THE N-TERMINAL SEQUENCE OF *ESCHERICHIA COLI* CL SYNTHASE: FUNCTION(S) OF THE CONSERVED RESIDUES

by

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2007

UMI Number: 3283204

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This manuscript has been read and accepted for the
Graduate Faculty in Biochemistry in satisfaction of the
dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

THE N-TERMINAL SEQUENCE OF *ESCHERICHIA COLI* CL SYNTHASE: FUNCTION(S) OF THE CONSERVED RESIDUES

by

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Escherichia coli (*E. coli*) cardiolipin synthase (CL synthase) was fused to a C-terminal 6 X histidine tag. The fusion protein migrates at the predicted apparent molecular mass when analyzed by SDS PAGE and western blot, and has little to no *in vivo* or *in vitro* activity. Removal of the tag by reintroduction of the C-terminal stop codon restores full activity to CL synthase *in vivo*.

An EE (EYMPME) epitope tag was introduced into the primary sequence of CL synthase by making three relatively conservative mutations. The EE epitope tagged enzyme migrates at the same apparent molecular mass as the wild type CL synthase when analyzed by SDS PAGE, and is recognized on western blot using an anti EE antibody, whereas the wild type is not. The EE tagged and wild type proteins have nearly identical activity *in vivo* and similar activity *in vitro*.

Mutations were made to amino acid residues within the N-terminus of the EE epitope tagged CL synthase, and their effect on the processing of CL

synthase was analyzed by SDS PAGE and western blot. The *in vivo* and *in vitro* activity of CL synthase with these mutations was also analyzed. Mutations made within the N-terminus of CL synthase resulted in five classes of mutants: (i) those with an increase in apparent molecular mass that have activity *in vitro* but not *in vivo*, (ii) those with an increase in apparent molecular mass that have no activity *in vivo* or *in vitro*, (iii) those with an increase in apparent molecular mass that have activity *in vivo* and *in vitro*, (iv) those with a decrease in apparent molecular mass that have no activity *in vivo* or *in vitro*, and (v) those mutations that have little to no effect on the processing or activity of CL synthase. A single mutant (pBQ72, LV7-8SS) was found that has *in vitro*, but not *in vivo* activity, suggesting an alteration in membrane topology. Furthermore, this mutant CL Synthase (LV7-8SS) migrates with an increase in apparent molecular mass relative to the EE tagged CL synthase with a wild type N-terminus, indicative of an alteration in processing.

ACKNOWLEDGEMENTS

I would like to thank the following people for their help during the course of my graduate studies.

Dr. Burton E. Tropp, for all of his help and guidance throughout this research, and for giving me this opportunity to complete the program.

Dr. Timothy Short, for use of his Hoefer Transphor electrophoresis system.

Most importantly, my mother, without whose support and encouragement I could not have completed this work.

ABBREVIATIONS

(CL)	Cardiolipin
(PG)	Phosphatidylglycerol
(SDS)	Sodium dodecyl sulfate
(BSA)	Bovine serum albumin
(CIP)	Calf intestinal phosphatase
(EDTA)	Ethylenediaminetetraacetate
(PAGE)	Polyacrylamide gel electrophoresis
(PE)	Phosphatidylethanolamine
(PLD)	Phospholipase D
(IPTG)	Isopropyl β-D-thiogalacto-pyranoside
(TEMED)	N,N,N',N'-tetramethylethylenediamine
(TWEEN20)	Polyethylene glycol sorbitan monolaurate

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1.0. Introduction

Escherichia coli (*E. coli*) cardiolipin synthase (CL synthase) catalyzes the reversible reaction that combines two molecules of phosphatidylglycerol (PG) to form one molecule of cardiolipin (CL) [1-3] as shown in Figure 1.1. The *E. coli* structural gene encoding CL synthase, *cls,* is located at min 28.02 of the *E. coli* genetic map [4].

Careful inspection of strains that have a defect in *cls,* known as *cls-1,* reveals that they are more resistant to the glycerol-3-phosphate analog 3,4-dihydroxybutyl-1-phosphonate (DBP) [5], and are more sensitive to novobiocin [6-8] than are their wild type parents. Further research shows that strains with a defect in *cls* (i) have increased doubling times, (ii) have lower final cell densities, and (iii) are less viable in the stationary phase than are their wild type parents [9]. Differences in doubling times are enhanced when the pH of the growth media is increased, suggesting that CL may be required for growth under alkaline conditions (Hwang, Y.W., and Tropp, B.E. unpublished data). The *cls-1* mutation also increases the temperature sensitivity of cells with the *pssA1* allele, which is a temperature-sensitive mutation for phosphatidylserine synthase formerly known as *pss-1* [9]. Strains with a *pssA1* mutation can grow on LB plates at 30°C and 42°C, while cells with *pssA1* and *cls-1* mutations can grow at 30°C but not at 42°C. (Heber, S., and Tropp, B.E., unpublished data).

The *cls* gene (GenBank accession numbers U15986, D38779, U01911 and L12044) has been cloned and placed under the control of a *tac* [10] or a T7 promoter [11], enabling overexpression of the protein. Translation of the 1,458-nucleotide *E. coli cls* open reading frame should produce a 54.8 kDa polypeptide (486 residues) with two hydrophobic segments at its N-terminus and none elsewhere [7,8]. These two N-terminal hydrophobic regions are predicted by TopPred II [14,15] to be membrane-spanning α-helices comprised of residues 5-25 and 36-56 respectively, having the topology shown in Figure 1.2 [14,15]. Translation of *cls* in a membrane free *in vitro* assay produces a polypeptide with a molecular mass of 53-55 kDa [7]. Mature cardiolipin synthase analyzed by SDS PAGE migrates with an apparent molecular mass of 46 kDa, which suggests that it is posttranslationally modified by removal of approximately 80 residues [11-13]. Deletion of 60 residues from the C-terminus of CL synthase results in a mutant that shows no enzymatic activity [16], while N-terminal deletion of 60 residues results in a mutant that maintains activity [17], indicating that the residues are removed N-terminally. Previous attempts to sequence the mature form of CL synthase by Edman degradation were unsuccessful because the N-terminus appears to be blocked [11].

CL synthase shares with other members of the phospholipase D (PLD) superfamily two HKD motifs of sequence $HXK(X)_4D(X)_6G(X)_2N$, where X represents any amino acid [18-21]. The tertiary structure of PLD members is such that these two conserved sequences form a single active site, allowing members of the PLD superfamily to bind molecules containing phosphodiester

bonds [22]. Nuc is an endonuclease first isolated from the pKM101 plasmid of *Salmonella typhimurium*, and is a member of the PLD superfamily that has only one copy of the HKD motif [23]. However, crystal structures obtained for this protein show that it exists as a dimer, with a single active site formed by HKD motifs from two monomers [23]. Although the crystal structure of CL synthase is unknown, it is very likely to have a tertiary structure which brings the two HDK motifs into close proximity, characteristic of a PLD superfamily member. The catalytic mechanism of PLD superfamily members proceeds via a covalent phosphohistidine intermediate [18]. The positively charged lysine residues within the active site formed by 2 HKD motifs stabilize interaction with the negatively charged substrate [18]. A conserved histidine from one domain serves as the nucleophile, attacking the phosphorous atom and forming a covalent phosphohistidine intermediate [18]. The conserved histidine from the other domain then acts as a general acid to protonate the leaving group. This same histidine then acts as a general base to abstract a proton from water, and the covalent phosphohistidine intermediate is hydrolyzed by nucleophyllic attack of hydroxide on phosphorous [18]. Both of these conserved histidine residues are essential for catalytic activity since mutation of either one renders the protein inactive [18].

In vivo, CL synthase is membrane associated [10-12] and can be extracted from the cell membrane fraction with buffers containing the nonionic detergent Triton® X-100 [10-12]. A method to express and purify CL synthase to homogeneity was previously developed in this laboratory [11]. Insertion of the *cls*

gene into pET3, which contains a T7 promoter and a T7 terminator, created plasmid pLR3 that contains *cls* downstream of a T7 promoter followed by a T7 terminator [11]. Plasmid pLR3 was then transformed to BL21(DE3), which is a strain that has a T7 RNA polymerase under the control of a *lac*UV5 promoter [24]. IPTG induced BL21(DE3)/pLR3 produce large amounts of CL synthase, and the membrane fraction of BL21(DE3)/pLR3 contains approximately 1200 times the amount of CL synthase activity compared to wild type cells [11].

CL synthase was purified to homogeneity by first extracting the membrane fraction of IPTG induced BL21(DE3)/pLR3 with Triton® X-114, and then subjecting the extract to further purification by diethylaminoethyl cellulose (DEAE cellulose) chromatography [11]. The first step in this purification took advantage of the fact that Triton® X-114, which is a nonionic detergent similar to Triton® X-100, "clouds out" or forms an oily droplet at 30°C resulting in an approximate 5-fold purification of CL synthase. CL synthase in this oily droplet was further purified to homogeneity using DEAE cellulose chromatography, and the purified enzyme was shown to migrate with an apparent molecular mass of 46 kDa when analyzed by SDS PAGE [11]. A mixed micelle assay was also developed [11] to characterize the *in vitro* activity of CL synthase, which was found to have a specific activity of 8400 units/mg protein, where 1 unit is defined as 1 nmol of glycerol released per minute [11]. The *in vitro* catalytic activity of CL synthase is stimulated by phosphate, and inhibited by the reaction product, cardiolipin (CL), and by phosphatidic acid (PA) [11].

The fact that CL synthase is membrane associated and migrates at an apparent molecular mass less than that predicted by the gene's open reading frame suggests that the protein is directed to the membrane and then subjected to posttranslational cleavage at approximately residue 80. Previous research has shown that a fusion of the first 320 residues of CL synthase to β-galactosidase, a protein of approximately 116 kDa that is normally found in the water-soluble fraction, results in a protein that migrates at approximately 136 kDa and is membrane associated [25]. β-Galactosidase activity in cells expressing this protein fusion was associated with the membrane fraction, while cells with a *cls lacZ* operon fusion, in which both genes were transcribed simultaneously but coded for the individual proteins, contained β-galactosidase activity in the water-soluble fraction [25]. The fact that the fusion protein is membrane associated and migrates with an apparent molecular mass 9 kDa less than predicted by the primary sequence of the fusion protein indicates that the N-terminus of CL synthase directs the fusion protein to the membrane and the fusion protein is undergoing cleavage.

A model system that may provide important insight into the posttranslational processing of CL synthase is that of *E. coli* leader peptidase. The N-terminal regions of leader peptidase and CL synthase both contain two hydrophobic segments separated by a positively charged hydrophilic segment. Experimental evidence locates the catalytic domain of both leader peptidase [26-27] and CL synthase [28] on the periplasmic side of the inner membrane. The two membrane segments of leader peptidase have been demonstrated to

partition one at a time into the lipid bilayer [27], resulting in the topology shown in Figure 1.4. However, CL synthase and leader peptidase differ in 2 important respects. First, CL synthase is posttranslationally modified, while leader peptidase is not. Second, amino acid residues 83-98 of the primary sequence of leader peptidase form a third hydrophobic region, which interacts with the periplasmic side of the inner membrane [26].

Twenty-two of the first 80 residues of CL synthase are conserved across 19 species of gram negative/positive bacteria (Figure 1.3). The fact that there is a high degree of conservation of amino acids in this region is puzzling because the mature protein migrates with an apparent molecular mass that suggests the first 80 or so residues are cleaved [11-13]. One possible explanation for this conservation is that these residues function as a signal that contains information necessary to direct CL synthase to the membrane and obtain proper topology with its catalytic site facing the periplasm [28]. Evidence for a periplasmic orientation of this enzyme comes from experiments showing that when wild type *E. coli* cells deficient in mannitol transport across the cytoplasmic membrane are incubated with 600 mM mannitol, two novel phospholipids, phosphatidylmannitol and bisphosphatidylmannitol are formed [28]. These mannitophospholipids are not formed in mannitol transport deficient strains with *cls-1* indicating that CL synthase is responsible for their synthesis [28].

The primary sequence of the N-terminus of CL synthase does not readily conform to any known signal template, yet experimental evidence confirms the translocation of the protein to the membrane [11-13], and the cleavage of the

protein *in vivo* but not *in vitro* [7, 10-13, 16,17]. Proper insertion into the membrane is probably required to ensure that the catalytic site will be located on the periplasmic side of the inner membrane [28].

This work will examine whether the conserved residues at the N-terminus of CL synthase comprise an unknown signal arrangement. To enable unambiguous identification of CL synthase with mutations to conserved residues within the N-terminus, an epitope tag will be introduced into the nucleotide sequence of *cls* in pLR3 [11]. Plasmid constructs coding for CL synthase with mutations to conserved residues within the N-terminus will be overexpressed in a T7 system, and the effects of these mutations on the processing of CL synthase will be visualized by SDS PAGE and western blot. Point mutations at conserved amino acids within this region will be used to identify residues involved in the targeting of the protein to the membrane and/or the posttranslational processing of CL synthase. Similarly, frameshift mutations will be introduced to the N-terminus to examine whether or not conserved amino acids within these regions are involved in the posttranslational processing of CL synthase. The effects of these mutations on the activity of CL synthase will be examined using *in vivo* [5] and *in vitro* [11] activity assays. The goal of this research is to isolate a mutant CL synthase with changes to conserved residues within the N-terminus, which migrates with an increase in apparent molecular mass, and has *in vitro* but not *in vivo* activity, characteristic of a CL synthase with an alteration in membrane topology.

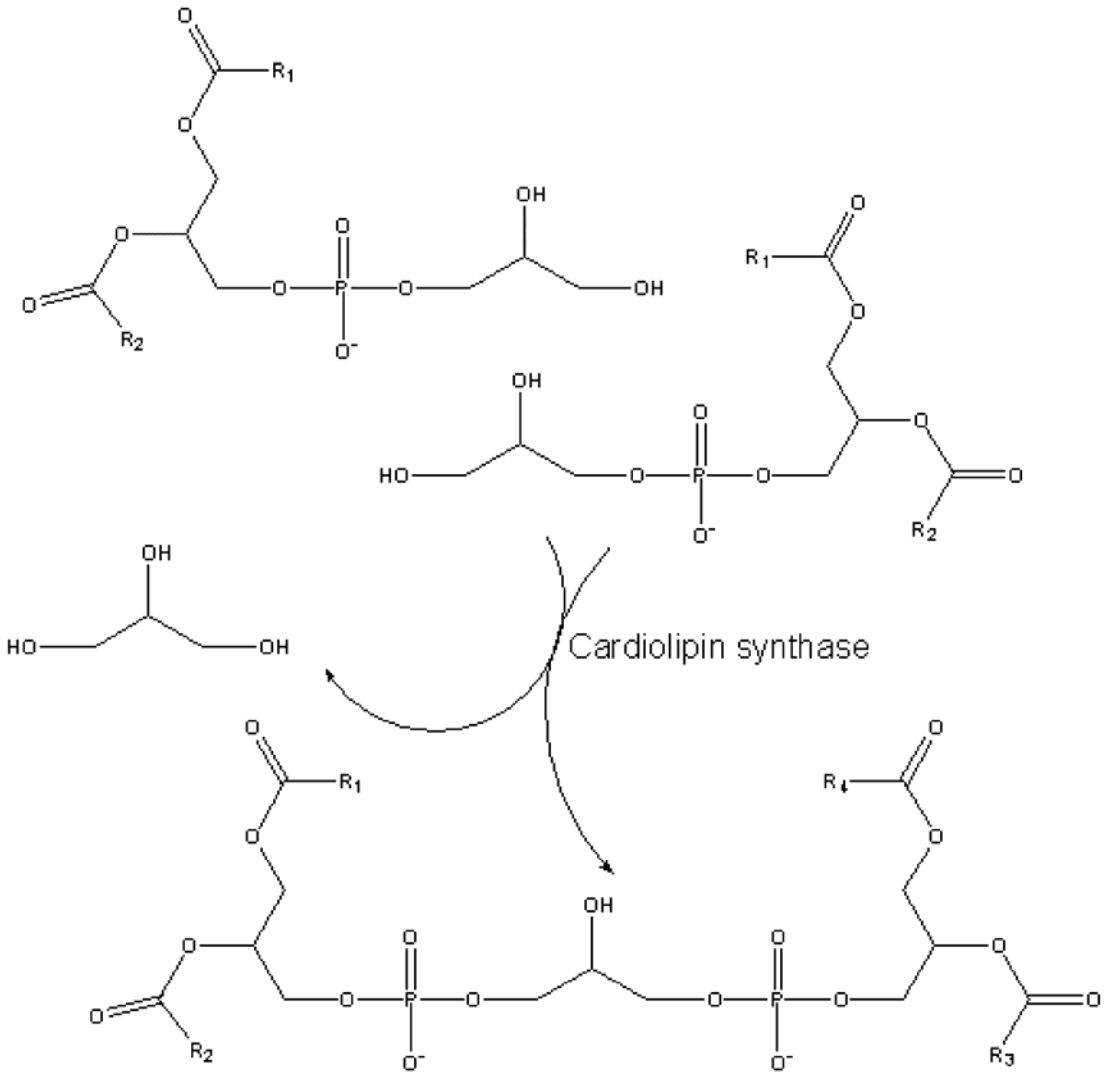


Figure 1.1. The reversible reaction catalyzed by *Escherichia coli (E. coli)* cardiolipin synthase (CL synthase).

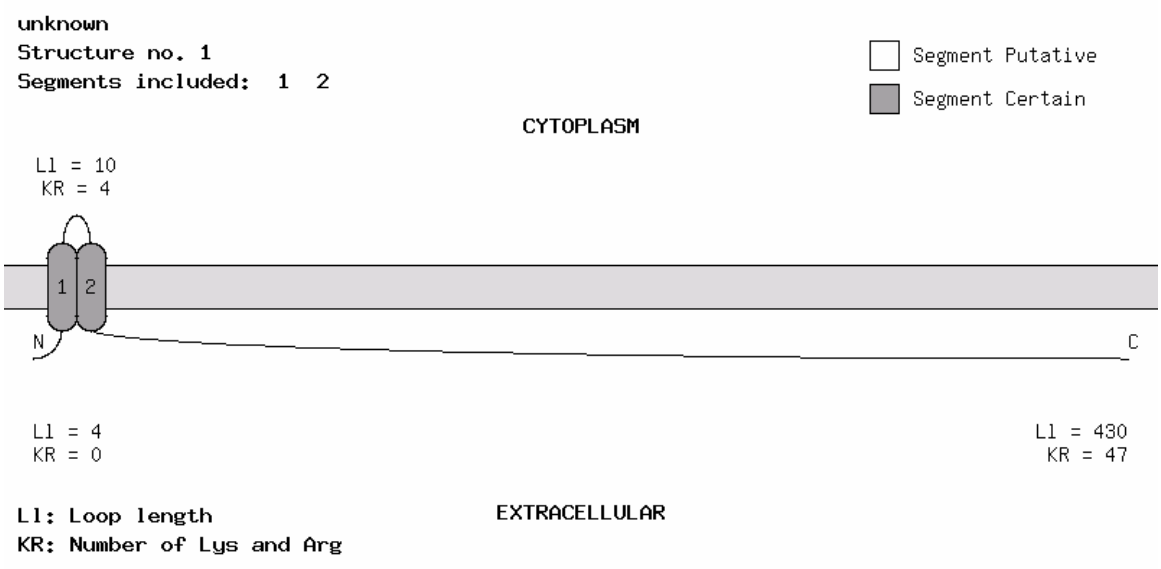


Figure 1.2. Theoretical membrane topology of *E. coli* CL synthase as predicted by TopPred II [14,15].

Figure 1.3. The first 80 amino acid residues of *E. coli* CL synthase. Residues conserved in CL synthases from 19 different bacterial species are shown in white letters in a black background. The first and second transmembrane regions extend from Y5 to L25 and S36 to L56 respectively [14,15]. Helix breaking residues G and P are present at the approximate midpoint of these regions.

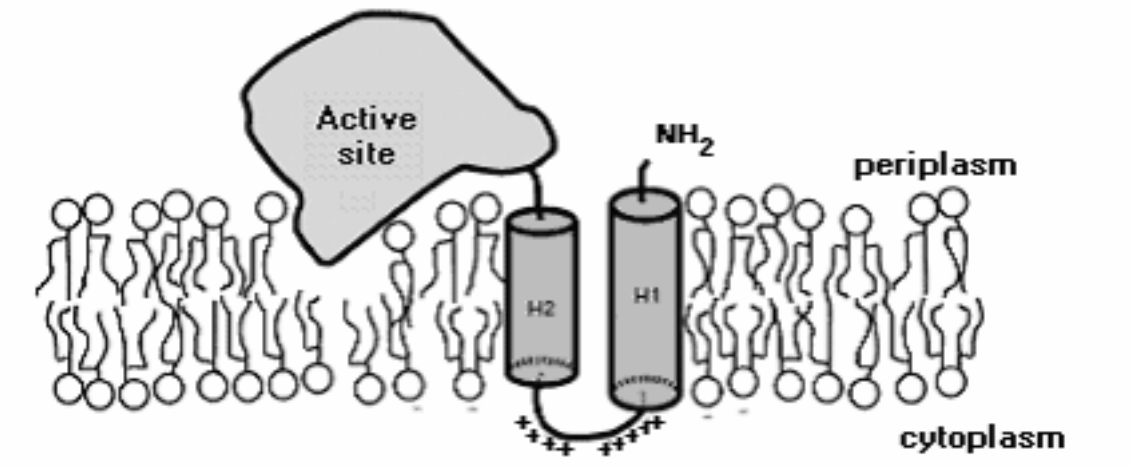


Figure 1.4. Orientation of leader peptidase in the membrane. Leader peptidase inserts with its active site in the bilayer. The positively charged residues in the cytosolic loop interact with the acidic lipids of the membrane. (Slightly modified from reference 29)

2. Materials and Methods

2.1. Chemicals

Ampicillin, chloramphenicol, ammonium persulfate, magnesium chloride, isopropyl β-D-thiogalactopyranoside (IPTG), XGAL (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside), brilliant blue G perchloric acid solution, SDS PAGE 5 X concentrate fixing solution, glycerol, Triton® X-100, NZ Amine A (Casein enzymatic hydrolysate), bovine serum albumin (fraction V), agarose, glycine (electrophoresis grade), SDS (Sodium dodecyl sulfate), phenol, the GenElute™ 5-minute plasmid miniprep kit, acrylamide:bis-acrylamide (29:1), TEMED (N,N,N',N'-tetramethylethylene-diamine), EDTA (Ethylenediaminetetraacetic acid disodium salt), MOPS (3-[N-Morpholino]propanesulfonic acid), TWEEN 20 (Polyethylene glycol sorbitan monolaurate), bromophenol blue, high molecular mass protein standard, Immobilon™-P 0.45 μm 15 X 15 cm polyvinylidene fluoride (PVDF) transfer membranes (Millipore), silica gel TLC general purpose (silica gel on polyester) plates, Polaroid 667 black and white film, Kodak developer/replenisher, Kodak fixer/replenisher, and Kodak Biomax 1 light film were purchased from Sigma-Aldrich, St. Louis, MO. Restriction endunucleases, T4 DNA ligase, calf intestinal phosphatase (CIP), and the 1 kb DNA Ladder, were purchased from New England Biolabs, Ipswich, MA. The goat polyclonal Glu-Glu tag antibody HRP conjugate (1 mg/mL) was purchased from Abcam, Cambridge, MA. The Western Lightning Enhanced Chemiluminescence Kit was purchased from PerkinElmer, Waltham, MA. Low melting temperature agarose, the Benchmark™ His-tagged Protein Standard, and the Anti-His(C-term)-HRP

mouse monoclonal antibody (1 mg/mL) were purchased from Invitrogen, Carlsbad, CA. The QuikChange® II Site-Directed Mutagenesis Kit was purchased from Stratagene, La Jolla, CA. The Qiagen Midi-prep kit was purchased from Qiagen, Valencia, CA. Primers used to introduce point mutations as well as frameshift mutations to *cls* were purchased from Sigma-Genosys, The Woodlands, TX. Primers used to introduce restriction sites for NdeI and XhoI to *cls* were purchased from Integrated DNA Technologies, Coralville, IA. DNA sequencing was performed by the Massachusetts General Hospital DNA Core Facility (Center for Computational and Integrative Biology), Cambridge, MA. UNSIL activated silicic acid (200-325 mesh) was purchased from Clarkson Chemical Company, Williamsport, PA. [2-^{14}C]Acetate was purchased from ICN, Irvine, CA. Millipore 0.45 μm MF-Millipore™ membrane filters were from Millipore, Billerica, MA. 2-[^{3}H]phosphatidylglycerol was provided by Dagang Guo. Ecoscint A biodegradable scintillation fluid was a product of National Diagnostics, Atlanta, GA. Tryptone, yeast extract, and agar were obtained from Difco Laboratories, Detroit, MI. All other chemicals were reagent grade or better.

2.2. Bacterial strains, plasmids, and media

Bacterial strains used in this study are listed in Table 2.1. Plasmids coding for a C-terminally 6 X histidine tagged CL synthase and its derivatives are listed in Table 2.2. Plasmids coding for EE (Glu-Glu) tagged (Epitope = EYMPME) CL synthase and its derivatives are listed in Table 2.3. LB, consisting of 1% tryptone, 0.5% NaCl, and 0.5% yeast extract, was used for routine growth

experiments. M9ZB (1% tryptone, 0.5% NaCl, 0.4% glucose, 2 mM $MgCl_2$, 18.7 mM NH_4Cl, 22 mM KH_2PO_4, 42.3 mM Na_2HPO_4) used in induction experiments was prepared as described by Studier and Moffatt [24]. Ampicillin and chloramphenicol were used at 125 μg/mL 20 μg/mL respectively. Where indicated, IPTG was added to a final concentration of 0.8 mM. Cell growth was monitored using a Klett-Summerson (660nm) photometer where 1 Klett unit corresponds to approximately 5 X 10^6 cells/mL.

2.3. Preparation of super competent strains and transformation

Cells were made competent by the method of Brian Seed as previously described [25] as follows. A repurified colony was incubated overnight at 37°C without shaking in 1 mL of TYM (2% tryptone, 0.5% yeast extract, 0.1 M NaCl, 10 mM $MgSO_4$ pH 7.0). The following morning, 50 μL of the overnight culture were transferred to 10 mL of TYM and cultured at 37°C with shaking at 250 rpm until reaching a turbidity of 60-70 Klett. The full 10 mL was then transferred to a 2 L flask containing 300 mL of TYM and incubated with shaking at 37°C until reaching a turbidity of OD_{600} 0.5-0.9, whereupon 190 mL TYM was added, and incubation was continued at 37°C with shaking until reaching an OD_{600} of 0.6. Unless otherwise specified, all of the following operations were performed at 4°C. Cells were harvested by centrifugation in a Sorvall RC5C centrifuge with a GSA rotor at 5000 X g for 5 minutes, and resuspended in 50 mL of TFB1 (30 mM KOAc, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$, 15% glycerol). Cells were centrifuged and resuspended in 10 mL of TFB2 (10 mM MOPS pH 7.0, 75 mM $CaCl_2$, 10 mM KCl, 15% glycerol), and immediately aliquoted in 500 μL volumes

to pre-chilled sterile Eppendorf tubes. Super competent cells prepared in this manner were stored frozen at –80°C until use. When the strain being made competent had a plasmid containing antibiotic resistance, the antibiotic was used throughout the procedure at the concentrations listed in section 2.2. Preparation of super competent strains having a *pssA1* mutation was performed as described above, with the exception that cells were cultured at 30°C.

Super competent cells prepared as above were transformed with plasmid DNA using the method given in the manual for the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) as follows. Fifty microliters of super competent cells were transferred to a pre-chilled Costar® Thermowell™ 0.5 mL thin-walled tube (Corning Incorporated, Corning, NY), combined with 10-250 ng of plasmid DNA and incubated on ice for 30 minutes. Cells were heat pulsed at 42°C for 45 seconds followed by incubation on ice for 2 minutes. Then 500 μL of NZY^+ (1% NZ Amine A, 0.5% yeast extract, 0.5% NaCl, 12.5 mM $MgCl_2$, 12.5 mM $MgSO_4$, 0.4% glucose pH 7.5) preheated to 42°C was added, and cells were incubated at 37°C with shaking (250 rpm) for 1 hour in an Aqua-Therm water bath shaker (New Brunswick Scientific, New Brunswick, NJ). Following outgrowth, cells were plated to LB containing the appropriate antibiotic(s) and incubated overnight at 37°C.

Transformation of super competent strains having a *pssA1* mutation was performed as described above with the following changes. The heat pulse used was 37°C for 90 seconds, NZY^+ was preheated to 30°C, and following plating, cells were incubated over night at 30°C.

2.4. DNA isolation and manipulation

Unless otherwise specified, plasmid DNA used for restriction analysis, transformation and sequencing was obtained from strain XL1 Blue (Stratagene) using a Qiagen midi prep kit (Qiagen, Valencia, CA) according the manufacturer's instructions.

Plasmid DNA was analyzed by absorbance at 260 nm and 280 nm, as well as by restriction analysis/agarose gel electrophoresis where appropriate. DNA fragments from restriction digests were analyzed using a 1% agarose gel with a TAE (40 mM Tris-acetate, 1 mM EDTA) buffer system [30], ethidium bromide at a concentration of 0.5 μg/mL, and a constant voltage setting of 100 V. Ethidium bromide was used in the gel only, and gels were destained in TAE for 30 minutes prior to photographing.

Dual restriction digests were carried out in buffer with maximum activity for both enzymes according to the information provided on the manufacturer's website (NEB). In the event that either restriction endonuclease had a BSA requirement, BSA was added to the assay according to the manufacturer's instructions. Nucleotide fragments were visualized and photographed using a Fotodyne UV enclosure with a Polaroid MP4 Land Camera and Polaroid 667 black and white film.

2.4.1. DNA sequencing

DNA was prepared for sequencing by ethanol precipitation following the method of Sambrook [30] as follows. A 2.0 μg plasmid DNA sample in TE was added to a sterile Eppendorf tube. Sodium acetate (3 M, pH 5.2) was added to a

final concentration of 300 mM followed by 2 total volumes of 100% ethanol at 4°C. Tubes were incubated at 4°C for 5 minutes followed by centrifugation at 12,000 rpm for 10 minutes at room temperature in an Eppendorf 5414 microfuge. The supernatants were aspirated and the DNA was washed twice with 500 μL of 70% ethanol and allowed to dry for 10-15 minutes under a sterile hood. Plasmid DNA was resuspended in 20 μL of sterile distilled water containing 100 ng of the desired sequencing primer and stored at –20°C until being sent out for sequencing.

