concentration of the both strains increased slowly in the following four hours and reached maximum level after overnight incubation. The L-carnitine yield for lipoprotein mutant O44K74Y reached 56%, about 30% increase over the yield of its parent strain, 43%.

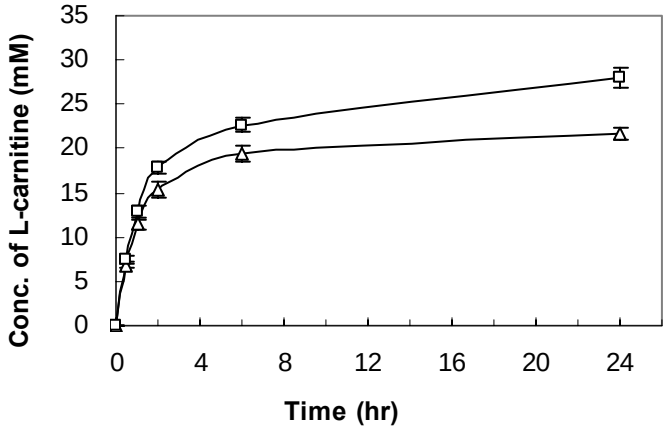

Figure 5–5. Time course of L-carnitine production by whole-cell *E. coli* O44K74Y (□) and O44K74 (△), with 50 mM initial crotonobetaine and ~20 g wet cell/liter biomass. Error bars represent standard deviations from the mean. All data are measured in three separate experiments.

From Figure 5–5, it is apparent that initial rates of both strains were very fast, with around 31% and 36% yield in the first hour for parent and mutant strains, respectively. Compared with the parent strain, the initial production rate of the mutant strain was only about 10% higher, which indicated that the membrane mutation did not have significant effect on permeability of hydrophilic substrate crotonobetaine and

product L-carnitine at the beginning of the reaction. Our earlier studies showed dramatic increases of 4– and 14–fold in the initial rates were achieved by the same lipoprotein mutation with hydrophilic substrates, nitrocefin and UDP-glucose, respectively[7]. However, this different observation with L-carnitine production is expected since both L-carnitine and crotonobetaine are relatively small molecules (MW~200) compared with nitrocefin and UDP-glucose (MW~600). The passage of these two small hydrophilic molecules through the outer membrane should be comparatively faster because they are far below the maximum size that allowable through the porin channels (600–700 Daltons).

The difference in the product concentrations between mutant and parent increased as the biotransformation continued. The final L-carnitine concentrations of mutant and parent strain were of 28.0 mM and 21.6 mM, respectively. Based on 50 mM crotonobetaine input, the product yield of the mutant strain was about 56%, which is 30% higher than that of the wild-type strain, 43%. The result suggests that the effect of lipoprotein mutation is more pronounced in the later phase of the reaction. Since the biotransformation catalyzed by L-carnitine dehydratase is a reversible reaction, we expect that the reaction would reach its equilibrium at a certain point depending on the intracellular concentrations of L-carnitine and crotonobetaine. Also, the L-carnitine yield is limited by the product degradation by side reaction. Therefore, the higher product yield by the mutant cells can be attributed to the faster product export that drives the reaction forward and reduces the product degradation. In order to demonstrate the advantage of mutant cells, further experiments were performed with multiple substrate replenishments.

5.3.4.2 Biotransformation with substrate feedings

To further evaluate the potential of the lipoprotein mutant for L-carnitine production, reactions with substrate feedings were performed as shown in Figure 5-6. The resting-cell reactions were able to continue for 48 hours with two 100 mM crotonobetaine replenishment at 4^{th} and 24^{th} hour. No further increase in the product concentrations were observed after a third addition of crotonobetaine at 48^{th} hour due to the depletion of cell material and energy for the biotransformation[, 64].

Figure 5-6 showed that the L-carnitine production rates were about 1.8 g l^{-1} h^{-1} and 1.6 g l^{-1} h^{-1} for the mutant and parent strains in the first two hours. The production rates reduced to 1.0 g l^{-1} h^{-1} and 1.2 g l^{-1} h^{-1} after the first substrate addition, 37% and 33% reduction for mutant and parent strains. After the first substrate addition, the L-carnitine concentrations reached 63 mM and 50 mM at 24^{th} hour for mutant and parent, respectively. Their respective product yields were 42% and 33% based on 150 mM crotonobetain input (after one substrate replenishment). The production rates after the second substrate addition further decreased to 0.1 g l^{-1} h^{-1}, less than 10% of the level of the first round. After 48 hours of reaction, the final L-carnitine concentrations of the mutant and parent strain were 84 mM and 59 mM, corresponding to yields of 34% and 24% (based on a total input of 250 mM after two substrate replenishments), respectively. From Figure 5-6, it's evident that the difference in the product yield between two strains increased as the reaction continued, with 42% higher yield achieved by the mutant. This also indicates that the lipoprotein membrane mutation is capable of enhancing the L-

carnitine yield without affecting the stability and durability of the whole cells as biocatalysts.

