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CARDIOLIPIN SYNTHASES IN BACTERIA

By

DAGANG GUO

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.

2001

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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

CARDIOLIPIN SYNTHASES IN BACTERIA

By

Dagang Guo

Advisor: Professor Burton E. Tropp

The gene that codes for cardiolipin (CL) synthase in the alkaliphilic bacteria *Bacillus firmus* was cloned and sequenced. The predicted amino acid sequence of the gene product is homologous to *Escherichia coli* CL synthase. Both enzymes belong to a protein superfamily that also includes phospholipase D. The *B. firmus* CL synthase was amplified in *E. coli*. A membrane fraction containing the overproduced enzyme converts phosphatidylglycerol to CL and glycerol. The *B. firmus* CL synthase shows similar kinetic characteristics as that of its *E. coli* counterpart.

A *B. firmus* strain containing a *cls* null mutation was constructed. Little, if any, CL was detected in the mutant strain. The *cls* null mutant grows normally in rich medium at pH10.5, indicating a high concentration of CL is not essential for *B. firmus* to grow under alkaline conditions. The mutant strain showed some growth difficulties when malate (a non-fermentable carbon source), but not glucose (a fermentable carbon source) was used as the carbon source.

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The *E. coli* open reading frame *f413*, which has the potential to code for a polypeptide homologous to CL synthase, was cloned. Its polypeptide product has a molecular mass of 48 kDa, is membrane-bound, and catalyzes CL formation but does not hydrolyze CL. A comparison of the sequences predicted for the polypeptides encoded by *f413* and *cls* indicates that the N-terminal residues specified by *cls* may be unnecessary for CL synthase activity. Construction of a truncated *cls* gene and characterization of its polypeptide product have confirmed this conclusion.

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Most of all my family. My parents, my brother, for their encouragement throughout my graduate studies.

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ABBREVIATIONS

(CL)	Cardiolipin
(PG)	Phosphatidylglycerol
(IPTG)	Isopropyl β-D-thiogalacto-pyranoside
(TEMED)	N,N,N',N'-tetramethylethylenediamine
(PEG)	Polyethylene glycol
(TX-100)	Triton X-100
(CDP)	Cytidine-5'-diphosphate
(SDS)	Sodium dodecyl sulfate
(EDTA)	Ethylenediaminetetraacetate
(PAGE)	Polyacrylamide gel electrophoresis
(PCR)	Polymerase chain reaction
(PE)	Phosphatidylethanolamine
(BSA)	Bovine serum albumin
(STS)	Trace salts solution
(PLD)	Phospholipase D
(NBD)	7-nitro-2-1,3-benzoxadiazol-4-yl)aminododecanoyl
(PA)	Phosphatidate

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CHAPTER 1

Cloning of the *Bacillus firmus* OF4 *cls* gene and characterization of its gene product.

1. Introduction

Cardiolipin (CL), also called bisphosphatidylglycerol, is a unique phospholipid found both in bacteria and in inner mitochondrial membranes of eukaryotes. Its chemistry and biochemistry have been extensively studied. Bacteria form CL by transferring a phosphatidyl group from one phosphatidylglycerol (PG) molecule to another [1-4]. In eukaryotes, CL is formed by moving a phosphatidyl group from CDP-diacylglycerol to PG by a mitochondrial enzyme [5]. The structure of CL is shown in Figure 1.1.

CL has a variety of distinct chemical and physical properties. Many of its functions have been reviewed [6]. Its unique structure suggests it is important for membrane integrity, giving the membrane a coherent structure [6]. The polar backbone glycerol of cardiolipin is found to be more rigid than other phospholipid headgroups. Thus the presence of cardiolipin increases order in the membrane surface [7]. Cardiolipin is one of the more acidic phospholipids in membranes and may help conduct protons laterally along the membranes with its fatty acyl saturation modulating this conduction [8]. Close examination reveals that cardiolipin has unusual pH-dissociation characteristics [9]. It is found to have two pK values, pK_1 at 2.8 and pK_2 at 7.5-9.5. The unusually high pK_2 may be due to the formation

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of a cyclic intramolecular hydrogen-bonded structure between the free hydroxyl group on the central glycerol and one protonated phosphate. This suggests that CL may function as a reservoir of protons at relatively high pH. A protonophore has been proposed to conduct protons across the membrane [10]. A bilayer to hexagonal H_{II} transition could be the mechanism for the formation of protonophores [10]. CL molecules rich in polyunsaturated fatty acid chains can easily form a H_{II} phase [10]. It is known that divalent cations [11] or NaCl [12] induce the H_{II} phase in cardiolipin model systems. However, membrane proteins apparently override ion effects [13]. CL has been found largely in mitochondrial inner membranes and bacterial plasma membranes (inner membranes for Gram-negative bacteria envelopes) [6]. The fact that both membranes are considered "living membranes" (biological membranes that actively transport cations, maintain a membrane potential and engage in energy transduction.) suggests a special role of cardiolipin in carrying out biological functions in living cell membranes besides its structural role. In this regard, it is interesting to observe that in mitochondria CL is associated with the major proteins of oxidative phosphorylation, as well as the carrier proteins for phosphate and adenine nucleotides [14, 15].

E. coli cardiolipin is synthesized by a membrane-bound enzyme, cardiolipin synthase. The structural gene for this enzyme, *cls*, has been cloned [16,17], sequenced (GenBank accession numbers U15986, D38779 and U01911), and placed under the control of a *tac* promoter [18] or a T7 promoter [19], allowing cells

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to overproduce CL synthase. The purified enzyme is stimulated by potassium phosphate and Triton X-100 [18]. When examined in a mixed micelle assay, *E. coli* CL synthase is inhibited by CL, the product of the reaction, and by phosphatidate [19].

The gram-positive strain *Bacillus firmus* OF4 is an alkaliphilic strain [20] in which CL accounts for approximately 25% of total glycerophospholipids [21]. The fact that *B. firmus* OF4 has a relatively high CL content and the ability to grow at pH 10.5 suggests that it might be an ideal strain for examining whether CL plays a role in bacterial growth under alkaline conditions. Besides, the study of *B. firmus* enzyme will enable us to compare it with its *E. coli* counterpart and gain new insights into enzyme structure-function relationships.

The present study reports the cloning of the *B. firmus* OF4 *cls* gene and the characterization of *B. firmus* OF4 CL synthase in the membranes of an *E. coli* strain that overproduces it.

2. Materials and Methods

2.1. Chemicals

Ampicillin; tetracycline-HCl; chloramphenicol; isopropyl β-D-thiogalactopyranoside (IPTG); bovine serum albumin (fraction V); DEAE-cellulose; glycerokinase (from *E. coli*); high molecular mass standard mixture for SDS gel electrophoresis; brilliant blue G-perchloric acid solution; Bradford reagent for protein determination; acrylamide; bis-acrylamide; TEMED (N,N,N',N'-

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tetramethylethylenediamine); bromophenol blue; cardiolipin from *E. coli*; L-α-phosphatidate, dioleoyl; restriction endonucleases; T4 DNA ligase; polyethylene glycol (PEG)-8000; ReadyMix™ Taq with $MgCl_2$; and fusaric acid were purchased from the Sigma Chemical, St. Louis, MO. Polymerase chain reaction primers were synthesized by CyberSyn, Inc., Aston, PA. Cytidine 5'-diphosphate-DL-diacylglycerol, dioleoyl and L-α-phosphatidyl-DL-glycerol, dioleoyl were products of Doosan Serdary Research Laboratories, Englewood Cliffs, NJ. Tryptone, yeast extract, and agar were obtained from Difco Laboratories, Detroit, MI. Agarose was purchased from BRL, Gaithersburg, MD. Triton X-100 (TX-100) and calf-intestine alkaline phosphatase were acquired from Boehringer-Mannheim Biochemicals, Indianapolis, IN. Polygram Sil G thin-layer chromatography plates were obtained from Brinkmann Instruments, Westbury, NY. [^{3}H]Glycerol was purchased from New England Nuclear, Wilmington, DE. [2-^{14}C]Acetate was a product of ICN, Irvine, CA. The Promega Wizard® Maxipreps kit was purchased from Promega, Madison, WI. All other chemicals were reagent grade or better.

2.2. Media and growth conditions

LB broth, consisting of 1.0% tryptone, 0.5% yeast extract, and 0.5% sodium chloride, was used for routine growth experiments. Fusaric acid plates were prepared according to Maloy and Nunn [22]. M9ZB broth was prepared as described by Studier and Moffatt [23]. Where indicated, IPTG was added to 0.8 mM and ampicillin, tetracycline-HCl, and chloramphenicol were used at 125, 20, and 20

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μg/mL, respectively. Cell growth was monitored by measuring the turbidity with a Klett-Summerson photometer (red filter). One Klett unit corresponds to approx. 5 x 10^6 cells/mL.

2.3. DNA isolation and manipulation

The PEG-$MgCl_2$ method [24] was used to isolate DNA for sequence studies. A 5-mL overnight culture of cells in LB broth plus the appropriate antibiotic was added to a 2-L Erlenmeyer flask containing 300 mL of the same medium and incubated at 37°C with shaking. When the growth reached a turbidity of 0.6-0.8 O. D. reading at 595-nm wavelength, choloramphenicol was added to a final concentration of 170 μg/ml. The culture was incubated overnight and harvested by centrifugation at 10,000 x g for 5 min. The pellet was washed with 8 ml of STE solution (100 mM NaCl, 10 mM Tris-HCl, pH 8.0 and 1mM EDTA) and resuspended in 8 ml of a cold GTE solution (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). To the cell suspension, 16 ml of NaOH-SDS solution (freshly prepared, containing 200 mM of NaOH and 1% of SDS) was added and the solution was gently mixed by inversion. After incubating the mixture on ice for 3 min, 12 mL of potassium acetate solution (prepared by mixing 60 ml of 5 M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of distilled water) was added and mixed by inversion. The mixture was incubated on ice for 5 min and centrifuged at 3000 x g for 10 min at 4°C. The supernatant was filtered through sterile gauze into a fresh tube followed by addition of 2 volumes of absolute ethanol at room temperature.

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The solution was incubated for at least 10 min at room temperature and centrifuged at 12,000 x g for 20 min at room temperature. The pellet was washed with 70% ethanol, air-dried and then resuspended in 5 mL of TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) containing 20 μg/mL of ribonulease A. The solution was transferred to a 15-mL centrifuge tube and incubated at room temperature for at least 20 min. After addition of 0.1 vol of 3 M sodium acetate, the solution was extracted once with phenol : chloroform : isoamyl alcohol (25:24:1), once with chloroform : isoamyl alcohol (24:1) and the DNA was precipitated by ethanol. After air-drying, the DNA pellet was dissolved in 1 mL of sterile water and the solution was transferred to a 1.5-mL microcentrifuge tube, followed by addition of 0.5 mL of 40% PEG-8000 in 30 mM $MgCl_2$. The mixture was incubated at room temperature for at least 10 min. DNA was pelleted by centrifugation at 12,000 x g for 20 min at 4°C. The pellet was washed twice carefully with 70% ethanol to remove PEG. At each washing step, the pellet was resuspended in 1 mL of ice-cold 70% ethanol and centrifuged at 12,000 x g for 5 min at 4°C. After air-drying, the pellet was resuspended in TE or sterile dH_2O. For routine plasmid preparations, the rapid SDS-alkaline lysis method [25] and the Promega Wizard® Maxipreps kit were used. Transformations were performed by the procedure of Brian Seed as previously described [16]. Restriction endonucleases, T4 DNA ligase, bacterial alkaline phosphatase, and calf intestine alkaline phosphatase were used according to their manufacturers' specifications. Plasmid DNA concentration was estimated by

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measuring UV absorption at 260-nm wavelength. Alternatively, agrose gel electrophoresis was used to determine the quality and quantity of plasmid DNA. A sample of 10-100 μg of plasmid DNA was linearized by digesting with a proper restriction endonuclease and loaded on a mini-gel containing 0.5 μg/mL of ethidium bromide, along with 300 ng of DNA marker (*Hin*dIII digest of λ DNA). After electrophoresis, DNA concentration was estimated by comparing the brightness of DNA band with the marker under UV light. DNA fragments were separated by gel electrophoresis in agarose and purified by the electroelution method described in the following section [25].

2.4. Electroelution

Dialysis tubing (2-cm diameter) was cut into pieces of 10-15 centimeters and boiled for 10 min in 400 mL of 2% (w/v) sodium bicarbonate and 1 mM EDTA (pH 8.0). After being rinsed thoroughly in distilled water, the tubing was boiled for 10 min in 1 mM EDTA (pH 8.0). The tubing was kept in the same solution and stored at 4°C. Before use, the tubing was washed inside and out with distilled water (the tubing was always handled with gloves).

Plasmid DNA was digested with the approriate endonuclease(s) to get the desired fragments. A mixture of 100 μL of digested DNA (10-50 μg of total DNA) and 50 μL of DNA loading buffer was loaded onto a regular agarose gel (1% agarose and 0.5 μg/mL ethidium bromide) with wide sample wells. The electrophoresis was run at either 100 volts for overday gels or 20 volts for overnight

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gels. DNA fragments were visualized under a UV lamp and the gel piece containing the desired DNA fragment was cut out with a razor blade. The gel piece was placed in the pretreated dialysis bag clipped on one end. A few milliliters of 1 x TAE buffer (40 mM Tris-HCl, 20 mM sodium acetate, and 2 mM EDTA at pH 8.0) was added to the dialysis bag. Excess TAE was carefully squeezed out so that the gel piece was just submerged in the buffer and no air bubbles were present. After the other end was clipped, the dialysis bag was placed in the middle of the electrophoresis tank filled with 1.0 L of TAE, oriented perpendicular to the current. The DNA was eluted for 1.5 h at 100 V.