All mutations made to *cls* in pBQ43 were confirmed by sequencing. Primers used (Table 2.11) to sequence plasmid constructs were between 18-24 bp in length, with a melting temperature between 50°C and 65°C, and were designed to anneal to a given plasmid approximately 100 nucleotides upstream of the desired nucleotide sequence. Mutations made to the nucleotide sequence of *cls* in pLR3 to introduce the EE epitope tag were sequenced using sequencing primer 1. All mutations made to *cls* in pBQ43 to construct plasmids encoding a CL synthase with mutations to the N-terminus were confirmed using sequencing primer 2. The nucleotide sequence of pBQ55, encoding a CL synthase with the mutation H224A, was sequenced using sequencing primer 3. The nucleotide sequence of pBQ74 was sequenced using sequencing primer 4. Sequencing in this manner routinely returned high quality sequences of 400-600 nucleotides.

2.4.2. Subcloning

Subcloning was performed by digesting plasmid DNA with the appropriate endonuclease(s) followed by purification of the resulting fragments with 0.8% low

melting temperature agarose gel electrophoresis [30], using ethidium bromide at half the normal concentration (0.25 μg/mL). Vector DNA (500 ng), and plasmid DNA (1 μg) containing the desired DNA fragment, were digested in separate tubes with a 20 X excess of endonuclease for 2 hours at 37°C in a buffer having maximum activity for the endonuclease(s). Immediately following the digest, the endonuclease in the vector digest tube was heat inactivated by incubation at 65°C for 20 minutes. After cooling to 4°C, 1 unit of calf intestinal phosphatase (CIP) was added to the vector DNA, and dephosphorylation proceeded at 37°C for 30 minutes. The manufacturer defines one unit of CIP as the amount of enzyme that hydrolyzes 1 μmol of p-nitrophenylphosphate to p-nitrophenol in a total reaction volume of 1 mL in 1 minute at 37°C in 1 M diethanolamine-HCl (pH 9.8) with 0.5 mM $MgCl_2$ and 10 mM p-nitrophenylphosphate.

The linearized vector and the desired *cls* insert were purified using low melting temperature (LMT) agarose gel electrophoresis [30] with a 0.8% gel and a TAE buffer system [30]. Samples were separated with a constant voltage setting of 50 V for approximately 30 minutes. Nucleotide fragments were visualized using a UVP (Ultra Violet Products, Upland, CA) LMW-20 White/2UV Transilluminator set to 365 nm taking all possible precautions to minimize UV exposure, which never exceeded 1 minute. Gel slices (usually ~100 μL each) containing the desired DNA fragments were excised with a clean razor blade, transferred to a sterile Eppendorf tube and stored at –20°C.

Nucleotide fragments were extracted from the LMT agarose gel slice as follows [30]. Five hundred microliters of 20 mM Tris-HCl pH 8.0, 1mM EDTA was

added to the gel slice in a 1.5 mL Eppendorf tube, and the mixture was incubated at 65°C for 5 minutes to melt the agarose. An equal volume of phenol, pre-equilibrated to pH 8.0 by washing twice each with 1 M Tris-HCl pH 8.0 and 0.1 M Tris-HCl pH 8.0, was added and the aqueous phase was isolated and extracted with equal volumes of phenol: chloroform 1:1 and chloroform. Fragments were ethanol precipitated as described above and resuspended in 20 μL of TE. Purified nucleotide fragment concentrations were estimated using agarose gel electrophoresis by comparison of bands in sample lanes to bands containing known DNA amounts in the 1 kb DNA standard ladder (NEB).

Ligation of relevant fragments was performed using T4 DNA ligase (NEB) at room temperature for 30 minutes using a vector: insert "molarity of ends ratio" of 1:3 calculated according to the manufacturer's instructions (NEB).

Contents of the ligation reaction (50 μL) were added to 400 μL of supercompetent XL1 Blue (Table 2.1) cells and transformation proceeded as described in section 2.3. The plasmids from twelve repurified colonies were obtained using the GenElute™ Five-Minute Plasmid Miniprep Kit (Sigma-Aldrich) and analyzed by restriction digest to select those with the desired insert. Four plasmids selected in this fashion were used in preliminary IPTG induction and *in vivo* activity experiments. If all clones showed identical properties, one was selected for further study.

2.5. Site directed mutagenesis

Introduction of restriction sites, frameshift mutations and point mutations to *E. coli cls* were introduced using a QuikChange® II Site-Directed Mutagenesis Kit

(Stratagene) according to the manufacturer's instructions. Primers used to introduce restriction endonuclease sites 5' and 3' of *cls* in pLR3 are listed in Table 2.4. Primers used for the introduction of frameshift regions to 6 X histidine tagged constructs are listed in Table 2.5. Primers used to remove the C-terminal 6 X histidine tag and to alter residue E487 are listed in Table 2.6. Primers used to construct an EE (Glu-Glu) epitope (EYMPME) tagged CL synthase are listed in Table 2.7. Primers used to insert alanine point mutations within the N-terminus of the EE tagged CL synthase encoded by pBQ43, as well as the mutation H224A, are listed in Table 2.8. Primers used to introduce aspartate, serine and threonine point mutations to within the N-terminus of CL synthase encoded by pBQ43 are listed in Table 2.9. Primers used to introduce frameshift regions within the N-terminus of CL synthase encoded by pBQ43 are listed in Table 2.10. Sense and antisense strands, usually 30-35 bp, were synthesized containing the desired mutation flanked by approximately 15 nucleotides on either side of the altered nucleotides. Primers were resuspended to a concentration of 10 mg/mL in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), and diluted to 100 ng/μL by serial dilution in sterile distilled H_20 that had been passed through a 0.45-micron filter (Millipore). Individual mutagenesis reactions were performed according to Stratagene's instructions, using 20 ng of plasmid template and 125 ng of each sense and antisense primer in a total reaction volume of 50 μL, in a Costar® Thermowell™ 0.5 mL thin-walled tube (Corning Incorporated, Corning, NY). To prevent evaporation during cycling, 30 μL of light mineral oil was layered onto the top each reaction. All other reaction

components, cycle segments and times, DpnI restriction digest of template strand, and transformation of competent cells were performed according to the manufacturer's instructions as follows. The reactions were first cycled to 95°C for 30 seconds followed by 16 cycles of: (i) 95°C for 30 seconds to denature the DNA, (ii) 55°C for 1 minute to anneal primers, and (iii) 68°C for 7.5 minutes for primer extension. Following amplification, reactions were incubated at 4°C for 5 minutes, and the non-mutant DNA was digested for 1 hour at 37°C with DpnI according to the manufacturer's instructions. Following digestion, competent cells were transformed as described in section 2.3. Due to a very high GC content in the primer used to recover the reading frame at position 68 (pBQ71), which caused the reaction to repeatedly fail, Stratagene recommended the addition of 4% dimethyl sulfoxide (DMSO) to the reaction mixture. Upon addition of DMSO the reaction completed successfully. Reactions were cycled using a Perkin Elmer 480 thermocycler (Perkin Elmer).

2.6. Construction of a 6 X histidine tagged CL synthase

To create pBQ3, an XhoI restriction site was introduced by site directed mutagenesis 3' of *cls* in pLR3 by modification of 3 nucleotides, shown as black letters in a gray background, (CTGTAA→CTCGAG) using the primers listed in Table 2.4 resulting in pBQ1. The introduction of this site involved the modification of the final L codon of *E. coli cls* to one of its wobble counterparts and introduced a glutamate (E) residue when this fragment was ligated in frame with the pET-23a(+) C-terminal 6 X histidine tag. The successful introduction of this site was confirmed by restriction analysis.

E. coli cls has an NdeI site approximately 50 nucleotides upstream of the initial AUG. The nucleotide sequence extending from this NdeI site to the introduced XhoI site contains the wild type *E. coli* RNA polymerase binding site, the *E*. *coli* ribosomal binding site (rbs) and *cls*. As shown in Figure 2.1, This *E*. *coli* chromosomal fragment from NdeI to XhoI was excised from pBQ1 and ligated into pET-23a(+) (Novagen) which had also been digested with NdeI and XhoI. The resulting plasmid (pBQ3) contains the *E*. *coli* chromosomal fragment described above immediately downstream of the pET-23a(+) T7 promoter and strong ribosomal binding site (rbs), and codes for the full-length amino acid sequence of CL synthase fused C-terminally to the sequence EHHHHHH. The fact that the *E. coli* chromosomal RNA polymerase promoter fragment is present allows transcription of *cls* in pBQ3 in wild type *E*. *coli* strains without a T7 RNA polymerase such as QC25 and QC30-15.

To create pBQ4, an NdeI site was introduced at the initial AUG codon of *cls* in pBQ1 by modification of 2 nucleotides (TCTATG→CATATG) 5' of *cls* using the primers listed in Table 2.4 to create pBQ2. As shown in Figure 2.2, this 1458 bp *cls* NdeI XhoI fragment was excised from pBQ2 and ligated into pET-23a(+) (Novagen), which had also been digested with NdeI and XhoI as described in section 2.4. Plasmid pBQ4 thus contains *cls* from the initial methionine (M) codon to the final Leucine (L) codon immediately downstream of the pET-23a(+) strong rbs fused C-terminally to the residues (EHHHHHH). Because this plasmid does not contain an *E. coli* chromosomal RNA polymerase promoter, it cannot be transcribed in strains without a T7 RNA polymerase.

To introduce frameshift regions comprising amino acid positions 2-20 (pBQ6) and 2-42 (pBQ7), a frameshift mutation was introduced at nucleotide position 4 of *cls,* the first nucleotide of the second amino acid codon in pBQ3, forming pBQ5 as shown (ACA→CGCA). The altered nucleotide is shown as a black letter in a gray background and the inserted nucleotide is underlined. The primers used to make this mutation (Table 2.5) introduced a new FspI site that was confirmed by restriction analysis.

To recover the reading frame at positions corresponding to amino acid residues 21 (pBQ6) and 43 (pBQ7) of *cls*, a single nucleotide deletion was made to pBQ5 using the primers listed in Table 2.5. To recover the reading frame at position 21, the deletion (ATTG→ATG) was made, resulting in pBQ6 (frameshift 2-20). The nucleotide deleted is shown as a white letter in a black background and the resulting sequence shows the nucleotide on either side of the deleted nucleotide in a box. To recover the reading frame at position 43, the deletion (GTTG→GTG) was made, resulting in pBQ7 (frameshift 2-42).

To remove the C-terminal 6 X histidine tag from pBQ3 and pBQ4, which are identical at the 3' of *cls*, the natural *cls* C-terminal Leucine codon and stop codon were reintroduced to pBQ3 (CTCGAG→CTGTAA) to form pBQ30 and to pBQ4 to form pBQ40 using the primers listed in Table 2.6. Altered nucleotides are shown as black letters in a gray background.

To obtain pBQ31 (E487G) and pBQ32 (E487L), the nucleotide codon for the glutamate residue introduced by ligation of *cls* in frame with the pET-23a(+) C-terminal 6 X histidine tag (E487) in pBQ3 was converted to glycine (G)

(GAG→GGG) (pBQ31) or Leucine (L) (GAG→TTG) (pBQ32) using the nucleotide substitutions shown, and the primers listed in Table 2.6. Altered nucleotides are shown as black letters in a gray background.

2.7. Construction of an EE (Glu-Glu) epitope (EYMPME) tagged CL synthase

To construct a plasmid coding for an EE tagged CL synthase (pBQ43), pLR3 was subjected to 3 sequential rounds of site-directed mutagenesis altering a total of 6 nucleotides of *cls* using the primers listed in Table 2.7. In the first round of mutagenesis, pLR3 was converted to pBQ41 (N296E) by altering two nucleotides in the *cls* N296 codon (AAT→GAG) so that it would be translated as E. In the second round, pBQ41 was converted to pBQ42 by altering two nucleotides of the I297 codon (ATT→TAT) so that it would be translated as Y. In the final mutation, pBQ42 was converted to pBQ43 by altering two nucleotides in the F299 codon (TTT→ATG) so that it would be translated as M. Nucleotides that have been altered are shown as black letters in a gray background. The result of these mutations was to convert the primary sequence of CL synthase as follows (NIMPFE→EYMPME). The resulting plasmid (pBQ43) is identical to pLR3 with the exception that the pBQ43 gene product has an EE (EYMPME) epitope at amino acid positions 296-301 of the primary sequence of CL synthase as shown in Figure 2.3.

2.8. Introduction of point mutations and frameshift regions to the N-terminus of CL synthase

2.8.1. Introduction of alanine point mutations

The introduction of alanine point mutations within the N-terminus of CL synthase were made using the primers listed in Table 2.8. Alterations made to *cls* in pBQ43 were directed at the amino acid codon of interest changing a maximum of 2 nucleotides in each case. In order to maximize the melting temperature of the primers used, the alanine codon chosen was the one that shared the greatest degree of homology with the codon being altered. Plasmid pBQ43 was converted to the alanine point mutants shown in Table 2.8 by making the following nucleotide substitutions, where altered nucleotides are shown as black letters in a gray background. Plasmid pBQ44 (W40A; TGG→GCG), pBQ45 (P48A; CCG→GCG), pBQ46 (Y55A; TAT→GCT), pBQ47 (G59A; GGC→GCC) pBQ61 (R66A; CGC→GCC) and pBQ62 (R70A; CGC→GCC). To construct pBQ52 (W40A P48A), pBQ44 (W40A) was altered at the P48 codon (CCG→GCG). Plasmid pBQ66 (R66A R70A) was constructed by altering the R70 codon of pBQ61 (CGC→GCC). In order to examine the effect of a mutation of one of the active site histidines, pBQ55 (H224A) was constructed by altering the H224 codon as follows (CAT→GCT).

2.8.2. Introduction of aspartate (D), serine (S), and threonine (T) mutations to the N-terminus of CL synthase

The introduction of aspartate, serine, and threonine point mutations within the N-terminus of CL synthase were made using the primers listed in Table 2.9.

The codon chosen in each case was the one that shared the greatest degree of homology with the codon being altered. Unless otherwise specified, the nucleotide changes, shown as black letters in a gray background, were made to the amino acid codon of interest of *cls* in pBQ43 as follows. Plasmid pBQ48 (S36D; TCC→GAC), pBQ67 (LII42-44SSS; TTGATTATT→TCGAGTAGT), pBQ68 (II52-53SS; ATTATT→AGTAGT), pBQ69 (L19S; CTC→TCC), pBQ72 (LV7-8SS; TTGGTG→TCGTCG). Plasmid pBQ73 (LI19-20SS) was made by alteration of the I20 codon of *cls* in pBQ69 (ATT→AGT). To create pBQ70 (KRR30-32TTT), a single nucleotide (underlined) was inserted at position 2 of the K30 codon of *cls* in pBQ43 (AAA→A<u>C</u>AA) resulting in pBQ64. This mutation was chosen because it shifted the reading frame from KRR to TTT. A single nucleotide deletion was then made to pBQ64 at position 3 of the T32 codon (AC**G**C→A[CC]) resulting in pBQ70 (KRR30-32TTT). The deleted nucleotide is shown as a white letter in a black background and the nucleotides on either side of the deleted nucleotide in the new sequence are shown in a box. Plasmid pBQ71 (KRR65-67SSA) was constructed by first shifting the reading frame of *cls* in pBQ43 and altering one nucleotide of an adjacent codon, so that the frameshift introduced would code for SSA, and then recovering the reading frame by deletion of 2 nucleotides. In the first step, two nucleotides were inserted between the G64 and R65 codons of *cls* in pBQ43 and a single nucleotide was altered in the adjacent codon as shown (GGC AAA CGC →GGC <u>TC</u> AAG CGC) resulting in pBQ65. Altered nucleotides are shown as black letters in a gray background and inserted nucleotides are underlined. The introduction of this mutation shifted

the translation of the reading frame from KRR to SSA. The reading frame was recovered at position 68 by deletion of 2 nucleotides of the alanine 68 codon in pBQ65 as shown (GCGCTG→GCGG) resulting in pBQ71 (KRR65-67SSA). The deleted nucleotides are shown as white letters in a black background and the nucleotides on either side of the deleted nucleotides in the new sequence are shown in a box.

2.8.3. Introduction of frameshift regions to the N-terminus of CL synthase

The introduction of frameshift regions within the N-terminus of CL synthase were made using the primers listed in Table 2.10. Unless otherwise specified, all nucleotide changes were made to *cls* in pBQ43. Frameshift mutations of amino acid residues 2-20 (pBQ56) and 2-42 (pBQ57) were introduced to pBQ43 as described for 6 X histidine constructs pBQ6 and pBQ7 in section 2.6. A frameshift mutation was introduced at nucleotide position 4 of *cls,* the first nucleotide of the second amino acid codon in pBQ43, forming pBQ51 as shown (ACA→CGCA). The altered nucleotide is shown as a black letter in a gray background and the inserted nucleotide is underlined. The primers used to make this mutation (Table 2.10) introduced a new FspI site that was confirmed by restriction analysis.

To recover the reading frame at positions corresponding to amino acid residues 21 (pBQ56) and 43 (pBQ57) of *cls*, a single nucleotide deletion was made to pBQ51 using the primers listed in Table 2.10. To recover the reading frame at position 21, the deletion (ATTG→ATG) was made, resulting in pBQ56 (frameshift 2-20). The nucleotide deleted is shown as a white letter in a black

background and the resulting sequence shows the nucleotide on either side of the deleted nucleotide in a box. To recover the reading frame at position 43, the deletion (GT**T**G→G[TG]) was made, resulting in pBQ57 (frameshift 2-42).

A non-epitope tagged CL synthase 2-20 frameshift mutant (pBQ63) was created by introduction of a frameshift region to *cls* in pLR3 as described above for pBQ56 (EE tagged 2-20 frameshift mutant) using the primers listed in Table 2.10. Introduction of a frameshift mutation at nucleotide position 4 of *cls* in pLR3 resulted in pBQ60, and recovery of the reading frame at a position corresponding to amino acid residue 21 resulted in pBQ63 (non-epitope tagged frameshift 2-20 mutant).

To create a frameshift mutant from amino acid residues 41-44 (pBQ50), the L41 codon was shifted out of frame by insertion of a single nucleotide (CTG→C<u>A</u>TG), to form pBQ49, where the underlined nucleotide has been inserted. This mutation introduced a new NspI site that was confirmed by restriction analysis. The reading frame was recovered at amino acid position 45 by deletion of a single nucleotide (A**T**TT→[AT]T) in pBQ49 to form pBQ50. The deleted nucleotide is shown as a white letter in a black background, and the nucleotide on either side of the deleted nucleotide in the new sequence is shown in a box. Plasmid pBQ59 (frameshift 72-82) was also constructed in 2 steps. First, a single nucleotide was inserted at position 2 of the R72 codon (AGA→A<u>C</u>GA) resulting in pBQ54. This mutation introduced a new BssSI restriction site that was confirmed by restriction analysis. In the second step, a single nucleotide deletion was made in pBQ54 as shown (CT**T**AA→C[TA]A)

resulting in pBQ59. The deleted nucleotide is shown as a white letter in a black background, and the nucleotide on either side of the deleted nucleotide in the new sequence is shown in a box. The frameshift regions introduced in pBQ50, pBQ56 and pBQ59 are shown as clustal W [36] alignments with the wild type CL synthase primary sequence in Figure 2.4.

2.8.4. Construction of a CL synthase missing amino acid residues 2-60

Plasmid pBQ74, which codes for an EE tagged CL synthase missing the first 60 amino acid residues, was constructed as follows. Plasmid pBQ43 was cut with SacI, and the 1.4 kb fragment containing *cls* from 180 nucleotides 3' of the initial AUG to just beyond the 3' end of the gene was ligated into pET-23a(+) that had also been cut with SacI as shown in Figure 2.5. Because a single restriction enzyme was used, 2 orientations were possible and both were obtained. This *cls* fragment codes for CL synthase from residue 60 to shortly beyond the C-terminus and includes the *cls* stop codon so that C-terminal 6 X histidine tag of pET-23a(+) is not fused to the protein. The resulting plasmid codes for an EE epitope tagged CL synthase missing the first 60 residues immediately downstream of the pET-23a(+) strong ribosomal binding site (rbs), fused in frame to the sixteen residues shown (MASMTGGQQMGRGSEF), where the residues comprising the N-terminal T7 tag of pET-23a(+) are shown as black letters in a gray background. The residues (RGSEF) result from translation of the pET-23a(+) multiple cloning site (MCS) up to the SacI restriction site into which the cls gene fragment was ligated.

2.9. TLC

Silica gel on polyester (Sigma-Aldrich) general-purpose thin layer chromatography (TLC) plates were washed prior to use with chloroform: methanol (1:1), and were heated for 40 minutes at 110°C immediately prior to use. All chromatography experiments used glass chromatography tanks that were lined with Whatmann 3MM filter paper and equilibrated with solvent for 2 hours prior to the start of the experiment. Samples were spotted to the plates using glass capillary tubes, and chromatographed in a chloroform: methanol: acetic acid 65:25:8 solvent system.

2.10. [^{14}C]Acetate in vivo glycerophospholipid incorporation

For *in vivo* activity assays involving pLR3, pBQ3, or pBQ43 based plasmids, which have the *E. coli* chromosomal RNA polymerase promoter, a repurified colony of strain QC30-15 (Table 2.1) containing the plasmid of interest was cultured overnight in 1 mL of LB with ampicillin (125 μg/mL) at 30°C without shaking. The following morning, 50 μL of the overnight culture were transferred to 10 mL of LB containing 125 μg/mL of ampicillin and incubated at 30°C with shaking at 250 rpm until reaching a turbidity of 30 Klett. Then 2.5 μCi of 2-[^{14}C]acetate (4.3 μCi/umol) was added and the cells were cultured a further 2 hours with shaking at 30°C. Phospholipids were isolated using a modification of the Bligh and Dyer method [31] as previously described [5] as follows. Cells were harvested using low speed centrifugation at 800 X g for 5 minutes at room temperature in glass tubes in an Adams DYNAC centrifuge (Clay-Adams Inc.) and the media was discarded. Cell pellets were resuspended in 1 mL of distilled

H_20, followed by the quick addition of 3.6 mL methanol: chloroform (2:1), 1.2 mL of chloroform, and 1.2 mL of 2 M $MgCl_2$ with brief vortexing after each addition. Phases were separated by centrifugation at 800 X g for 5 minutes at room temperature, and the chloroform layer was washed twice with 1.2 mL of distilled water. A glass transfer pipette was inserted through the protein layer at the interphase and the chloroform layer was passed through a glass wool column that had been freshly washed with chloroform, to a clean glass vial. Samples were blown down with N_2 and resuspended in 50 μL of chloroform. Fifteen to 20 μL of each sample was subjected to TLC as described in section 2.9, and the plates were dried under a fume hood for 1 hour at room temperature. Phospholipids were visualized by staining dried plates for 2-3 minutes in an iodine tank. Spots corresponding to individual lipids were circled with pencil and the plate was allowed to sit for 1 hour under a fume hood to remove the iodine. Phospholipids were excised from the TLC plate, transferred to a scintillation vial containing approximately 6 mL Ecoscint A biodegradable scintillation fluid, and the radioactivity was determined using a Packard Tri-Carb 2200CA scintillation counter. When plasmids that did not have an E. *coli* RNA polymerase promoter, such as pBQ4, pBQ40, pBQ74, and pET-23a(+) were analyzed for glycerophospholipid production, a modification of this procedure was used [17]. DG6/pLysS containing the plasmid of interest was cultured overnight in 1 mL of M9ZB as described above, and the following morning 50 μL of the overnight culture were transferred to 10 mL of M9ZB and incubated at 37°C to 40 Klett with shaking at 250rpm. IPTG was added to a final concentration of 0.8 mM to induce

expression of the protein of interest, and cells were incubated at 37°C with shaking for 30 minutes. Then 2.5 μCi of [^{14}C]acetate (4.3 μCi/μmol) were added and the cells were incubated a further 2 hours. Strains were then isolated and analyzed for glycerophospholipid content as described above.

2.11. Preparation and purification of 2-[^{3}H]phosphatidylglycerol

2-[^{3}H]-Phosphatidylglycerol was prepared previously by Dagang Guo [17] as follows. A 4 mL aqueous solution containing 300 mM sodium acetate (pH 5.6), 120 mM $CaCl_2$, 4 g dihydroxyacetone and 200 units of cabbage phospholipase D was mixed with 31 mL of diethyl ether containing 300 mg of phosphatidylcholine. The solution was stirred vigorously for 10 h at 26^{o}C in a closed flask, followed by removal of diethyl ether by evaporation. The aqueous phase was extracted with 30 mL chloroform, and the chloroform was then washed twice with 10 mL of distilled water and dried. The lipid product was resuspended in 1 mL chloroform and loaded onto a 1.2 x 20 cm activated silicic acid column. The column was washed with 30 mL of chloroform followed by 30 mL of chloroform: methanol (9:1), and the desired product, phosphatidyldihydroxyacetone (PDHA), was eluted with 30 mL of chloroform: methanol (3:1). The purified lipid migrated as a single spot on TLC when analyzed in a chloroform: methanol: acetic acid 65:25:8 solvent system. Approximately 50 mg of dry phosphatidyldihydroxyacetone was dissolved in 1 mL of distilled 2-propanol at 70°C and the mixture was immediately added to a vial containing 5 mCi of [^{3}H]sodium borohydride (sp. act. 222.3 mCi/mmol) and stored overnight at room temperature. The following morning 1 mL of water was added

and the lipid product, phosphatidyl[2-^{3}H]glycerol, was extracted with chloroform. 2-[^{3}H]-Phosphatidylglycerol produced in this fashion co-chromatographed with authentic PG on TLC in the solvent system listed above and was fully active when used as a substrate for *E. coli* CL synthase.

Due to degradation of this lipid over time, it was necessary to repurify 2-[^{3}H]-Phosphatidylglycerol for use in *in vitro* activity assays, using a silicic acid column as previously described [17]. Approximately 7.5 g of silicic acid (200-325 mesh) was activated by heating at 120°C for 2 hours, and resuspended in 25 mL of chloroform. A 1.2 x 18 cm column was cast and washed with 5 column volumes of chloroform until it became transparent. 2-[^{3}H]-Phosphatidylglycerol in chloroform was blown down with nitrogen, resuspended in 10 mL chloroform and loaded onto the column. The column was then washed with 50 mL chloroform, 50 mL chloroform: methanol 9:1, 50 mL chloroform: methanol 3:1, 50 mL chloroform: methanol 2:1 50 mL chloroform methanol 1:1 and 50 mL methanol. The total volume of all fractions were saved and analyzed by TLC as described in section 2.9. Following chromatography, lanes corresponding to individual column fractions were cut into 1.5 cm strips and were transferred to scintillation vials containing 6 mL of Ecoscint A scintillation fluid. Then, samples were counted in a Packard Tri-Carb 2200CA scintillation counter. 2-[^{3}H]-Phosphatidylglycerol, which eluted in the chloroform: methanol 3:1 fraction, migrated as a single band with an R_f of approximately 0.56 (data not shown).

2.12. In vivo activity analysis in QC25

Plasmids were introduced into QC25 (Table 2.1) by transformation as described in section 2.3. Growth experiments were performed in duplicate at 30°C and 42°C by streaking a separate repurified colony of each strain to be tested to 2 identical LB/ampicillin plates and incubating one plate at 30°C and the other at 42°C. Plasmids that coded for a functional CL synthase allowed QC25 to grow as a broad streak at both 30°C and 42°C.

2.13. Amplification of gene products

Expression studies were performed in M9ZB medium [24] supplemented with the appropriate antibiotics. For overexpression of DG6/pBQ43 derivatives, ampicillin was used at the concentration of 125 μg/mL. When pLysS was present, both ampicillin and chloramphenicol were used at concentrations of 125 μg/mL and 20 μg/mL respectively. Chloramphenicol in overnight cultures incubated at 37°C without shaking was increased to 40 μg/mL. Fifty microliters of a 1 mL overnight culture were transferred to 10 mL of M9ZB with appropriate antibiotic(s) and cells were cultured at 37°C with shaking at 250 rpm until reaching a turbidity of approximately 120 Klett. IPTG was then added to a final concentration of 0.8 mM, and cells were cultured for a further 3 hours.

Whole cells were prepared for SDS PAGE and western blot as described in section 2.14. Crude membrane and aqueous fractions used in activity assays and SDS PAGE and western blot experiments were isolated as follows [11]. Unless otherwise specified, all operations were performed at 4°C. Following

induction with IPTG, cells were centrifuged at 5000 X g for 5 minutes using a Sorvall® RC5C centrifuge, and resuspended in sonication buffer (100 mM Tris-HCl pH 8.0, 5 mM EDTA, 10 mM β-mercaptoethanol). Cells were disrupted by sonication for 3 X 30 seconds with 1 minute pauses between sonications using a Sonifier® Cell Disruptor Model W140 (Heat Systems-Ultrasonics Inc., Plainview, NY.) at an output setting of 8, corresponding to 50% power. Samples were centrifuged at 5000 X g to remove unbroken cells and cell debris, and the supernatant was transferred to an ultracentrifuge tube and centrifuged at 150,000 X g for 1 hour using a Sorvall® RC-80 ultracentrifuge with a T865 rotor.

Membranes used for *in vitro* activity assays were resuspended in 100 mM KH_2PO_4 pH 7.5, 10 mM β-mercaptoethanol, 1% Triton® X-100 using a Teflon® homogenizer. The membrane resuspension was centrifuged at 150,000 X g to remove insoluble material and the supernatant containing Triton® X-100 soluble protein was stored as 50 μL aliquots at –80°C.

Membranes used in SDS PAGE and western blot experiments were resuspended using a Teflon® homogenizer in 100 mM Tris-HCl pH 8.0, 5 mM EDTA, 10 mM β-mercaptoethanol, 1% SDS. Five times concentrate SDS sample buffer [29] was added to a final concentration of 1 X, and the samples were heated to 100°C for 10 minutes and stored at –80°C until use.