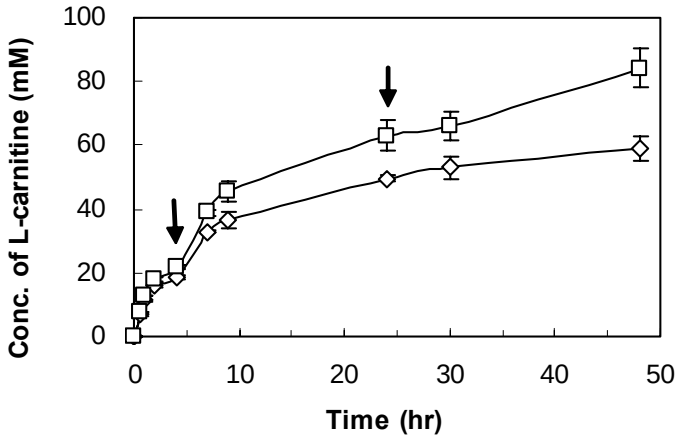

Figure 5–6. Time course of L-carnitine production catalyzed by whole-cell *E. coli* O44K74Y (□) and O44K74 (△) with 50 mM initial crotonobetaine and two100 mM crotonobetaine replenishments at 4th and 24th hour. Arrows represent two substrate feedings. Error bars represent standard deviations from the mean. All data are measured in three separate experiments.

5.4 Discussion

A Braun's lipoprotein transposon mutation was found to be effective in accelerating the reaction rates of whole-cell biocatalysis utilizing substrates with different size and hydrophobicity[,]. The enhancement in the reaction rates ranges from 1.5–fold to as high as striking 14–fold. In order to find out the genetic changes corresponding to this phenotype, the *lpp* region of this lipoprotein mutation E609L were sequenced and compared with control strain. It turned out the *lpp* is completely inactivated by a 59-bp insertion containing two double-stop codon at the beginning of *lpp* gene. Therefore, we believe that the same phenotype could be obtained by deleting the *lpp* gene from the chromosome DNA of any *E. coli* strain of interest. A generally applicable methodology was established for accelerating whole-cell biocatalysis employing a high efficient gene inactivation procedure. The characterization of *lpp* deletion strain showed that the mutant grew as fast as the parent strain in both rich and minimal medium and exhibited the same glucose consumption rate as the parent strain under both aerobic and anaerobic conditions. The mechanisms related to these observations have yet to be discovered. Some minor changes in the fatty acid compositions were detected (<5%), including the decrease of short-chain (C14:0~C16:0) and cyclopropane fatty acids levels and the increase of long-chain fatty acids (C17:0~C18:0) level. It suggestes that the lipoprotein mutant cells could manage to modulate the proportions of fatty acids as a response to the lipoprotein defect. The results also imply that compensation mechanisms are likely to exist in different aspects of growth and metabolism to help maintenance and function for the mutant cells.

This study demonstrates that a useful *lpp* lipoprotein mutation can be applied to solve the general permeability problems in whole-cell biocatalysis process. The *lpp* knockout strains can be generated with a straightforward and high efficient gene inactivation method. In this study, the *lpp* deletion was successfully introduced into *E. coli* strains with different genetic background for accelerating whole-cell biocatalysis. An example was illustrated using L-carnitine production strain *E. coli* O44K74 with 30~42% increase in product yield.