2.5. Bacterial strains and plasmids

Bacterial strains, constructed by standard P1 transduction techniques [26], are listed in Table 1.1. As shown in Figure 1.2, a mini F Kan^r plasmid was introduced into HB101 to form QC21, a derivative with a functional *rec*A gene that could be transduced. The *cls*::miniCam10 and *pcn*B80*zad*::Tn10 were then introduced by standard transduction techniques to form QC22 and QC23, respectively. Strain QC24, a tetracycline-sensitive derivative of QC23 strain was selected on fusaric acid plates [22] and transduced to form QC25. The plasmids used in this study are listed in Table 1.2.

2.6. B. firmus *gene library*

The *B. firmus* OF4 gene library containing bacterial DNA fragments in a pSPT19 vector was generously provided by Terry Krulwich. This library was

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prepared as described by Ivey et al. [27]. In brief, the vector, plasmid pSPT19, and the *B. firmus* DNA were cut with *Sal*I and *Mbo*I, respectively. The staggered ends were partially filled in by incubating linearized plasmid DNA with dCTP and dTTP and bacterial DNA fragments with dATP and dGTP in the presence of DNA polymerase. The bacterial fragments were mixed with the linearized plasmid and incubated with DNA ligase to form a gene library in which each recombinant plasmid has a *B. firmus* OF4 DNA fragment inserted into the *Sal*I site (between T7 and Sp6) of plasmid pSPT19.

2.7. Selection of plasmid pQC1 bearing the B. firmus cls *gene*

The *B. firmus* gene library was introduced into QC25 (*pcn*B80 *pss*A1 *cls*::miniCam*10*) and transformants were selected for their ability to grow at 30°C on LB agar medium containing ampicillin. The approx. 200-300 transformant colonies that were present on each plate were suspended in 2 mL of LB broth to form a single pool of cells. A sample from each pool was diluted so that about 10^4 cells were spread on LB agar supplemented with ampicillin and the plates were incubated at 42°C. Strain QC25/pQC1 (Figure 1.3) appeared as large colonies on these plates.

2.8. Construction of plasmids pDG1 and pDG2

Plasmid pQC1 was digested with *Bam*HI and *Bgl*II and the restriction fragments were separated by agarose gel electrophoresis. A segment of the gel containing a 2.5-kb fragment of *B. firmus* OF4 DNA was placed in dialysis tubing

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containing TAE buffer and the DNA was released by electroelution and purified by phenol extraction as described by Sambrook et al. [25]. Plasmid pET3 was digested by *Bam*HI to form a linear duplex, which was treated with calf-intestine alkaline phosphatase to remove the 5'-phosphate groups and then purified by gel electrophoresis. The linear pET3 DNA and the 2.5 kb DNA fragment were joined by T4 DNA ligase. The resulting recombinant plasmids were introduced into competent HB101 cells and transformants were selected on LB plates supplemented with ampicillin at 37°C. Recombinant plasmids were isolated from the transformants by the SDS-alkaline lysis method. Analysis of the restriction endonuclease digests revealed two classes of recombinant plasmids with the *cls* gene inserted into the vector pET3 in opposite orientations. Plasmids pDG1 and pDG2, representing each orientation, were isolated for further study. Each plasmid was introduced into competent BL21(DE3) and QC30-15.

2.9. Lipid Analysis

Cells were cultured in 10 mL of LB broth at 30°C. When the culture reached a turbidity of 30 Klett units, 2.5 μCi of [2-^{14}C]acetate (sp. act. 4.3 mCi/mmol) were added to the culture. Cells were harvested after 2 h and glycerophospholipids were isolated and chromatographed as previously described [28]. Radioactive lipids were detected and quantified by cutting the silica gel plates into strips and counting the strips in a liquid scintillation counter.

2.10. DNA sequence analysis

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Plasmid pQC1 DNA was prepared for sequencing by the PEG-$MgCl_2$ method [24]. The DNA samples were sequenced at the DNA Sequencing Core Laboratory at the University of Florida (Gainesville, FL) using automated fluorescent cycle sequencing protocols and ABI 373 Stretch DNA sequencer (Perkin Elmer/ABI Division, Forster City, CA).

2.11. Computer analysis of sequence data

The computer program BLAST [29, 30] was used to search the entire sequence database for homologous sequences. The multiple sequence alignments of the homologous sequences were produced with the use of the computer programs CLUSTAL X [31] and BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). The Kyte-Doolittle algorithm [32] was used to generate a hydropathy plot.

2.12. Amplification of B. firmus *CL Synthase in* E. coli

BL21(DE3)/pDG2 cells were cultured in M9ZB media supplemented with ampicillin. When the culture reached a turbidity of 150 Klett units, IPTG was added to induce CL synthase expression. Cells were harvested 3 h later. Crude membranes were isolated as previously described [19], stored in 100 μL aliquots, and frozen at -70°C. Protein concentrations of the crude membranes were determined using the Bradford reagent [33] in accordance with the manufacturers instructions.

2.13. SDS-polyacrylamide gel electrophoresis (PAGE)

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SDS-PAGE was used to determine the molecular mass of the overproduced protein in crude membrane according to the method of Laemmli [34]. A Hoeffer Scientific Instruments (San Francisco, CA) Sturdier Slab Gel Electrophoresis Unit was used.

The gel mold made with two glass plates was assembled following the instruction manual. A Separating Gel monomer solution was made as described in Table 1.3. Ammonium persulfate and TEMED were added at the last minute before pouring the gel. The gel solution was quickly pipetted into the gel mold, with about 5 cm of space left for the stacking gel. About 0.5 mL of distilled water was carefully layered onto the gel surface and the gel was allowed to solidify at room temperature for an hour. After polymerization was complete, the water on the top layer was removed by decanting and the comb was set in place. A Stacking Gel solution was prepared as described in Table 1.3 with ammonium persulfate and TEMED added last. The solution was pipetted into the gel mold and allowed to polymerize. When polymerization was complete, the comb was carefully removed. The gel sandwich was placed into the lower buffer chamber and the upper chamber was assembled according to the manufacturer's instruction. About 250-300 mL of tank buffer was added to each chamber. Protein samples were prepared by adding equal volumes of 2X treatment buffer and then heating in a boiling water bath for 2 min. After cooling, each sample was loaded with a 50 μL syringe. A typical sample is 50 μL and contains between 10-100 μg of protein. The safety lid was then attached to the

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unit before the unit was connected to the power supply. Electrophoresis was performed either overnight or overday. When the electrophoresis was performed overnight, 70 V for both stacking and resolving gels was maintained. When performed overday, it was maintained at 125 V for the stacking gel and then shifted to 200 V for the resolving gel.

When the eletrophoresis finished, the unit was disassembled and the gel was detached from glass plates. The gel was placed in a plastic tray and rinsed for 10-30 seconds in distilled water. It was then treated with with Sigma's gel fixing solution following the instructions. The gel was rinsed in several changes of distilled water to completely remove the fixing solution and then incubated in Brilliant Blue G-Perchloric Acid Solution (Sigma) for 30-60 min. After staining, the gel was washed with dH_2O until the background became clear.

2.14. CL synthase assay

CL synthase was assayed at 37°C by adding enzyme to a mixture containing phosphatidyl[2-^{3}H]glycerol and following the release of [2-^{3}H]glycerol as previously described [19]. The phosphatidyl[2-^{3}H]glycerol used in this assay was prepared as described previously [19]. The standard assay mixture for *E. coli* CL synthase was also as previously described [19]. The standard assay mixture for the *B. firmus* CL synthase was the same as that for the *E. coli* enzyme except that the potassium phosphate concentration was decreased to 160 mM and the pH was increased to 7.5. After 5 min preincubations, reactions were initiated by adding either 0.05 μg

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crude membranes for *E. coli* CL synthase or 1 μg crude membranes for the *B. firmus* enzyme.

3. Results

3.1. Selection and characterization of plasmid pQC1

The approach described by Ohta et al. [17] was used to select a recombinant plasmid bearing the *cls* gene from a *B. firmus* OF4 gene library. This approach takes advantage of the fact that *pss*A1 *cls* double mutants are much more temperature-sensitive than are *pss*A1 mutants while minimizing the possibility of selecting for *pss* revertants. Nonselective conditions are used to isolate transformants, which are then tested for the ability to grow at 42°C. Previous studies demonstrated that *pss*A1 *cls* double mutants do not grow on LB agar plates at 42°C, whereas *pss*A1 mutants do (Heber, S. and Tropp, B. E., unpublished data). This observation suggested that a recipient strain with a *pss*A1 *cls*::minCam*10* genotype should be able to grow on LB agar at 42°C after being transformed with a plasmid bearing the *B. firmus cls* gene. The recipient strain, QC25 (*pcn*B *pss*A1 *cls*::miniCam*10*) was constructed from HB101(a strain that is known to be readily transformed). The *pcn*B gene was introduced because previous studies suggested that *E. coli* do not grow well when transformed with a relaxed plasmid bearing the *cls* gene [17]. The *B. firmus* OF4 DNA gene library was introduced into QC25 and transformants were selected for growth at 30°C on LB agar containing ampicillin. Of the approximately 25,000 transformants isolated, only two produced large

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colonies when tested for growth at 42°C. The recombinant plasmids from these two transformants were isolated and introduced into strain QC30-15. Only one of the plasmids, pQC1 (Figure 1.3), restored the ability of this strain to grow on LB agar at 42°C. The other recombinant plasmid was not studied further.

Plasmid pQC1's ability to support the growth of the two different *pss*A1 *cls* double mutants (QC25 and QC30-15) suggests that it contains a *pss* or a *cls* gene. QC30-15/pQC1 was tested on agar medium containing half the normal concentration of LB medium. These plates, which support the growth of *cls* mutants but not *pss*A1 mutants at 42°C, did not support the growth QC30-15/pQC1. These results suggest that pQC1 bears the *B. firmus* OF4 *cls* gene. More direct support for this hypothesis was provided by analyzing the radioactive lipids that were formed by QC30-15/pBR322, QC30-15/pSH103, and QC30-15/pQC1 when cultures were incubated in the presence of [2-^{14}C]acetate in LB broth at 30°C for 2h. As shown in Table 1.4, cells bearing either plasmid pSH103 or pQC1 convert 12-14% of the radioactive label to material that cochromatographed with CL. In contrast, cells bearing plasmid pBR322 convert about 1.5% of the radioactive label into material that cochromatographs with CL. All three strains convert approximately 71% of the label to PE. These results indicate that plasmid pQC1 has the *B. firmus cls* gene and not the *pss* gene.

3.2. Sequence of B. firmus *OF4* cls *gene*

The nucleotide sequence of the *B. firmus* fragment in plasmid pQC1 was

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determined (GenBank accession number U88888). Two open reading frames were detected. Open reading frame 1, which contains 1509 bp, codes for a polypeptide with 503 amino acid residues and a molecular mass of 57.9 kDa. A possible Shine-Dalgarno (SD) sequence, GGAG, is located 6 bases upstream from the initiation codon, ATG. As shown in Figure 1.3, plasmid pQC1 has three *Hind*III cleavage sites. Two of these sites are in open reading frame 1 and the third is near the T7 promoter in the pSPT19 vector. Cleavage of pQC1 with *Hind*III endonuclease removes a 1.2 kb DNA fragment, containing the last 893 nucleotides in open reading frame 1 (corresponding to 297 amino acid residues at the carboxyl terminus) along with small nucleotide segments from the remaining *B. firmus* DNA insert and from pSPT19. The truncated linear plasmid, which retained open reading frame 2 in its intact form, was treated with DNA ligase to form plasmid pQC2 and introduced into QC30-15 (*pss*A1 *cls*::miniTet*10*). None of the resulting transformants were able to grow on LB agar at 42°C, supporting the notion that open reading frame 1 is the *B. firmus cls* gene.

DNA sequence analysis revealed that the polypeptide encoded by the *B. firmus cls* gene (open reading frame 1) is homologous to *E. coli* CL synthase. A total of 129 identical amino acids and 100 similar amino acids were found in the two polypeptides. The *B. firmus* CL synthase is also homologous to polypeptide sequences from *B. subtilis* (accession number Z49884, and Z99122), *Pseudomonas putida* (accession number X55704), *Clostridium perfringens*

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(accession number Q9ZNC6), *B. haloduran* (accession number BAB06577), *Lactococcus lactis* (accession number AE006300), *Pseudomonas aeru* (accession number B82971), *Bacillus cereus* (accession number CAB69815), *Vibrio cholerae* (accession number C82171), *Ureaplasma urealy* (accession number G82872), *Bucnera* sp. APS (accession number P57361), *Xylella fastidiosa* (accession number H82711) and *Zymomonas mobilis* (accession number AF176207). Comparisons of amino acid sequences from the bacterial strains revealed six highly conserved regions (Figure 1.4). Two groups of conserved amino acid residues, $RN(Q)HRK(x)_4D(x)_6G(x)_2N$ and $HXK(x)_4D(x)_6G(x)_2N$ (Region III and Region V in Figure 1.4), are of special interest because they may be part of the active site. A hydropathy plot for the polypeptide encoded by *B. firmus* OF4 *cls* is shown in Figure 1.5. Open reading frame 2 contains 651 bp and is homologous to the *B. subtilis* *mec*A gene [35].