2.14. Analysis of whole cells by SDS PAGE and western blot

Whole cells were analyzed for the production of gene product by a slight modification of the method of Studier and Moffat [24] as follows. Five hundred microliters of a 10 mL M9ZB cell culture induced for 3 hours with 0.8 mM IPTG was harvested by centrifugation at 12,000 rpm for 1 minute in an Eppendorf 5414 microfuge at 25°C. The pellet was washed once with an equal volume of wash buffer (100 mM Tris-HCl pH 8.0, 10 mM β-mercaptoethanol, 5 mM EDTA) at 4°C and was resuspended by vigorous vortexing in 1 X SDS sample buffer [30]. Total protein concentrations were roughly normalized by resuspension in a volume of 1 X SDS sample buffer determined by multiplying the volume of the sample in mL by the final Klett of the sample. For example, 0.5 mL of an induced sample with a final Klett of 300 would be resuspended in 150 μL of 1 X SDS buffer. Samples were then heated to 100°C for 10 minutes and centrifuged for 30 seconds at 12,000 rpm in an Eppendorf 5414 microfuge at 25°C. Approximately 80% of the volume determined for resuspension was transferred to a new tube stored at –80°C until being used in SDS PAGE and western blot experiments.

2.15. Protein determination

Protein concentration in samples determined using the method of Lowry, as modified by Peterson [32] as follows. Reagent A (50 mL) was prepared by combining 25 mL of distilled H_20 containing 0.078 g $CuSO4•5H_20$ and 0.1 g of potassium tartarate•$1/2H_20$ and 25 mL of a solution containing 5.0 g Na_2CO_3 with

gentle stirring. This solution was stored at room temperature in a light tight bottle. On the day of the experiment, reagent A was diluted with equal volumes of distilled H_20, 0.2 M NaOH and 10% SDS. Reagent B, Folin & Ciocalteu's Phenol Reagent, 2.0 N (Sigma-Aldrich), was diluted with 5 volumes of water. Membrane samples (2 μL) and 10 to 50 μL of a 1mg/mL standard BSA solution were transferred in duplicate to separate Eppendorf tubes and brought to a final volume of 1 mL with distilled H_2O. Then 0.1 mL of 0.15% sodium deoxycholate was added and the samples were vortexed briefly and incubated at room temperature for 10 minutes. One hundred microliters of 72% trichloroacetic acid (TCA) was then added and the samples were vortexed briefly and incubated at room temperature for 15 minutes. Samples were centrifuged for 10 minutes at room temperature at 12,000 rpm in an Eppendorf 5414 microfuge, the supernatants were decanted, and the samples were dried at room temperature until all traces of liquid were gone. Then 400 μL of distilled H_2O were added. After briefly vortexing, 400 μL of reagent A were added and samples were vortexed again and incubated at room temperature for 10 minutes. Then 200 μL of reagent B were added and samples were vortexed and incubated at room temperature for 30 minutes. Absorbance was read at 750 nm using a PerkinElmer Lambda Bio UV/VIS Spectrometer (PerkinElmer, Waltham, MA).

Experiments were conducted to determine whether Triton® X-100 interfered with the accurate determination of sample concentration using this method. A 1 mg/mL BSA standard was made in resuspension buffer (100 mM phosphate pH 7.5, 10 mM β-mercaptoethanol, 1% Triton® X-100), and analyzed

as described above with and without sodium deoxycholate addition and TCA precipitation. Samples that were not TCA precipitated were analyzed as follows. Ten to 50 μL of a 1 mg/mL BSA solution, used to make a standard curve for use in determination of unknown sample concentrations, were transferred in duplicate to separate Eppendorf tubes and brought to a total volume of 400 μL with distilled H_2O. Similarly, membrane samples of unknown protein concentration (2 μL) were transferred in duplicate to separate Eppendorf tubes containing 398 μL of distilled H_2O. Then 400 μL of reagent A were added and samples were vortexed. After a 10 minute incubation period at room temperature, 200 μL of reagent B were added and samples were vortexed briefly and incubated for 30 minutes at room temperature. Absorbance at 750 nm was read, and the results were compared to TCA precipitated samples that had been run in parallel. No difference was found in the concentrations of the BSA standards made from the 1 mg/mL BSA solution in distilled H_2O or the 1 mg/mL BSA solution in resuspension buffer with or without TCA precipitation. Similarly, membrane samples tested with or without TCA precipitation were found to have the same concentrations (data not shown) indicating that Triton® X-100 does not interfere in the accurate determination of sample concentration.

2.16. SDS PAGE

SDS PAGE was performed using a discontinuous buffer system with a stacking gel of pH 6.8 and a resolving gel of pH 8.8 according to the method of Laemmli [33]. Protein samples from membranes (section 2.13) or whole cells (section 2.14) were analyzed using both large format and mini gels. When

samples were intended for western blot, all reagents were prepared using 0.45 μm (Millipore) filtered distilled H_2O.

Large format gels were run using a Hoefer SE400 (“The Sturdier”) Vertical Slab Gel Unit (Hoefer Scientific Instruments, San Francisco, CA) with a NOVEX 3540 programmable power supply (NOVEX San Diego, CA) at a constant voltage setting of 70 V for approximately 24 hours. Sample lanes contained 10-30 μL of whole cell lysate normalized for protein concentration as described in section 2.14. Ten microliters of a 1:50 Benchmark 6 X histidine molecular mass standard (Invitrogen) were loaded onto a separate lane to provide a standard ladder. For membrane samples of known concentration, approximately 80 μg of sample were loaded per lane. Gel plates were assembled according to the manufacturer’s instructions and resolving and stacking gels were constructed as shown in Table 2.12. The resolving gel was made first, adding TEMED immediately prior to casting. Two hundred and fifty microliters of distilled H_20 was gently layered onto the top of each side of the resolving gel, and it was allowed to polymerize for 30 minutes at room temperature. When polymerization was complete, the 500 μL of water was decanted and the gel comb was set into place, leaving approximately 2 cm of space between the bottom of the comb and the resolving gel. The stacking gel was prepared, adding TEMED immediately prior to casting, and was gently pipeted into the gel mold being careful to avoid the formation of bubbles under the gel comb in any of the lanes. Following polymerization at room temperature for 30 minutes, the comb was removed, and the lanes were washed twice with 1 X SDS PAGE tank buffer [30] (25 mM Tris pH 8.3, 250 mM glycine,

0.1% SDS). The gel was transferred to the lower buffer chamber and the upper chamber was secured into place. SDS PAGE tank buffer was added to the upper and lower reservoirs and samples were loaded using a pipetman with a long-nose sample tip.

Mini-gels were run using a Bio-Rad Mini Protean 2 electrophoresis unit (Bio-Rad) and a NOVEX 3540 programmable power supply (NOVEX San Diego, CA) at a constant setting of 175 V for 40 minutes. Resolving and stacking gels were constructed as shown in Table 2.12. A 4 mL resolving gel was cast and 50 μL of distilled H_20 was gently layered onto each side, and the gel was polymerized at room temperature for 30 minutes. The water was decanted, the comb was set into place, and the stacking gel was constructed as described above using the volumes listed in Table 2.12. A 5 to 10 μL sample of whole cell lysate was loaded per lane, along with one lane containing 5 μL of a 1:50 Benchmark 6 X histidine molecular mass standard (Invitrogen). When sample concentrations could be determined, as in the case of a membrane preparation, approximately 15 μg of total protein was loaded to each sample lane.

Following electrophoresis, gels intended for western blot were rinsed for 30 seconds in distilled H_2O that had been passed through a 0.45 μm filter (Millipore), followed by equilibration in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 20% v/v methanol) [34] for at least 10 minutes, and transfer to an Immobilon^{TM}-P PVDF membrane (Millipore) was performed as described in section 2.17.

Gels that were to be stained using coomassie blue were washed for 30 seconds in distilled H_2O followed by 2 washes, of 2 minutes each, in distilled H_2O with gentle shaking at 75 rpm. Gels were fixed in 1 X fixing solution (Sigma-Aldrich) for 30 minutes at room temperature followed by 2 washes of 2 minutes each with distilled H_2O. Gels were stained for 30 minutes at room temperature using a Brilliant Blue G Perchloric Acid Solution (Sigma-Aldrich), followed by an overnight destain in distilled H_2O. Apparent molecular masses were estimated by comparison of distance migrated by samples to standards of known molecular mass.

2.17. Western blot and chemiluminescence

Samples in large format gels were transferred to Immobilon^{TM}-P PVDF membranes (0.45 μm, Millipore) in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 20% v/v methanol) [34] using a Hoefer TE62 Transphor Electrophoresis tank with power supply lid at a constant current setting of 250 mA for a duration of 2.5 hours with vigorous stirring.

Samples in minigels were transferred to Immobilon^{TM}-P PVDF membranes (0.45μm, Millipore) in transfer buffer pre-cooled to 4°C at a constant voltage setting of 100 V for a duration of 1.5 hours using a Bio-Rad Mini Trans-Blot electrophoresis system (Bio-Rad), a NOVEX 3540 programmable power supply (NOVEX San Diego, CA.), with a Bio-Ice cooling unit in place, and vigorous stirring.

Following transfer, PVDF membranes were immediately transferred to TBST blocking buffer [34] (137 mM NaCl, 15.4 mM Tris-HCl pH 7.6, 0.15%

TWEEN 20, 5% nonfat dry milk (NFDM)), and incubated for 30 minutes at room temperature with gentle shaking (~75 rpm). Following blocking, PVDF membranes were incubated in a plastic sandwich bag with 10 mL (minigel) or 20 mL (large format) of a 1:10,000 dilution of a 1 mg/mL anti-Glu-Glu HRP-conjugate goat polyclonal antibody (Abcam) in blocking buffer for 30 minutes with gentle agitation at room temperature. Because no standard protein ladder is available for the Glu-Glu tag, a 1:5000 dilution of a 1 mg/mL Anti-His(C-term) HRP-conjugate mouse monoclonal antibody (Invitrogen) was added to the primary antibody incubation mixture to visualize the 6 X histidine tagged standard ladder (see section 2.16). This antibody recognizes a 6 X histidine amino acid sequence at the carboxy- terminus of proteins. The free carboxy-terminus of the terminal histidine residue is a key element of the epitope recognition for this antibody.

The Anti-His(C-term) Antibody recognizes the sequence:

-His-His-His-His-His-His-COOH.

The addition of the second antibody did not affect the results of the western or introduce any extraneous bands based upon comparisons of western blot signals obtained using one or both antibodies. Following incubation with primary antibody, PVDF membranes were washed with 2 X 200 mL (large format) or 2 X 50 mL (minigel) of TBST (137mM NaCl, 15.4mM Tris-HCl pH 7.6, 0.15% TWEEN 20) [34] for 5 minutes at room temperature with gentle shaking, removed from the

sandwich bag, and excess buffer was removed by blotting the membrane with Whatmann 3MM filter paper.

Chemiluminescent detection was performed using a PerkinElmer Western Lightning Enhanced Chemiluminescense Kit (PerkinElmer, Waltham, MA) according to the manufacturer's instruction. Following a 1 minute incubation in chemiluminescence reagent, excess reagent was removed by blotting well with Whatmann 3MM filter paper and the membrane was enclosed in a thin layer of saran wrap to prevent contact of the membrane with the film. Signals were immediately recorded using Kodak Biomax Light I film with approximately a 2 minute exposure time. Film exposed in this fashion was incubated for approximately 30 seconds in Kodak GBX developing solution (Sigma-Aldrich) until signals became visible. The film was then washed briefly with distilled H_2O and then incubated in Kodak GBX fixing solution (Sigma-Aldrich) for approximately 1-2 minutes. The film was rinsed well with d-H_2O and dried overnight at room temperature. Apparent molecular masses CL synthase constructs producing signals recorded in this fashion were estimated by comparison of the distance migrated by the constructs to those of the 6 X histidine standards of known molecular mass.

2.18. Staining of PVDF membranes

To visualize protein bands on PVDF, membranes were stained with coomassie brilliant blue G-250 as follows. After analysis by western blot, PVDF membranes were soaked in distilled H_20 with gentle shaking (~75 rpm) for 10 minutes at room temperature followed by 2 washes of distilled water 5 minutes

with gentle shaking. Membranes were then incubated for 5 minutes at room temperature with gentle shaking in staining solution (50% Methanol, 0.05% Coomassie Brilliant Blue G-250).

Membranes were destained in 50% methanol long enough for bands to become visible, which was usually not longer than 1 minute. Following destain, PVDF membranes were washed twice for 5 minutes in d-H_20 with gentle shaking and dried at room temperature.

2.19. In vitro activity analysis

Cell membranes prepared as described in section 2.13 were examined for the ability to catalyze the formation of CL as previously described [11]. Mixed micelle assays (50 μL), consisting of 320 mM KH_2PO_4 pH 7.1, 10 mM β-mercaptoethanol, 40 nM phosphatidyl[2-^{3}H]glycerol 16,700 dpm/nmol, and 0.03% Triton® X-100, were conducted in duplicate using 600 ng of crude membrane isolated as described in section 2.13. Reaction contents were preincubated at 37°C for 5 minutes and 5 μL of crude enzyme, diluted approximately 10-20 fold to 120 ng/μL with dilution buffer (100 mM KH_2PO_4 pH 7.1, 10 mM β-mercaptoethanol), was added to start the reaction. The tubes were gently tapped to mix the contents, and the assays were incubated for the desired time at 37°C. The reaction was stopped by adding 50 μL of a solution containing 20 mg/mL BSA and 10 mg/mL glycerol and briefly vortexing. Then, 100 μL of 72% TCA was added, and samples were vortexed again and centrifuged at room temperature for 5 minutes at 12,000 rpm in an Eppendorf 5414 microfuge. A 150 μL sample of supernatant was transferred to a

scintillation vial containing 6 mL of Ecoscint A scintillation fluid, and samples were counted using a Packard Tri-Carb 2200CA scintillation counter.

To determine the linearity of the assay with time, duplicate assays were run in a scaled up reaction volume of 300 μL. Aliquots were withdrawn at 0, 2, 4, 6, 8, and 10 minutes, and added to a new tube containing a BSA/glycerol solution as described above. Then, samples were TCA precipitated and counted as described above. The activity assay [11] was found to be linear over a ten minute period with respect to membrane isolated from DG6/pBQ43 as shown in Figure 3.7.

2.20. Computer analysis of sequence data.

BLAST [35-36], TopPred II [37], ClustalW [38], SignalP3.0 [39-40], iPSORT [41], Translate [42], PeptideMass [43], Antheprot [44-46] and FinchTV (Geospiza Inc. Seattle, WA) were used to analyze DNA/Protein sequence data.

Table 2.1.

Bacterial Strains

Strain	Genotype or description	Source or reference
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tetr)]	Product of Stratagene, La Jolla, CA, USA
DG6	F- *omp*T *hsd*SB ($r_B^-m_B^-$), *dcm gal* (DE3) *cls*::Tn*10*dTet3	17
QC25	*pcn*B80 *pssA1 zee*::miniTet*10*	47
QC30-15	*glpR glpD pssA1 cls*::*Tn10dTet3*;	47

Table 2.2.

Plasmids Coding for a C-terminally 6 X Histidine Tagged CL Synthase and its Derivatives

Plasmid	Description	Source or reference
pBQ3[a]	6 X histidine tagged CL synthase in pET-23a(+)	This work
pBQ4[a]	6 X histidine tagged CL synthase in pET-23a(+)	This work
pBQ6	6X histidine tagged CL synthase frameshift 2-20 in pET-23a(+)	This work
pBQ7	6X Histidine tagged CL Synthase frameshift 2-42 in pET-23a(+)	This work
pBQ30[b]	CL synthase in pET23a(+)	This work
pBQ31	6X histidine tagged CL synthase E487G in pET-23a(+)	This work
pBQ32	6X histidine tagged CL synthase E487L in pET-23a(+)	This work
pBQ40[b]	CL synthase in pET-23a(+)	This work
pET-23a(+)	T7 expression vector with C-terminal 6 X histidine tag	Product of Novagen

[a] pBQ3 differs from pBQ4 as described in section 2.6.

[b]pBQ30 differs from pBQ40 as described in section 2.6.

Table 2.3.

Plasmids Coding for an EE (Glu-Glu) Epitope (EYMPME) Tagged CL Synthase and Derivatives

Plasmid	Description/Mutation	Source or reference
pBQ43	EE (Glu-Glu) tagged (EYMPME) CL Synthase	This work
pBQ44	W40A	This work
pBQ45	P48A	This work
pBQ46	Y55A	This work
pBQ47	G59A	This work
pBQ48	S36D	This work
pBQ50	Frameshift 41-44	This work
pBQ52	W40A,P48A	This work
pBQ55	H224A	This work
pBQ56	Frameshift 2-20	This work
pBQ57	Frameshift 2-42	This work
pBQ59	Frameshift 72-82	This work
pBQ61	R66A	This work
pBQ62	R70A	This work
pBQ63*	Non epitope tagged frameshift 2-20	This work
pBQ66	R66A,R70A	This work

pBQ67	LII42-44SSS	This work
pBQ68	II52-53SS	This work
pBQ69	L19S	This work
pBQ70	KRR30-32TTT	This work
pBQ71	KRR65-67SSA	This work
pBQ72	LV7-8SS	This work
pBQ73	LI19-20SS	This work
pBQ74	EE tagged 2-60 deletion in pET-23a(+)	This work
pLR3	CL Synthase in pET3	11
pET3	T7 Expression vector	24
pET-23a(+)	T7 expression vector with 6 X histidine tag	Product of Novagen

Table 2.4.

Primers Used to Introduce Restriction Endonuclease Sites 5' and 3' of *cls* in pLR3

Plasmid Conversion	Primer Sequence*	Mutation
pLR3→pBQ1	5'-CTTCTTCAGTCCGTTGCTCGAGAACG TCGCCAACAGAGGTTAAACAGG-3'	Insert XhoI site
	5'-CCTGTTTAACCTCTGTTGGCGACGTT CTCGAGCAACGGACTGAAGAAG-3'	3' cls
pBQ1→pBQ2	5'-GCTTTCAAAAGGATTTCTAACATATGACAA CCGTTTATACGTTGGTGAGTTGG-3'	Insert NdeI site
	5'-CCAACTCACCAACGTATAAACGGTTGTC ATATGTTAGAAATCCTTTTGAAAGC-3'	5' cls

* The sense primer listed on top, and the antisense primer is listed on the bottom of each pair.
Nucleotides that have been altered as described in section 2.6 are shown as black letters in a gray background.

Table 2.5.

Primers Used to Introduce Frameshift Mutations to the C-terminally 6 X Histidine Tagged CL Synthase

Plasmid Conversion	Primer Sequence*	Mutation
pBQ3→pBQ5	5'-GCTTTCAAAAGGATTTCTAATCTATGCG CAACCGTTTATACGTTGGTGAG-3' 5'-CTCACCAACGTATAAACGGTTGCGCATA GATTAGAAATCCTTTTGAAAGC-3'	Insert frameshift at position 2
pBQ5→pBQ6	5'-GGATACTGGTTGCTCATGCAGGCGTAACTTTACG-3' 5'-CGTAAAGTTACGCCTGCATGAGCAACCAGTATCC-3'	Recover reading frame at position 21
pBQ5→pBQ7	5'-GCGATGGCCTGGCTGTGATTATTTACATTCTGCC-3' 5'-GGCAGAATGTAAATAATCACAGCCAGGCCATCGC-3'	Recover reading frame at position 43

* The sense primer listed on top, and the antisense primer is listed on the bottom of each pair.
Nucleotides that have been altered as described in section 2.6 are shown as black letters in a gray background.
Underlined nucleotides have been inserted.
Single Nucleotide deletions have been made between the boxed nucleotides.

Table 2.6.

Primers Used to Remove the C-terminal 6 X Histidine Tag and Alter Residue E487

Plasmid Conversion	Primer Sequence*	Mutation
pBQ3→pBQ30	5'-CTTCTTCAGTCCGTTGCTGTAACACCACCACCACCACCAC-3' 5'-GTGGTGGTGGTGGTGGTGTTACAGCAACGGACTGAAGAAG-3'	Remove 6 X histidine tag from pBQ3 an pBQ4
pBQ3→pBQ31	5'-CTTCAGTCCGTTGCTCGGGCACCACCACCACCACC-3' 5'-GGTGGTGGTGGTGGTGCCCGAGCAACGGACTGAAG-3'	E487G
pBQ3→pBQ32	5'-CTTCAGTCCGTTGCTCTTGCACCACCACCACCACC-3' 5'-GGTGGTGGTGGTGGTGCAAGAGCAACGGACTGAAG-3'	E487L

* The sense primer listed on top, and the antisense primer is listed on the bottom of each pair.
Nucleotides that have been altered as described in section 2.6 are shown as black letters in a gray background.

Table 2.7.

Primers Used to Construct an EE (Glu-Glu)

Epitope Tagged CL Synthase

Plasmid Conversion	Primer Sequence*	Mutation
pLR3→pBQ41	5'-CCGCCACCACCAGATGTCGAGATTATGCCGTTTGAACAGG-3' 5'-CCTGTTCAAACGGCATAATCTCGACATCTGGTGGTGGCGG-3'	N296E
pBQ41→pBQ42	5'-CCACCAGATGTCGAGTATATGCCGTTTGAACAGGC-3' 5'-GCCTGTTCAAACGGCATATACTCGACATCTGGTGG-3'	I297Y
pBQ42→pBQ43	5'-GTCGAGTATATGCCGATGGAACAGGCCAGCGG-3' 5'-CCGCTGGCCTGTTCCATCGGCATATACTCGAC-3'	F299M

* The sense primer listed on top, and the antisense primer is listed on the bottom of each pair.
Nucleotides that have been altered as described in section 2.7 are shown as black letters in a gray background.

Table 2.8.

Primers Used to Insert Alanine Point Mutations within the EE Epitope Tagged CL Synthase

Plasmid Conversion	Primer Sequence*	Mutation
pBQ43→pBQ44	5’-CCTCCGCGATGGCCGCGCTGTTGATTATTTAC-3’ 5’-GTAAATAATCAACAGCGCGGCCATCGCGGAGG-3’	W40A
pBQ43→pBQ45	5’-GGCTGTTGATTATTTACATTCTGGCGTTAGTCGGAATTATTGC-3’ 5’-GCAATAATTCCGACTAACGCCAGAATGTAAATAATCAACAGCC-3’	P48A
pBQ43→pBQ46	5’-GTCGGAATTATTGCCGCTCTTGCCGTTGGCGAG-3’ 5’-CTCGCCAACGGCAAGAGCGGCAATAATTCCGAC-3’	Y55A
pBQ43→pBQ47	5’-GCCTATCTTGCCGTTGCCGAGCTCCATTTAGGC-3’ 5’-GCCTAAATGGAGCTCGGCAACGGCAAGATAGGC-3’	G59A
pBQ44→pBQ52	5’-GGCTGTTGATTATT TACATTCTGGCGTTAGTCGGAATTATTGC-3’ 5’-GCAATAATTCCGACTAACGCCAGAATGTAAATAATCAACAGCC-3’	W40A, P48A
pBQ43→pBQ55	5’-GGACCTGCGCCAAGCTCGCAAGATGATCATGATCG-3’ 5’-CGATCATGATCATCTTGCGAGCTTGGCGCAGGTCC-3’	H224A
pBQ43→pBQ61	5’-GCTCCATTTAGGCAAAGCCCGCGCTGAGCGCGCC-3’ 5’-GGCGCGCTCAGCGCGGGCTTTGCCTAAATGGAGC-3’	R66A
pBQ43→pBQ62	5’-CGCCGCGCTGAGGCCGCCAGAGCGATGTGG-3’ 5’-CCACATCGCTCTGGCGGCCTCAGCGCGGCG-3’	R70A
pBQ61→pBQ66	5'-GCGCTGAGGCCGCCAGAGCGATGTGG-3' 5'-CCACATCGCTCTGGCGGCCTCAGCGC-3’	R66A, R70A

* The sense primer listed on top, and the antisense primer is listed on the bottom of each pair.
Nucleotides that have been altered as described in section 2.8.1 are shown as black letters in a gray background

Table 2.9.
Primers Used to Insert Aspartate Serine and Threonine Mutations within the EE Epitope Tagged CL Synthase

Plasmid Conversion	Primer Sequence*	Mutation
pBQ43→pBQ48	5'-CGACGCGCAGTTCCCGACGCGATGGCCTGGCTG-3' 5'-CAGCCAGGCCATCGCGTCGGGAACTGCGCGTCG-3'	S36D
pBQ43→pBQ64	5'-GCATTCTAATGACAACGACGCGCAGTTCCCTCC-3' 5'-GGAGGGAACTGCGCGTCGTTGTCATTAGAATGC-3'	KRR 30-32 TTT frameshift position 30
pBQ43→pBQ65	5'-CTCCATTTAGGCTCAAGCGCCGCGCTGAGC-3' 5'-GCTCAGCGCGGCGCTTGAGCCTAAATGGAG-3'	KRR65-67SSA frameshift position 65
pBQ43→pBQ67	5'-CGATGGCCTGGCTGTCGAGTAGTTACATTCTGCCG-3' 5'-CGGCAGAATGTAACTACTCGACAGCCAGGCCATCG-3'	LII42-44SSS
pBQ43→pBQ68	5'-CTGCCGTTAGTCGGAAGTAGTGCCTATCTTGCC-3' 5'-GGCAAGATAGGCACTACTTCCGACTAACGGCAG-3'	II52-53SS
pBQ43→pBQ69	5'-GGATACTGGTTGTCCATTGCAGGCGTAACTTTACG-3' 5'-CGTAAAGTTACGCCTGCAATGGACAACCAGTATCC-3'	L19S
pBQ64→pBQ70	5'-CATTCTAATGACAACGACCGCAGTTCCCTCC-3' 5'-GGAGGGAACTGCGGTCGTTGTCATTAGAATG-3'	KRR 30-32 TTT recover reading frame position 32
pBQ65→pBQ71	5'-CGCCGCGGAGCGCGCCAGAGC-3' 5'-GCTCTGGCGCGCTCCGCGGCG -3'	KRR 65-67 SSA Recover reading frame position 68
pBQ43→pBQ72	5'-GACAACCGTTTATACGTCGTCGAGTTGGTTGG-3' 5'-CCAACCAACTCGACGACGTATAAACGGTTGTC-3'	LV7,8SS
pBQ69→pBQ73	5'-GGATACTGGTTGTCCAGTGCAGGCGTAACTTTACG-3' 5'-CGTAAAGTTACGCCTGCACTGGACAACCAGTATCC-3'	LI19,20SS

* The sense primer is listed on top, and the antisense primer is listed on the bottom of each pair.
Nucleotides that have been altered as described in section 2.8.2 are shown as black letters in a gray background.
Underlined nucleotides have been inserted.
Nucleotide deletions have been made between the boxed nucleotides.

Table 2.10.

Primers Used to Introduce Frameshift Mutations within the EE Epitope Tagged CL Synthase

Plasmid Conversion	Primer Sequence*	Mutation
pBQ43→pBQ51 and pLR3→pBQ60	5'-GCTTTCAAAAGGATTTCTAATCTATGCG CAACCGTTTATACGTTGGTGAG-3'	Insert frameshift at
	5'-CTCACCAACGTATAAACGGTTGCGCATA GATTAGAAATCCTTTTGAAAGC-3'	position 2
pBQ51→pBQ56 and pBQ60→pBQ63	5'-GGATACTGGTTGCTCATGCAGGCGTAACTTTACG-3'	Recover reading frame
	5'-CGTAAAGTTACGCCTGCATGAGCAACCAGTATCC-3'	at position 21
pBQ51→pBQ57	5'-GCGATGGCCTGGCTGTGATTATTTACATTCTGCC-3'	Recover reading frame
	5'-GGCAGAATGTAAATAATCACAGCCAGGCCATCGC-3'	at position 43
pBQ43→pBQ49	5'-GCGATGGCCTGGCATGTTGATTATTTACATTCTGC-3'	Insert frameshift
	5'-GCAGAATGTAAATAATCAACATGCCAGGCCATCGC-3'	at position 41
pBQ49→pBQ50	5'-GGCCTGGCATGTTGATTATTACATTCTGCCGTTAG-3'	Recover reading frame
	5'-CTAACGGCAGAATGTAATAATCAACATGCCAGGCC-3'	at position 45
pBQ43→pBQ54	5'-GCTGAGCGCGCCACGAGCGATGTGGCCTTCC-3'	Insert frameshift
	5'-GGAAGGCCACATCGCTCGTGGCGCGCTCAGC-3'	at position 72
pBQ54→pBQ59	5'-CCACCGCAAAATGGCTAACGACCTTAAAGCCTG-3'	Recover reading frame
	5'-CAGGCTTTAAGGTCGTTAGCCATTTTGCGGTGG-3'	at position 82

* The sense primer listed on top, and the antisense primer is listed on the bottom of each pair.
Nucleotides that have been altered as described in section 2.8.3 are shown as black letters in a gray background.
Underlined nucleotides have been inserted.
Nucleotide deletions have been made between the boxed nucleotides.

Table 2.11.

Sequencing Primers

Primer	Primer Sequence	Purpose
Sequencing primer 1	5'-CAATGGATTGATCTGATGG-3'	pBQ43 epitope sequencing primer
Sequencing primer 2	5'-CTATGCAATAACAGAATGGTC-3'	Sequencing of all *cls* constructs encoding mutations within the N-terminus
Sequencing primer 3	5'-CATTCATTGCCGATTGATG-3'	pBQ55 (H224A) sequencing primer
Sequencing primer 4	5' TAATACGACTCACTATAGGG-3'	T7 sequencing primer for pET-23a(+) vector

Table 2.12.

Reagents for SDS PAGE

A

Reagent	10 % Resolving gel	5% Stacking gel
d-H2O	17.82 mL	6.8 mL
1.5M Tris-HCL pH8.8	11.25 mL	-----
1.0 M Tris-HCl pH 6.8	-----	1.25 mL
30% Acrylamide/bis-Acrylamide 29:1	15.03 mL	1.7 mL
10% m/V SDS	450 uL	100 uL
10% Ammonium persulfate	450 uL	100 uL
TEMED	18 uL	10 uL
Final Volume	45 mL	10 mL

B

Reagent	10 % Resolving gel	5% Stacking gel
d-H2O	3.96 mL	3.4 mL
1.5M Tris-HCL pH8.8	2.5 mL	-----
1.0 M Tris-HCl pH 6.8	-----	0.625 mL
30% Acrylamide/bis-Acrylamide 29:1	3.34 mL	.85 mL
10% m/V SDS	100 uL	50 uL
10% Ammonium persulfate	100 uL	50 uL
TEMED	4 uL	5 uL
Final Volume	10 mL	5 mL

Reagents used for the construction of SDS PAGE gels in large format (A) and small format (B).

