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# *E. coli* cardiolipin synthase: Function of N-terminal conserved residues

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ABSTRACT

The *E. coli cls* open reading frame (ORF) predicts a 54.8 kDa polypeptide, whereas mature cardiolipin (CL) synthase is 46 kDa. The N-terminal region extending to residue 60 contains several conserved residues but is not essential for enzyme activity. A deletion mutant that is missing residues 2–60 produces a fully active protein. These findings raise the question of why several residues in a region that is not required for enzyme activity are conserved. Recombinant DNA technology was used to introduce an EYMPE epitope (EE) tag into the interior of CL synthase. The EE tagged polypeptide retained the biological properties of wild type CL synthase, including full enzymatic activity. Site-directed mutagenesis was used to alter conserved residues in the N-terminal region. An EE tagged CL synthase in which Leu-7 and Val-8 were both replaced by Ser residues retains in vitro activity but loses most of its in vivo activity. Furthermore, the mutant protein has a higher apparent molecular mass than its parent protein. Taken together, these findings suggest that conserved residues L7 and V8 play a role in polypeptide processing, topology, or both.



## 1. Introduction

*Escherichia coli* (*E. coli*) cardiolipin synthase (CL synthase) catalyzes the reversible reaction between two phosphatidylglycerol molecules to form cardiolipin (CL) and glycerol [1–3]. The *E. coli* structural gene encoding CL synthase, *cls*, is located at min 28.02 of the *E. coli* genetic map [4]. The *cls* gene (GenBank accession numbers U15986, D38779, U01911 and L12044) has been cloned and placed under the control of a *tac* [5] or a T7 promoter [6], enabling over-expression of the protein. Translation of the 1458-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 to be membrane-spanning α-helices comprised of residues 5–25 and 36–56 [9,10]. 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 [6,11,12]. When 60 residues are deleted from the C-terminus, CL synthase has no enzymatic activity [13]. In contrast, when the first 60 residues are deleted from the N-terminus, CL synthase retains activity [14]. Taken together, these observations suggest that the N-terminal region is removed during mature CL synthase formation.

Many of the N-terminal residues of CL synthase are conserved in gram-negative bacteria (Fig. 1). The conservation of these amino acids is puzzling because the mature protein migrates with an apparent molecular mass that suggests the first 80 or so residues are cleaved [6,11,12]. 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 [15]. 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 cell membrane are incubated with 600 mM mannitol, two novel glycerophospholipids, phosphatidylmannitol and bisphosphatidylmannitol are formed [15]. These mannitol glycerophospholipids are not formed in mannitol transport deficient *cls-1* mutants, indicating that CL synthase is responsible for their synthesis [15].

The primary sequence of the CL synthase N-terminus does not readily conform to any known signal sequence, yet experimental evidence confirms that CL synthase is a membrane protein [6,11,12]. Proper insertion into the cell membrane is probably required to ensure that the catalytic site will be located on the periplasmic side of the inner membrane. The leader peptidase, another inner membrane associated protein that has its catalytic site in the periplasmic space [16] may be an instructive model when considering CL synthase orientation in the cell membrane. Two hydrophobic N-terminal segments of the leader peptidase are embedded in the cell membrane. Comparison of the N-terminal bacterial CL synthase sequence with

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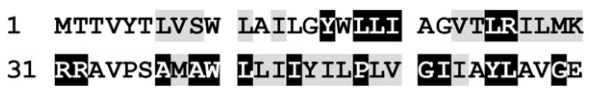


**Fig. 1.** The first 60 amino acid residues of *E. coli* CL synthase. Conserved residues are shown as white letters in a black background. Residues that are similar are shown as black letters in a gray background. The first and second transmembrane regions are predicted to extend from Y5 to L25 and S36 to L56, respectively [9,10]. Residue conservation was determined by CLUSTALW analysis of the following gram-negative bacteria: *Escherichia coli*, *Planctomyces maris*, *Desulfotalea psychrophila*, *Vibrio campbellii*, *Yersinia pestis*, *Buchnera sp.*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Xylella fastidiosa*, *Zymomonas mobilis subsp. mobilis*, and *Ureaplasma urealyticum*.

N-terminal leader peptidase sequences from nine gram-negative bacteria reveals four conserved residues[2]: L7, V8, R32 and G59. One or more of these conserved residues may be required for proper protein insertion into the cell membrane. Other residues that are conserved in CL synthase but not present in leader peptidase may be essential for posttranslational cleavage, because the *E. coli* leader peptidase is not modified in this fashion.