3.3. Overproduction of B. firmus *OF4 CL synthase*

In order to obtain substantial amplification of the *B. firmus* OF4 CL synthase, the *B. firmus cls* gene was placed under the control of the T7 promoter in pET3. This was accomplished by taking advantage of the fact that plasmid pQC1 has a *Bam*HI site in the pSPT19 vector near the Sp6 promoter and a *Bgl*II site just beyond the end of the *cls* gene. Cleavage with *Bam*HI and *Bgl*II restriction endonucleases produced a 2.5 kb fragment and a 3.4 kb fragment. After resolution by agarose gel electrophoresis, the 2.5 kb fragment, which contained most of the *B. firmus* DNA,

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was extracted from the gel by electroelution. This fragment was mixed with pET3 that had been cut at its *Bam*HI restriction site just downstream from the T7 promoter and the two DNA molecules were joined by T4 DNA ligase. Two types of recombinant plasmids, pDG1 and pDG2, were recovered after transformation of HB101. Each type contained the expected insert but in an opposite orientation. When plasmids pDG1 and pDG2 were introduced into QC30-15 (*pss-1 cls*), both types of transformants were able to grow on LB agar at 42°C.

Plasmids pDG1 and pDG2 were also introduced into BL21(DE3). The resulting transformants were cultured in 20 ml of M9ZB medium supplemented with ampicillin and then induced with IPTG. The cells were harvested, disrupted by sonication, and centrifuged to obtain the crude membrane fraction as described in Materials and Methods. The protein composition of the crude membranes from BL21(DE3)/pDG1, BL21(DE3)/pDG2, and BL21(DE3)/pET3 were analyzed by SDS-PAGE gel electrophoresis. A single dark band corresponding to a molecular mass of about 51 kDa was observed in the membrane fraction from BL21(DE3)/pDG2 but was not present in either of the other membrane fractions (Figure 1.6). The molecular mass of this protein is about 7 kDa smaller than that predicted by the amino acid sequence of the polypeptide chain specified by the *B. firmus cls* gene.

3.4. CL synthase assay

Crude membranes from IPTG-induced BL21(DE3)/pDG1, BL21(DE3)/pDG2, BL21(DE3)/pET3, and BL21(DE3)/pLR3 were assayed for CL synthase activity. As

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shown in Table 1.5, the CL synthase activity from BL21(DE3)/pDG2 membranes was approximately 40 times higher than that from BL21(DE3)/pDG1 or BL21(DE3)/pET3 membranes. However, its activity was about 20-40 fold lower than that observed in membranes from BL21(DE3)/pLR3, a strain that overproduces the normal *E. coli* CL synthase.

The CL synthase assay used for the experiments shown in Table 1.5, based upon [^{3}H]glycerol release from phosphatidyl[2-^{3}H]glycerol, would also give a positive test for phospholipases C or D. Therefore, it was necessary to show the product formed actually was CL. This was accomplished by incubating phosphatidyl[2-^{3}H]glycerol with crude membranes from BL21(DE3)/pDG2 and isolating the glycerophospholipids, and then analyzing the isolated lipids by thin-layer chromatography. Approximately 11% of the radioactive phosphatidylglycerol was converted to a radioactive glycerophospholipid that cochromatographed with CL. The remaining 89% cochromatographed with phosphatidylglycerol and no other products were detected. In a parallel experiment, performed by the standard glycerol release assay, 12% of the [^{3}H]glycerol was released.

3.5. Characterization of the crude B. firmus *CL synthase activity*

B. firmus CL synthase produced by IPTG induced BL21(DE3)/pDG2 was considerably less active than the comparable *E. coli* CL synthase produced by IPTG induced BL21(DE3)/pLR3 (Table 1.5). The *B. firmus* CL synthase catalyzed reaction was linear for about 20 min at 37°C (data not shown). The pH optimum for

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B. firmus CL synthase is pH 7.5 as compared to pH 7.0 for the *E. coli* enzyme (Figure 1.7). Enzyme activity is stimulated by potassium phosphate with optimum acitivity observed at about 160 mM potassium phosphate (Figure 1.8). *B. firmus* CL synthase activity was not affected by the addition of up to 10 mM magnesium chloride, calcium chloride, or manganese chloride or by the addition of 5 mM EDTA. Previous studies indicated that the *E. coli* CL synthase is inhibited by CL and phosphatidate [8]. As evident from Figure 1.9, CL is a more powerful inhibitor than phosphatidate for both the *B. firmus* and the *E. coli* CL synthases. Moreover, *E. coli* CL synthase appears to be more sensitive to CL inhibition than does the *B. firmus* enzyme.

4. Discussion

The plasmid pQC1 contains a 3-kb fragment of the *B. firmus* OF4 chromosome that has two open reading frames. The first of these codes for CL synthase and the second for a homolog of the *B. subtilis mec*A gene. The evidence for identifying the first open reading frame as CL synthase is as follows: (1) *pss*A1 *cls* double mutants that contain plasmid pQC1 can grow at 42°C on LB agar and synthesize CL, (2) a *pss*A1 *cls* double mutant with a pQC1 derivative that has a deletion corresponding to the carboxyl terminus of the *cls* gene cannot grow at 42°C, (3) the *cls* gene is homologous to the *E. coli cls* gene, and (4) BL21(DE3) which contains pDG2, a plasmid with the *B. firmus cls* gene, produces CL synthase when induced with IPTG. When the membranes from IPTG induced

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BL21(DE3)/pDG2 were analyzed by SDS-PAGE electrophoresis, a single intense band was observed that was not present in the membranes of IPTG induced BL21(DE3)/pDG1 or BL21(DE3)/pET3 (Figure 1.6). This band corresponds to a protein with a molecular mass of 51 kDa. Molecular mass calculations based upon the *cls* nucleotide sequence predict a polypeptide with a molecular mass of 57.9 kDa. These results suggest that the *B. firmus* CL synthase may be modified after translation. Previous studies show that the *E. coli* CL synthase appears to be modified by removal of an 8-kDa fragment [9]. Perhaps posttranslational modification is essential for full activity. The observed activity difference between the *E. coli* and *B. firmus* OF4 CL synthases may be due to incorrect modification of the *B. firmus* enzyme in *E. coli* or the absence of some required factor(s) in the *E. coli* membrane. The second open reading frame encodes a polypeptide with 217 amino acid residues. However, this polypeptide, which is homologous to the *B. subtilis* MecA protein, was not present in membranes prepared from IPTG induced BL21(DE3)/pDG2 or BL21(DE3)/pDG1 (Figure 1.6). The absence of this polypeptide is probably due to the fact that it, like the *B. subtilis* Mec A protein, is not a membrane protein.

The hydropathy plot of the *B. firmus* OF4 CL synthase (Figure 1.5) indicates that the amino end of the protein is highly hydrophobic, containing three possible transmembrane domains. The hydropathy plot of the *E. coli* CL synthase shows that the *E. coli* enzyme also contains a hydrophobic N-terminal with two potential

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transmembrane β-sheets [36]. However, it is possible that this N-terminal region is lost after posttranslational modification. Remarkably, the *B. firmus* OF4 CL synthase does not have any cysteine residues, eliminating the possibility of a diacylglycerylcysteine anchor.

Six conserved segments were found when the sequence of *B. firmus* OF4 CL synthase was compared with that from *E. coli* CL synthase and with other putative CL synthase sequences (Figure 1.4). Conserved histidines are of great interest because they might participate in phosphatidyl group transfer. If so, one might expect that the histidine would be surrounded by basic residues such as lysine or arginine that could interact with the phosphate group of the glycerophospholipid. There are two conserved histidine residues in the homologous sequences. One, His-243, is part of a pentapeptide RN(Q)HRK in Region III in all seven sequences. Another, His-421, of the *B. firmus* CL synthase is in the Region V. A lysine is located two residues after this histidine in all seven sequences. The motif $HxK(x)_4D(x)_6G$ observed in Region III and Region V is present in all seven CL synthases as well as in phospholipase D sequences from several organisms and some other proteins, suggesting that they belong to an enzyme superfamily [37,38]. Region II and IV are also present in some other members of the phospholipase D superfamily. It is interesting to note that these two regions, each followed by an HKD motif, share weak homology with each other, suggesting that the cls gene may have arisen by gene duplication. Region I is of interest because it is unique to CL

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synthases and may contain a signal for posttranslation modification. Region VI is also unique to CL synthases.

Studies by Shibuya et al. suggest that the *E. coli* CL synthase catalytic site is located on the outer surface of the cell membrane [39]. If the *B. firmus* enzyme's catalytic site were also located on the outer surface of the cell membrane, one might expect the two enzymes to have different pH optima. As shown in Figure 1.7, the pH optimum for the *B. firmus* enzyme is only slightly higher than that for the *E. coli* enzyme, suggesting that its catalytic site may be located on the cytoplasmic side of the *B. firmus* OF4 cell membrane. The *B. firmus* CL synthase is inhibited by CL, the reaction product. However, it is less sensitive to CL than is the *E. coli* CL synthase (Figure 1.9). Perhaps this is why *B. firmus* OF4 CL content is higher than that of *E. coli*.

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TABLE 1.1.

Bacterial Strains

Strain	Parent	Genotype or description	Source or Reference
BL21(DE3)		F^- *ompT hsdSB* ($r_B^- m_B^-$), *dcm gal* (DE3)	[23]
ECL930		*pcnB80 zad*::Tn10 *endA hsdR*Δ(*argF-lac*)U169, *thi*	[40]
GE944		MC4100 *srl*:: Tn10Δ*recA*	[41]
GE1634		GE944/mini F Kan^R *recA*$^+$	[41]
HB101		F' *recA13 supE44 rpsL20* (sm^r) *hsd20* (r_B^-, m_B^-) *ara14 galK2 lacY1 proA2 xyl15 leu mtl1* λ^-	[42]
HW55		*HfrC glpR cls*-1	[28]
JC7623		*recB21 recC22 sbcB15*	[43]
LK30	JC7623	*cls*::miniCam*10*	Lei Ke
MC4100		F^- *araD139Δ(argF-lac)* U169 *rpsL150 relAl deoC1 PtsF25 rbsR flB5301*	[44]
QC21	HB101	mini F Kan^r *recA*$^+$	This study
QC22	QC21	*cls*::miniCam*10*	This study
QC23	QC22	*pcnB80 zad*::Tn10	This study

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QC24	QC23	*pcn*B80 Tet^s	This study
QC25	QC24	*pss*-1 *zee*::miniTet*10*	This study
QC30-15	QC30	MC4100 *glpR glpD pss*-1 *cls::Tn10dTet3;*	[45]
SOH142		HfrKL16 *thi*-1 *rel*A1 *spo77 pss*-1 *zee*::miniTet*10*	[16]

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TABLE 1.2.

Plasmids

Plasmid	Description	Reference
pBR322	Amp^r Tet^r	[46,47]
pDG2	*B. firmus cls* inserted within pET3	This study
pET3	Over-expression vector with T7 promotor	[23]
pQC1	*B. firmus cls* inserted within pSPT19	This study
pSH103	Amp^r cls^+	[16]
pSPT19	Vector with both T7 and Sp6 promotors	[a]

[a] Product of Boehringer-Mannheim Biochemicals, Indianapolis, IN.

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TABLE 1.3.

Gel Recipes for a 1.5 mm thick slab gel

Reagents (see below*)	Separating Gel (10%T 2.7%C)	Stacking Gel (4%T 2.7%C)
30%T 2.7%C (1)	15 mL	1.33 mL
Buffer (2)	11.25 mL	-------
Buffer (3)	-------	2.5 mL
10% SDS (4)	0.45 mL	0.1 mL
dH_2O	18 mL	6.1 mL
Ammonium persulfate (5)	225 µL	50 µL
TEMED	15 µL	5 µL
Final Volume	45 mL	10 mL

*Reagents for SDS-PAGE:

1. Monomer Solution (30% T 2.7% C_{Bis}):
   Acrylamide (Neurotoxic, handle with care) 58.4 g; Bis-acrylamide 1.6 g; H_2O to 200 mL. Filter and store at 4°C in the dark.

2. Resolving Gel Buffer (1.5 M Tris-HCl pH 8.8):
   Tris 36.3 g. Adjust to pH 8.8 with HCl; H_2O to 200 mL.

3. Stacking Gel Buffer (0.5 M Tris-HCl pH 6.8):
   Tris 3.0 g. Adjust to pH 6.8 with HCl; H_2O to 50 mL.

4. 10% SDS:
   SDS 10 g; H_2O to 100 mL.

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5. Initiator (10% ammonium persulfate, freshly made):
   Ammonium persulfate 0.1 g; H_2O to 1.0 mL.

6. 2x Treatment Buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) : Solution (3) 2.5 mL; Solution (4) 4.0 mL; Glycerol 2.0 mL; 2-mercaptoethanol 1.0 mL; Bromophenol blue 2.5 mg; H_2O to 10.0 mL. Divide in aliquotes and freeze.