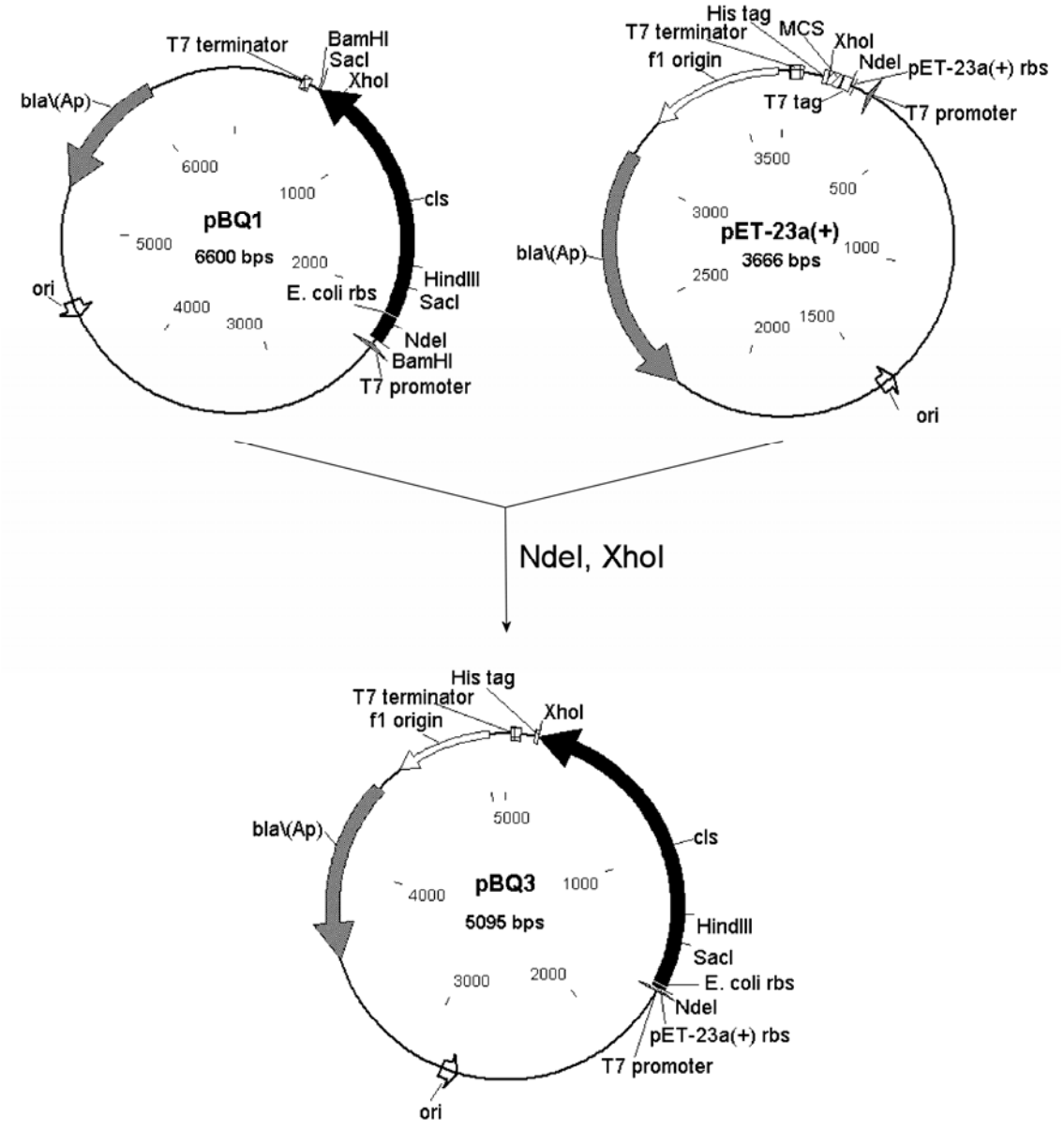


Figure 2.1. The construction of pBQ3. The 1508 bp NdeI XhoI *cls* fragment was excised from pBQ1 and ligated into pET-23a(+) (Novagen) that had also been cut with NdeI and XhoI as described in section 2.6.

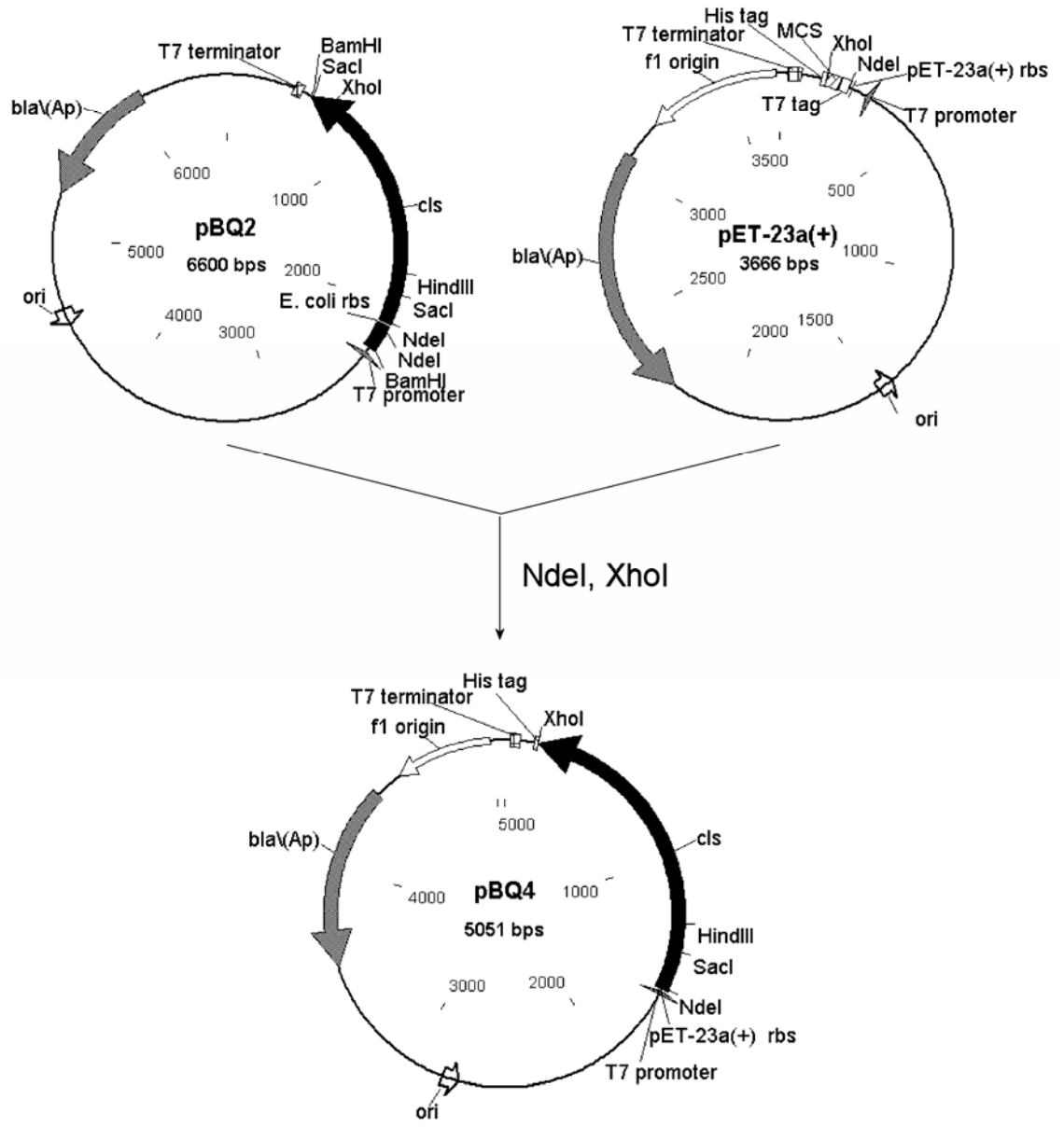


Figure 2.2. The construction of pBQ4. The 1458 bp NdeI XhoI *cls* fragment was excised from pBQ1 and ligated into pET-23a(+) (Novagen) which had also been cut with NdeI and XhoI as described in section 2.6.

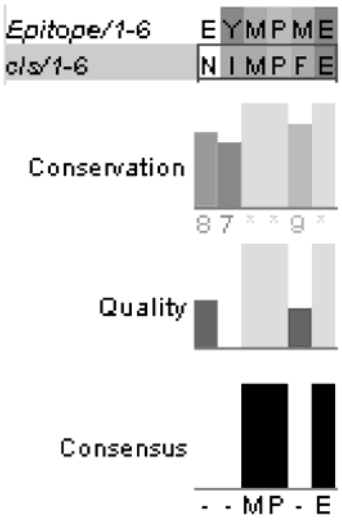


Figure 2.3. Clustal W alignment showing amino acids 296-301 of the CL synthase primary sequence altered to introduce the EE epitope as described in section 2.7. The 6 amino acid residue epitope tag (EYMPME) is shown in the first line of the figure (Epitope/1-6), while the CL synthase primary sequence in this area is shown immediately below it (cls/1-6). Residue conservation, quality and consensus are shown below the clustal W [36] alignment.

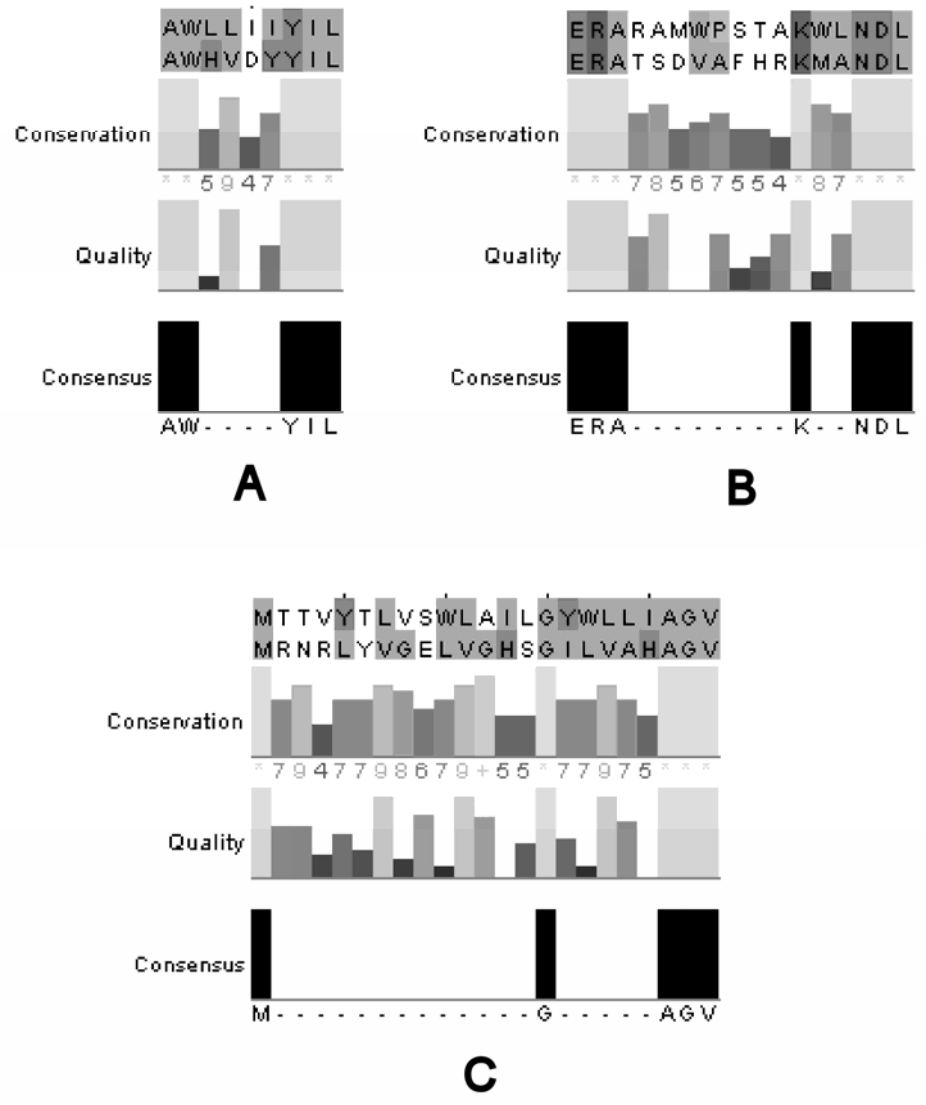


Figure 2.4. Clustal W alignments of frameshift regions introduced to CL synthase. The primary amino acid sequence of wild type CL synthase is shown on top and the primary amino acid sequence of the frameshift mutant on the bottom in each pair. Where possible, two to three residues N and C terminal of the mutation are shown for clarity. Residue conservation, quality and consensus sequence as determined by clustal W [36] are shown under each alignment. (A) pBQ50 (frameshift 41-44), (B) pBQ59 (frameshift 72-82), (C) pBQ56 (frameshift 2-20).

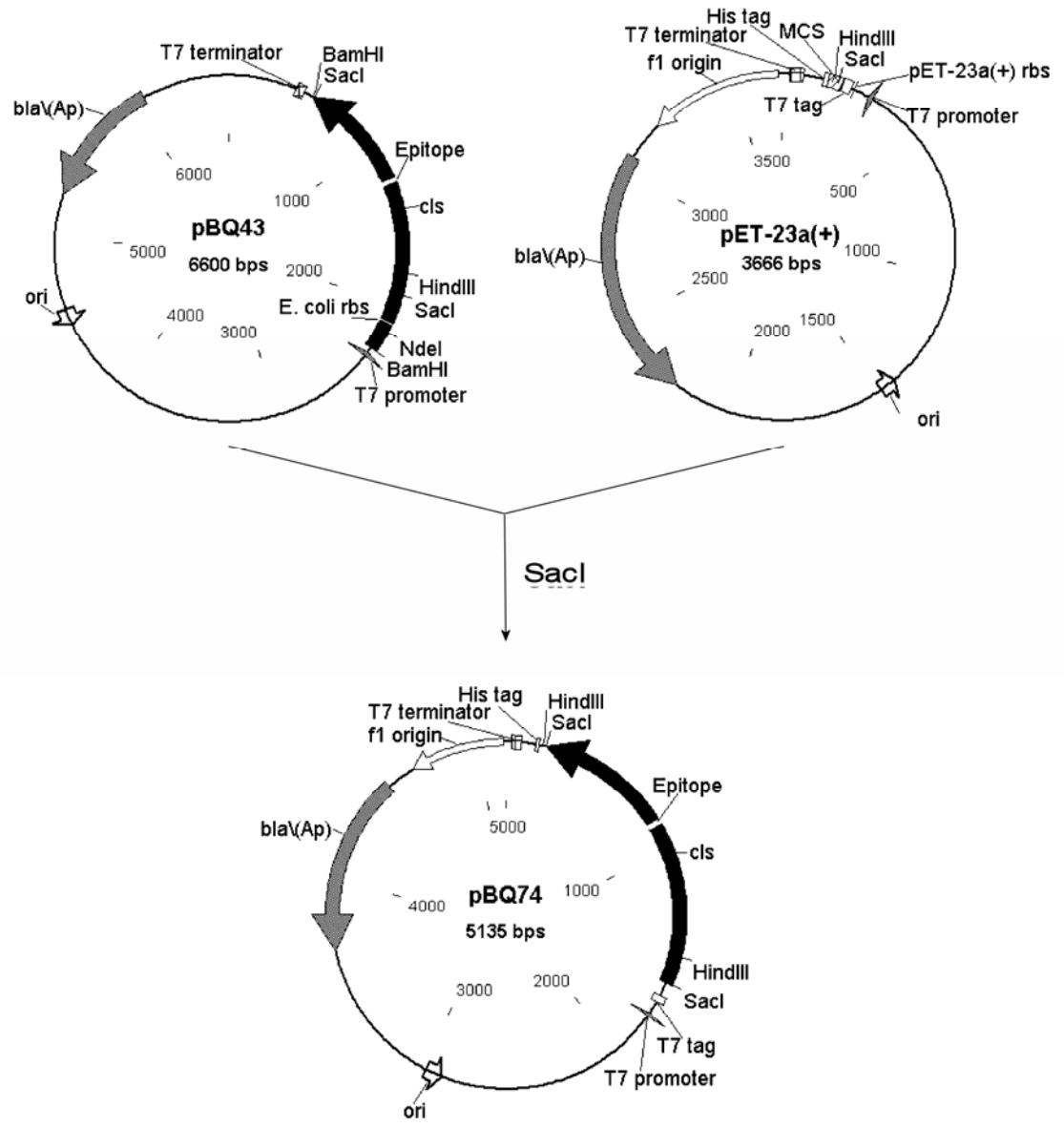


Figure 2.5. The construction of pBQ74. The 1.4 kb SacI fragment containing *cls* from nucleotide 180 to slightly beyond the 3' of *cls* was excised from pLR3 and ligated into pET-23a(+) (Novagen) that had also been cut with SacI.

3.0. Results

3.1. Construction of a 6 X histidine tagged CL synthase

Plasmid pLR3 contains *E. coli cls* behind a T7 promoter [11]. In order to determine what effects, if any, the alteration of conserved residues within the N-terminus of CL synthase would have on the posttranslational processing of the protein, it was necessary to introduce an epitope tag so that mutant proteins could be unambiguously identified by western blot. Plasmids pBQ3 and pBQ4 were originally created for this purpose by ligation of *cls* into the pET-23a(+) vector (Novagen) in frame with the C-terminal 6 X histidine tag as described in section 2.6, and shown in Figure 2.1 for pBQ3 and in Figure 2.2 for pBQ4.

3.2. In vivo analysis of C-terminally 6 X histidine tagged constructs

Two methods were used to check for the activity of constructs *in vivo*. The first method was based on previous studies showing that *pssA1 cls* double mutants do not grow on LB agar plates at 42°C whereas *pssA1* mutants do grow (Heber, S., and Tropp, B.E., unpublished data). Therefore, plasmids with mutant *cls* genes were introduced into QC25 (Table 2.1) and the transformants were tested for growth at 30°C and at 42°C. QC25 was used for these studies because it is *pcn*B and therefore maintains only one or two copies of the plasmid. Although the growth experiment is fast and very convenient, failure to grow at 42°C does not provide absolute proof that a plasmid contains a mutant *cls* gene that codes for a nonfunctional protein. The second method, which monitors [^{14}C]acetate incorporation into cardiolipin, provides a more reliable measure of *in*

vivo CL synthase activity. Plasmids bearing the *cls* gene were introduced into QC30-15 (Table 2.1) and then [^{14}C]acetate incorporation into cardiolipin was monitored as described in section 2.10.

The CL synthase encoded by pBQ3 differs from wild type CL synthase encoded by pLR3. The modified CL synthase has an EHHHHHH tail attached to the C-terminal leucine residue in wild type cardiolipin synthase. It was therefore necessary to demonstrate that the CL synthase encoded by pBQ3 retained activity before attempting to use pBQ3 for further studies. QC25/pBQ3 forms a broad band when streaked on LB agar at 30°C but forms discrete colonies when streaked at 42°C. In contrast, QC25/pLR3 forms broad streaks at both 30°C and 42°C. The discrete colonies probably arise as a result of mutations. Similar speckled streaks are often observed when antibiotic-sensitive cells are streaked on medium containing the antibiotic. Thus, it seems likely that QC25/pBQ3 does not synthesize CL. The interpretation that pBQ3 does not code for an active CL synthase is confirmed by failure of QC30-15/pBQ3 to incorporate [^{14}C]acetate into CL (Figure 3.2).

The growth of strains QC25/pBQ6 (frameshift 2-20) and QC25/pBQ7 (frameshift 2-42) were also examined at 30°C and 42°C. Both strains grow normally at the lower temperature but fail to produce any colonies at the higher temperature, suggesting that the CL synthases encoded by these plasmids lack *in vivo* activity (Table 3.1).

As described in section 2.6, pBQ3 codes for a CL synthase fused C-terminally to the sequence (EHHHHHH). Removing the 6 X histidine tag by

reintroducing the natural *cls* C-terminal stop codon converts pBQ3 to pBQ30 (see section 2.6). As expected, QC25/pBQ30 grows normally at both 30°C and 42°C (Table 3.1). Furthermore, QC30-15/pBQ30 incorporates [^{14}C]acetate to CL to the same degree as QC30-15/pLR3 (Figure 3.2). These results indicate that the addition of the 6 X histidine tag to CL synthase is responsible for the loss of activity of the fused protein *in vivo*.

To determine whether or not the introduced glutamate residue, rather than the 6 X histidine tag, was responsible for the loss of activity, the glutamate residue (E487) was changed to glycine (G) and to leucine (L) as described in section 2.6. Members of the phospholipase D superfamily have a tertiary structure that brings the 2 HKD motifs into proximity to form a single active site [22]. Glycine was chosen to see whether an amino acid with no side chain would allow the tag to rotate away from the active site in the event that loss of activity was due to steric interference caused by the tag in the folded protein.

The final 9 residues at the C-terminus of CL synthase (LFYFFSPLL) comprise a relatively hydrophobic region. The introduction of a glutamate and 6 histidine residues decreased the hydrophobicity of this region. Glutamate 487 was converted to leucine to examine whether or not the addition of a hydrophobic residue would restore activity by increasing the C-terminal hydrophobicity of the fusion protein to a level nearer that of wild type CL synthase. As shown in Table 3.1, neither QC25/pBQ31 (E487G) nor QC25/pBQ32 (E487L) form an active CL synthase *in vivo* as evidenced by lack of growth at 42°C. The results of [^{14}C]acetate incorporation experiments with QC30-15/pBQ31 and QC30-

15/pBQ32 were fully consistent with growth experiments in QC25. Replacing the glutamate residue just before the 6 X histidine tag with a glycine or leucine residue does not restore the tagged CL synthase's ability to incorporate [^{14}C]acetate into CL (Figure 3.2).

3.3. Characterization of 6 X histidine tagged constructs in DG6/pLysS and DG6/pLysE

Plasmid pBQ3 causes a loss in viability when transformed into strain DG6 (F^- *ompT hsdSB* ($r_B^-m_B^-$), *dcm gal* (DE3) *cls*::Tn*10*dTet3), whereas pLR3 does not do so. To obtain viable colonies, pBQ3 and its derivatives were introduced into DG6/pLysS. Plasmid pLysS allows growth in the absence of IPTG because it contains the T7 lysozyme gene in the antisense orientation to the *tet* promoter. The low transcription level of the T7 lysozyme gene permits sufficient T7 lysozyme formation to block transcription at T7 promoters that results from the leaky production of T7 RNA polymerase. Strain DG6/pLysS/pBQ3 shows complete growth inhibition and a slight decrease in turbidity when induced with IPTG, whereas DG6/pLysS/pLR3 shows no growth inhibition and increased turbidity, consistent with continued growth under the same conditions (Figure 3.1, Table 3.2). IPTG induced DG6/pLysS/pBQ3 produces a protein that migrates on SDS PAGE with an apparent molecular mass of 46.5 kDa (Table 3.2), consistent with the C-terminal fusion of the sequence (EHHHHHH), which adds a mass of approximately 1 kDa to the wild type protein. This C-terminally 6 X histidine tagged CL synthase is readily detected by western blot using an anti-histidine (C-term)-HRP antibody that recognizes only 6 X histidine tagged proteins at the C-

terminus (see section 2.16). As expected, no band is detected when extracts from Strain DG6/pLysS/pLR3 are examined in the same way (data not shown).

DG6/pLysS/pBQ6 (frameshift 2-20) exhibits no growth inhibition upon induction with IPTG (Table 3.2). However, a new protein is visible on coomassie stained SDS PAGE or when the anti-histidine (C-term)-HRP antibody is used to detect the 6 X histidine tag. The new CL synthase migrates with an apparent molecular mass of 47.5 kDa, which is about 1 kDa heavier than the histidine tagged CL synthase with a wild type 2-20 residue sequence (Table 3.2). Similarly, IPTG induced DG6/pLysS/pBQ7 (frameshift 2-42) do not show growth inhibition, and a new band is visible on coomassie SDS PAGE and western blot that migrates with an apparent molecular mass of 49.1 kDa, which is 2.6 kDa heavier than the histidine tagged CL synthase protein with a wild type 2-42 residue sequence (Table 3.2).

Plasmid pBQ4 differs from plasmid pBQ3 in two important respects: (1) pBQ4 has a stronger ribosome binding site and (2) pBQ4 lacks the normal *cls* promoter and must therefore rely on the T7 RNA polymerase for transcription. It was not possible to isolate pBQ4 transformants of DG6/pLysS. Presumably, the low level of T7 RNA polymerase activity that escaped lysozyme inhibition was sufficient to produce high levels of CL synthase that was toxic to the cells. It was therefore necessary to introduce pBQ4 into DG6/pLysE. Plasmid pLysE contains the T7 lysozyme gene in the sense orientation relative to the *tet* promoter, and codes for large amounts of T7 lysozyme. Even in the presence of pLysE, cells transformed with pBQ4 show complete growth inhibition and decreased turbidity

consistent with cell death when induced with IPTG (Figure 3.1). As expected, IPTG induced DG6/pLysE/pBQ4 show a protein on coomassie stained SDS PAGE that migrates with an apparent molecular mass of 45.3 kDa and is identical to that produced by DG6/pLysS/pBQ3 (data not shown). Site directed mutagenesis was used to convert pBQ4 to pBQ40, which codes for CL synthase with a normal C-terminus (see section 2.6). DG6/pLysE/pBQ40 has the same growth characteristics as DG6/pLysE/pBQ4. Therefore, these growth characteristics are due to the overproduction of CL synthase rather than to modifications in the C-terminus.

3.5. In vitro activity assay of a C-terminally 6 X histidine tagged CL synthase

Membranes were isolated from IPTG induced DG6/pLysS/pBQ3 (see section 2.13) and analyzed for their ability to catalyze CL formation *in vitro* (see section 2.19). As shown in Figure 3.3, the activity of the 6 X histidine tagged CL synthase coded for by pBQ3 is approximately 15% that of the wild type protein *in vitro*.

3.6. Summary of 6 X histidine constructs

IPTG induced strain DG6/pLysS/pBQ3 produces a CL synthase that migrates on SDS PAGE with an apparent molecular mass of 46.5 kDa, which is 1.2 kDa heavier than the CL synthase produced by IPTG induced DG6/pLysS/pLR3 (wild type CL synthase), consistent with the fusion of the residues (EHHHHHH) to the C-terminus. The recognition of this protein on western blot at the apparent molecular mass expected for the fusion protein by an antibody specific for 6 histidines with a free carboxy terminus indicates that

the C-terminal 6 X histidine tag is correctly fused to CL synthase. Furthermore, the recognition of a CL synthase missing approximately 80 residues migrating at this apparent molecular mass indicates that the residues missing from CL synthase are removed N-terminally. The CL synthase encoded by pBQ3 is inactive *in vivo* and has little to no activity *in vitro*. Further confirmation that the fusion protein is produced in the cell but is inactive comes from the restoration of activity to the protein when the 6 X histidine tag is removed by reintroduction of the natural C-terminal stop codon.

The fact that mutant proteins with frameshift regions 2-20 and 2-42 show no growth inhibition when induced with IPTG and migrate with an increase in apparent molecular mass relative to the histidine tagged CL synthase indicates that the normal processing of these proteins is altered. This difference in processing may result from the alteration of conserved residues in these regions. However, it is also possible that the addition of the 6 X histidine tag in conjunction with these frameshift regions is responsible for the altered processing. The fact that neither of these constructs is active *in vivo* is not surprising since the histidine tagged protein is itself inactive. Plasmids coding for CL synthase with frameshift regions were constructed at the same time as pBQ3 and pBQ4 because the loss of activity in the fusion protein was not anticipated.

The fact that none of the C-terminal 6 X histidine tagged constructs have activity *in vivo* in QC25 or in QC30-15, along with the fact that membranes from IPTG induced DG6/pLyS/pBQ3 have little to no activity *in vitro* prohibit the use of this epitope tag to study CL synthase. The use of a C-terminal 6 X histidine tag to

study the effects of mutations to conserved residues within the N-terminus of CL synthase was therefore discontinued, and another epitope was introduced to CL synthase (see sections 2.7 and 3.6).

The 6 X histidine tag is a very common epitope used to study many proteins without any deleterious effect. Members of the phospholipase D superfamily have a tertiary structure that brings the two HKD motifs together to form a single active site [22], and it is possible that the introduction of the histidine tag disrupts the activity of the folded CL synthase by preventing proper tertiary structure formation or by sterically hindering catalysis. One possible way to explain the fact that the addition of a glutamate and six histidine residues to the relatively hydrophobic C-terminus of wild type CL synthase causes the loss of activity *in vivo* is that normal membrane translocation cannot take place, preventing the enzyme from attaining proper membrane topology with its catalytic site facing the periplasm [28]. However, the fact that this construct has such low *in vitro* activity indicates that CL synthase probably cannot fold into an active tertiary structure because of steric interference, an alteration in processing of the pre-protein, or some combination of these or factors caused by the addition of the tag.

3.6. Construction of an EE (Glu-Glu) epitope (EYMPME) tagged CL synthase

Due to the loss of activity *in vivo* and the substantial reduction or elimination of activity *in vitro* upon addition of a C-terminal 6 X histidine tag to CL synthase, it was necessary to find a new epitope tag in order to investigate the effects of mutations of conserved residues within the N-terminus of this protein.

Based on the studies with the 6 X histidine tag, the following criteria were established for finding a satisfactory epitope tag. First, the new tag should not be located in or alter the primary sequence in residues 1-100 because this region comprises the N-terminus of CL synthase under investigation. Second, introduction of the tag should not alter any residues within either of the 2 HKD motifs essential for catalysis. Third, the epitope should not be attached to C-terminus or located in this region. Fourth, the tag should be introduced into the protein with minimal perturbation of the primary sequence and without altering elements such as the tetra proline (residues 290-293), which may make significant contributions to the folding pattern. Fifth, introduction of the tag should minimally alter the hydrophobicity of a given region. Finally, and most importantly, insertion of the tag should not significantly reduce or eliminate activity of the mutant protein.