We applied the useful lipoprotein mutation to L-carnitine production strain *E. coli* O44K74 to achieve higher product yield. The fast initial production rates of both strains indicated that the effect of membrane mutation on the permeability of product and substrate (L-carnitine and crotonobetaine) was not significant at the beginning of the reaction due to their low MW. Only 10% difference in the initial production rates was observed in the first two hours. The effect of lipoprotein mutation was more pronounced in the later phase of the reaction, with a product yield of 56% achieved by the mutant strain, 30% increase over the yield of the parent strain, 43% (based on an input of 50 mM crotonobetaine). The results suggest that the lipoprotein mutant cells are able to achieve higher product yield by faster product export, which is crucial for forwarding the reversible reaction and releasing the product from degradation in the L-carnitine production. Further substrate feeding experiments confirmed the beneficial effect of mutation on the L-carnitine production. The reactions were continued for 48 hours, and the final L-carnitine yield after two substrate feedings were 42% higher than that of the parent strain. This also indicates that the lipoprotein membrane mutation is capable of

enhancing the L-carnitine yield while maintaining the stability and durability of the whole cells as biocatalysts. These results demonstrate that a useful phenotype can now be transferred to any *E. coli* strain of interest with a straightforward genetic manipulation to effectively enhance the rate of a whole-cell biocatalysis reaction. This method might be generally applicable to biocatalysis processes as the permeability change effected by the mutation is of global nature.

CHAPTER 6 Summary

The past two decades has seen tremendous progress in the research of using biocatalysts for the synthesis of high-value fine chemicals. Compared with isolated enzymes, whole-cell biocatalysts can be much more readily and inexpensively prepared on an industrial scale. Whole-cell biocatalysts are more stable than their isolated counterparts and allow reaction involving multi-enzymes even multi-pathways[1]. However, one major problem in whole-cell catalysis is the mass transfer limitation imposed by the cell envelopes. As a result, the reaction catalyzed by the whole-cells is often orders of magnitude slower than that of the isolated enzymes. Until recently, the only effective way to address this was to permeabilize cells by chemical (such as detergent, solvent, and chelators) or physical (such as extreme temperature fluctuations) treatment[2]. These methods are far from being desirable, especially for large scale applications, since they create additional steps in manufacturing processes, complicate downstream processing, and incur unintended damage to cells.

In this study, we successfully reduced the membrane permeability barrier of *E. coli* strains by genetically altering the membrane structure. A lipopolysaccarides *E. coli* mutant (SM101) and a Braun's lipoprotein mutant (E609L) were used in this study. As our first test, two large model substrates (MW~600) that differ substantially in hydrophobicity, nitrocefin, and a tetrapeptide N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide were employed to interrogate the outer membrane of the cells. The reduction of the outer membrane permeability by genetic methods led to significant increases (up to 380%) in

reaction rates of whole-cell catalyzed reactions. The magnitude of the increase in biocatalysis rates was also found to be dependent on the substrates and on the nature of the mutations introduced in the outer membrane structure. Notably, the lipopolysaccarides mutations can render the outer membrane completely permeable to the tetrapeptide, a barrier-less condition that maximizes the reaction rate. Subsequently, the toluene dioxyengase (TDO)-catalyzed reaction was investigated to investigate the effect of lipoprotein mutation on small, hydrophobic substrates. Dramatic enhancement of the reaction rate, an increase of up to six fold, was observed over a wide range of substrate concentrations. We also studied the effects of outer membrane mutations on a hydrophilic large molecule, UDP-glucose (MW~600) in whole-cell systems. Owing to the severe limitation in substrate permeability, the wild type cells only exhibited as low as 4% of available enzyme activities. Reducing the barriers of the outer membrane permeability led to a striking acceleration (up to 14–fold) of reaction rate in cells expressing UDP-glucose dehydrogenase. Mutations in the lipopolysaccharide synthesis pathway or Braun's lipoprotein are both effective. An application of outer membrane mutants was illustrated with the synthesis of a disaccharide (*N*-acetyllactosamine) from UDP-glucose. Both reaction rate and product yield were enhanced significantly (more than two fold) in the lipoprotein mutant, demonstrating the importance of the outer membrane permeability barrier and the advantages of using outer membrane mutants in synthesis.

The impact of membrane mutations was compared with a known potent permeabilizer PMBN (polymixin B nonapeptide). It showed that membrane modifications to enhance the permeability of hydrophilic molecules should target the

Lipid A region. However, molecular engineering strategies other than reduction of Lipid A synthesis should be considered. Our study also illustrated that the mutations were much more effective than the commonly used EDTA treatment and freeze-thaw (FT) permeabilizing procedure. Therefore, our results demonstrated the clear advantages of using genetic modification in cellular membrane engineering for improved whole-cell catalysts.