7. Tank Buffer (0.025 M Tris pH8.3, 0.192 M glycine, 0.1% SDS):
   Tris 12 g; Glycine 57.6 g; Solution (4) 40 mL; H_2O to 4.0 L

8. Gel Fixing solution (45% methanol, 10% acetic acid):
   Methanol 450 mL; Acetic acid 100 mL; dH2O to 1.0 L.

9. Stain: Coomassie Blue G Perchloric Acid (Sigma).

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TABLE 1.4.

Glycerophospholipid Analyses

Strain	% ^{14}C Distribution		
	PE	PG	CL
QC30-15/pQC1	69	16	15
QC30-15/pSH103	72	16	12
QC30-15/pBR322	70	28	1.6

Table 1.4. Bacteria were cultured in 10 ml of LB broth at 30°C. At a turbidity of 30 Klett units, 2.5 μCi of [2-^{14}C]acetate (sp. Act. 4.3 mCi/mmol) were added and the culture was incubated for another 2 h. Glycerophospholipids were isolated and analyzed as described in Materials and Methods.

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TABLE 1.5.

Cardiolipin Synthase Activity

Strains	Sp. activity (units/mg protein)
BL21(DE3)/pDG2	20
BL21(DE3)/pDG1	0.5
BL21(DE3)/pET3	0.5
BL21(DE3)/pLR3	450

Table 1.5. The strains were induced with 0.8 mM IPTG and crude membranes were obtained and assayed as described in Meterials and Methods. One unit of enzyme activity is defined as 1 nmol of [2-^{3}H]glycerol released per min [18].

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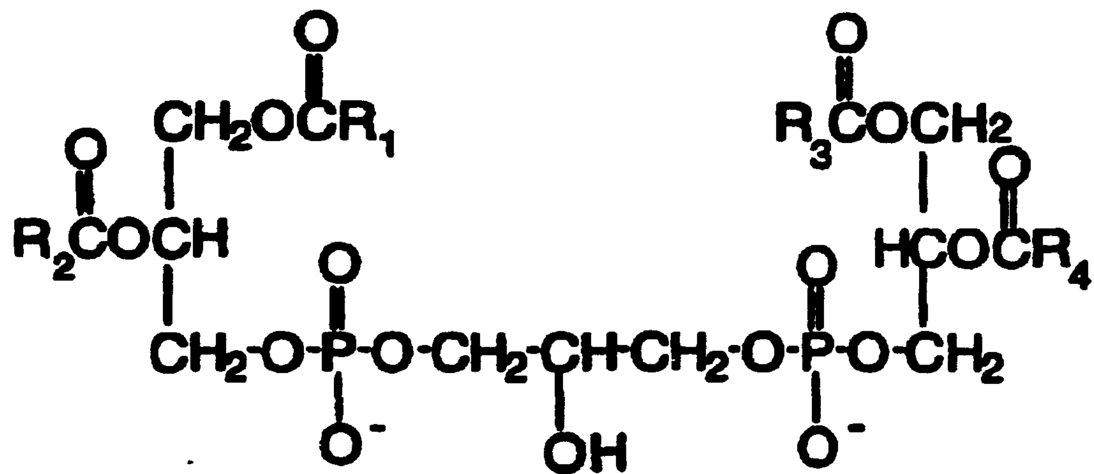

Fig. 1.1. Structure of Cardiolipin.

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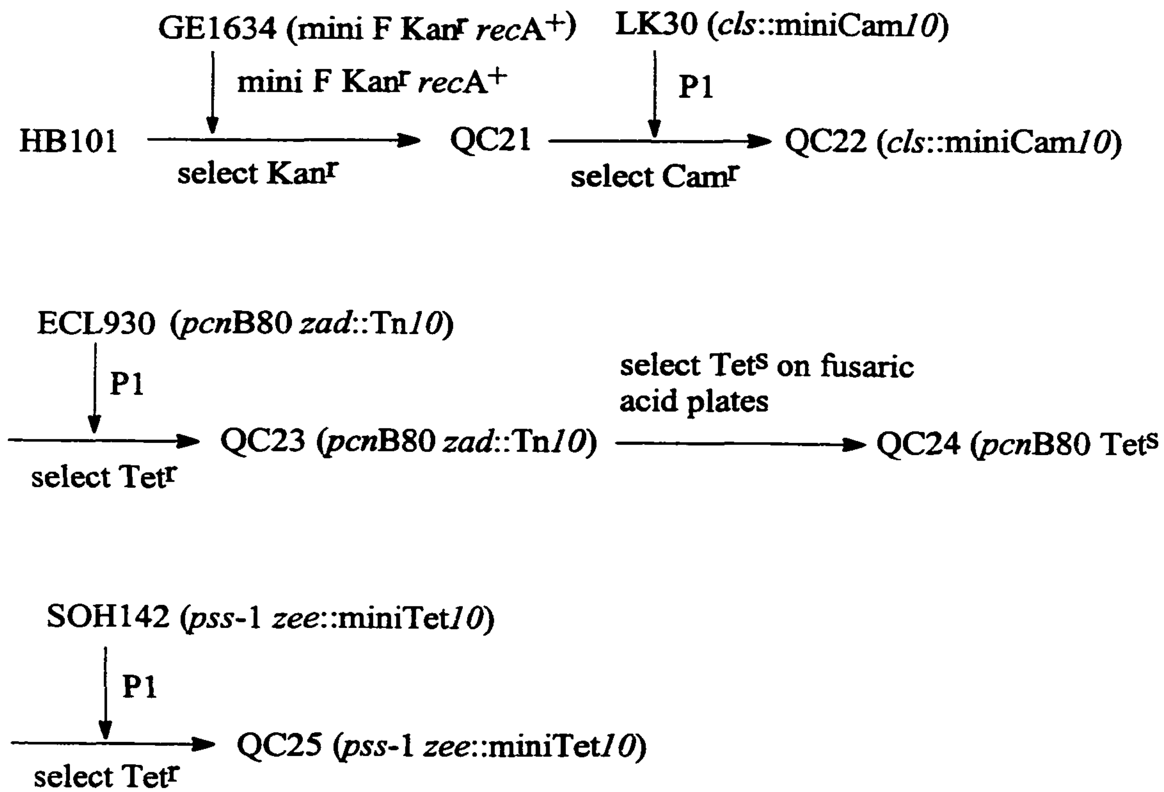

Fig. 1.2. Pedigrees of strains QC21, QC22, QC23, QC24 and QC25.

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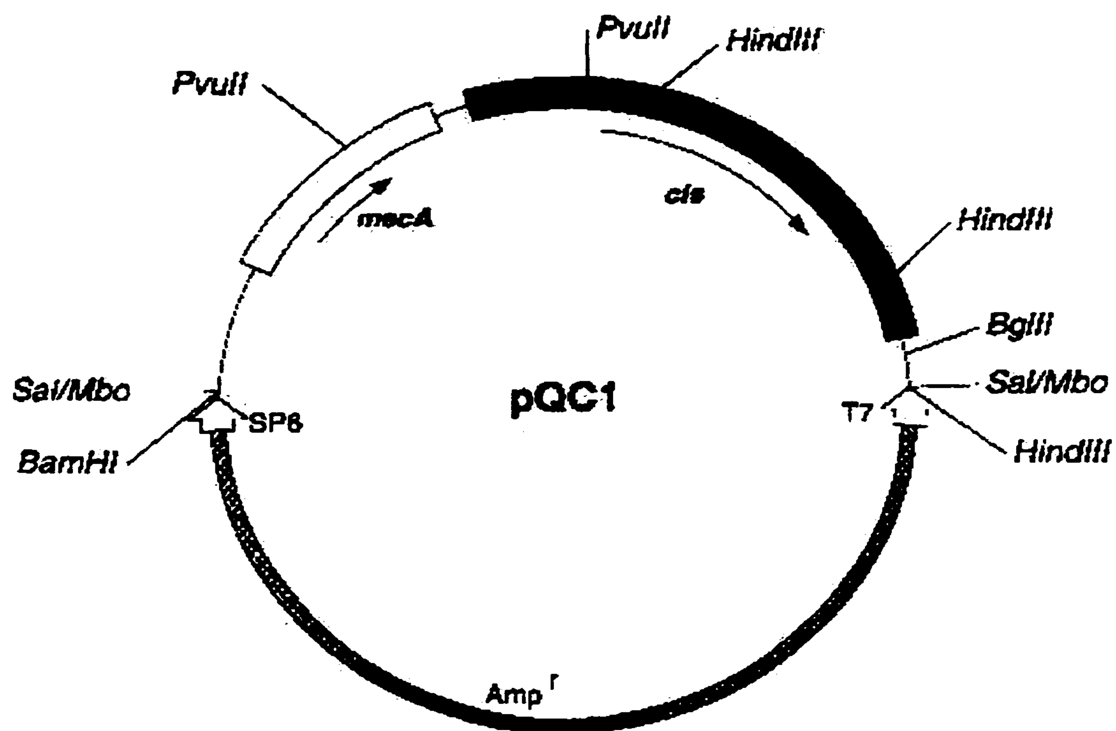

Fig. 1.3. Structure of plasmid pQC1. The *cls* gene and the *mecA* gene are as indicated. The promoters Sp6 and T7 are indicated by (⇨).

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```
CLS_B_FI    1 MKNRLNVLAFFALLFAALYISRGFLQSW VGT S FT S-  G  F N HPTK
CLS_E_CO    1 ----------------------MTTVYT VSW A GYWLL AG T R LMKR AVPSAM
YLP2_P_P    1 ------------------MHMDYHSPYFFGYL G HL G- AA HA T-V TAQGA
C_PERFRI    1 -----------------------------HLF N FL N- S I R NPQT W
YWIE_B_S    1 MLKRRLEFFFLYMMLIGAYVIWFFPVSR EFYGG CY SI S YS IL N TSQH
YWNE_B_S    1 --------------------------MSISSIL S FFI N- LA K R DASA
YWJE_B_S    1 --------------------------------------K-- I----------

CLS_B_FI   60 T AAF FF QNHR S R S--- A EDERAFQ IEGQRQLNEEQ KK
CLS_E_CO   39 YI I A AV ELHLGK RAER----ARAMWPSTA W NDLKACKHIFAE
YLP2_P_P   41 A P F Y TL P ARSFYAYIKAR-- QANQEMHVAMAN NWRPWVEEA TA
C_PERFRI   32 T I QNIT E N ---- K LDDKTKQ Y NSFKS-HYK DN
YWIE_B_S   61 L H V F F FS QLYV G L K---T R YNREKLR LFDKEET--PE TG
YWNE_B_S   35 F L HNLR KHL QWED K GIERLLKHQ EDLETKQFQFNN
YWJE_B_S   11 ---- I FA --LLD FM RAGY K A ---------------------E--PVFSK
```

Region I

```
CLS_B_FI  117 -MGGHQQL FR AH LGKNPISFSSETK T K T AH QA KM EHH VRH
CLS_E_CO   95 EN SVAAP FK CERRQGIAGVKG Q Q T SD VMQA R QL RHN E V QP
YLP2_P_P   99 RE ESYAA RA P- LGRMPCLAN Q K VN KAT DA FAA E RDV L Q F IHD
C_PERFRI   87 -I LKYKD IM NFNNDNSTYTQR D D YF NSL EE D N EKF E F S
YWIE_B_S  116 -LKDNQERFFTYSI AAHMNINTKSN K KN E PD FKA R ESY E MF S
YWNE_B_S   95 RA FDNKD IY LI NNHAVFTED S D T R K QR S S KDH Q G
YWJE_B_S   41 ------K----------------KSD E HC A LVER N RQ ASS M N

CLS_B_FI  176 NQ K S K H F G WK S Y EE P-D E VS S V- LP
CLS_E_CO  155 GG DQ A S AA R HC L SA VAFF SPWPE MRN E VEALKVNLMRV
YLP2_P_P  158 T A QQL R A Q F R HA PA YSQQ - Q HA ATR- GW
C_PERFRI  146 K LQA TK E V S N-S H KD DK -A G FKI F G-FCK
YWIE_B_S  175 M G M E Q E F AA MK A RD MR - VP S L- YG
YWNE_B_S  155 K R Q Q E RT R FFKE - GH EV F S- LRP
YWJE_B_S   79 SHN YT KT QA S Y L WA CRA K A QT - H HVMNRP- FP
```

Region II