The EE tag (Glu-Glu tag) [48] was selected because of its homology with the primary sequence of CL synthase (Figure 2.3), its conformation to the above specifications, and the relatively conservative substitutions that would need to be made in order to introduce the epitope. As shown in Figure 2.3, this 6 amino acid epitope tag allowed the use of three amino acids already present in the primary sequence of wild type CL synthase. To complete the epitope, three relatively conservative substitutions were made to CL synthase (see section 2.7). The use of this tag enabled the unambiguous identification of CL synthase with mutations to conserved residues within the N-terminus by western blot.

3.6.1. Characterization of the EE tagged CL synthase

To examine whether or not the introduction of the EE epitope tag altered the properties of CL synthase, the epitope tagged CL synthase coded for by pBQ43 was compared to the wild type CL synthase coded for by pLR3 as follows. First, the ability to produce a functional CL synthase *in vivo* was compared using growth studies of QC25/pLR3 and QC25/pBQ43 at 30°C and 42°C (see section 2.12). Second, incorporation of [^{14}C]acetate to CL in QC30-15 was used to quantify the relative *in vivo* activities of these proteins (section 2.10). Third, the proteins were expressed in strain DG6 [17] and their migration on coomassie stained SDS PAGE and western blot was compared to be sure that (a) they both migrated with the same apparent molecular mass and (b) only the EE tagged CL synthase gave a signal when probed on western blot with an anti EE antibody. Finally, the *in vitro* activity of the wild type and EE epitope tagged CL synthases were compared (see section 2.19). The EE epitope tagged CL synthase encoded by pBQ43 was found to be similar to the wild type CL synthase encoded by pLR3 in all respects.

Both QC25/pLR3 and QC25/pBQ43 form broad streaks at 30°C and 42°C indicating that the epitope tagged CL synthase is active *in vivo* (Table 3.3).

QC30-15/pLR3 and QC30-15/pBQ43 incorporate [^{14}C]acetate into cardiolipin to the same degree (Figure 3.4), and the doubling times of these strains are the same (data not shown). Furthermore the doubling time of these strains is approximately 75% that of QC30-15/pET3, which does not have an active CL synthase.

The CL synthase expressed by IPTG induced DG6/pBQ43 and DG6/pLR3 are membrane associated and migrate with an apparent molecular mass of 45.3 kDa when viewed in coomassie stained SDS PAGE experiments (Table 3.3, Figure 3.5 A).

A comparison of *in vitro* activity (see section 2.19) shows that the CL synthase activity in membranes isolated from DG6/pBQ43 is slightly greater than that of membranes isolated from DG6/pLR3 (Figure 3.6).

The one major difference between the CL synthases produced by DG6/pBQ43 and DG6/pLR3 is observed when the extracts are subjected to electrophoresis and anti EE antibody [48] is used to detect proteins with the EE epitope. Only the DG6/pBQ43 extracts have a detectable band that migrates as CL synthase (Figures 3.5 B, 3.8, 3.9). The upper arrows in Figure 3.5 A and B show the location of CL synthase encoded by pLR3 and pBQ43 respectively as described in the legend of that figure. The lower arrows in Figure 3.5 A and B show the location of the degradation products of both epitope and non epitope tagged proteins as described in the legend. The degradation products of these CL synthases show a signal of decreased intensity, which probably results from nonspecific binding of the antibody to this degradation product because there is a large amount of it and it is hydrophobic. The antibody does not recognize the wild type non-epitope tagged CL synthase encoded by pLR3 (Figure 3.5 B, lane 1 upper arrow), so it can therefore not recognize the degradation product of the non-epitope tagged CL synthase frameshift 2-20 mutation encoded by pBQ63 (Figure 3.5 B, lane 2 lower arrow). The antibody does recognize the epitope in

the CL synthase encoded by pBQ43 and gives a dark signal as shown in Figure 3.5 B (lane 3, upper arrow). The fact that there is a faint signal at the position corresponding to the degradation product of the epitope tagged CL synthase frameshift 2-20 mutant encoded by pBQ56 (Figure 3.5 B, lane 4 lower arrow), identical to that of the non epitope tagged 2-20 frameshift mutant (Figure 3.5 B, lane 2 lower arrow), indicates that like the non-epitope tagged degradation product, it is not recognized by the antibody. Similarly, a signal resulting from nonspecific binding of a protein with an apparent molecular mass of approximately 30 kDa, believed to be β-lactamase, is present in all lanes in Figures 3.8 and 3.9.

Plasmid pBQ55 (H224A) encodes a CL synthase mutant in which the conserved histidine residue [18-21] in the first HKD motif of CL synthase is changed to alanine. As expected, the mutant protein is inactive *in vivo* as evidenced by lack of growth of QC25/pBQ55 at 42°C (Table 3.3) and failure of QC30-15/pBQ55 to incorporate [^{14}C]acetate into CL (Figure 3.4). When induced with IPTG, DG6/pBQ55 produces a CL synthase that migrates on SDS PAGE at the same apparent molecular mass as the epitope tagged CL synthase encoded by pBQ43, and is recognized by the anti EE antibody on western blot (data not shown).

3.7. Rationale for mutagenesis of amino acid residues within the N-terminus of CL synthase

Replacement of conserved amino acids with alanine, serine, or threonine and introduction of frameshift regions within residues 1-80 was used to identify

point mutations or regions within the N-terminus of CL synthase involved in the posttranslational processing of CL synthase. Replacement of amino acids with alanine was performed to examine the effect of substitution of the side chains of conserved amino acids with a methyl group. The replacement of amino acids with serine or threonine was performed to examine the effect of replacing either a nonpolar side chain with a polar uncharged side chain of similar length, or a charged polar side chain with a polar uncharged side chain. The introduction of frameshift mutations was used to determine whether a region within the N-terminus was necessary for the correct processing and activity of CL synthase, and to identify amino acids within those regions as possible candidates for further mutation. The replacement of serine 36 with aspartate was performed because analysis of the N-terminus of CL synthase with Antheprot [44-46] predicted that this change would eliminate cleavage of CL synthase at one of two sites identified by the program. Throughout the remainder of this report, pBQX will be used to refer to the plasmid coding for a particular protein while Bqx will refer to the CL synthase derivative.

3.8. Construction of alanine point mutations within the N-terminus of E. coli CL synthase and a CL synthase active site mutant

Alanine point mutants were introduced by site directed mutagenesis (section 2.5) using the primers shown in Table 2.8. Plasmids pBQ44 (W40A), pBQ45 (P48A), pBQ46 (Y55A), pBQ47 (G59A), pBQ55 (H224A), pBQ61 (R66A), and pBQ62 (R70A) were constructed by altering the nucleotide sequence of *cls* in pBQ43 in a single round of site directed mutagenesis. Plasmids pBQ52

(W40A, P48A) and pBQ66 (R66A, R70A) were constructed by altering the nucleotide sequence of *cls* in pBQ44 and pBQ61 respectively.

3.9. Construction of aspartate (D), serine (S), and threonine (T) mutations within the N-terminus of E. coli CL synthase

Mutations of residues to D, S, or T were introduced by site directed mutagenesis (section 2.5) with the primers listed in Table 2.9. Plasmids pBQ48 (S36D), pBQ67 (LII42-44SSS), pBQ68 (II52-53SS), pBQ69 (L19S), and pBQ72 (LV7-8SS) were made in a single round of site directed mutagenesis by altering the nucleotide sequence of *cls* in pBQ43. Plasmid pBQ73 (LI19-20SS) was obtained by altering the nucleotide sequence of *cls* in pBQ69. Plasmids pBQ70 (KRR30-32TTT), and pBQ71 (KRR65-67SSA) were made by introducing a frameshift mutation to *cls* in pBQ43 followed by the recovery of reading frame at the desired location in the resulting plasmid.

3.*10. Construction of frameshift regions within the N-terminus of E. coli CL synthase*

Frameshift mutations were introduced by site directed mutagenesis (section 2.5) with the primers listed in Table 2.10. Plasmids pBQ50 (frameshift 41-44), pBQ56 (frameshift 2-20), pBQ57 (frameshift 2-42), and pBQ59 (frameshift 72-82), were made by introducing a frameshift mutation to the *cls* gene in pBQ43 followed by the recovery of the reading frame at the desired location. Any new restriction sites that were introduced (see section 2.8.3) were confirmed by restriction analysis (see section 2.4).

3.11. Characterization of EE tagged constructs

Plasmids coding for an EE tagged CL synthase with mutations to the N-terminus were compared to the EE tagged CL synthase with the wild type signal region encoded by pBQ43 to examine the effects of the introduced mutations on the processing and activity of the enzyme.

As previously stated, *pssA1 cls* double mutants do not grow on LB agar plates at 42°C while *pssA1* mutants do so (Heber, S., and Tropp, B.E., unpublished data). QC25 (Table 2.1) bearing a plasmid coding a functional CL synthase would grow at 30°C and 42°C while one bearing an inactive CL synthase or one with reduced activity would grow at 30°C but not at 42°C.

Examination of the amount of [^{14}C]acetate incorporated to CL in QC30-15 (Table 2.1) transformed with plasmids coding for a mutant CL synthase would confirm whether or not the mutants were active *in vivo*, and show whether the activity of the mutant was decreased or increased relative to the EE tagged CL synthase with a wild type N-terminus.

Analysis by western blot of proteins expressed in strain DG6 [17] (F$^-$ *ompT hsdSB* ($r_B^-m_B^-$), *dcm gal* (DE3) *cls*::Tn*10*dTet3) with an anti EE antibody would show differences in migration resulting from altered processing of the mutant CL synthase.

These methods were employed to find a CL synthase with a mutation to conserved residues within the N-terminus that migrated at an increased apparent

molecular mass, and had a loss of activity *in vivo* but not *in vitro*, consistent with the protein having an altered membrane topology in the cell.

Use of the methods described above resulted in five classes of mutants that are categorized as follows: (i) mutations resulting in an increase in apparent molecular mass, and a loss of activity *in vivo* but not *in vitro*, suggesting an alteration in membrane topology, (ii) mutations resulting in an increase in apparent molecular mass and a loss of activity *in vivo* and *in vitro*, (iii) mutations resulting in an increase in apparent molecular mass but not a loss of activity, (iv) mutations resulting in a decrease in apparent molecular mass and a loss of activity *in vivo* and *in vitro*, and (v) mutations resulting in little to no effect on the apparent molecular mass or activity of the mutant CL synthase. Not all mutants obtained fit exactly into these categories, so they will be grouped into the category that best fits them.

Although the most interesting mutant encoded by pBQ72 (LV7-8SS) is presented first, it was found toward the end of this research, after many other CL synthase mutants had been examined.

3.11.1. A mutation to conserved residues within the N-terminus of CL synthase resulting in an increase in apparent molecular mass, and a loss of activity in vivo but not in vitro, suggesting an alteration in membrane topology

1. pBQ72 (LV7-8SS)

As shown in Table 3.4, strain QC25/pBQ72 grows normally at 30°C but does not form colonies at 42°C, suggesting that the protein has reduced activity or is inactive *in vivo*.

QC30-15/pBQ72 shows a 77% decrease in CL formation (Figure 3.10) along with increased cellular phosphatidylglycerol. This decrease in CL accompanied by an increase in the amount of phosphatidylglycerol in the cells is expected, since cellular phosphatidylglycerol is the precursor for CL (Figure 1.1). Furthermore, the doubling time of QC30-15/pBQ72 in LB at 30°C is approximately 2.3 times that of QC30-15/pBQ43 under the same conditions (data not shown). The cause of this increased doubling time is not clear. One possibility is that the mutant protein somehow jams the transport or processing machinery.

IPTG induced strain DG6/pBQ72 analyzed by SDS PAGE and western blot produces a CL synthase that migrates with an apparent molecular mass of 47.1 kDa, which is 1.8 kDa heavier than the epitope tagged CL synthase, indicating that the normal processing of this mutant is altered (Figure 3.8).

When analyzed *in vitro*, the CL synthase activity of the membrane fraction isolated from DG6/pBQ72 is greater than that of the membrane fraction isolated from cells expressing the epitope tagged CL synthase (DG6/pBQ43) (Figure 3.6). The fact that this protein migrates with an increase in apparent molecular mass, and has *in vitro* but not *in vivo* activity suggests that it exists in the cell with an altered membrane topology. As described in chapter 1, strains deficient in mannitol transport that have a functional CL synthase form phosphatidylmannitol and bisphosphatidylmannitol, while those with a *cls-1* mutation do not. This suggests that the catalytic site of CL synthase is located on the periplasmic side of the cell membrane [28]. The results of experiments performed with this mutant

CL synthase (Bq72) suggest that it exists in the cell with its catalytic site facing the cytoplasm, accounting for its lack of activity *in vivo,* but its full activity *in vitro*.

3.11.2. Mutations to conserved residues within the N-terminus of CL synthase resulting in an increase in apparent molecular mass and loss of activity in vivo and in vitro

1. pBQ50 (frameshift 41-44)

As shown in Table 3.5, QC25/pBQ50 fails to form colonies at 42°C, suggesting that CL synthase with this mutation is inactive *in vivo*. This result is confirmed by lack of incorporation of [^{14}C]acetate into CL, and increased cellular phosphatidylglycerol in QC30-15/pBQ50 (Figure 3.11). The introduction of pBQ50 to QC30-15 causes a decrease in cell viability and makes the cellular doubling time in LB at 30°C difficult to determine. As a result, the normal [^{14}C]acetate glycerophospholipid incorporation experiment was modified as follows. A repurified colony of QC30-15/pBQ50 was added to 10 mL of LB containing 125 μg/mL ampicillin and incubated overnight at 30°C with shaking at 250 rpm. The following morning, when the cells had reached a turbidity of 20-30 Klett, 2.5 μCi of 2-[^{14}C]acetate (4.3 μCi/umol) was added and the cells were incubated for 4 hours at 30°C with shaking. Lipids were then isolated and analyzed as described in section 2.10. This increased doubling time was not seen in strains QC25/pBQ50, DG6/pBQ50, or XL1/pBQ50, although DG6/pBQ50 did show inhibition of growth when induced with IPTG as described below.

IPTG induced DG6/pBQ50 shows full inhibition of growth, and produces a CL synthase that migrates on SDS PAGE and is detected by western blot at an

apparent molecular mass of 47.4 kDa (Table 3.5, Figure 3.8), which is 2.1 kDa heavier than the EE tagged CL synthase, indicating that the posttranslational processing of Bq50 is altered. Of all pBQ43 based plasmid constructs containing mutations to the N-terminus of CL synthase, DG6/pBQ50 is the only strain that shows growth inhibition when induced with IPTG. Furthermore, the amount of Bq50 produced by DG6/pBQ50 (frameshift 41-44) is decreased relative to the amount of Bq43 produced by DG6/pBQ43 (EE tagged CL synthase), as judged by visual inspection of coomassie stained SDS PAGE gels.

As shown in Figure 3.6, this mutant CL synthase does not catalyze the formation of CL *in vitro*.

Of the four amino acids altered in this mutant, the three shown as black letters in a gray box (LLII→HVDY) are conserved. As shown in Figure 2.4, the most nonconservative changes are the replacement of leucine with histidine at position one and isoleucine with aspartate at position three of the frameshift region. The introduction of this frameshift region alters three conserved residues and disrupts the hydrophobic nature of the second, more hydrophobic, transmembrane α-helix.

2. pBQ52 (W40A P48A)

QC25/pBQ52 grows normally at 30°C, but fails to grow at 42°C, suggesting that this protein has reduced activity or is inactive *in vivo*. The inactivity of this CL synthase mutant *in vivo* is confirmed by lack of [^{14}C]acetate incorporation to CL and increased levels of cellular phosphatidylglycerol in QC30-15/pBQ52 (Figure 3.11). The doubling time of QC30-15/pBQ52 in LB at

30°C is approximately twice that of QC30-15/pBQ43 (data not shown). The cause of this increased doubling time is not known.

IPTG induced DG6/pBQ52 analyzed by SDS PAGE and western blot produces a CL synthase that migrates with an apparent molecular mass of 46.9 kDa, which is 1.6 kDa heavier than the EE tagged CL synthase (Figure 3.8), indicating that this protein is processed abnormally.

As shown in Figure 3.6, this mutant CL synthase does not catalyze the formation of CL *in vitro*.

Both W40 and P48 are conserved residues (Figure 1.3) within the N-terminus of CL synthase. Proline 48 is predicted to be a helix breaking residue which may be responsible for proper α-helical structure and membrane insertion of the second transmembrane segment. Although mutation of each of these residues by themselves had some effect on the processing and activity of CL synthase (see section 3.11.3), neither prevented growth of QC25 at 42°C (Table 3.6), the incorporation of [^{14}C] acetate to CL in QC30-15 (Figure 3.12), or altered the doubling time of QC30-15 (data not shown). As is the case for the mutant CL synthase coded for by pBQ50, the amount of Bq52 present in IPTG induced DG6/pBQ52 appears to be decreased relative to the amount of epitope tagged CL synthase produced by IPTG induced DG6/pBQ43 as determined by visual inspection of coomassie stained SDS PAGE gels.

3.11.3. Mutations to the N-terminus of CL synthase resulting in an increase in apparent molecular mass but not a loss of activity

Table 3.6 shows the growth of QC25 containing plasmids encoding a CL synthase with mutations to the N-terminus resulting in an increase in apparent molecular mass but not a loss of activity. Figure 3.12 shows the results of *in vivo* analysis of these constructs in QC30-15. Figures 3.8 and 3.9 show the results of western blots.

1. pBQ44 (W40A)

QC25/pBQ44 grows normally at both 30°C and 42°C, indicating that this CL synthase is active *in vivo* (Table 3.6). However, QC30-15/pBQ44 only incorporates [^{14}C]acetate into CL at 50% the level of QC30-15/pBQ43, and has increased levels of cellular phosphatidylglycerol (Figure 3.12).

IPTG induced strain DG6/pBQ44 analyzed by SDS PAGE and western blot produces a CL synthase that migrates at 46.9 kDa, which is approximately 1.6 kDa heavier than the EE tagged CL synthase (Figure 3.8). These results indicate that alteration of this conserved residue has an effect on the normal processing and activity of CL synthase.

2. pBQ45 (P48A)

QC25/pBQ45 grows normally at both 30°C and 42°C indicating that this CL synthase is active *in vivo*.

QC30-15/pBQ45 shows an approximate 25% increase in cellular CL and a corresponding decrease in cellular PG relative to the amount of these lipids

produced by QC30-15/pBQ43, which contains the epitope tagged CL synthase with a wild type N-terminus.

IPTG induced DG6/pBQ45 analyzed by SDS PAGE and western blot produces a CL synthase that migrates with an apparent molecular mass of 46.4 kDa, which is 1.1 kDa heavier than the EE tagged CL synthase with a wild type N-terminus (Figure 3.8).

As with pBQ44, these results indicate that alteration of this conserved residue has an effect on the normal processing and the activity of CL synthase. It is interesting to note that when the mutations made in pBQ44 (W40A) and pBQ45 (P48A) are combined in pBQ52 (W40A P48A), the resulting CL synthase migrates with an apparent molecular mass very close to that of Bq44 and is inactive *in vivo* and *in vitro*.

3. pBQ48 (S36D)

QC25/pBQ48 grows normally at both 30°C and 42°C indicating that this mutant CL synthase retains activity *in vivo*.

Strain QC30-15/pBQ48 shows a 50 % reduction in the amount of CL formed along with a corresponding increase in cellular phosphatidylglycerol relative to QC30-15/pBQ43.

As shown in Figure 3.9, IPTG induced DG6/pBQ48 analyzed by SDS PAGE and western blot produces a protein that migrates with an apparent molecular mass of 45.8 kDa, which is 0.5 kDa heavier than the EE tagged CL synthase with a wild type N-terminus produced by IPTG induced DG6/pBQ43.

Although S36 is not conserved, Bq48 shows altered electrophoretic mobility and decreased *in vivo* activity. When analyzed with Antheprot [44-46], two potential cleavage sites, the first at amino acid position 40 and the second at amino acid position 80, were identified within the N-terminus CL synthase. The mutation S36D was predicted by Antheprot [44-46] to eliminate cleavage at position 40 but not affect cleavage at position 80.

4. pBQ67 (LII42-44SSS)

QC25/pBQ67 grows normally at 30°C, but does not form colonies at 42°C suggesting that CL is not being formed *in vivo*.

Contrary to expectation, strain QC30-15/pBQ67 shows a slight increase in the amount of CL synthesized (Figure 3.12) relative to strain QC30-15/pBQ43 (EE tagged CL synthase). Previous results have shown that QC25 strains that do not grow at 42°C do not contain an active CL synthase. The reason for the unexpected growth properties of QC25/pBQ67 requires further study. The doubling time of strain QC30-15/pBQ67 in LB at 30°C is increased approximately three fold relative to strain QC30-15/pBQ43 (data not shown). The reason for this increase in doubling time is not known. One possibility is that the cellular processing machinery is jammed by a CL synthase with this mutation to conserved residues within the N-terminus.

When DG6/pBQ67 is induced with IPTG, a CL synthase is produced that migrates with an apparent molecular mass of 46.5 kDa when analyzed by SDS PAGE and western blot (Figure 3.9), which is 1.3 kDa heavier than the EE tagged CL synthase with a wild type N-terminus; indicating that the substitution of

the polar uncharged amino acid serine for these conserved hydrophobic residues has an effect on the processing of CL synthase.

5. pBQ68 (II52-53SS)

QC25/pBQ68 (II52-53SS) grows normally at 30°C, but shows abnormal growth at 42°C, forming lightly speckled colonies, meaning that very small individual colonies are visible. The growth of strain QC25/pBQ68 suggests that the CL synthase encoded by pBQ68 is inactive *in vivo*.

Contrary to expectation, QC30-15/pBQ68 shows a 20% increase in CL formation relative to QC30-15/pBQ43, along with a decrease in cellular phosphatidylglycerol. The cellular doubling time of QC30-15/pBQ68 in LB at 30°C is approximately 2.3 times that of strain QC30-15/pBQ43 (data not shown). As with QC30-15/pBQ67 (LII42-44SSS), the fact that QC30-15/pBQ68 (II52-53SS) produces CL *in vivo* is at odds with the fact that QC25/pBQ68 grows abnormally at 42°C but grows normally at 30°C. The reason for this discrepancy requires further study.

IPTG induced DG6/pBQ68 produces a protein that migrates at an apparent molecular mass of 46.5 kDa when analyzed by SDS PAGE and western blot (Figure 3.9), which is 1.3 kDa heavier than the epitope tagged CL synthase encoded by pBQ43.

It is interesting to note that both pBQ67 and pBQ68 encode a CL synthase with mutations that convert conserved hydrophobic residues in the second transmembrane α-helix to polar uncharged residues, and both result in proteins that migrate at the same apparent molecular mass and show a similar alteration

of *in vivo* activity. Furthermore, both mutations increase the cellular doubling time of QC30-15 transformed with these plasmids by approximately 2-fold in LB at 30°C (data not shown).

6. pBQ69 (L19S)

QC25/pBQ69 (L19S) grows normally at 30°C and 42°C, indicating that this plasmid codes for CL synthase that is functional *in vivo*.

QC30-15/pBQ69 shows a 20% increase in CL formation along with a decrease in cellular phosphatidylglycerol relative to QC30-15/pBQ43 (EE tagged CL synthase).

IPTG induced DG6/pBQ69 produces a protein that migrates with an apparent molecular mass of 46.1 kDa, which is 0.8 kDa heavier than the EE tagged CL synthase encoded by pBQ43. It is interesting to note that when both L19 and I20 are mutated to serine (Bq73, described below) this increase in apparent molecular mass roughly doubles to 1.8 kDa. Both L19 and I20 are conserved residues that are located within the first N-terminal transmembrane α-helix of CL synthase.

7. pBQ73 (LI19-20SS)

QC25/pBQ73 grows normally at 30°C and 42°C, indicating that this plasmid codes for CL synthase that is functional *in vivo.*

QC30-15/pBQ73 shows a reduction of CL synthesis by approximately 30%, along with a slight increase in cellular phosphatidylglycerol relative to QC30-15/pBQ43 (EE tagged CL synthase).

IPTG induced DG6/pBQ73 produce a CL synthase that migrates with an apparent molecular mass of approximately 47.1 kDa, which is 1.8 kDa heavier than that produced by DG6/pBQ43 (EE tagged CL synthase). It is interesting to note that the difference in migration between the EE tagged CL synthase with a wild type signal region (Bq43) and a CL synthase with the mutation L19S (Bq69) roughly doubles to 1.8 kDa when both L19 and I20 are mutated to serine (Bq73). Both L19 and I20 are conserved residues that are located in the first transmembrane region of the N-terminus. It is also interesting to note that both Bq69 and Bq73 migrate with an increase in apparent molecular mass relative to Bq43, and retain *in vivo* CL synthase activity, while Bq72 (LV7-8SS) migrates with an increase in apparent molecular mass but is inactive *in vivo*. Both pBQ72 (LV7-8SS) and pBQ73 (LI19-20SS) encode a CL synthase with mutations of conserved hydrophobic residues within the first transmembrane α-helix, comprised of residues 5-25, to serine. Furthermore both Bq72 and Bq73 migrate at approximately 47.1 kDa when analyzed by SDS PAGE and western blot. It is interesting to note that Bq72 is active *in vitro* but not *in vivo* while Bq73 is active *in vitro* and *in vivo*.

8. pBQ74 (CL synthase with a 2-60 deletion)

Plasmid pBQ74 codes for a CL synthase missing the first 60 residues, fused in frame to the N-terminal T7 tag of pET-23a(+) (see section 2.8.4). As a consequence of this fusion, the residues (MASMTGGQQMGRGSEF) are fused to the N-terminus of CL synthase at amino acid position 60 of the primary sequence. The amino acid residues comprising the T7 tag are shown as black

letters in a gray background. The residues (RGSEF) result from the translation of the pET-23a(+) multiple cloning site (MCS) up to the SacI restriction site, into which *cls* was ligated to create this construct (see section 2.8.4). The N-terminal fusion coded for by this plasmid has a predicted molecular mass 49.9 kDa, as determined by PeptideMass [41] and previously reported [17]. This construct does not have an *E. coli* promoter (see section 2.8.4) and it therefore cannot be tested for *in vivo* activity in QC25 or QC30-15.

Plasmid pBQ74 causes a loss in viability when transformed to strain DG6 (F^- *ompT hsdSB* ($r_B^-m_B^-$), *dcm gal* (DE3) *cls*::Tn*10*dTet3) while pBQ43 does not. In order to obtain viable colonies, DG6/pLysS was used for expression studies involving this plasmid. Incorporation of [^{14}C]acetate to glycerophospholipid experiments involving this plasmid were also performed in DG6/pLysS, as described in section 2.10 for plasmids lacking an *E. coli* promoter. As shown in Figure 3.12, when examined for activity *in vivo* as described in section 2.10, the amount of [^{14}C]acetate incorporated into CL by DG6/pLysS/pBQ74 is almost double that of QC30-15/pBQ43 (EE tagged CL synthase).

IPTG induced DG6/pLysS/pBQ74 shows no growth inhibition and produces a CL synthase that migrates with an apparent molecular mass of 47.1 kDa when analyzed by SDS PAGE and western blot (Figure 3.8), which is 1.8 kDa heavier than the apparent molecular mass of the EE tagged CL synthase encoded by pBQ43. Since Bq74 migrates with an apparent molecular mass less than the predicted value of 49.9 kDa, it may still be undergoing some type of posttranslational processing.

Membranes isolated from IPTG induced DG6/pLysS/pBQ74 have full *in vitro* activity relative to those obtained from DG6/pBQ43 (EE tagged CL synthase). When comparing the *in vivo* and *in vitro* activities of Bq43 and Bq74, it is important to note that (i) the amount of CL synthase produced in IPTG induced DG6/pLyS/pBQ74 is approximately twice that produced in IPTG induced DG6/pBQ43, as determined by visual inspection of coomassie stained SDS PAGE gels, and (ii) the *in vivo* activity of Bq74 was determined in a strain that uses T7 RNA polymerase for transcription. An even closer comparison of the *in vivo* properties of a CL synthase missing residues 2-60 (Bq74) to wild type is made by examination of the growth properties, and CL synthase activity of DG6/pLysS/pBQ74 and DG6/pLysS/pBQ40. As described in section 2.6, pBQ40 results from the removal of the 6 X histidine tag from pBQ4, and codes for a non-epitope tagged CL synthase from the first AUG the final leucine in the pET-23a(+) vector. Both pBQ4 and pBQ74 lack the *E. coli* chromosomal RNA polymerase promoter and the *E. coli cls* ribosomal binding site (rbs). Furthermore, both constructs contain the *cls* gene located immediately downstream of the pET-23a(+) strong rbs. IPTG induced DG6/pLysS/pBQ74 shows no growth inhibition, and produces approximately twice the amount of CL synthase of IPTG induced DG6/pLysS/pBQ40, as determined by visual inspection of SDS PAGE gels. DG6/pLysS/pBQ40 shows full inhibition of growth and a decrease in Klett indicative of cell death (data not shown) when induced with IPTG. Although Bq40 is a wild type CL synthase that is not epitope tagged and Bq74 contains the EE tag, the main difference between these 2 proteins is

that Bq40 has the first 60 N-terminal residues of CL synthase and Bq74 does not. It is also interesting to note that Bq56, which contains a frameshift mutation encompassing residues 2-20, is degraded in the cell, while Bq74 which is missing the first 60 residues of CL synthase and has the 16 residues listed above fused N-terminally to its primary sequence is not. Furthermore although Bq74 is missing both N-terminal hydrophobic transmembrane regions, it is still directed to the membrane, is membrane associated and is able to catalyze formation of CL *in vivo,* indicating that its catalytic site has correct tertiary structure and membrane topology. The reason why CL synthase missing the first 60 amino acid residues should be correctly translocated to the membrane, have activity *in vivo* and *in vitro*, and have correct tertiary structure and membrane topology, while mutants that have single or multiple point mutations show alterations in processing and loss of activity requires further investigation.

3.11.4. Mutations to the N-terminus of CL synthase resulting in a decrease in apparent molecular mass and loss of activity in vivo and in vitro

Table 3.7 shows the growth at 30°C and 42°C of strain QC25 transformed with plasmids encoding a CL synthase that is inactive *in vivo* and migrates with a decrease in apparent molecular mass relative to the EE tagged CL synthase (Bq43). Figure 3.13 shows the results of *in vivo* analysis of these constructs in QC30-15, and Figures 3.8 and 3.9 show the results of western blots.