If conserved N-terminal residues are required for N-terminal cleavage and proper CL synthase insertion in the cell membrane, then altering these residues should affect cleavage, insertion, or both. Efforts to study CL synthase processing would be facilitated by the use of a tagged protein. Attempts to use a C-terminal 6 X Histidine tag were unsuccessful (B. R. Quigley in The N-terminal sequence of *E. coli* CL synthase: function(s) of the conserved residues. Ph.D. Thesis, City University of New York, 2007). Although the 6 X His tagged CL synthase is membrane associated, migrates on SDS PAGE at the predicted molecular mass of the mature protein, and is visible on western blot using an anti-6 X His antibody, the protein is inactive in vivo and in vitro. The fact that the inactive fusion protein retains the C-terminal tag even though it appears to undergo normal post-translational processing provides further support for the hypothesis that cleavage takes place at the N-terminus. The present report describes the construction of an EYMPE epitope (EE) tagged CL synthase and the use of this tagged protein to study the effects that altering conserved N-terminal residues have on CL synthase structure and function.

## 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), Folin and Ciocalteu's phenol reagent, brilliant blue G perchloric acid solution, 5× concentrate fixing solution, glycerol, Triton® X-100, NZ Amine A (casein enzymatic hydrolysate), BSA (bovine serum albumin fraction V), agarose, glycine (electrophoresis grade), SDS (sodium dodecyl sulfate), phenol, acrylamide:bis-acrylamide (29:1), TEMED (N,N,N′,N′-tetramethylethylenediamine), 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 15×15 cm PVDF (polyvinylidene difluoride) 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 endonucleases, T4 DNA ligase, calf intestinal phosphatase, and the 1 kb DNA Ladder were purchased from New England Biolabs, Ipswich, MA. Goat polyclonal Glu–Glu tag antibody horse radish peroxidase (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 was purchased from Invitrogen, Carlsbad, CA. The QuikChange® II Site-Directed Mutagenesis Kit was purchased from Stratagene, La Jolla, CA. The Qiagen Plasmid Midi Kit was purchased from Qiagen, Valencia, CA. Primers used to introduce mutations in *cls* were purchased from Sigma-Genosys, Woodlands, TX. [2-$^{14}$C] Acetate was purchased from ICN, Irvine, CA. Millipore 0.45 μm MF-Millipore™ membrane filters were from Millipore, Billerica, MA. Phosphatidyl[2-$^{3}$H]glycerol was prepared and purified as previously described [14]. Ecoscint 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 1. Plasmids used in this study are listed in Table 2. LB medium (1% bactotryptone, 0.5% NaCl, and 0.5% yeast extract) was used for most growth experiments. M9ZB medium [17] was used in IPTG induction experiments. Strain QC30-15 [8] and its transformants were cultured at 30 °C. Unless otherwise stated, all other strains were cultured at 37 °C. Ampicillin and chloramphenicol were used at 125 μg/mL and 20 μg/mL, respectively. Chloramphenicol concentrations were increased from 20 μg/mL to 40 μg/mL when cultures were incubated overnight at 37 °C without shaking. Where indicated, IPTG was added to a final concentration of 0.8 mM. Cell growth was monitored using a Klett-Summerson photometer fitted with a 660 nm filter. A Klett unit corresponds to approximately $5\times10^6$ cells/mL. Cells

**Table 1**
Bacterial strains used in this study.

| Strain | Parent | Genotype or description | Source or reference |
|---|---|---|---|
| XL1-Blue | | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacIqZΔM15* Tn*10* (TetR)] | Product of Stratagene, La Jolla, CA, USA |
| BL21 (DE3) | | F$^-$ *ompT hsdSB* ($r^-_B m^-_B$), *dcm gal* (DE3) | [17] |
| DG6 | BL21 (DE3) | *cls*::Tn*10*miniTet*3* | [14] |
| BQ1 | DG6 | Plasmid pLysS was introduced into DG6 | This study |
| QC30-15 | QC30 | MC4100 *glpR glpD pssA1 cls*::Tn*10* miniTet*3*; | [8] |

**Table 2**
Plasmids used in this study.

| Plasmid | Description/mutation | Source or reference |
|---|---|---|
| pET3 | T7 expression vector | [17] |
| pET-23a(+) | T7 expression vector containing T7 and 6× His tags | Product of Novagen |
| pLysS | Encodes T7 lysozyme that inhibits T7 RNA polymerase | [39] |
| pLR3 | CL synthase in pET3 | [6] |
| pBQ41 | CL synthase N296E | This work |
| pBQ42 | CL synthase N296E, I297Y | This work |
| pBQ43 | CL synthase N296E, I297Y, F299M (EE tagged) | This work |
| pBQ47 | EE tagged CL synthase G59A | This work |
| pBQ70 | EE tagged CL synthase KRR30-32TTT | This work |
| pBQ72 | EE tagged CL synthase LV7-8SS | This work |
| pBQ74[a] | EE tagged CL synthase 2-60 deletion | This work |
| pBQ75[a] | Non-epitope tagged CL synthase 2-60 deletion | This work |

[a] Transcription is dependent on the T7 RNA polymerase and translation is under the control of the pET-23a(+) strong RBS.

[2] *Escherichia coli*, *Shigella dysenteriae*, *Salmonella enterica*, *Enterobacter*, *Klebsiella pneumoniae*, *Serratia proteamaculans*, *Erwinia carotovora*, *Photorhabdus luminescens*, and *Vibrio harveyi*.