```
CLS_B_FI  234 LTHT V L - K AYF Y Y R E RT L
CLS_E_CO  215 FLR DL NY T SM V PR FKQ AG Q I LMA E PIATA GI
YLP2_P_P  216 NRFQ L H - GHPQ SP Q S PV AC E
C_PERFRI  203 INL I SK F - KN H T K L ND EG
YWIE_B_S  233 FNQ F KT L K- S PY F L E EI QT HA
YWNE_B_S  213 INL MT F - LNPKF Y Q T HA T
YWJE_B_S  137 FFFH K T K F - KAKF N E Y I E HD T
```

Region III

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CLS_B_FI 293
CLS_E_CO 275
YLP2_P_P 275
C_PERFRI 262
YWIE_B_S 292
YWNE_B_S 272
YWJE_B_S 196

CLS_B_FI 349
CLS_E_CO 332
YLP2_P_P 327
C_PERFRI 322
YWIE_B_S 346
YWNE_B_S 328
YWJE_B_S 244

Region IV

CLS_B_FI 409
CLS_E_CO 392
YLP2_P_P 387
C_PERFRI 382
YWIE_B_S 406
YWNE_B_S 388
YWJE_B_S 304

Region V

CLS_B_FI 469
CLS_E_CO 452
YLP2_P_P 447
C_PERFRI 442
YWIE_B_S 466
YWNE_B_S 448
YWJE_B_S 364

Region VI

Fig. 1.4. Alignments of the derived amino acid sequences of the CL synthases from *B. firmus* OF4 (CLS_B_FI), *E. coli* (CLS_E_CO), *P. putida* (YLP2_P_P), *C. refringens* (C_PERFRI) and *B. subtilis* (YWIE_B_S, YWNE_B_S and YWJE_B_S). The number of the first amino acid residue of each of the highly conserved sequences is given to the left of the sequence. Homologous regions are indicated as Region I-VI.

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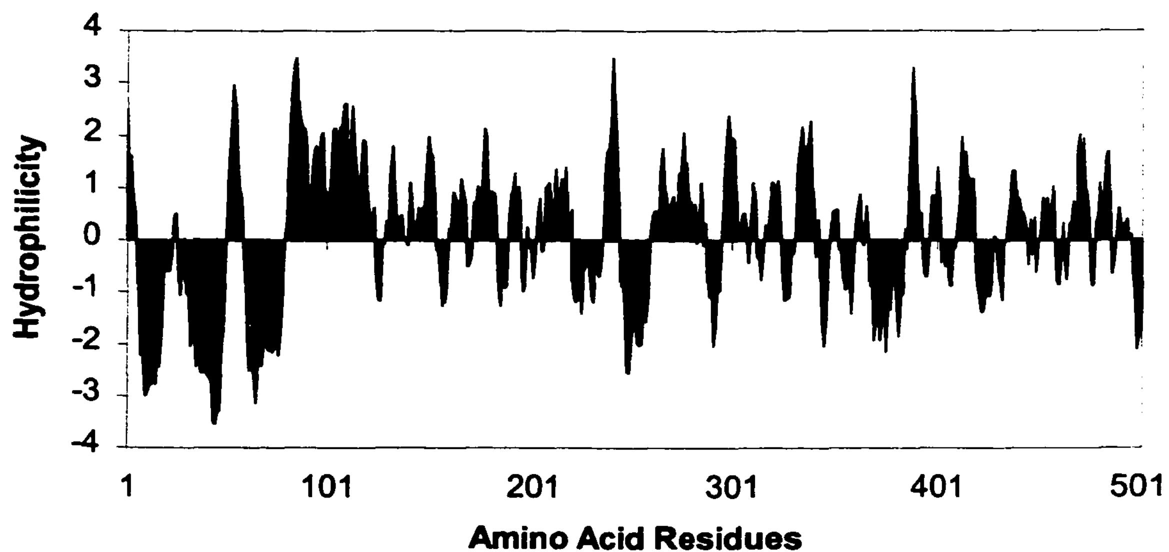

Fig. 1.5. Hydropathy plot of the predicted amino acid sequence of *B. firmus* OF4 CL synthase. Hydrophilic sequences are on the positive side of the scale and hydrophobic on the negative side.

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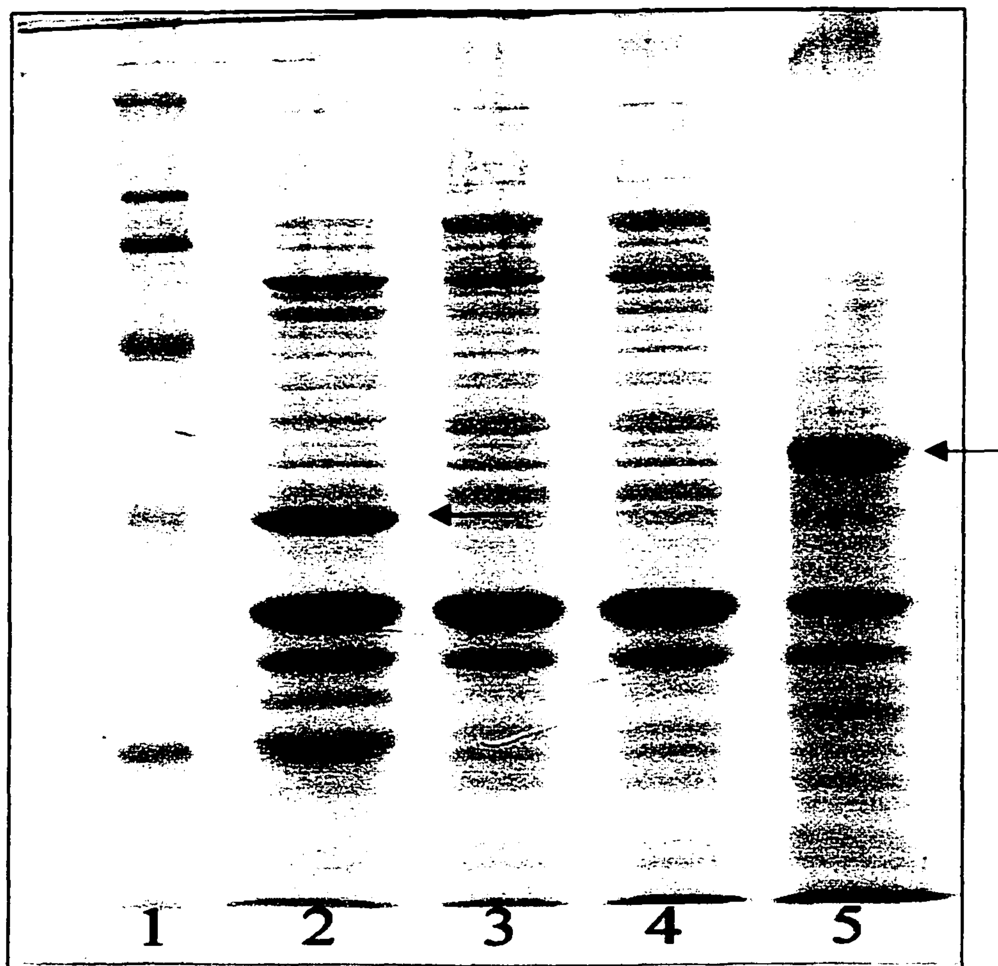

Fig. 1.6. Overexpression of the *B. firmus* OF4 CL synthase. Crude membranes containing about 50 μg proteins were analyzed by SDS-PAGE. Lane 2, BL21(DE3)/pLR3; Lane 3, BL21(DE3)/pET3; Lane 4, BL21(DE3)/pDG1; Lane 4, BL21(DE3)/pDG2. Overexpressed proteins are indicated by arrows. Lane 1 is the high molecular mass protein standard mixture.

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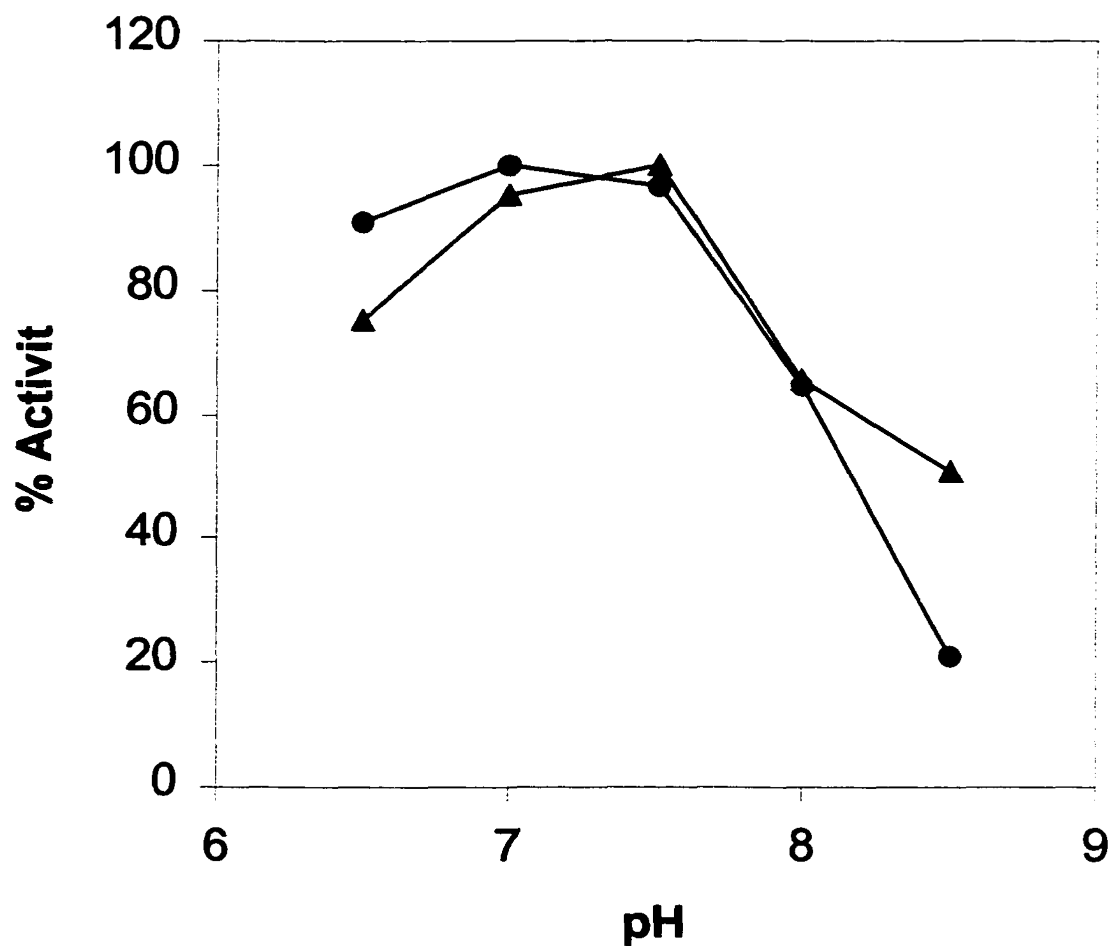

Fig. 1.7. pH profiles of the CL synthases. Activities of *E. coli* crude membranes containing *B. firmus* OF4 (▲) and *E. coli* (●) CL synthases were measured after 10 min at 37°C as described in Materials and Methods. The crude enzymes were used at the final concentrations of 20 μg/ml for *B. firmus* and 1 μg/ml for *E. coli*, respectively.

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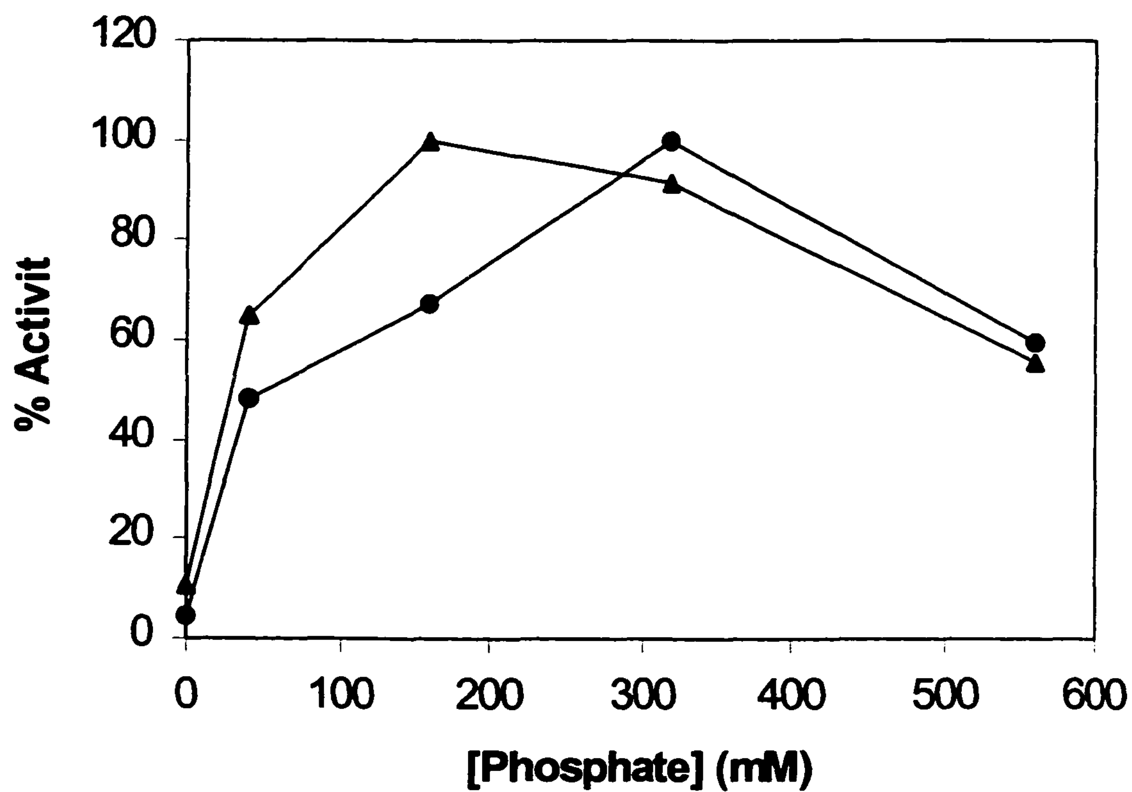

Fig. 1.8. Effect of phosphate concentrations on *E. coli* crude membranes containing *B. firmus* OF4 (▲) and *E. coli* (●) CL synthases. Phosphate buffers (pH7.0) were used at the indicated concentrations. CL synthase assays were performed as described in Materials and Methods. Glycerol release was measured after 5 min at 37°C.

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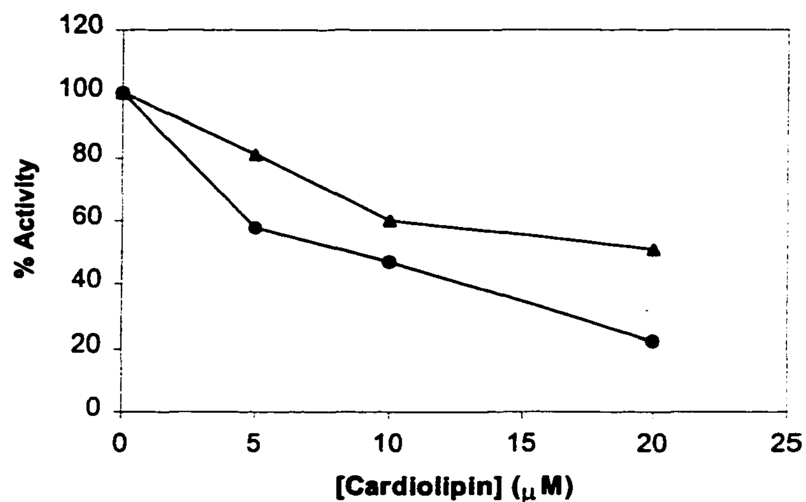
A



B

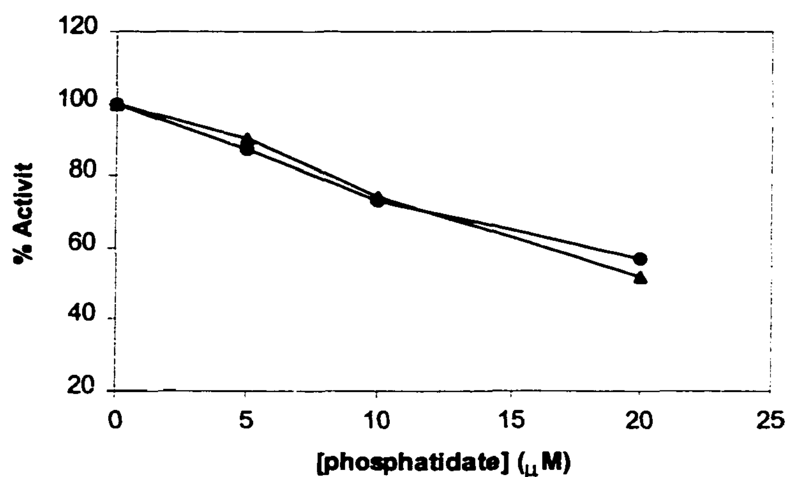


Fig. 1.9. Effect of (A) CL and (B) phosphatidate on *E. coli* crude membranes containing *B. firmus* OF4 (▲) and *E. coli* (●) CL synthases. CL and PA were added at the indicated concentrations. The crude enzymes were used at the final concentrations of 20 μg/mL for *B. firmus* and 1 μg/mL for *E. coli*, respectively. Glycerol release was measured after 10 min at 37°C.

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CHAPTER 2

Construction of a *B. firmus* OF4 strain with a null mutation in *cls* and its growth characteristics

1. Introduction

The uniqueness of the structure and properties of cardiolipin and its presence in the "living membranes" suggest it may play important roles in cell metabolism. However, despite extensive study, the true biological functions of cardiolipin still remain largely unknown. In fact, it is still not clear whether cardiolipin is essential for cell growth. Studies by Jiang et al [48] showed that a yeast strain bearing a null mutation in cardiolipin synthase had no cardiolipin synthase activity and no cardiolipin in its membranes. Nevertheless the yeast cells were viable in media containing fermentable or non-fermentable carbon sources, indicating that cardiolipin is not essential for growth under those conditions. However, it was observed that the yeast CL synthase null mutant cannot grow in a medium containing non-fermentable carbon sources at an elevated temperature [48], suggesting that CL may play important roles under some growth conditions or when the cells are under stress. Further studies revealed that at elevated temperature, the CL synthase mutant cells lose viability in both fermentable and non-fermentable media [49]. In addition, the mutant cells have decreased mitochondrial membrane potential and are deficient in maximum respiratory rate, ATPase and cytochrome oxidase activities, and protein import, indicating CL is essential in maintaining these physiological functions [49]. In *E.*

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coli, cls gene disruption is not lethal. However, a small amount of cardiolipin is still synthesized in *cls* null mutants by other enzymes. *E. coli* cells that lack CL synthase or have a defective enzyme have characteristic physiological properties. When *cls* mutants are compared to their wild type parents, they are more sensitive to novobiocin [45], are more resistant to 3,4-dihydroxybutyl-1-phosphonate [28], have a lower survival rate in the stationary phase [18], and have a slightly longer doubling time [50]. The difference in doubling time becomes even more pronounced when the pH of the growth medium is increased (Hwang, Y.-W. and Tropp, B. E., unpublished data), suggesting that CL may play a role in growth under alkaline conditions. The *cls* mutation also increases the temperature-sensitivity of cells with a *pss*A1 allele (a temperature-sensitive mutation for phosphatidylserine synthase formerly known as *pss*-1) [51].

Alkaliphilic strain *Bacillus firmus* OF4 exhibits rapid growth on malate over the pH range from 7.5 to 10.6 [52]. To maintain pH homeostasis, *B. firmus* cells utilize Na^+/H^+ antiporters in an alkaline environment. When the external pH rises, a reverse pH gradient (ΔpH, acid inside) is formed, causing the chemiosmotic driving force (Δp) to fall. Nonetheless, the cells are able to maintain a steady rate of oxidative phosphorylation with a submaximal Δp. Furthermore, the F_1F_0-ATP synthase of *B. firmus* OF4 is exclusively proton-translocating and functions at both pH7.5 and pH10.5 [53]. The mechanisms for alkaliphile oxidative phosphorylation are unknown. One possibility is that protons moving laterally

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along the membrane surface are used for ATP synthesis before they are lost to the bulk phase [52]. If this is true, one can see the importance of the buffering ability of membranes and possible roles of acidic phospholipids, especially cardiolipin.

The difficulty in growth at elevated pH shown by *E. coli cls* null mutants and the fact that the alkaliphilic strain *B. firmus* has an unusually high CL content led us to question whether CL is required for growth at high pH. In this study, a *cls* null mutation was introduced into *B. firmus* OF4 strain. The growth studies show that a high CL concentration is not essential for *B. firmus* to grow under alkaline conditions.

2. Materials and methods

2.1. Chemicals

Kanamycin; ampicillin; erythromycin; streptomycin; agarose; E. coli cardiolipin; polyethylene glycol (PEG)-8000, ReadyMix™ Taq PCR reaction mix (containing Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffer in a 2x concentrate), and mineral oil were purchased from Sigma Chemical, St. Louis, MO.

Bacto-tryptone, Bacto-yeast extract, Bacto-penassay broth (Antibiotics Medium No. 3), Bacto-agar and purified agar were obtained from Difco Laboratories, Detroit, MI. Restriction enzymes, and T4 DNA ligase were purchased from BRL, Gaithersburg, MD. Triton X-100 (TX-100) and calf-intestine alkaline phosphatase were acquired from Boehringer-Mannheim Biochemicals,

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Indianapolis, IN. Polygram Sil G thin-layer chromatography plates were obtained from Brinkmann Instruments, Westbury, NY. [^{3}H]Glycerol was purchased from New England Nuclear, Wilmington, DE. [2-^{14}C]Acetate was a product of ICN, Irvine, CA.