1. pBQ56 (frameshift 2-20)

QC25/pBQ56 (frameshift 2-20), fails to form colonies at 42°C, but grows normally at 30°C suggesting that this plasmid codes for a CL synthase that is inactive *in vivo*.

The absence of *in vivo* activity is confirmed by the fact that QC30-15/pBQ56 fail to incorporate [^{14}C]acetate into CL.

IPTG induced DG6/pBQ56 do not produce an epitope tagged CL synthase visible on western blot. Instead, a new protein, which is not present in IPTG induced DG6/pET3 (vector control), appears on coomassie stained SDS PAGE migrating with an apparent molecular mass of 20 kDa (Figure 3.5). Due to the fact that this CL synthase construct is degraded and has no activity *in vivo*, it was not tested further.

The residues altered in Bq56 are shown as a clustal W alignment with wild type CL synthase in Figure 2.4. Within the first 20 amino acids of the primary sequence of CL synthase there are 4 conserved amino acids: L7, V8, L19 and I20. It is interesting to note that as a result of the introduction of a frameshift to this region, highly conservative substitutions are made to two of the conserved residues (L7V and L19V). The degradation of this CL synthase frameshift 2-20 mutant in DG6 is in direct contrast to results obtained using the C-terminally 6 X histidine tagged frameshift 2-20 construct (pBQ6) which encodes a CL synthase that produces a signal on western blot migrating with an apparent molecular mass approximately 1 kDa heavier than the histidine tagged wild type protein expressed from pBQ3 (data not shown).

2. pBQ57 (frameshift 2-42)

QC25/pBQ57 grows normally at 30°C but fails to grow at 42°C suggesting that like the frameshift 2-20 mutant, this CL synthase mutant is inactive *in vivo*.

The results of analysis of IPTG induced DG6/pBQ57 are the same as those of IPTG induced DG6/pBQ56. No epitope tagged CL synthase is visible on western blot, and a new protein that is not present in DG6/pET3 (vector control) appears on coomassie stained SDS PAGE gels migrating with an apparent molecular mass of 20 kDa (data not shown). The degradation of this CL synthase 2-42 frameshift mutant *in vivo* is also in direct contrast to results obtained using the C-terminally 6 X histidine tagged frameshift 2-42 construct (pBQ7), which migrates on SDS PAGE and gives a signal on western blot with an apparent molecular mass 2.6 kDa heavier than the histidine tagged wild type CL synthase encoded by pBQ3.

Due to the formation of identical degradation products in IPTG induced DG6/pBQ56 (frameshift 2-20) and DG6/pBQ57 (frameshift 2-42), the CL synthase 2-42 frameshift mutation was not examined further. The clustal W [38] alignment of this mutant to wild type CL synthase is not shown in Figure 2.4 for the same reason. Both pBQ56 and pBQ57 were constructed at the same time because results from the histidine tagged CL synthase indicated that introduction of these frameshift regions resulted in mutants that migrated with an increase in apparent molecular mass relative to the 6 X histidine tagged CL synthase encoded by pBQ3 (Table 3.2).

3. pBQ63, a non-epitope tagged 2-20 frameshift mutant

In order to determine whether or not the degradation of CL synthase with a frameshift 2-20 region was due to the introduction of the EE epitope into CL synthase, a plasmid coding for a non-epitope tagged 2-20 frameshift mutant (pBQ63) was constructed (see section 2.8.3).

QC25/pBQ63 grow normally at 30°C but fail to grow at 42°C suggesting that like the epitope tagged frameshift 2-20 mutant, the wild type protein with a frameshift region 2-20 is inactive *in vivo*.

IPTG induced DG6/pBQ63 produce a new protein that is not present in DG6/pET3 (vector control), that is identical to the CL synthase produced by IPTG induced DG6/pBQ56 (EE tagged frameshift 2-20), which migrates with an apparent molecular mass of 20 kDa when analyzed by SDS PAGE (Figure 3.5). These results indicate that the wild type CL synthase with a 2-20 frameshift region behaves identically to the epitope tagged CL synthase with a 2-20 frameshift region, and thus the epitope tag is not responsible for the observed degradation and loss of activity of CL synthase with this mutation.

Due to the degradation of CL synthase transcribed from plasmids pBQ56 (frameshift 2-20), pBQ57 (frameshift 2-42), and pBQ63 (non-epitope tagged frameshift 2-20), these constructs were not investigated further.

4. pBQ70 (KRR30-32TTT)

QC25/pBQ70 (KRR30-32TTT) grows normally at 30°C, but does not form colonies at 42°C suggesting that the CL synthase encoded by this plasmid has reduced activity or is not active *in vivo*.

When analyzed for [^{14}C]acetate incorporation to glycerophospholipids, strain QC30-15/pBQ70 shows a 70% decrease in CL synthesis relative to strain QC30-15/pBQ43 and an increase in cellular phosphatidylglycerol.

IPTG induced DG6/pBQ70 analyzed by SDS PAGE and western blot produces a CL synthase that migrates with an apparent molecular mass of 44.1kDa, which is 1.2 kDa less than the EE tagged CL synthase encoded by pBQ43.

As shown in Figure 3.6, membranes isolated from DG6/pBQ70 do not catalyze formation of CL *in vitro*.

Of the three residues altered in this mutant, only R32 is a conserved residue. However, this residue is located between a lysine and an arginine residue in the primary sequence of CL synthase (Figure 1.3). In order to be certain that an adjacent residue would not compensate for the mutation of this conserved residue, all three were mutated. The fact that alteration of these residues results in a protein that undergoes altered posttranslational processing and is inactive *in vivo* and *in vitro* demonstrates the involvement of one or all of residues K30, R31 and R32 in the correct processing and activity of CL synthase. The effect, if any, of the mutation of conserved residue R32 by itself remains to be investigated further.

3.11.5. Mutations to the N-terminus of CL synthase resulting in little to no effect

Table 3.8 shows the results of growth experiments at 30°C and 42°C of QC25 containing plasmids encoding CL synthase mutants that retain activity *in vivo*, and migrate with an apparent molecular mass similar to that of the epitope

tagged CL synthase when analyzed by SDS PAGE and western blot. Figure 3.14 shows the results of *in vivo* analysis of these constructs in QC30-15. Figures 3.8 and 3.9 show the results of western blots conducted with these constructs.

1. pBQ46 (Y55A)

QC25/pBQ46 grows normally at both 30°C and 42°C indicating that CL synthase with a mutation to this conserved residue retains activity *in vivo*.

QC30-15/pBQ46 incorporates [^{14}C]acetate into CL at a level close to that of strain QC30-15/pBQ43 (EE tagged CL synthase), indicating that this mutation has little to no effect on the activity of CL synthase *in vivo*.

IPTG induced DG6/pBQ46 analyzed by SDS PAGE and western blot shows a CL synthase that migrates with an apparent molecular mass of 45.6 kDa, 0.3 kDa heavier than the EE tagged CL synthase encoded by pBQ43, indicating that this mutation does have a slight effect on the processing of CL synthase (Figure 3.9). The amount of Bq46 produced by IPTG induced DG6/pBQ46 appears to be slightly decreased relative to the amount of CL synthase produced by IPTG induced DG6/pBQ43 (EE tagged CL synthase) as determined by visual inspection of coomassie stained SDS PAGE gels.

2. pBQ47 (G59A)

QC25/pBQ47 grows normally at both 30°C and 42°C indicating that the CL synthase produced by this construct is active *in vivo*.

QC30-15/pBQ47 shows an approximate 20% decrease in CL formation relative to QC30-15/pBQ43 (EE tagged CL synthase) with a corresponding

increase in cellular phosphatidylglycerol indicating that the mutation of this conserved residue has an effect on the *in vivo* activity of CL synthase.

IPTG induced DG6/pBQ47 analyzed by SDS PAGE and western blot shows a CL synthase that migrates at approximately the same apparent molecular mass as the EE tagged CL synthase (Bq43), indicating that this mutation has little to no effect on the processing of CL synthase (Figure 3.9).

Although G59 is conserved, the replacement of a glycine residue with an alanine residue may not be enough of a change to see an effect on the processing of CL synthase.

3. pBQ59 (frameshift 72-82)

QC25/pBQ59 grows normally at 30°C and 42°C indicating that CL synthase with a frameshift mutation to residues 72-82 of CL synthase is active *in vivo*.

QC30-15/pBQ59 incorporates radioactive label from [^{14}C]acetate into CL in at levels very similar to that of QC30-15/pBQ43 (EE tagged CL synthase).

IPTG induced DG6/pBQ59, analyzed by SDS PAGE and western blot produces a protein that migrates at the same apparent molecular mass as the EE tagged CL synthase encoded by pBQ43 (Figure 3.9). These results demonstrate that amino acids in this region are probably not involved in the posttranslational processing of CL synthase. This result is interesting because although none of the altered residues are conserved (Figure 1.3), this frameshift mutation (Figure 2.4) spans the suspected cleavage site of CL synthase, which is believed to be in the vicinity of residue 80 based on the difference in migration of the processed

protein to the molecular mass of CL synthase predicted by the *cls* open reading frame [11-13].

4. pBQ61 (R66A)

QC25/pBQ61 grows normally at 30°C and 42°C indicating that the CL synthase produced by this construct retains activity *in vivo*.

QC30-15/pBQ61 incorporates radioactive label from [^{14}C]acetate into CL at levels very similar to that of QC30-15/pBQ43 (EE tagged CL synthase).

IPTG induced DG6/pBQ61 analyzed by SDS PAGE and western blot shows a CL synthase that migrates at the same apparent molecular mass as the EE tagged CL synthase (Bq43) indicating that this mutation results in little to no effect on the processing of CL synthase (Figure 3.9).

5. pBQ62 (R70A)

QC25/pBQ62 grows normally at 30°C and 42°C indicating that like Bq61 (R66A), CL synthase with a mutation to this conserved residue retains activity *in vivo*.

QC30-15/pBQ62 incorporates [^{14}C]acetate into CL at levels very similar to that of QC30-15/pBQ43 (EE tagged CL synthase) indicating that this mutation has little to no effect on the *in vivo* activity of CL synthase.

IPTG induced DG6/pBQ62 analyzed by SDS PAGE and western blot shows a CL synthase that migrates at the same apparent molecular mass as the EE tagged CL synthase (Bq43) indicating that this mutation results in little to no effect on the processing of CL synthase (Figure 3.9).

6. pBQ66 (R66A, R70A)

QC25/pBQ66 grows normally at 30°C and 42°C indicating that CL synthase with a mutation to these conserved residues retains activity *in vivo.*

QC30-15/pBQ66 incorporates [^{14}C]acetate into CL at a level close to that of strain QC30-15/pBQ43 (EE tagged CL synthase), indicating that this mutation has little to no effect on the activity of CL synthase *in vivo.*

IPTG induced DG6/pBQ66 analyzed by SDS PAGE and western blot shows a CL synthase that migrates at the same apparent molecular mass as the EE tagged CL synthase (Bq43) indicating that this mutation results in little to no effect on the processing of CL synthase (Figure 3.9).

Residues R66 and R70 are located within a positively charged region of the CL synthase primary sequence (Figure 1.3). If this region is involved in the posttranslational processing of CL synthase, it is possible that adjacent positively charged residues are able to compensate for the mutations made to the conserved arginine residues in the mutant CL synthases encoded by plasmid constructs pBQ61 (R66A), pBQ62 (R70A) and pBQ66 (R66A, R70A), allowing these mutants to be processed normally.

7. pBQ71 (KRR65-67SSA)

QC25/pBQ71 grows normally at 30°C and 42°C indicating that the CL synthase produced by this construct is active *in vivo*.

QC30-15/pBQ71 incorporates radioactive label from [^{14}C]acetate into CL at levels very similar to that of QC30-15/pBQ43 (EE tagged CL synthase) indicating that this mutation has little to no effect on the *in vivo* activity of CL synthase.

IPTG induced DG6/pBQ71 analyzed by SDS PAGE and western blot shows a CL synthase that migrates at the same apparent molecular mass as the EE tagged CL synthase with a wild type N-terminus (Bq43) indicating that this mutation results in little to no effect on the processing of CL synthase (Figure 3.9).

As shown in Figure 1.3, the region mutated in this mutant is located within a highly charged region of the CL synthase primary sequence containing many lysine and arginine residues. The results of this mutation are similar to those obtained from examination of mutations made to pBQ61 (R66A), pBQ62 (R70A) and pBQ66 (R66A, R70A). As discussed for pBQ66 (R66A, R70A), if this positively charged region comprised of residues 65-72 of the CL synthase primary sequence is involved in the posttranslational processing of the protein, it may be necessary to mutate all of the positively charged residues within this area to see an effect on processing and activity. Results obtained with pBQ71 suggest that the substitution of two different amino acids for the same conserved residue results in the formation of what appears to be the same protein, since both Bq61(R66A) and Bq71 (KRR65-67SSA) encode what appears to be a normally processed CL synthase that has similar *in vivo* activity to the EE tagged CL synthase (Bq43).

Table 3.1.

Growth of QC25 containing C-terminally 6 X Histidine Tagged CL Synthase Constructs at 30°C and 42°C

Plasmid	Description	Growth at 42°C	Growth at 30°C	Reference
pBQ3	6 X histidine tagged CL synthase in pET-23a(+)	DS[a]	+	This work
pBQ4[b]	6 X histidine tagged CL synthase in pET-23a(+)	NA	NA	This work
pBQ6	6 X histidine tagged CL synthase frameshift amino acid position 2-20	-	+	This work
pBQ7	6 X histidine tagged CL synthase frameshift amino acid position 2-42	-	+	This work
pBQ30	CL synthase in pET-23a(+)	+	+	This work
pBQ31	6 X histidine tagged CL synthase E487G	-	+	This work
pBQ32	6 X histidine tagged CL synthase E487L	-	+	This work
pBQ40[b]	CL synthase in pET-23a(+)	NA	NA	This work
pLR3	CL synthase in pET3	+	+	11
pET-23a(+)	T7 Expression Vector containing T7 and 6 X histidine tags	-	+	Product of Novagen

Plasmids were introduced to QC25 as described in section 2.3 and examined for the ability to produce a functional CL synthase in vivo as described in section 2.12.

[a] Formation of darkly speckled colonies, meaning that individual colonies are visible.

[b] pBQ4 differs from pBQ3 and pBQ40 differs from pBQ30 as described in section 2.6.

Table 3.2.

IPTG Inhibition and Apparent Molecular Mass of 6 X Histidine Constructs in DG6/pLysS

Plasmid	Description	Inhibition 0.8 mM IPTG	Apparent Molecular Mass (kDa)[a]	Reference
pBQ3	6 X histidine tagged CL synthase in pET-23a(+)	+	46.5	This work
pBQ4[b,c]	6 X histidine tagged CL synthase in pET-23a(+)	+	46.5	This work
pBQ6	6 X histidine tagged CL synthase frameshift 2-20	-	47.5	This work
pBQ7	6 X histidine tagged CL synthase frameshift 2-42	-	49.1	This work
pBQ30	CL synthase in pET-23a(+)	+	45.3	This work
pBQ31	6 X histidine tagged CL synthase E487G	NA	NA	This work
pBQ32	6X histidine tagged CL synthase E487L	NA	NA	This work
pBQ40[b]	CL synthase in pET-23a(+)	+	45.3	This work
pLR3	CL synthase in pET3	-	45.3	This work
pET-23a(+)	T7 expression vector containing 6 X histidine tag	-	NA	Product of Novagen
pLR3	CL synthase in pET3	-	45.3	11

Gene products were amplified as described in section 2.13 and analyzed as described in sections 2.14-2.17.

[a] products were amplified as described in section 2.13 and analyzed as described in sections 2.14-2.17.

[b]pBQ4 differs from pBQ3 and pBQ40 differs from pBQ30 as described in section 2.6.

[c] Experiment performed in DG6/pLysE.

NA= Experiment not performed / data not available.

Table 3.3.

Comparison of the Wild Type to the EE Tagged CL Synthase

Plasmid	Description	Apparent molecular mass (kDa)[a]	Growth at 42°C[b]	Growth at 30°C[b]
pLR3	CL synthase in pET3	45.3	+	+
pBQ43	EE epitope tagged CL synthase	45.3	+	+
pBQ55	H224A	45.3	-	+

[a] products were amplified as described in section 2.13 and analyzed as described in sections 2.14-2.17.
[b]Experiments performed in QC25 as described in section 2.12.

Table 3.4.

A Mutation to the N-terminus of CL synthase Resulting in an Increase in Apparent Molecular Mass and a Loss of Activity *in vivo* but not *in vitro*

Plasmid	Description	Apparent molecular mass (kDa)[a]	Growth at 42°C[b]	Growth at 30°C[b]
pBQ43	Epitope tagged CL synthase	45.3	+	+
pBQ72	LV7-8SS	47.1	-	+

[a] Gene products were amplified as described in section 2.13 and analyzed as described in sections 2.14-2.17.
[b]Experiments performed in QC25 as described in section 2.12.

Table 3.5.

Mutations to the N-terminus of CL synthase Resulting in an Increase in Apparent Molecular Mass and a Loss of Activity *in vivo* and *in vitro*

Plasmid	Description	Apparentmolecular mass (kDa)[a]	Growth at 42°C[b]	Growth at 30°C[b]
pBQ43	Epitope tagged CL synthase	45.3	+	+
pBQ50	Frameshift 41-44	47.4	-	+
pBQ52	W40A, P48A	46.9	-	+

[a] Gene products were amplified as described in section 2.13 and analyzed as described in sections 2.14-2.17.
[b]Experiments performed in QC25 as described in section 2.12.

Table 3.6.

Mutations to the N-terminus of CL synthase Resulting in an Increase in Apparent Molecular Mass but not a Loss of Activity

Plasmid	Description	Apparent molecular mass (kDa)[a]	Growth at 42°C[b]	Growth at 30°C[b]
pBQ43	Epitope tagged CL synthase	45.3	+	+
pBQ44	W40A	46.9	+	+
pBQ45	P48A	46.4	+	+
pBQ48	S36D	45.8	+	+
pBQ67	LII42-44SSS	46.5	-	+
pBQ68	II52-53SS	46.5	LS[c]	+
pBQ69	L19S	46.1	+	+
pBQ73	LI19-20SS	47.1	+	+
pBQ74*	2-60 deletion in pET23a(+)	47.1	NA*	NA*

[a] Gene products were amplified as described in section 2.13 and analyzed as described in sections 2.14-2.17.
[b]Experiments performed in QC25 as described in section 2.12.
[c]LS=lightly speckled meaning that a small number of small individual colonies were visible.
*NA=Experiment not performed.

Table 3.7.

Mutations to the N-terminus of CL synthase Resulting in a Decrease in Apparent Molecular Mass and a Loss of Activity

Plasmid	Description	Apparent molecular mass (kDa)[a]	Growth at 42°C[b]	Growth at 30°C[b]
pBQ43	Epitope tagged CL synthase	45.3	+	+
pBQ56	frameshift 2-20	20	-	+
pBQ57	frameshift 2-42	20	-	+
pBQ63*	Non epitope tagged frameshift 2-20	20	-	+
pBQ70	KRR30-32TTT	44.1	-	+

[a] Gene products were amplified as described in section 2.13 and analyzed as described in sections 2.14-2.17.
[b]Experiments performed in QC25 as described in section 2.12.
* pBQ63 differs from pBQ56 as described in section 2.8.3.

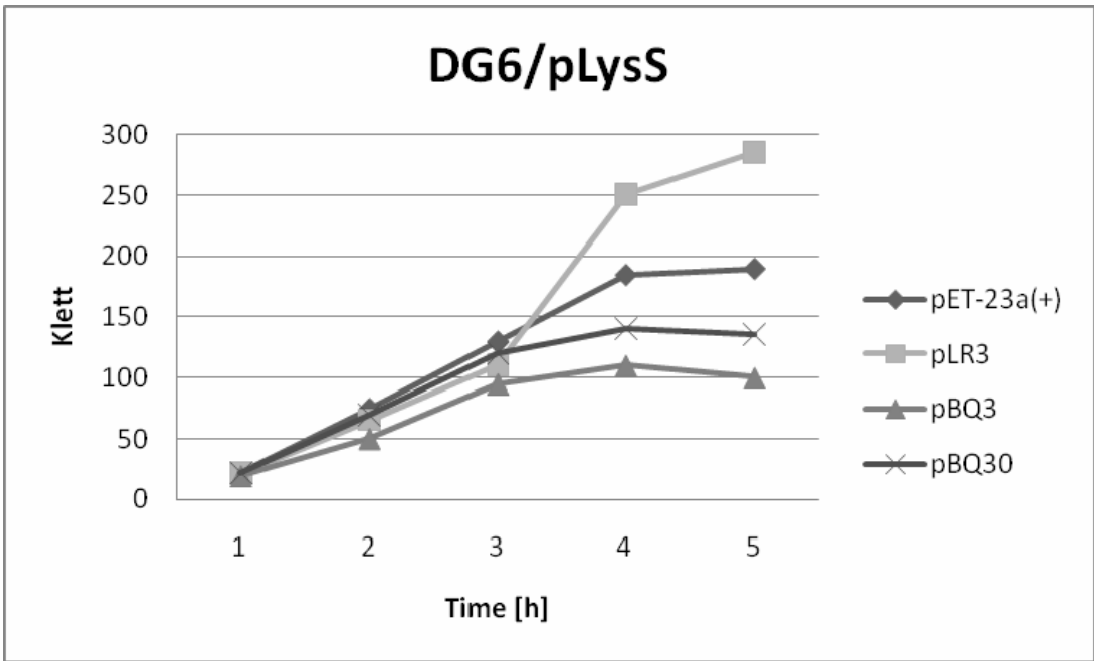
Table 3.8.

Mutations to the N-terminus of CL synthase Resulting in Little to no Effect

Plasmid	Description	Apparent molecular mass (kDa)[a]	Growth at 42°C[b]	Growth at 30°C[b]
pBQ43	Epitope tagged CL synthase	45.3	+	+
pBQ46	Y55A	45.61	+	+
pBQ47	G59A	45.3	+	+
pBQ59	frameshift 72-82	45.3	+	+
pBQ61	R66A	45.3	+	+
pBQ62	R70A	45.3	+	+
pBQ66	R66A,R70A	45.3	+	+
pBQ71	KRR65-67SSA	45.3	+	+

[a] Gene products were amplified as described in section 2.13 and analyzed as described in sections 2.14-2.17.
[b] Experiments performed in QC25 as described in section 2.12.

A



B

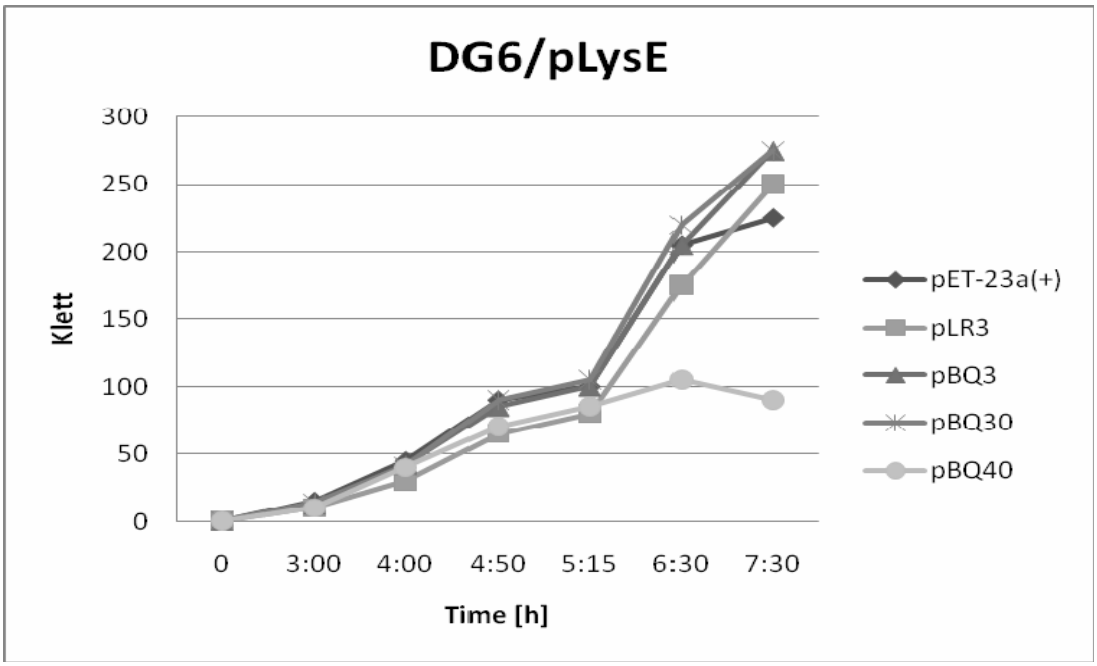


Figure 3.1. Growth properties of selected strains in M9ZB. (A) DG6/pLysS containing the plasmid shown was cultured in M9ZB for the indicated time and induced with IPTG at a total growth time of 4:45 as described section 2.13. (B) DG6/pLysE was cultured as above and induced with IPTG at a total growth time of 5:15.

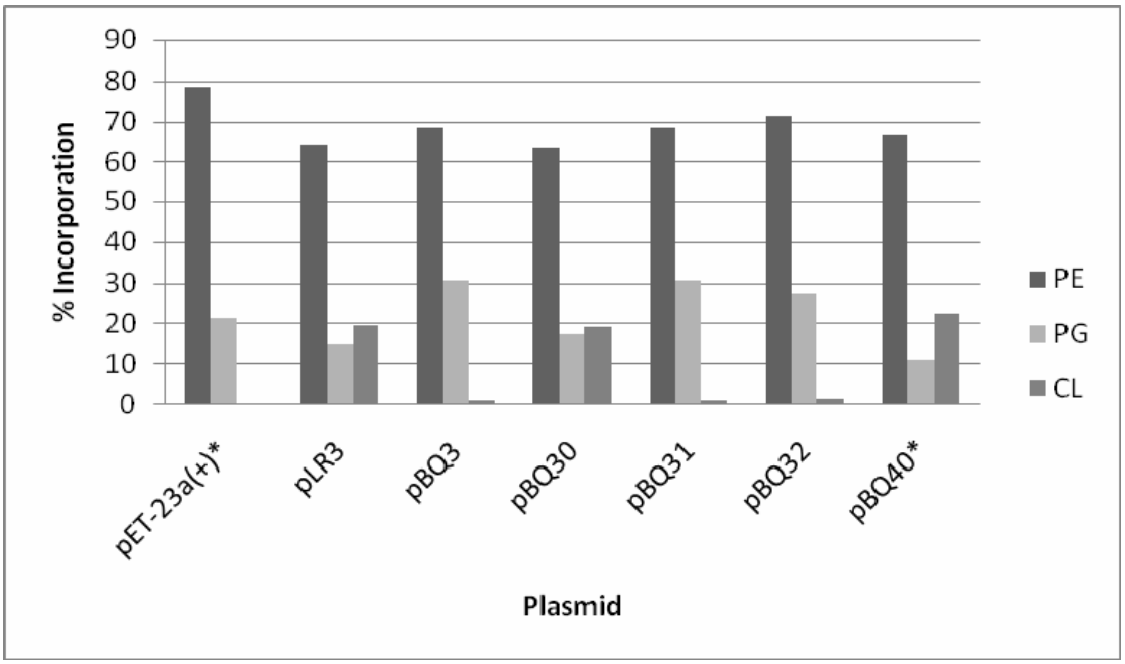


Figure 3.2. [^{14}C]Acetate incorporation into glycerophospholipid of a C-terminally 6 X histidine tagged CL synthase construct and its derivatives. Strain QC30-15 or DG6/pLysS containing the plasmids listed was analyzed for in vivo activity as described in section 2.10. Plasmids: 1. pET-23a(+), vector control; 2. pLR3, wild type CL synthase; 3. pBQ3 C-terminally 6 X histidine tagged CL synthase; 4. pBQ30, wild type CL synthase; 5. pBQ31, C-terminally 6 X histidine tagged CL synthase E487G; 6. pBQ32, C-terminally 6 X histidine tagged CL synthase E487L; 7. pBQ40 wild type CL synthase.

Plasmid pBQ40 differs from pBQ30 as described in section 2.6.

*****Experiment performed in DG6/pLysS.

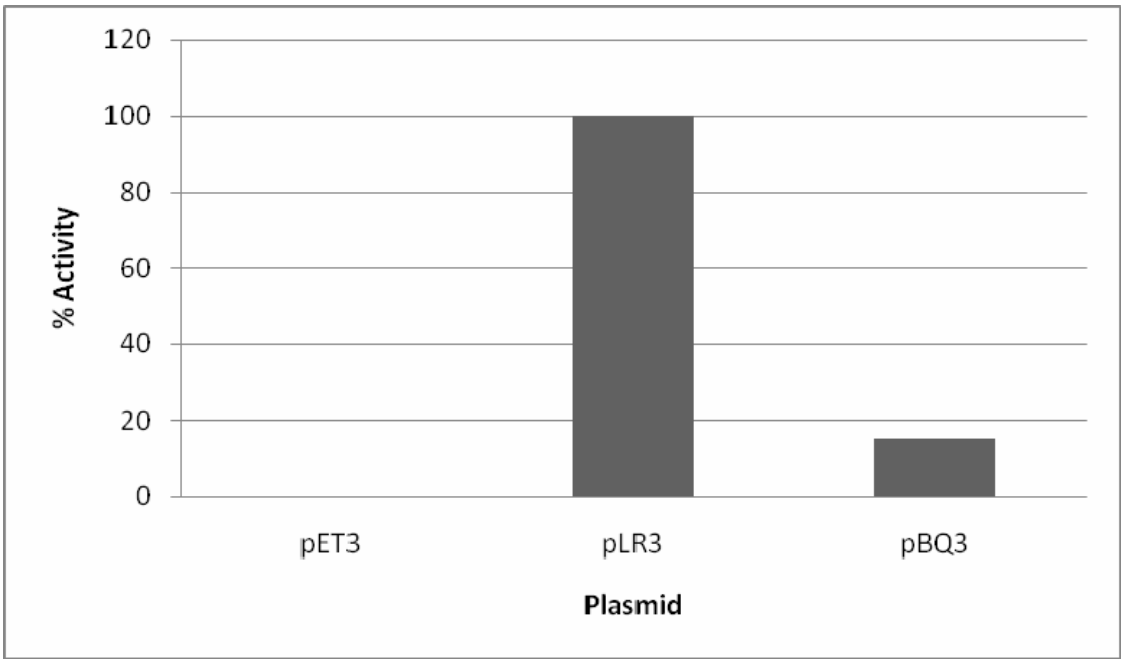


Figure 3.3. ***In vitro*** **activity of a C-terminally 6 X histidine tagged CL synthase.** Membranes from IPTG induced DG6/pLysS containing the plasmids shown were isolated as described in section 2.13, and *in vitro* activity assays were run as described in section 2.19. Activity was determined relative to pLR3. Plasmids: 1. pET3, vector control; 2. pLR3, wild type CL synthase; 3. pBQ3, C-terminally 6 X histidine tagged CL synthase.