Our studies[,,] with a Braun's lipoprotein mutant E609L demonstrated that Braun's lipoprotein transposon mutation in outer membrane could be an effective method to increase permeability of a variety of substrates. The mutant exhibited a normal growth rate and expressed the recombinant multi-component enzyme as well as the isogenic parent strain. The results showed that reactions with all six substrates were dramatically accelerated by this lipoprotein mutation (up to 14–fold increase in reaction rate). In order to establish this as a generally applicable methodology for accelerating whole-cell biocatalysis, we investigated an alternative genetic modification to obtain the same phenotype. The *lpp* gene deletion was successfully introduced into *E. coli* strains from different genetic background and characterized. It showed that the deletion had the same effect on substrate permeability as the original transposon mutation. Also, the *lpp* deletion was found to have no significant effect on cell growth, carbon metabolism, and lipid compositions. As an example, the *lpp* deletion strain *E. coli* O44K74Y was successfully applied in L-carnitine production with 42% increase in product yield. These results demonstrate that a useful phenotype can now be transferred to any *E. coli* strain with a straightforward genetic manipulation to effectively enhance the rate of a whole-

cell biocatalysis reaction. This method might be generally applicable to biocatalysis processes as the permeability change effected by the mutation is of global nature.

This research and the results outlined in this dissertation point to a valid strategy in addressing permeability issues in whole-cell biocatalysis. It also highlights a need for an assessment of substrate permeability in biocatalysis research and development. Future study should be focused on,

1. Application of the lipoprotein mutation in non-*E. coli* Gram-negative bacteria strains.
2. Investigation of the relationship between the lipoprotein mutation to other important substrate properties, such as spatial structure, group size and charge.
3. Investigation of the potential effect of outer membrane mutations at other loci, such as integral protein of outer membrane, peptidoglycan.

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APPENDIX 1.1

A short Glossary: Terminology Used in the Dissertation

Mutation: A change of the DNA sequence within a gene or chromosome of an organism resulting in the creation of a new character or trait not found in the parent cell. Natural mutations can occur spontaneously or in response to environmental factors such as radiation and mutagenic chemicals. In this study, mutations were introduced into *E. coli* strains using molecular engineering techniques.

Mutant: A cell or organism that has been genetically changed as a result of mutation. The original cell is called the parent or wild-type cell.

Substrate: Substrate refers to the reactant of an enzymatic reaction.

Membrane permeability: The capability of cell membrane to allow the passage of substrates tested. Permeability depends on both the nature of the membrane structure and the physical properties of the substrate. In this study, the permeability is evaluated by the reaction rate of a whole-cell catalyzed reaction.

Reaction rate: In this study, the term reaction rates refers to apparent or observed rates of substrate utilization by host cells. Reaction rate was inferred from product measurement.

APPENDIX 1.2

VITA

Education

08/2002—05/2006	**Virginia Commonwealth University**
Major:	Chemical and Life Science Engineering
GPA:	4.0/4.0
Degree:	Ph.D.
09/1999—07/2002	**East China University of Science and Technology (ECUST)**
Major:	Biochemical Engineering
GPA:	88/100
Degree:	M.E.
09/1992—07/1996	**East China University of Science and Technology (ECUST)**
Major:	Biochemical Engineering
GPA:	86/100
Degree:	B.E.

Research Experience

08/2002—05/2006 Chemical and Life Science Engineering, Virginia Commonwealth University

Accelerating Whole-cell Biocatalysis By Reducing Outer Membrane Permeability Barrier Through Genetic Modification of Membrane Structure

- Application of membrane mutants as whole cell biocatalysts

- Rationally design and generate various outer membrane mutants that favor different types of reactions
- Characterization membrane mutant with different reaction system and their application

08/2002—4/2003 Chemical and Life Science Engineering, Virginia Commonwealth University

Cloning of *Sinorhizobium meliloti rkpK* Gene Encoding UDP-Glucose Dehydrogenase and Its Expression in *Escherichia coli*

- Cloning of *rkpK* Gene from *Sinorhizobium meliloti* and subcloning of rkpK into expression vector system
- High level expression of rkpK gene

09/1999-07/2002 Biochemical Engineering Department, East China University of Science and Technology

- Isolation and identification of an aryl ketone reductase producing strain, *Rhodotorula* sp. AS2.2241
- Purification and characterization of aryl ketone reductase
- Application of *Rhodotorula* sp. AS2.2241 in large-scale production of (*S*)-(-)-1-phenylethanol and (*R*)-(-)-2-bromo-1-phenylethanol

09/1995-07/1996 Biochemical Engineering Department, East China University of Science and Technology

- Optimization of high density cultivation condition of bifidobacterium

Working Experience

08/2002-05/2006 Research Assistant, Virginia Commonwealth University

08/2002-05/2006 Teaching Assistant, Virginia Commonwealth University

09/1999-07/2000 Research Assistant, East China University of Science and Technology