Polymerase chain reaction primers were synthesized by CyberSyn, Inc., Aston, PA. The primers used in this study were 5'-CGA AGT CAT ATG TCG AAG AAC TGC-3' and 5'-GAC ATC AAT CCC GCT TAA AGC GGC A-3'.

2.2. Bacterial strains, plasmids and growth conditions

E. coli strain HB101 was cultured in LB medium. Reagents and media for growth and transformation of *Bacillus firmus* are described in Appendix 1. *B. firmus* OF4 811M (*met Str^r*) was kindly provided by Dr. Terry A. Krulwich [54]. The media used to culture *B. firmus* OF4 811M are designated medium A (also known as complex medium) and medium B (also known as malate-containing medium) [55]. The mutant strain DG301 was cultured in medium A at pH7.5. When necessary, kanamycin was added at 50 μg/mL and ampicillin was used at 100 μg/mL. Unless noted otherwise, erythromycin was used at 0.3 μg/mL for B. firmus and 150 μg/mL for *E. coli.* Plasmid pDG782, containing a kanamycin resistance cassette, was provided by the Bacillus Genetic Stock Center. Plasmid pG$^+$host5 was obtained from Appligene, Inc., Pleasanton, CA. Transformation of *E. coli* was performed by the procedure of Brian Seed as previously described [16]. Protoplasts preparation and transformation of *B. firmus* was performed

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using a protocol described by Ito, et al [56] (see Section 2.4 for detailed description).

2.3. DNA isolation and Plasmid construction

E. coli plasmids were isolated as described in Chapter 1. When isolating plasmid DNA from *B. firmus*, 10 mL of medium A was inoculated with *B. firmus* cells from a single colony. The culture was incubated overnight at 30°C in a shaker at 200 rpm. The cells were harvested by centrifugation and the pellet was resuspended in 400 μL of GTE solution (see Ch.1) containing 1 mg/mL lysozyme. After incubation at 37°C for 10 min, plasmid was isolated by the SDS-alkaline lysis method as described by Sambrook et al.[25].

The construction of plasmids is shown in Figure 2.1. The techniques for restriction enzyme digestion, calf intestine phosphatase treatment, DNA fragment purification and DNA ligation are as described in Chapter 1. Plasmid pQC1 [57] was digested with *Cla*I to cut the two *Cla*I sites, which are both within the *B. firmus cls* gene and 300 bp apart. The digested plasmid was then treated with calf intestine phosphatase to prevent self-ligation. The 300 bp *Cla*I-*Cla*I fragment of the plasmid was replaced by a 1.4 kb kanamycin resistant cassette, obtained by digesting plasmid pDG782 with *Cla*I. The resulting plasmid pPS1 was digested with *Bam*HI and *Pst*I to yield a 4.1 kb fragment containing the disrupted *B. firmus cls* gene. This fragment was inserted into plasmid pG$^+$host5 that had been cleaved by *Bam*HI and *Pst*I to construct plasmid pSYJ.

2.4. Preparation and transformation of B. firmus *OF4 811M protoplasts*

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Fifty milliliters of medium A in a 250 mL flask was inoculated with cells from a single *B. firmus* OF4 811M colony. The culture was incubated overnight at 30°C with constant shaking (about 200 rpm). A half milliliter of the overnight culture was transferred into 50 mL of fresh medium A in a 250 mL flask and the culture was incubated in a 30°C shaker (200 rpm). When the OD_{660} reached 0.4-0.6, the cells were harvested by centrifugation at 3000 g for 10 min at 4°C. The cells were resuspended in 10 mL of cold SMMP (see Appendix 1) and centrifuged again at 3000 x g for 10 min at 4°C. After pouring off the supernatant, the cell paste was resuspended in 3 mL of SMMP. To remove the cell walls, 10 μL of lysozyme solution (3 mg/mL) was added and the mixture was incubated at 37°C for 30 min without shaking. Then a 10 μL sample was removed and examined by phase-contrast microscopy. Approximately 99% of the cells were converted to protoplasts (sphere shape), which were harvested by centrifugation at 5000 rpm for 10 min at room temperature. The pellet was gently resuspended in 500 μL of cold SMMP and divided into 5 tubes. Although the protoplasts can be stored at -80°C, fresh preparations were used in this work.

Plasmid pSYJ, containing the disrupted *cls*, was introduced into *B. firmus* OF4 811M protoplasts by transformation. An aliquot (100 μL) of protoplasts was mixed with 4 μL of pSYJ DNA (~1 μg/μL) followed by 300 μL of 30% PEG (see Appendix 1). To get the best result, plasmid DNA was laid on the wall of the microtube and then carried into the protoplast suspension by a quick injection of PEG solution with a disposable syringe. The mixture was mixed and left for 3 min

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at room temperature and then 1 mL of SMMP was added and the suspension mixed. All mixings were done gently by slowly pipetting the contents with a pipetman. The protoplasts were harvested by centrifuging at 3000 rpm for 15 min at room temperature in a microfuge. The supernatant was discarded and the protoplasts were resuspended in 500 μL of cold SMMP. About 100 μL of this suspension was plated on the modified DM3 plates (see Appendix 1) supplemented with 0.1 μg/mL erythromycin. The plates were incubated at 30°C and colonies appeared after 3 days.

2.5. Construction of a cls *null mutant strain of* B. firmus *OF4*

The method of Biswas et al [58] was used to construct a *cls* null mutant of *B. firmus* OF4. This method is based on a thermosensitive (Ts) broad-host-range rolling-circle plasmid pG$^+$host5. The plasmid also contains a pBR322 replicon, allowed recombinant plasmid pSYJ to be constructed and maintained in *E. coli*. Plasmid pSYJ was introduced into *B. firmus* OF4 811M cells as described above. Cell walls of the transformed cells were regenerated on the modified DM3 plates containing 0.1 μg/mL erythromycin at 30°C. Colonies appeared after 3 days and were purified on medium A (pH7.5) containing 0.3 μg/mL erythromycin. The presence of pSYJ was verified by electrophoretic analysis of plasmid DNA isolated from the transformed cells. To obtain single-crossover integrants, the transformed cells were cultured overnight in medium A containing 0.3 μg/mL erythromycin at 28°C, diluted and plated on medium A supplemented with 50 μg/mL kanamycin. The plates were incubated at 39°C overnight. Recombination

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between the plasmid and *B. firmus* chromosome DNA occurred at homologous regions, resulting in the integration of plasmid pSYJ into *B. firmus* chromosome. The acquired single-crossover integrants were resistant to both kanamycin and erythromycin at 37°C. The gene replacement was completed by a second recombination. A single-crossover integrant was cultured overnight at 28°C. The shift of temperature activated the replication of the integrated plasmid that led to plasmid excision and gene replacement. The culture was then diluted and plated on medium A supplemented with kanamycin at 37°C. Colonies that appeared were transferred onto plates of medium A containing kanamycin and tested for erythromycin sensitivity at 30°C. Out of about 500 colonies tested four were erythromycin sensitive. One of the four, designated DG301, was selected for further examination.

2.6. Polymerase chain reaction

The gene replacement in the mutant cells was confirmed by colony PCR analysis. A small portion of a colony was transferred with a toothpick to 50 μL of distilled water in a microfuge tube. The tube was heated in boiling water bath for 5 min. Insoluble materials were removed by centrifugation for 30 sec at 12000 rpm and the supernatant was used for PCR reaction. The following reagents were added to a thin-wall PCR microfuge tube: 25 μL of PCR ReadyMix, 1 μL each of the forward and the reverse primers, 10 μL of the colony supernatant and 13 μL of distilled water. The tube was vortexed and centrifuged briefly to force all contents to the bottom of the tube. About 50 μL of mineral oil was added to the

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top of the tube to prevent evaporation. A DNA Thermal Cycler (Perkin Elmer) was used to run the PCR reaction. The settings were as follows: (1) denature the template at 94°C for 1 min; (2) anneal primers at 55°C for 2 min; (3) extend at 72°C for 3 min (amplify 25 cycles); (4) soak at 4°C. The last soaking step was used in case reaction mixtures are not to be removed immediately from the machine at the completion of the amplification cycles. After the reaction was complete, a 10 μL sample was carefully taken from the bottom layer and mixed with 5 μL of DNA loading buffer. The amplified DNA fragments were analyzed by agarose gel electrophoresis.