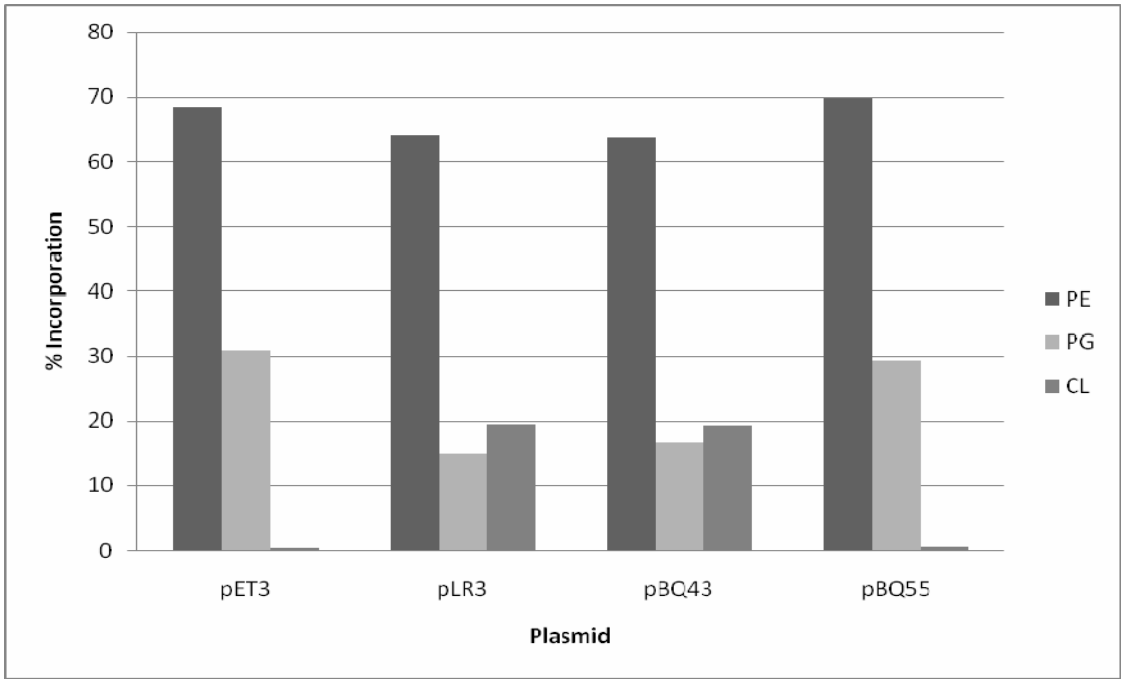


Figure 3.4. [^{14}C]Acetate incorporation into glycerophospholipid of QC30-15 containing wild type or EE epitope tagged CL synthase. Strain QC30-15 containing the plasmids listed was analyzed for in vivo activity as described in section 2.10. Plasmids: 1. pET3, vector control; 2. pLR3, wild type non epitope tagged CL synthase; 3. pBQ43, EE epitope tagged CL synthase; 4. pBQ55, EE epitope tagged CL synthase with H224A.

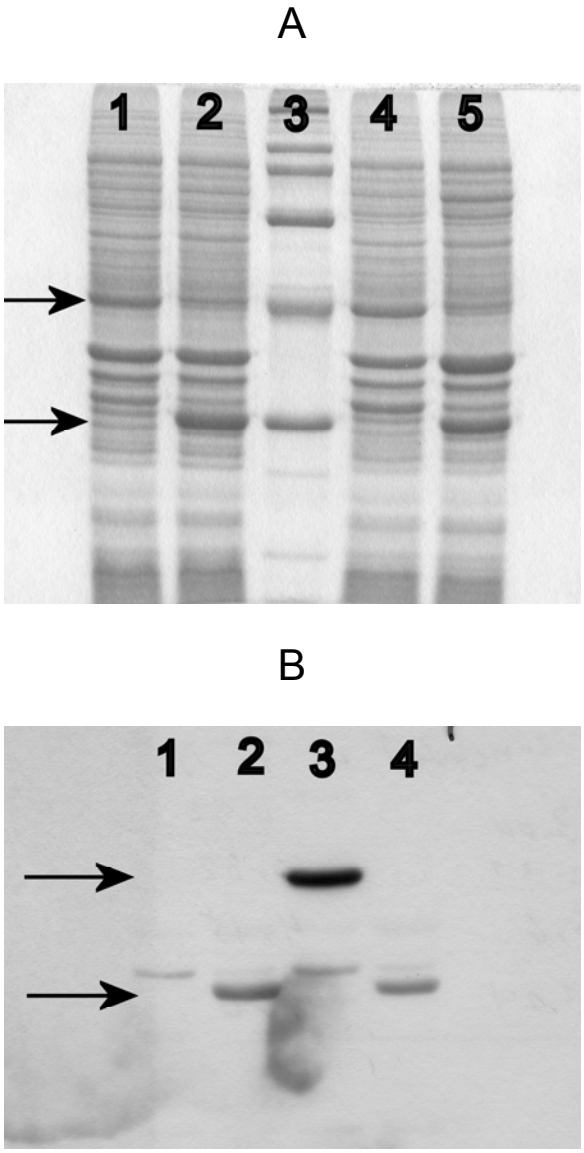


Figure 3.5. SDS PAGE and western blot analysis of wild type and EE epitope tagged CL synthase and frameshift 2-20 mutants. Approximately 15 μg of membrane from IPTG induced DG6 containing the plasmid of interest were analyzed by SDS PAGE (A) and western blot (B) as described in sections 2.16 and 2.17 respectively. The upper arrow in both figures shows the migration of CL Synthase coded by pLR3 (wild type CL synthase) and pBQ43 (EE epitope tagged CL synthase). The lower arrow shows a new band that migrates with an apparent molecular mass of approximately 20 kDa in pBQ63 (non epitope tagged frameshift 2-20) and pBQ56 (EE epitope tagged frameshift 2-20) as described in section 3.11.4. (A) Lanes: 1. pLR3, wild type CL synthase; 2. pBQ63, wild type CL synthase with frameshift 2-20 mutation; 3. Molecular mass standard, 4. pBQ43, 5. pBQ56. (B) Lanes: 1. pLR3, 2. pBQ63, 3. pBQ43, 4. pBQ56.

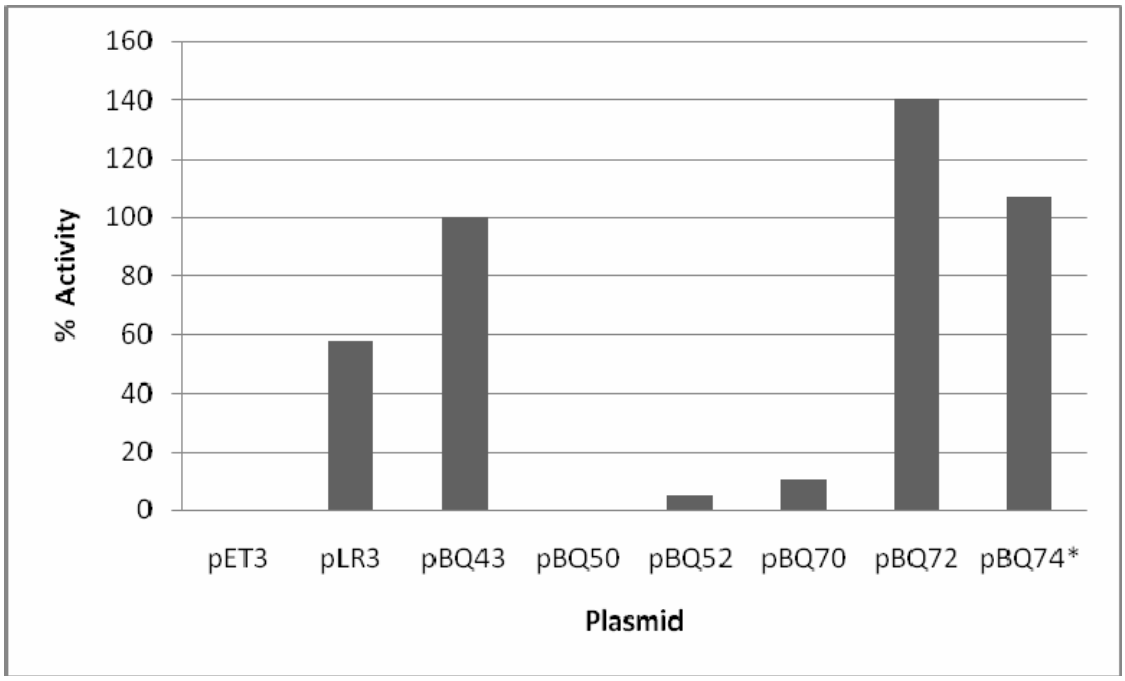


Figure 3.6. ***In vitro*** **activity of selected EE epitope tagged CL synthase constructs showing no** ***in vivo*** **activity.** DG6 containing the plasmid of interest was induced with IPTG and the membranes were prepared and assayed as described in materials and methods. Assays were performed in quadruplicate using 600 ng of TX-100 soluble membrane protein as described in section 2.19. Percent activity was determined relative to pBQ43. Plasmids: 1. pET3, vector control; 2. pLR3, wild type CL synthase; 3. pBQ43, EE epitope tagged CL synthase; 4. pBQ50, EE epitope tagged CL synthase frameshift 41-44; 5. pBQ52, W40A P48A; 6. pBQ70, KRR30-32TTT; 7. pBQ72, LV7-8SS; 8. pBQ74, CL synthase 2-60 deletion.
*Membranes were isolated from IPTG induced DG6/pLysS.

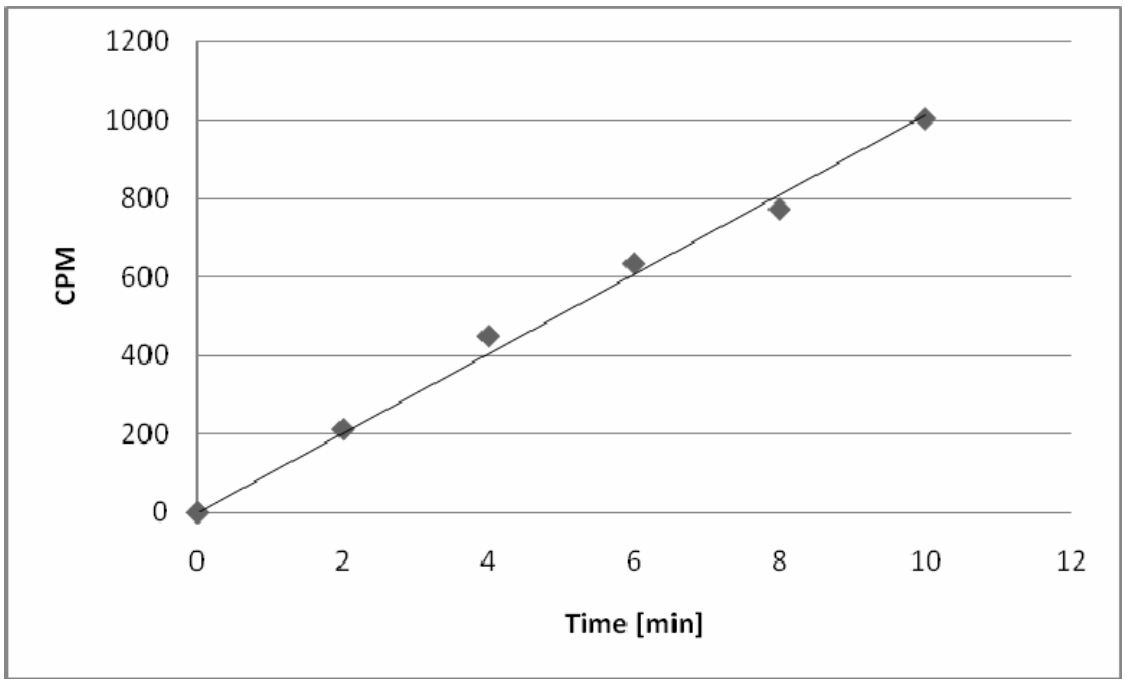


Figure 3.7. Linearity with time of the *in vitro* CL synthase activity assay. Assays to determine the linearity of reaction were performed as described in section 2.19.

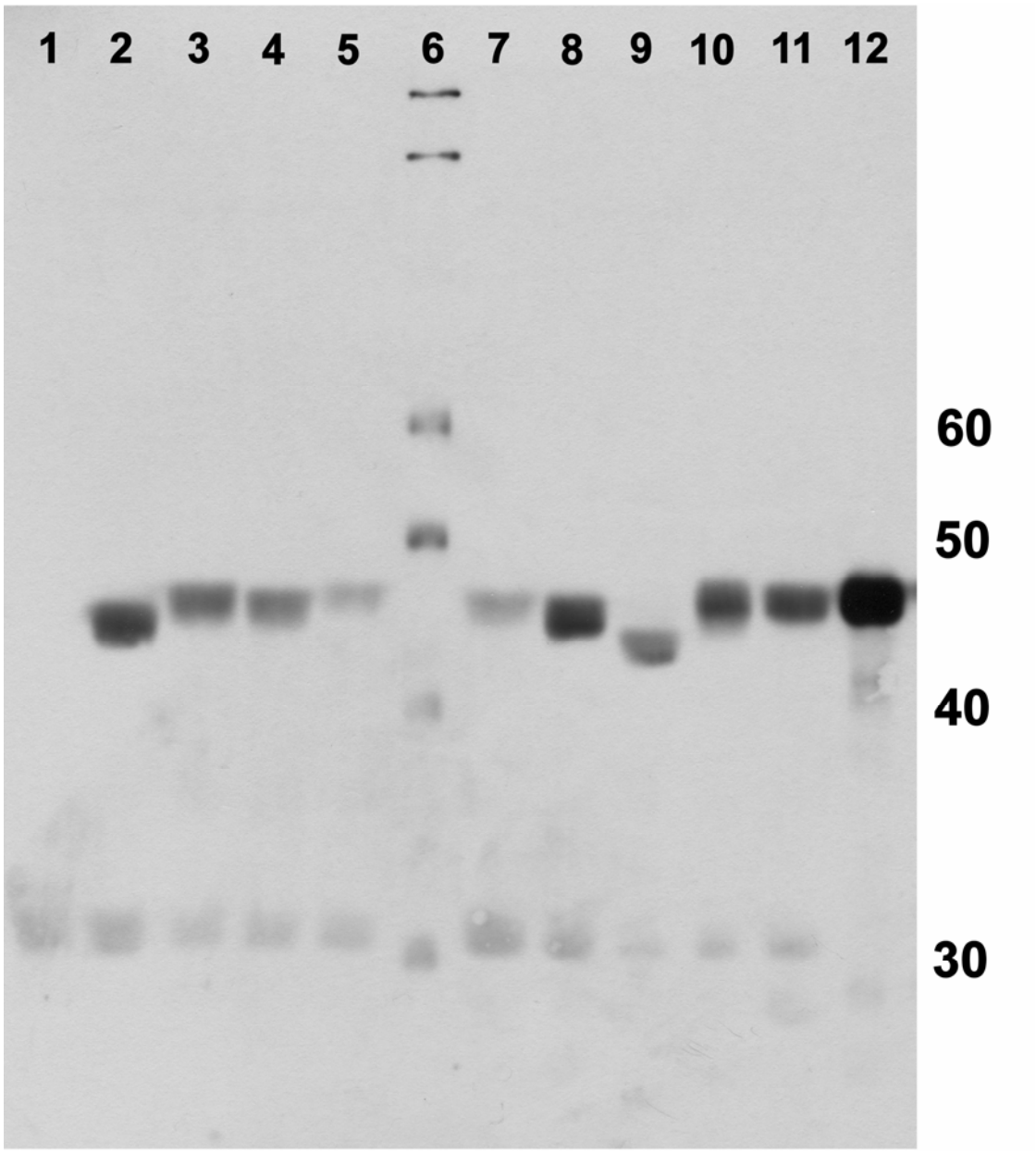


Figure 3.8. **Western blot 1 of EE epitope tagged CL synthase constructs.** DG6 containing the plasmid of interest was induced with IPTG and whole cells were analyzed for expression of an EE tagged CL synthase as described in materials and methods. Selected molecular masses of the Benchmark 6 X histidine standard ladder [kDa] are listed on the right side of the figure. Lanes : 1. pLR3, non-epitope tagged wild type CL synthase; 2. pBQ43, EE epitope tagged CL synthase; 3. pBQ44, W40A; 4. pBQ45, P48A; 5. pBQ50, frameshift 41-44; 6. 6 X Histidine standard ladder, 7. pBQ52, W40A P48A; 8. pBQ69, L19S; 9. pBQ70, KRR30-32TTT; 10. pBQ72, LV7-8SS; 11. pBQ73, LI19-20SS; 12. pLysS/pBQ74, CL synthase 2-60 deletion.

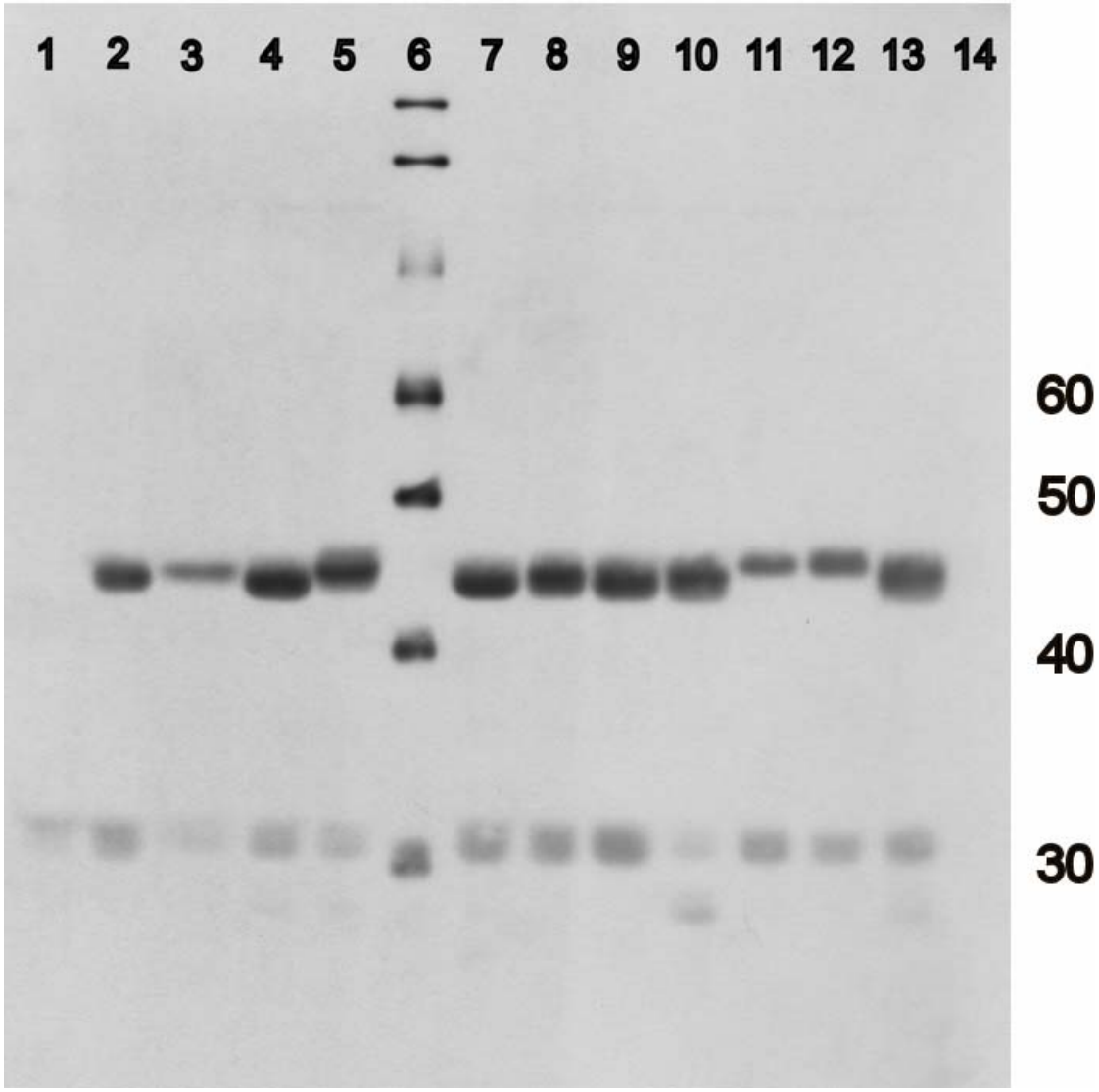


Figure 3.9. Western blot 2 of EE epitope tagged CL synthase constructs. DG6 containing the plasmid of interest was induced with IPTG and whole cells were analyzed for expression of an EE tagged CL synthase as described in materials and methods. Selected molecular masses of the Benchmark 6 X histidine standard ladder [kDa] are listed on the right side of the figure. Lanes: 1. pLR3, non-epitope tagged wild type CL synthase; 2. pBQ43, EE epitope tagged CL synthase; 3. pBQ46, Y55A; 4. pBQ47, G59A; 5. pBQ48, S36D; 6. 6 X histidine standard ladder, 7. pBQ59, frameshift 72-82; 8. pBQ61, R66A; 9. pBQ62, R70A; 10. pBQ66, R66A R70A; 11. pBQ67, LII42-44SSS; 12. pBQ68, II52-53SS; 13. pBQ71, KRR65-67SSA; 14. pLysS/pBQ40, non-epitope tagged wild type CL synthase

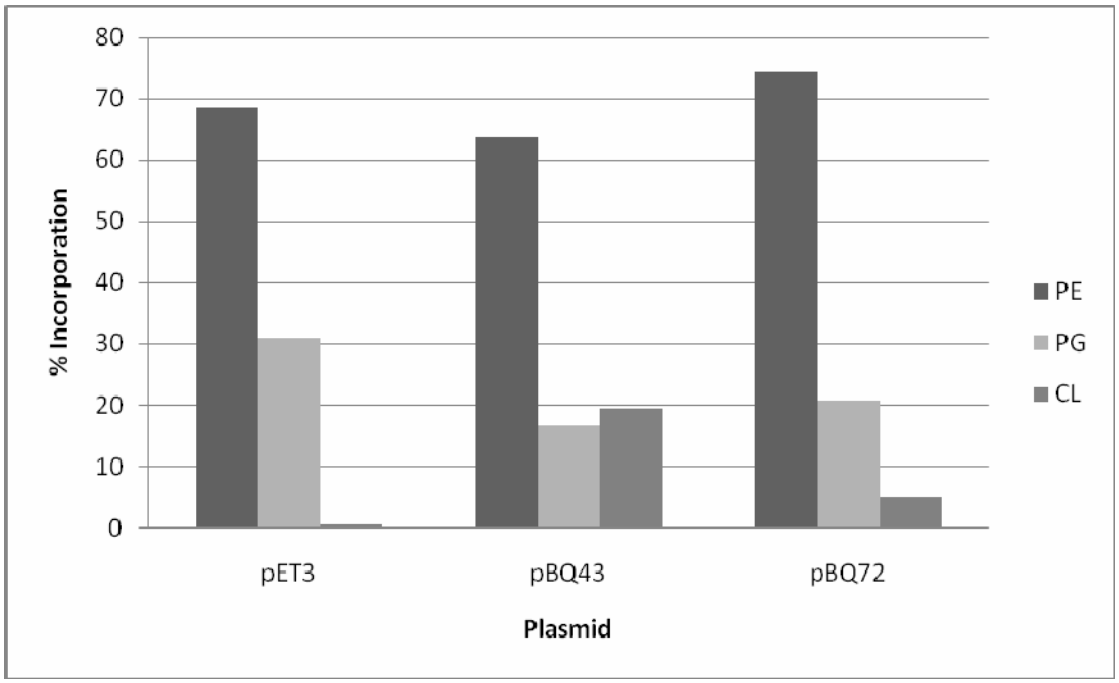


Figure 3.10. [^{14}C]Acetate incorporation into glycerophospholipid of QC30-15 containing a CL synthase with a mutation to the N-terminus resulting in an increase in apparent molecular mass and a suspected alteration in membrane topology. Strain QC30-15 containing the plasmids listed was analyzed for *in vivo* activity as described in section 2.10. Plasmids: 1. pET3, vector control; 2. pBQ43, EE epitope tagged CL synthase; 3. pBQ72, LV78-SS.

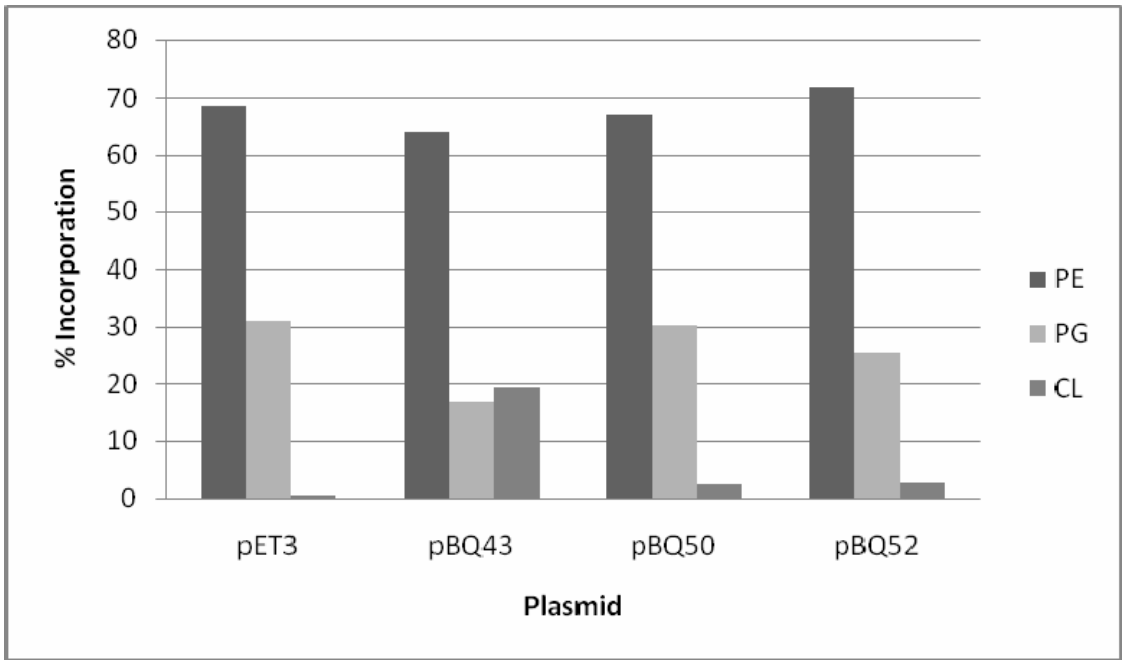


Figure 3.11. **[^{14}C]Acetate incorporation into glycerophospholipid of QC30-15 containing a CL synthase with a mutation to the N-terminus resulting in an increase in apparent molecular mass and a loss of activity *in vivo* and *in vitro*.** Strain QC30-15 containing the plasmids listed was analyzed for *in vivo* activity as described in section 2.10. Plasmids: 1. pET3, vector control; 2. pBQ43, EE epitope tagged CL synthase; 3. pBQ50, frameshift 41-44; 4. pBQ52, W40A P48A.