07/1996—07/1999 Lecturer, Applied Chemistry Department, Southern Yangtze University, Wuxi, Jiangsu, P. R. China

Honors and Awards

- American Society for Microbiology 2005 Corporate Activities student Travel Grant Award, Virginia Commonwealth University, 2005
- Phi Kappa Phi scholarship, Virginia Commonwealth University, 2004
- Graduate Student Association Spring 2004 Travel Award, Virginia Commonwealth University, 2004
- Graduate Assistantship, Virginia Commonwealth University, 2002-2006
- Excellent Prize for Graduate Thesis Symposium, East China University of Science and Technology, 2001
- Award for Outstanding Research Assistant, East China University of Science and Technology, 2000
- Award for Outstanding teacher, Southern Yangtze University, Wuxi, Jiangsu, P. R. China, 1998
- Excellent Course Scholarship for four consecutive years, East China University of Science and Technology, 1992-1996

Professional Activities

- Member of American Society for Microbiology, since 2005
- Member of American Chemical Society, since 2004
  Division of Biochemical Technology
- Member of Phi Kappa Phi, since 2004

Presentations and Posters

- 231th ACS National Meeting, Atlanta, GA, March 26-30, 2006. “Outer Membrane Mutation Effects on UDP–Glucose Permeability and Whole-Cell Catalysis Rate", Ye Ni, Zichao Mao and Rachel R. Chen, poster
- AIChE Annual Meeting, Cincinnati, OH, Oct. 30-Nov. 4, 2005. “Enhance and modulate substrate permeability for whole-Cell biocatalysis through cellular membrane engineering”, Ye Ni, Xuan Guo, John Reye and Rachel Ruizhen Chen, oral presentation
- 105th ASM General Meeting, Atlanta, GA, June 5-9, 2005. “Lipoprotein Mutation Accelerates Substrate Permeability-Limited Toluene Dioxygenase–Catalyzed Reaction”, Ye Ni, Rachel R. Chen, oral presentation and poster
- 7th annual graduate research symposium and exhibit, Virginia Commonwealth University, April 8 2004. “Modulating cell permeability for whole-cell biocatalysis”, Ye Ni, Xuan Guo, Rachel R. Chen, poster
- 227th ACS National Meeting, Anaheim, CA, March 28-April 1, 2004."Reducing outer membrane permeability barrier in whole-cell biocatalysis", Ye Ni, Xuan Guo, Rachel R. Chen, oral presentation
- Mid-Atlantic Biochemical Engineering Consortium (MABEC), University of Maryland, Baltimore County, March 22, 2004. “Accelerating Whole-cell Biocatalysis by Reducing Outer Membrane Permeability Barrier”, Ye Ni, Xuan Guo, Rachel R. Chen, oral presentation
- AIChE Annual Meeting, San Francisco, Nov. 16-21, 2003 Accelerating Whole-cell Biocatalysis by Reducing Outer Membrane Permeability, Rachel R. Chen, Ye Ni, oral presentation
- National Biochemical Engineering Meeting, Hangzhou, Zhejiang, 2002 Isolation and identification of an aryl ketone reductase producing strain, *Rhodotorula* sp. AS2.2241,

Ye Ni, Jian-he Xu, oral presentation

Publications

- Ye Ni, Zichao Mao, and Rachel R. Chen. "Outer Membrane Mutation Effects on UDP–Glucose Permeability and Whole-Cell Catalysis Rate" *Applied Microbiology & Biotechnology* (in press)
- Ye Ni and Rachel R. Chen. "Lipoprotein Mutation Accelerates Substrate-permeability-limited Toluene Dioxygenase–catalyzed Reaction," *Biotechnology Progress* 21 (2005) 799-805
- Ye Ni and Rachel R. Chen. "Accelerating Whole-cell Biocatalysis by Reducing Outer Membrane Permeability Barrier," *Biotechnology & Bioengineering* 87 (2004) 804-811
- Ye Ni and Jian-He Xu. "Asymmetric reduction of aryl ketones with a new isolate Rhodotorula sp. AS2.2241," *Journal of Molecular Catalysis B: Enzymatic* 18 (2002) 233-241
- Ye Ni and Jian-He Xu. "Isolation and identification of an aryl ketone reductase producing strain, *Rhodotorula* sp. AS2.2241," National Biochemical Engineering Meeting (Chinese) 2002