2.7. Lipid analysis

B. firmus cells were cultured in 10 mL of medium A at 30°C with shaking. When the culture reached a turbidity of 30 Klett units, 6 μCi of [2-^{14}C]acetate (sp. act. 4.3 mCi/mmol) were added to the culture. Cells were harvested after 2 h and glycerophospholipids were isolated and analyzed as described in section 2.9 of Chapter 1.

2.8. Growth experiments

Growth experiments were performed using both liquid media and agar plates. To study the growth of the *B. firmus* strain containing a *cls* null mutation under alkali conditions, medium A and B were prepared at different pH values from 7.5-10.5. To test the effect of different carbon sources, growth media were prepared with either glucose or malate as the carbon source while the other ingredients were kept the same.

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3. Results and Discussion

3.1. Construction of plasmid pSYJ

The construction of plasmid pSYJ is shown in Figure 2.1. The *B. firmus* *cls* gene on plasmid pQC1 was disrupted by replacing the 300 bp fragment between the two *Cla*I site with a 1.4 kb kanamycin resistant cassette from pDG782. The resulting plasmid pPS1 was introduced into HB101 by standard transformation procedure. The transformed cells were resistant to 50 μg/mL kanamycin. Plasmid pPS1 was then digested with *Bam*HI and *Pst*I. A 4.1 kb fragment containing the disrupted cls gene and the intact *mec*A gene was isolated and inserted into the shuttle vector pG^{+}host5 that also was treated with *Bam*HI and *Pst*I. The ligation mixture was used to transform competent HB101 cells. Transformants that were resistant to both kanamycin and erythromycin were selected. Recombinant plasmid pSYJ was isolated from one of the transformants. The structure of pSYJ was verified by agarose gel electrophoresis after cleavage with restriction enzymes.

3.2. Construction of the B. firmus *OF4* cls *null mutant DG301*

Preparation and transformation of *B. firmus* OF4 811M protoplasts were performed as described in Section 2.5. A modified DM3 was used to plate the transformation mixture for cell wall regeneration. Kanamycin was found to be problematic when used in DM3 plates for selection. The drug showed little effect even when the concentration used was as high as 300 μg/mL. The transformant cells however were very sensitive to erythromycin. Initial use of erythromycin at

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0.6 μg/mL didn't produce any transformant colonies after 3 days of incubation at 30°C. The concentration of erythromycin was then lowered to 0.1 μg/mL. About 15 colonies appeared after the transformation plates were incubated at 30°C for 3 days. The colonies were purified on medium A plates supplemented with 0.3 μg/mL erythromycin at 30°C. Two thirds of the colonies were able to grow under these conditions. These transformants were also resistant to kanamycin at a concentration of 50 μg/mL on medium A plates. Plasmid DNA was isolated from two of these transformants as described in section 2.3 and verified to be pSYJ by restriction enzyme digestion and agrose gel eletrophoresis (data not shown).

Plasmid pSYJ, like its parent plasmid pG$^+$host5, has a temperature-sensitive replicon for replication in gram-positive bacteria and a pBR322 replicon for replication in *E. coli*. After the plasmid was transformed into *B. firmus*, the transformed cells, *B. firmus* OF4 811M/pSYJ, were incubated in medium A supplemented with 50 μg/mL kanamycin at 28°C overnight. The overnight culture was diluted 10^3, 10^4, and 10^5 fold into medium A. One hundred microliters of each dilution was plated on medium A plates containing kanamycin. The plates were incubated at 39°C for about 20 h. The temperature increase shut off plasmid replication, forcing plasmids to be integrated into the host chromosome at the homologous regions flanking the kanamycin cassette insertion site in the *cls* gene. One of the single-crossover integrants, designated DG300, was selected for further study. DG300 was cultured in 10 mL medium A supplemented with 50 μg/mL kanamycin at 28°C to activate rolling circle

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replication leading to plasmid excision. After overnight incubation, the culture was diluted in medium A and plated on medium A agar plates supplemented with 50 μg/mL kanamycin at 39°C. The colonies that appeared on the second day were transferred to fresh medium A plates supplemented with 50 μg/mL kanamycin and incubated overnight at 28°C. The double-crossover integrants were then tested for erythromycin sensitivity. The second recombination occurs at the homologous regions, so the plasmid excision could either restore the original chromosome or result in gene replacement at *cls*. The latter should produce a strain that is kanamycin-resistant and erythromycin-sensitive. Of about 500 double-crossover integrants tested, four didn't grow on plates containing 0.3 μg/mL erythromycin. One of the four, strain DG301, was purified and saved for further study.

3.3. Gene replacement was verified by analysis of PCR products.

A pair of PCR primers (see Materials and Methods) was selected just outside the two ClaI sites within the *B. firmus cls* gene. The two primer sites are about 500 base pairs apart in the wild type *cls* gene. In the disrupted *cls* gene, the two sites are about 1.7 kb apart because of the insertion of the kanamycin cassette. The result of the PCR reaction is shown in Figure 2.2. The product obtained from DG301 appeared as a single band with a size of about 1.7 kb (lane 4) while the product obtained from wild type *B. firmus* OF4 811M appeared as a single band of 0.5 kb (lane 5). This result clearly shows that the *cls* gene in DG301 was altered. To ensure the mutant strain was indeed *B. firmus*, a pair of

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PCR primers kindly provided by Dr. Terry A. Krulwich were also used for DNA amplification. The product from these primers belongs to an unidentified *B. firmus* gene and should have a size of about 600 bp when *B. firmus* DNA is used as the template (unpublished results of Dr. Krulwich's laboratory). The results shown in lane 1 and 2 indicated that strain DG301 gave the same positive result as the wild type strain.

3.4. The mutant strain DG301 doesn't make cardiolipin

DG301 and OF4 811M membrane lipids were radio-labeled by incubating the cell culture with [2-^{14}C]acetate as described in section 2.7. The phospholipids were isolated and each resuspended in 20 μL of chloroform. About half of each lipid solution was applied to a Silica G thin-layer chromatography plate. Authentic phospholipids CL, PE and PG were used as markers. The plate was developed in methanol : chloroform : acetic acid (25 : 65 : 8) solvent system. The plate was air-dried and put into I_2 Tank. The lipid samples appeared as yellow spots after 1 min. Three spots appeared in the lanes with the wild type *B. firmus* lipids. These spots had the same Rf values as the authentic CL, PE and PG, respectively. Only two spots were visible in the lanes with samples from DG301. The spot corresponding to CL was missing. Radioactive lipids were quantified by cutting the silica plate into strips and counting the strips in a liquid scintillation counter. As shown in Table 2.1, less than 1% of the radioactive label in the lipid extract of DG301 (the *cls* null mutant) migrated in the CL region. The comparable value for the lipid extract from wild type cells was 12%. This result shows that the mutant

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strain synthesizes little, if any, cardiolipin. As expected, the mutant has a higher PG level. It also has a somewhat lower PE level.

Homologous sequence search reveals that several organisms, including *E, coli* (Fig. 3.1, Chapter 3) and *B. subtilis* (Fig. 1.4, Chapter 1), have more than one genes coding for proteins that belong to the CL synthase family. Whether or not this is also the case for *B. firmus*, the results from the lipid analysis of DG301 supports the conclusion that the isolated gene actually codes for *B. firmus* CL synthase.