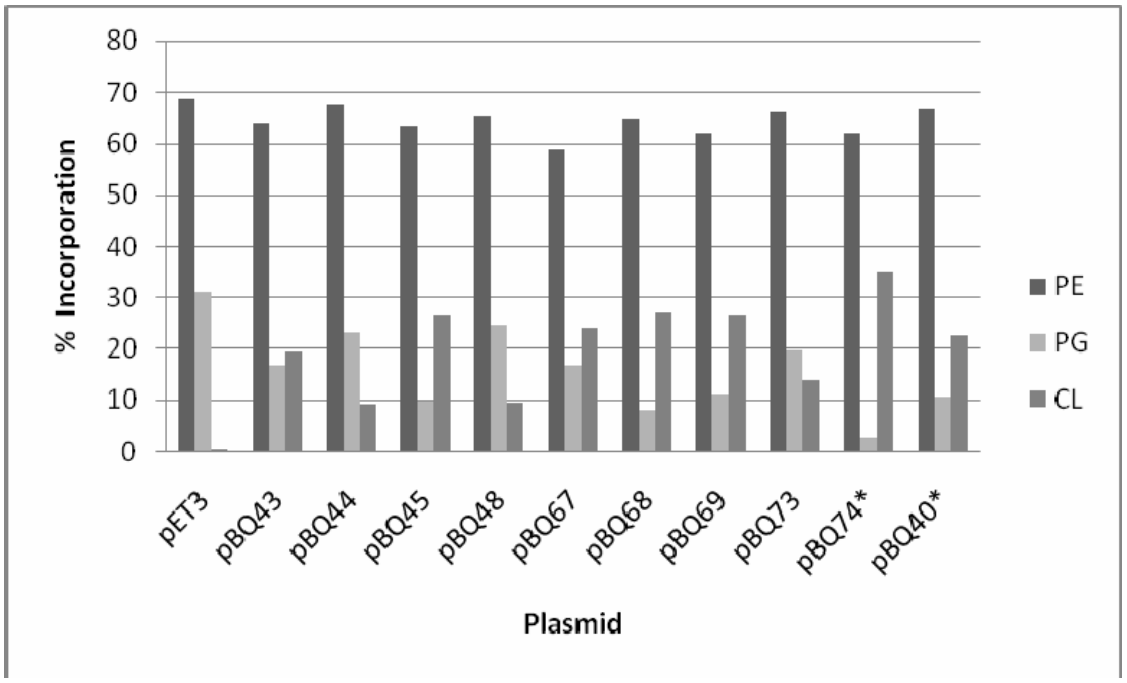


Figure 3.12. [^{14}C]Acetate incorporation into glycerophospholipid of QC30-15 containing a CL synthase with a mutation to the N-terminus resulting in an increase in molecular mass but not a loss of activity *in vivo*. Strain QC30-15 with the plasmids listed was analyzed for *in vivo* activity as described in section 2.10. Plasmids: 1. pET3, vector control; 2. pBQ43, EE epitope tagged CL synthase; 3. pBQ44, W40A; 4. pBQ45, P48A; 5. pBQ48, S36D; 6. pBQ67 LII42-44SSS; 7. pBQ68, II52-53SS; 8. pBQ69, L19S; 9. pBQ73, LI19-20SS; 10. pBQ74, CL synthase with a 2-60 deletion. 11. pBQ40, non-epitope tagged CL synthase in pET-23a(+).
*Experiment performed in DG6/pLysS

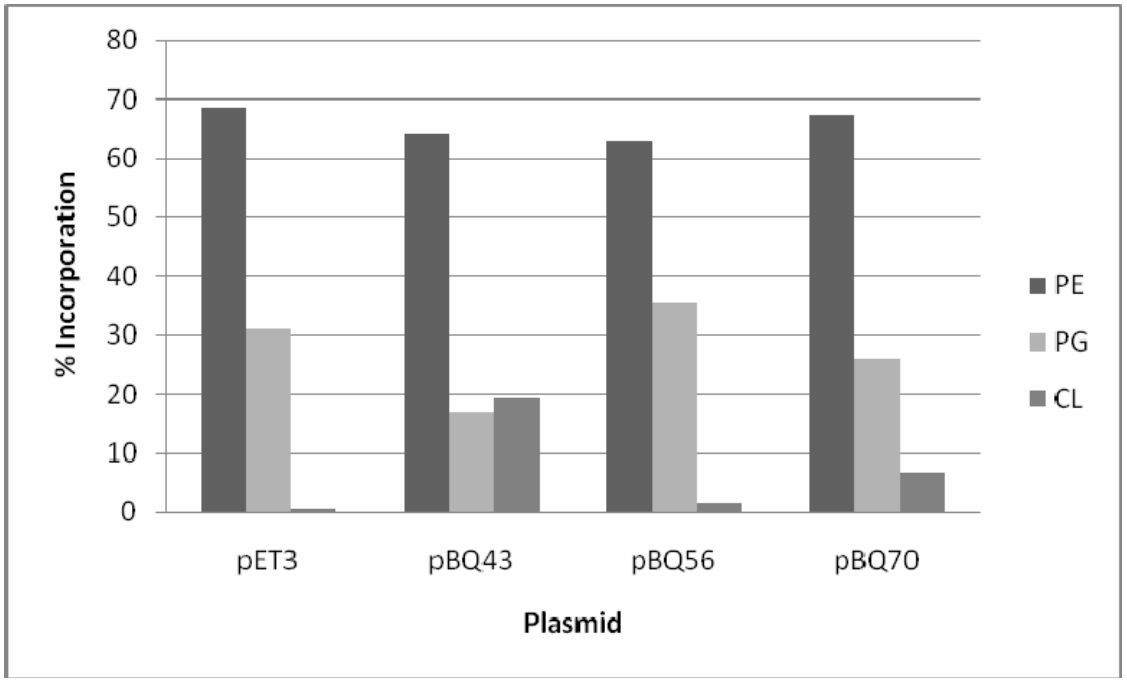


Figure 3.13. [^{14}C]Acetate incorporation into glycerophospholipid of QC30-15 containing a CL synthase with a mutation to the N-terminus resulting in a decrease in molecular mass and a loss of activity *in vivo*. Strain QC30-15 with the plasmids listed was analyzed for *in vivo* activity as described in section 2.10. Plasmids: 1. pET3, vector control; 2. pBQ43, EE epitope tagged CL synthase; 3. pBQ56, frameshift 2-20; 4. pBQ70, KRR30-32TTT.

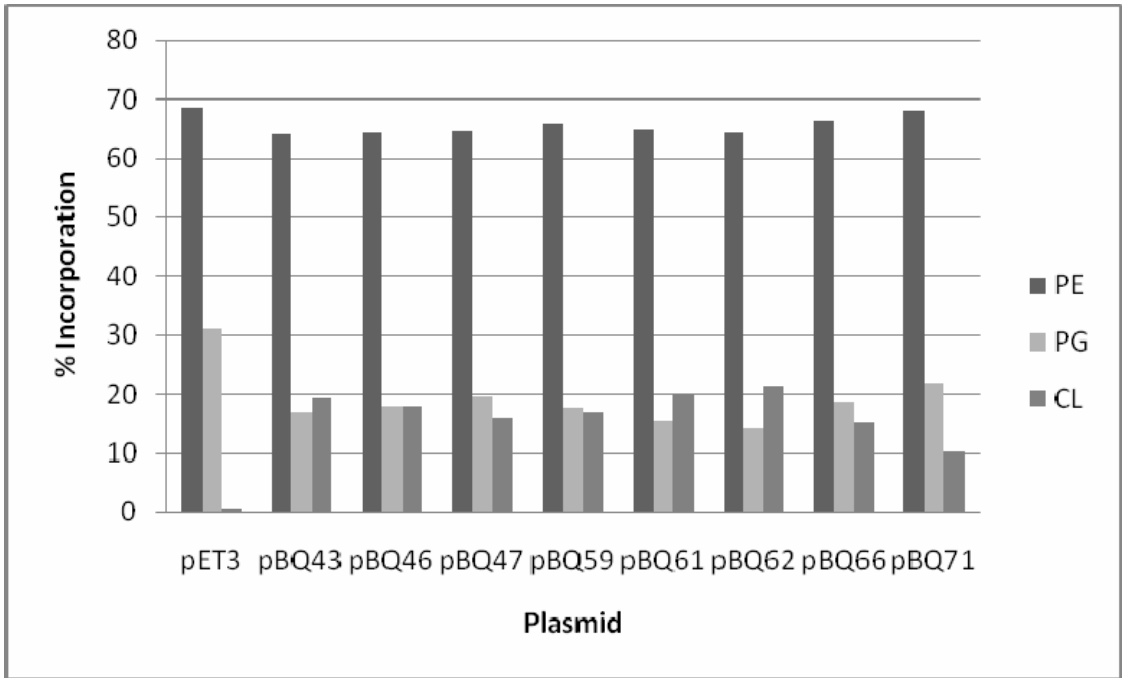


Figure 3.14. [^{14}C]Acetate incorporation into glycerophospholipid of QC30-15 containing a CL synthase with a mutation to the N-terminus resulting in little to no effect. Strain QC30-15 with the plasmids listed was analyzed for *in vivo* activity as described in section 2.10. Plasmids: 1. pET3, vector control; 2. pBQ43, EE epitope tagged CL synthase; 3. pBQ46, Y55A; 4. pBQ47, G59A; 5. pBQ59, frameshift 72-82; 6. pBQ61, R66A; 7. pBQ62, R70A; 8. pBQ66, R66A R70A; 9. pBQ71, KRR65-67SSA.

4.0. Discussion

In order to be catalytically active, CL synthase likely adopts a tertiary structure [22] characteristic of members of the phospholipase D (PLD) superfamily, bringing the 2 HKD ($HXK(X)_4D(X)_6G(X)_2N$) motifs [18-21] within the primary sequence into close proximity. This correct tertiary structure allows the PLD superfamily member CL synthase to bind two identical phosphatidylglycerol molecules, both of which have a charged phosphate group and two relatively long acyl chains. The two HKD motifs of CL synthase span amino acid residues 224-242 and 404-422 of the primary sequence respectively. The C-terminal 6 X histidine tag occupies positions 487-492 of the primary sequence of the fusion protein. The loss of activity of the 6 X histidine tagged CL synthase *in vitro* and *in vivo* may be the result of (i) an inability of the enzyme to achieve proper tertiary structure caused by addition of the tag, (ii) steric interference at the active site between the hydrophilic tag and the substrate molecule(s), (iii) improper membrane topology due to an increased energy barrier of translocation of seven hydrophilic residues (EHHHHHH) across the cell membrane, or some combination of these or other factors. The fact that (i) activity cannot be restored by alteration of the introduced glutamate residue, (ii) full activity is restored to the protein upon removal of the tag, and (iii) the fusion protein has low *in vitro* activity suggests that an incorrect tertiary structure rather than incorrect membrane topology is the cause of lack of activity.

The formation of lightly speckled colonies when QC25/pBQ3, is cultured at 42°C suggests that a small amount of CL synthase activity might be present. It is

possible that these colonies contain plasmids with a mutant *cls* that codes for a protein with one or more altered amino acids, which allows the enzyme to be catalytically active. However, it is also possible that these mutants are *pssA1* revertants, or mutations in which the histidine tag has been removed by restoration of the natural C-terminal stop codon. Another possibility is that a secondary mutation in the cell chromosome has occurred that somehow allows altered proteins to function, or allows the cell to process cellular proteins other than the abnormal CL synthase in a normal way. Due to the uncertainties in testing and obtaining a plasmid coding for a catalytically active C-terminally histidine tagged CL synthase by selection of plasmids with unknown mutations, extensive characterization of these mutants was not performed.

The fact that the C-terminally 6 X histidine tagged CL synthase migrates on SDS PAGE with the apparent molecular mass expected for the fusion protein (46.5 kDa), and is visible by western blot, suggests that the construct is being expressed and processed by the cellular machinery normally. The detection of a posttranslationally processed C-terminally 6 X histidine tagged CL synthase migrating with this apparent molecular mass also indicates that the missing residues are removed from CL synthase N-terminally. As with the non-histidine tagged wild type enzyme, the fusion protein is membrane associated, and can be extracted with the nonionic detergent Triton® X-100, indicating that it does not exist within the cell as inclusion bodies. However, the normal processing of the histidine tagged CL synthase encoded by pBQ3 is brought into question by experiments involving 6 X histidine and EE tagged 2-20 and 2-42 frameshift

mutants. As shown in Table 3.2, IPTG induced DG6/pLysS/pBQ6 (6X histidine tagged frameshift 2-20) and DG6/pLysS/pBQ7 (6 X histidine tagged frameshift 2-42) show no inhibition and produce a CL synthase that migrate with an apparent increase in molecular mass of 1 kDa and 2.6 kDa respectively, relative to the 6 X histidine tagged CL synthase produced by IPTG induced strain DG6/pLysS/pBQ3, which shows full inhibition of growth. As shown in Table 3.7, IPTG induced DG6/pBQ56 (EE tagged frameshift 2-20), DG6/pBQ57 (EE tagged frameshift 2-42) and DG6/pBQ63 (non-epitope tagged frameshift 2-20) all produce a CL synthase that is degraded *in vivo,* and appears as a new protein migrating with an apparent molecular mass of 20 kDa on coomassie stained SDS PAGE gels. This new protein is not present in vector control samples, and is not recognized by an anti EE antibody, as shown Figure 3.5 for Bq56 and Bq63. This data indicates that the addition of the residues EHHHHHH to the C-terminus of CL synthase is responsible for the difference in processing of wild type and histidine tagged frameshift 2-20 and 2-42 mutants *in vivo*. As such, it may be the case that the C-terminally 6 X histidine tagged CL synthase with a wild type signal region is processed in a manner different from that of the wild type enzyme encoded by pLR3.

It has been shown that removal of 60 residues from the C-terminus of CL synthase renders the protein inactive [16], while an N-terminal deletion of 60 residues retains activity [17]. The fact that the addition of 7 residues to the C-terminus of this protein results in the loss of activity and incorrect processing of

CL synthase was unexpected, and demonstrates the importance of the C-terminus of this enzyme for proper enzymatic function.

The introduction of an EE epitope (EYMPME) to the primary sequence of CL synthase as described in section 2.7 overcame the problems associated with the use of a C-terminal 6 X histidine tag to study CL synthase. The EE epitope tagged CL synthase encoded by pBQ43 behaved similarly to the untagged wild type CL synthase encoded by pLR3 as described in section 3.6.1.

Mutations to the N-terminus of CL synthase resulted in proteins that were grouped into the 5 categories: (i) mutations resulting in an increase in apparent molecular mass, and a loss of activity *in vivo* but not *in vitro*, suggesting an alteration in membrane topology, (ii) mutations resulting in an increase in apparent molecular mass and a loss of activity *in vivo* and *in vitro,* (iii) mutations resulting in an increase in apparent molecular mass but not a loss of activity, (iv) mutations resulting in a decrease in apparent molecular mass and a loss of activity *in vivo* and *in vitro,* and (v) mutations resulting in little to no effect on the apparent molecular mass or activity of the mutant CL synthase.

The most interesting mutation found (Bq72) replaced two conserved hydrophobic residues in the first transmembrane region with polar uncharged residues of similar side chain length (LV7-8SS). The resulting CL synthase migrates at an increased apparent molecular mass relative to the EE epitope tagged CL synthase (Bq43), and is inactive *in vivo*, but active *in vitro*. Furthermore, the amount of *in vitro* CL synthase activity in membranes isolated from IPTG induced DG6/pBQ72 is greater than that of membranes obtained from

IPTG induced DG6/pBQ43 (EE tagged CL synthase). These results suggest that CL synthase with this mutation exists in the cell with an altered membrane topology. One method to examine whether or not CL synthase with the mutation LV7-8SS exists within the cell with an alteration in membrane topology would be to examine IPTG induced DG6/pBQ43 and DG6/pBQ72 with a protease assay [26]. It is interesting to note that the CL synthase encoded by pBQ72 (LV7-8SS) is active *in vitro* but has little to no activity *in vivo,* while the CL synthase encoded by pBQ73 (LI19-20SS) has full activity *in vivo.* The doubling time of QC30-15/pBQ72 is approximately 2.3 times that of QC30-15/pBQ43 (EE tagged CL synthase), while the doubling time of QC30-15/pBQ73 is identical to that of QC30-15/pBQ43. Both of these plasmids encode a CL synthase with mutations of 2 adjacent conserved hydrophobic residues within the first transmembrane α-helix, comprised of residues 5-25, to serine. Furthermore, IPTG induced DG6/pBQ72 and DG6/pBQ73 produce a CL synthase that migrates with an apparent molecular mass of 47.1 kDa when analyzed by SDS PAGE and western blot. Experiments with these constructs suggest that Bq72 has an altered membrane topology while Bq73 has normal membrane topology.

A CL synthase with a deletion of residues 2-60 (Bq74) is missing 20 out of the 22 conserved residues within the N-terminus, yet it remains membrane associated, and retains *in vitro* and *in vivo* activity. The conserved residues within the N-terminus of CL synthase that are not removed by this mutation, R66 and R70, have been shown to have no effect independently (pBQ61, R66A; pBQ62, R70A), or together (pBQ66, R66A R70A), on the processing and activity

of CL synthase. It is interesting that CL synthase missing such a large number of conserved residues is directed to the membrane, translocated, and then correctly folded to an active tertiary structure. The results of this construct also confirm that CL synthase missing these residues should be able to function, consistent with the post translational removal of approximately 80 residues in the wild type protein. However, the question of how CL synthase missing the first 60 residues is correctly translocated to the membrane remains to be answered. It is interesting to note that IPTG induced DG6/pLysS/pBQ74 does not show inhibition of growth, while DG6/pLysS/pBQ40, which codes for the full length CL synthase (see section 2.6) shows full inhibition and a decrease in Klett, consistent with cell death. Furthermore, IPTG induced DG6/pLysS/pBQ74 produces approximately twice the amount of CL synthase compared to DG6/pLysS/pBQ40 or DG6/pBQ43 under the same conditions, as determined by visual inspection of coomassie stained SDS PAGE gels. It has been suggested that decreased cell viability upon IPTG induction of strains bearing an inducible CL synthase is a consequence of the accumulation of CL synthase in the cell membrane [10,12]. However, the experimental evidence that CL synthase missing residues 2-60 (Bq74) is membrane associated and does not cause a loss in cell viability, even when expression levels are twice that of wild type CL synthase, disputes this hypothesis. This data is consistent with the theory that the decrease in cellular viability can be correlated to a jamming of cellular processing machinery responsible for the posttranslational processing of not only CL synthase, but other proteins essential to cell growth and survival [49]. The

results obtained for pBQ74 (EE tagged 2-60 deletion mutant) are slightly at odds with previous research performed in this laboratory with a non-epitope tagged CL synthase 2-60 deletion mutant, which showed activity *in vitro* but not *in vivo* [17]. The reason for this discrepancy requires further investigation. One possibility is that the introduction of the epitope slightly alters the properties of CL synthase. In order to test this hypothesis, a plasmid encoding a non-epitope tagged 2-60 deletion mutant will be constructed as previously described [17]. The resulting protein will be examined by SDS PAGE and western blot to determine whether the epitope and non-epitope tagged CL synthase 2-60 deletions migrate with the same apparent molecular mass. The *in vivo* and *in vitro* activity of epitope and non-epitope tagged CL synthase 2-60 deletion mutants will also be compared as described in sections 2.10 and 2.19.

Strains QC30-15/pBQ50 (frameshift 41-44), QC30-15/pBQ52 (W40A P48A), QC30-15/pBQ67 (LII42-44SSS), QC30-15/pBQ68 (II52-53SS) and QC30-15/pBQ72 (LV7-8SS) show a decreased growth rate relative to that of QC30-15/pBQ43 (EE tagged CL synthase). The decreased growth rates of these strains are due to the presence of a CL synthase with one or more mutations within the N-terminus. The fact that these differences in growth are seen in QC30-15 and not in QC25 transformed with the same plasmid is likely due to the plasmid copy numbers in these strains. QC25 is *pcn*B80, which lowers the plasmid copy number to one or two copies in cells. Therefore, results with this strain more closely reflect transcription from a single chromosomal gene. The fact that QC25 has fewer plasmid copies than QC30-15 means that fewer copies

of the mutant protein are transcribed in the cell relative to QC30-15. Therefore, it is possible that the increase in doubling time seen in these strains is due to a jamming of the cellular processing machinery [49] due to an increase in transcription of CL synthase mutants in QC30-15 resulting from increased number of plasmids.

Some mutations made to the N-terminus of CL synthase appear to affect the *in vivo* half-lives of the CL synthase mutants produced in strain DG6. Mutant CL synthases encoded by pBQ56 (frameshift 2-20), pBQ57 (frameshift 2-42), and pBQ63 (non-epitope tagged frameshift 2-20) are degraded immediately in the cells, while others, like pBQ50 (frameshift 41-44) and pBQ52 (W40A P48A) show reduced signals on SDS PAGE and western blot as determined by visual inspection. This suggests that the *in vivo* half-lives of the CL synthases encoded by pBQ50 and pBQ52 may be reduced relative to that of the EE epitope tagged CL synthase encoded by pBQ43.

The cellular function performed (if any) by the signal peptide itself remains to be investigated. It is possible that this peptide, comprised of the approximately 80 residues cleaved from the N-terminus of CL synthase, plays a role in the regulation of CL synthase or performs some other function in the cell.

This research has shown that mutations to conserved residues within the N-terminus of CL synthase result in alteration of the molecular mass and activity of the mutant protein. The most interesting mutant, Bq72 (LV7-8SS), migrates at an increased apparent molecular mass relative to the EE epitope tagged CL synthase (Bq43) and has activity *in vitro* but not *in vivo*, suggesting that the

protein exists in the cell with an altered membrane topology. Further research with this mutant will employ the use of a protease assay [26] to examine its membrane topology. Protease assays take advantage of the fact that the protease cannot cross an intact cell membrane. If Bq72 exists with an altered membrane topology, with its catalytic site facing the cytoplasm, then it should not be degraded by the protease. The EE tagged CL synthase with a wild type N-terminus (Bq43), which is predicted to have a periplasmic orientation [28], should be degraded by the protease. Thus, analysis by western blot of whole cells treated with protease will show a signal corresponding to CL synthase with a cytoplasmic membrane topology, but not for CL synthase with a periplasmic orientation.

An attempt will be made to purify both the EE epitope tagged CL synthase (Bq43) and the CL synthase encoded by pBQ72 (LV78-SS) using an agarose immobilized anti EE antibody (Abcam). If successful, a new attempt will be made to sequence these proteins by Edman degradation or some other means.

APPENDIX

Table A1
Properties of CL synthase encoded by plasmid constructs

Plasmid	Description/mutation	Apparent molecular mass [kDa][a]	Inhibition 0.8 mM IPTG[a]	Growth 42°C[b]	Growth 30°C[b]	% CL[c]	% PG[c]	% PE[c]
pBQ43	EE epitope tagged CL Synthase	45.3	-	+	+	19.35	16.76	63.88
pBQ44	W40A	46.9	-	+	+	9.43	23.19	67.36
pBQ45	P48A	46.4	-	+	+	26.62	9.84	63.53
pBQ46	Y55A	45.6	-	+	+	17.86	17.78	64.35
pBQ47	G59A	45.3	-	+	+	15.82	19.57	64.6
pBQ48	S36D	45.8	-	+	+	9.58	24.83	65.57
pBQ50	Frameshift 41-44	47.4	+	-	+	2.65	30.25	67.09
pBQ52	W40A,P48A	46.9	-	-	+	2.84	25.29	71.86
pBQ55	H224A	45.3	-	-	+	0.77	29.33	69.88
pBQ56	Frameshift 2-20	20	-	-	+	1.4	35.55	63

pBQ57	Frameshift 2-42	20	-	-	+	NA	NA	NA
pBQ59	Frameshift 72-82	45.3	-	+	+	16.67	17.47	65.85
pBQ61	R66A	45.3	-	+	+	19.71	15.61	64.66
pBQ62	R70A	45.3	-	+	+	21.39	14.33	64.27
pBQ63	Non-epitope tagged Frameshift 2-20	20	-	-	+	NA	NA	NA
pBQ66	R66A, R70A	45.3	-	+	+	15.15	18.58	66.26
pBQ67	LII42-44SSS	46.5	-	-	+	24.26	16.81	58.72
pBQ68	II52-53SS	46.5	-	LS	+	27.09	8.04	64.85
pBQ69	L19S	46.1	-	+	+	26.66	11.27	62.06
pBQ70	KRR30-32TTT	44.1	-	-	+	6.71	25.95	67.32
pBQ71	KRR65-68SSA	45.3	-	+	+	10.16	21.84	67.99
pBQ72	LV7-8SS	47.1	-	-	+	4.78	20.69	74.52
pBQ73	LI19-20SS	47.1	-	+	+	13.95	19.9	66.13

pBQ74[d]	2-60 deletion mutant in pET-23a(+)	47.1	-	NA	NA	34.91	3	62.08
pLR3[g]	CL Synthase in pET3	45.3	-	+	+	19.62	14.85	64.16
pET3[h]	T7 Expression vector	NA	-	-	+	0.4815	30.99	68.52
pBQ3[d.e]	6 X His tagged CL Synthase in pET-23a(+)	46.5	+	LS	+	1.01	30.6	68.38
pBQ4[e,f]	6 X His tagged CL Synthase in pET-23a(+)	46.5	+	NA	NA	NA	NA	NA
pBQ6	6 X His tagged CL Synthase frameshift 2-20	47.5	+	-	+	NA	NA	NA
pBQ7	6 X His tagged CL Synthase frameshift 2-42	49.1	+	-	+	NA	NA	NA
pBQ30[d,e]	CL Synthase in pET-23a(+)	45.3	+	+	+	19.39	17.24	63.36
pBQ31[d]	6 X His tagged CL Synthase E487G	NA	NA	-	+	0.911	30.75	68.33
pBQ32[d]	6 X His tagged CL Synthase E487L	NA	NA	-	+	1.23	27.43	71.32

pBQ40[d,e]	CL Synthase in pET-23a(+)	45.3	+	NA	NA	22.58	10.8	66.6

[a] Experiment Performed in DG6 unless otherwise noted.
[b] Experiment performed in QC25.
[c] Experiment performed in QC30-15 unless otherwise noted.
[d] Experiment Performed in DG6/pLysS.
[e] Plasmid pBQ3 differs from pBQ4 and plasmid pBQ30 differs from pBQ40 as described in section 2.6.
f Experiment performed in DG6/pLysE
[g] See reference [11].
[h] See reference [24].
NA=Data not available / experiment not performed

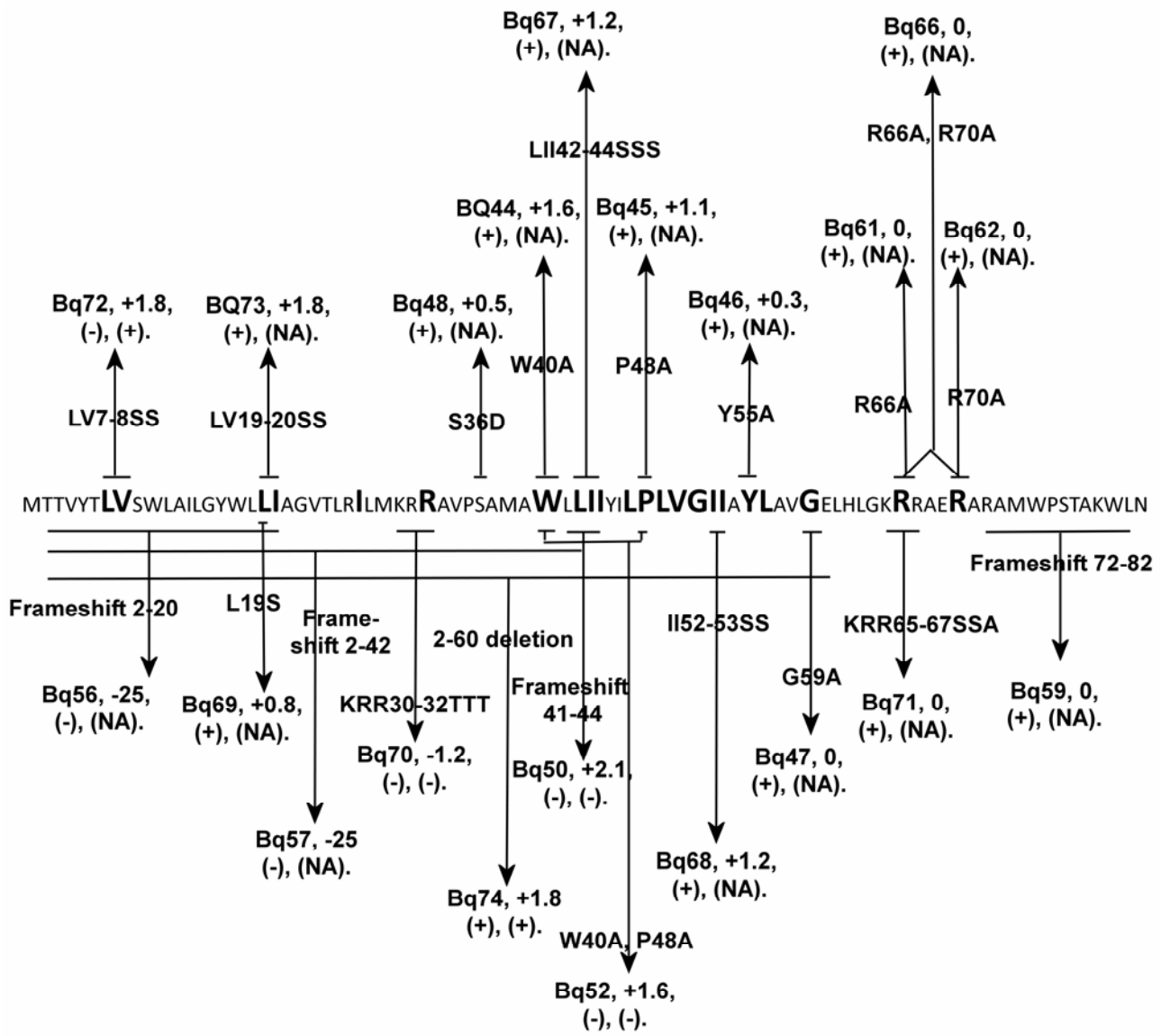

Bq67, +1.2,
(+), (NA).
Bq66, 0,
(+), (NA).
LII42-44SSS
R66A, R70A
BQ44, +1.6,
(+), (NA).
Bq45, +1.1,
(+), (NA).
Bq61, 0,
(+), (NA).
Bq62, 0,
(+), (NA).
Bq72, +1.8,
(-), (+).
BQ73, +1.8,
(+), (NA).
Bq48, +0.5,
(+), (NA).
Bq46, +0.3,
(+), (NA).
W40A
P48A
LV7-8SS
LV19-20SS
S36D
Y55A
R66A
R70A
MTTVYTLVSWLAILGYWLLIAGVTLRILMKRRAVPSAMAWLLIIYILPLVGIIAYLAVGELHLGKRRAERARAMWPSTAKWLN
Frameshift 72-82
Frameshift 2-20
L19S
Frame-
shift 2-42
2-60 deletion
II52-53SS
KRR65-67SSA
G59A
Bq56, -25,
(-), (NA).
Bq69, +0.8,
(+), (NA).
KRR30-32TTT
Frameshift
41-44
Bq71, 0,
(+), (NA).
Bq59, 0,
(+), (NA).
Bq70, -1.2,
(-), (-).
Bq50, +2.1,
(-), (-).
Bq47, 0,
(+), (NA).
Bq57, -25
(-), (NA).
Bq68, +1.2,
(+), (NA).
Bq74, +1.8
(+), (+).
W40A, P48A
Bq52, +1.6,
(-), (-).

Figure A1. Results of mutations made to the N-terminus of *E. coli* CL synthase. Conserved amino acid residues are shown as large font bold letters. Each horizontal line above or below the sequence indicates the area mutated in each construct. The mutation made appears along the arrow that points to the result. Key: Protein produced, difference in apparent molecular mass relative to the EE epitope tagged CL synthase (+/- kDa), activity *in vivo* (+/-), and activity *in vitro* (+/-). Data for EE tagged CL synthase encoded by pBQ43: Bq43, 45.3 kDa, (+), (+). With the exception of Bq74, mutant constructs showing activity *in vivo* were not analyzed for activity *in vitro*. The results observed for Bq63 are the same as those observed for Bq56 (see section 3.11.4). NA = Data not available / experiment not performed.

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