3.5. Growth characteristics of DG301

The fact that a *cls* null mutant strain of *B. firmus* could be constructed indicated that *cls* is not an essential gene. To test the effect of deactivation of *cls* on the growth of *B. firmus*, especially on the growth of cells at alkali condition, medium A and B were both prepared at pH7.5 and pH10.5. Overnight cultures of OF4 811M and DG301 in medium A were diluted 50 fold respectively into 10 mL of fresh medium A in a 250 mL side-arm flask. The cultures were incubated at 30°C with shaking at 200 rpm. Growth was monitored by checking the turbidities with a Klett-Summerson photometer. As shown in Figure 2.3, both the wild type strain and the mutant strain grow at similar rates in medium A. All the cultures reached stationary phase after about 7 hours incubation. The turbidities at stationary phase were about 100 Klett. To our surprise, there were no significant differences in growth for the mutant strain at pH 7.5 and pH10.5. The results with medium B are shown in Figure 2.4. The wild type cells grow

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normally in this medium and at a similar rate as when cultured in medium A and reach stationary phase at about 100 Klett in 7 hr. However, the mutant strain grows much more poorly in this medium. The cells reach 15 Klett after about 4 hr, but then the growth slows down substantially. The turbidity only reaches about 20 Klett after a 12 hr incubation. And once again, the difference in media pH does not have any effect on the growth of the mutant cells. The mutant has virtually the same growth curve at pH7.5 and pH10.5.

The growth experiments were repeated on agar plates containing the same media (data not shown). Fresh colonies of DG301 and OF4 811M from agar plates containing medium A or B (all at pH7.5) were streaked onto plates containing the same media with pH 7.5 or pH10.5. After a 16-h incubation at 30°C, the plates were examined for growth. Both strains appeared as thick white lines on medium A plates, indicating normal growth. No differences were observed on the plates with different pH values for either strain. On medium B plates, the wild type strain OF4 811M appeared as thick white lines, indicating healthy growth. However the mutant strain DG301 appeared as thin white lines, indicating poor growth. The pH difference was found to have no effect on the growth of DG301.

As shown in Figure 2.3 and 2.4 and results of growth experiments on agar plates, the *cls* null mutant DG301 grows normally in medium A but poorly in medium B while the wild type strain grows normally in both media. The two media are very different. One of the major differences is the carbon source.

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Medium A contains glucose, a fermentable carbon source and medium B contains non-fermentable malate. It is possible *B. firmus* cells with a *cls* null mutation may not be able to utilize malate as an energy source as well as the wild type cells do. To test this possibility, cell growth with different carbon sources was compared in both medium A and B. The results are shown in Figures 2.5 and 2.6. In media A, when glucose was used as the carbon source (Figure 2.5A), both strains showed normal growth. Cells reached stationary phase when the turbidities were above 100 Klett. In medium A containing malate (Figure 2.5B), after a slightly longer lag phase, DG301 grew at a similar rate to that of OF4 811M. Wild type cells reached stationary phase at about 160 Klett and stopped growing completely after approximately 9 h of incubation. However, the growth of the mutant DG301 started to level off at about 40 Klett. The cells continued to grow at a very slow rate for the rest of the monitored time period and reached about 70 Klett after 15 h of incubation. In medium B (Figure 2.6) though, no significant differences were found for the growth of either strain when they were cultured in media containing glucose or malate as the carbon source. However, wild type cells showed much healthier growth than the *cls* mutant did.

Further comparisons between the two media (see Appendix 1) reveals some other differences besides the carbon sources: (1) medium A contains 0.5% of Bacto-peptone but medium B does not; (2) medium B contains STS while medium A contains citrate; (3) medium A has a higher concentration of Bacto-yeast extract and Mg^{2+}. To study the reason or reasons for the growth difficulty of

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the mutant in medium B, contents of both media were altered to test for the growth of both the mutant and the wild type strains. These experiments were performed on agar plates and the results are summarized in Table 2.2. In these experiments, changes were made to the presence or absence of Bacto-peptone, STS and citrate as well as to the concentrations of Bacto-yeast extract and Mg^{2+} in both media. The results shown in Table 2.2 indicate that none of the changes made significantly affects the growth ability of either strain in either media. The media used in Table 2.2 were prepared at both pH7.5 and pH10.5 and both Na^{+} and K^{+} concentrations were kept at 0.2 M. No pH effects were observed in any of the experiments.

The growth experiments described above were usually carried out at 30°C. However, the strains were also tested for growth at 42°C (data not shown). No significant changes were observed except that the growth of both strains was poorer at the elevated temperature.

One interesting result is that DG301, the *cls* null mutant, lost streptomycin resistance that is present in the wild type strain *B. firmus* OF4 811M [59]. Streptomycin is an aminoglycoside antibiotic. It can bind to 30S ribosomal proteins and block protein synthesis. Mechanisms for streptomycin resistance include failure of the drug to reach the target due to changes in membrane transport system or the target proteins and destruction of the drug by enzymes such as phosphorylases. However, the mechanism for streptomycin-resistance by OF4 811M is not clear. Further study is needed to clarify the mechanism of

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streptomycin resistance by OF4 811M and the loss of resistance to the drug by DG301. It is possible that lack of cardiolipin in the mutant membranes alters the cell membrane structure, causing increased accessibility to the drug by the cells.

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Table 2.1.

Glycerophospholipid analysis

Strain	% ^{14}C distribution		
	PE	PG	CL
DG301	17.7	81.4	0.87
OF4811M	23.1	65.1	11.8

Table 2.1. Bacteria were cultured in 10 mL of medium A at 30°C with shaking. At a turbidity of 30 Klett, 6 μCi of [2-^{14}C]acetate (sp. Act. 4.3 mCi/mmol) were added and the culture was incubated for another 2 h. Glycerophospholipids were isolated and analyzed as described in Materials and Methods.

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Table 2.2

Growth experiments on agar plates

	Media	**Growth Ability**	
		DG301	**OF4 811M**
Medium A	Remove Bacto-peptone	+	+
	Add STS	+	+
	Remove citrate	+	+
	Use 0.1% yeast extract	+	+
	Use 0.1 mM Mg^{2+}	+	+
Medium B	Add Bacto-peptone	-	+
	Remove STS	-	+
	Add Citrate	-	+
	Use 0.2% yeast extract	-	+
	Use 0.2 mM Mg^{2+}	-	+

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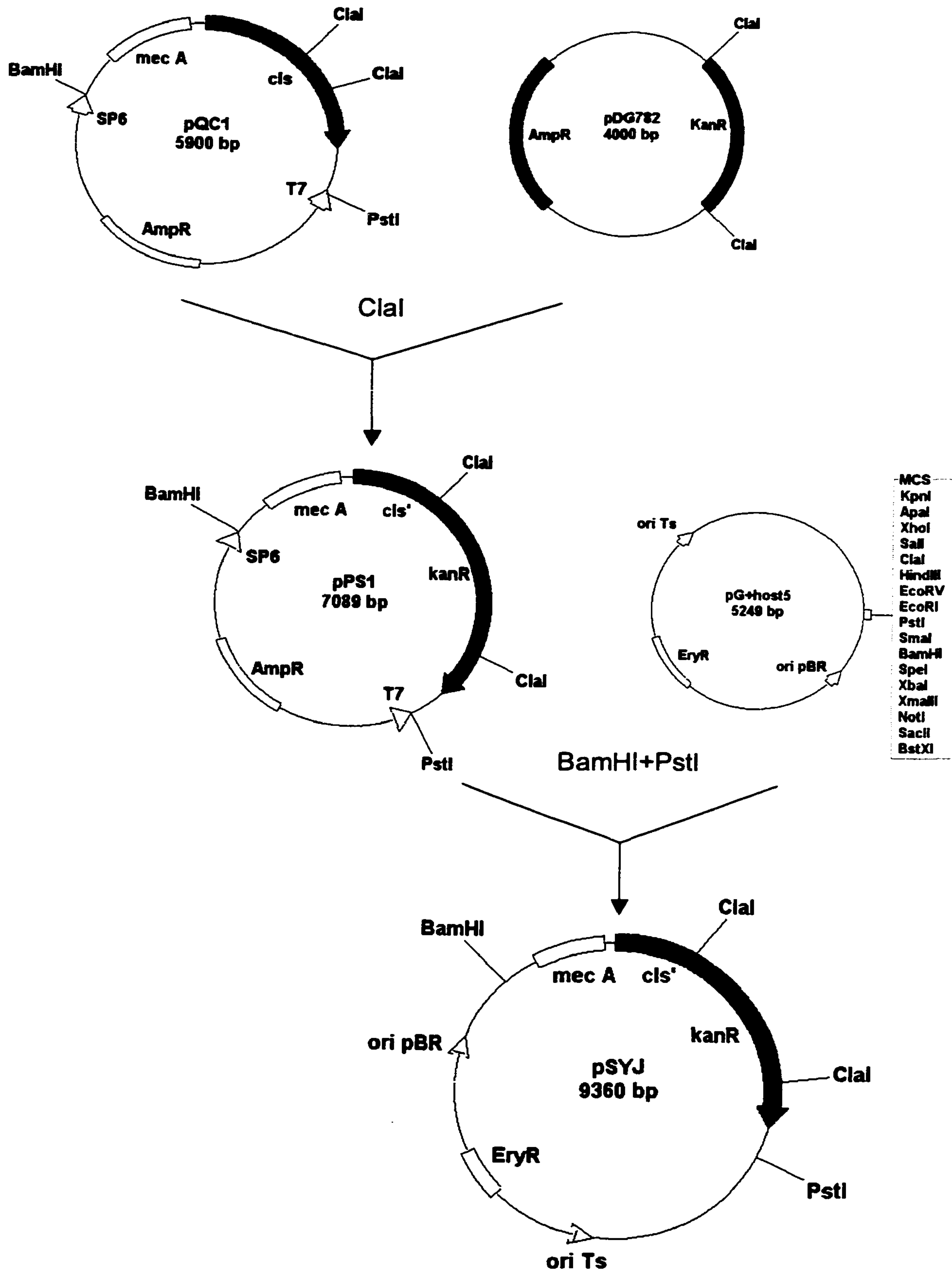

Fig. 2.1. Construction of plasmid pSYJ.

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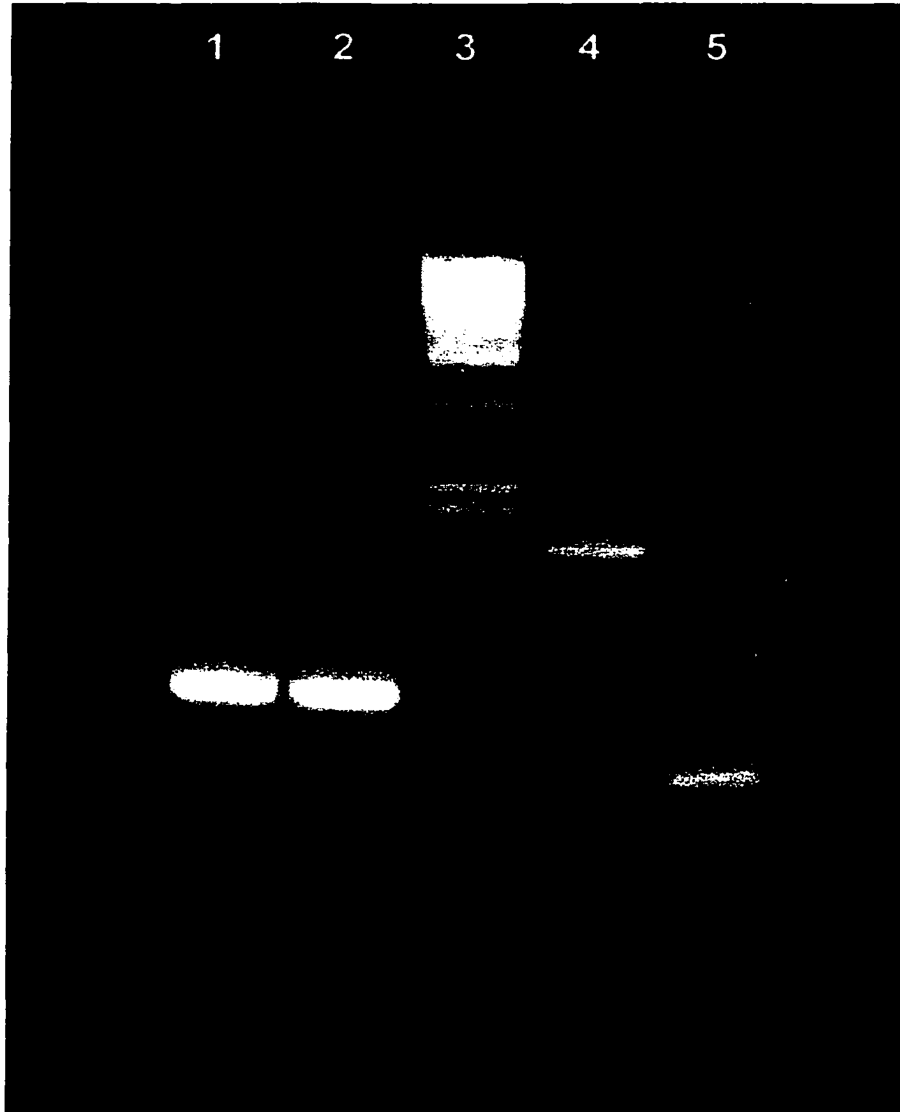

Fig. 2.2. Agarose gel electrophoresis analysis of PCR products. Lane 1, DG301 with the primers provided by Dr. Krulwich; Lane 2, OF4 811M with the primers provided by Dr. Krulwich; Lane 3, DNA marker (λ/Hind III); Lane 4, DG301 with the primers for *cls*; Lane 5, OF4 811M with the primers for *cls*.

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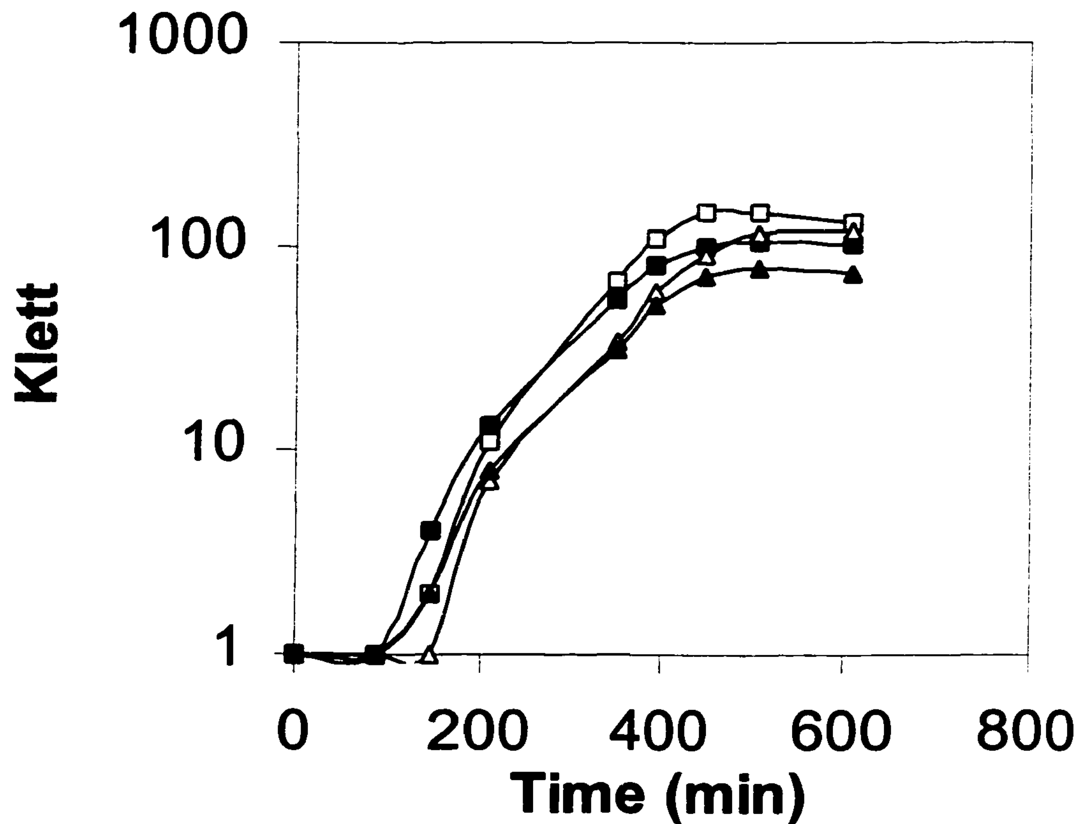

Fig. 2.3. Effect of pH on growth in mediiu A. Strains *B. firmus* OF4 811M (□, pH7.5 and ■, pH10.5) and DG301 (△, pH7.5 and ▲, pH10.5) were grown in media A, shaking at 30°C and 250 rpm. Cell growth was measured using a Klett-Summerson photometer (660-nm filter). One Klett unit corresponds to approximately 5×10^6 cells/mL.

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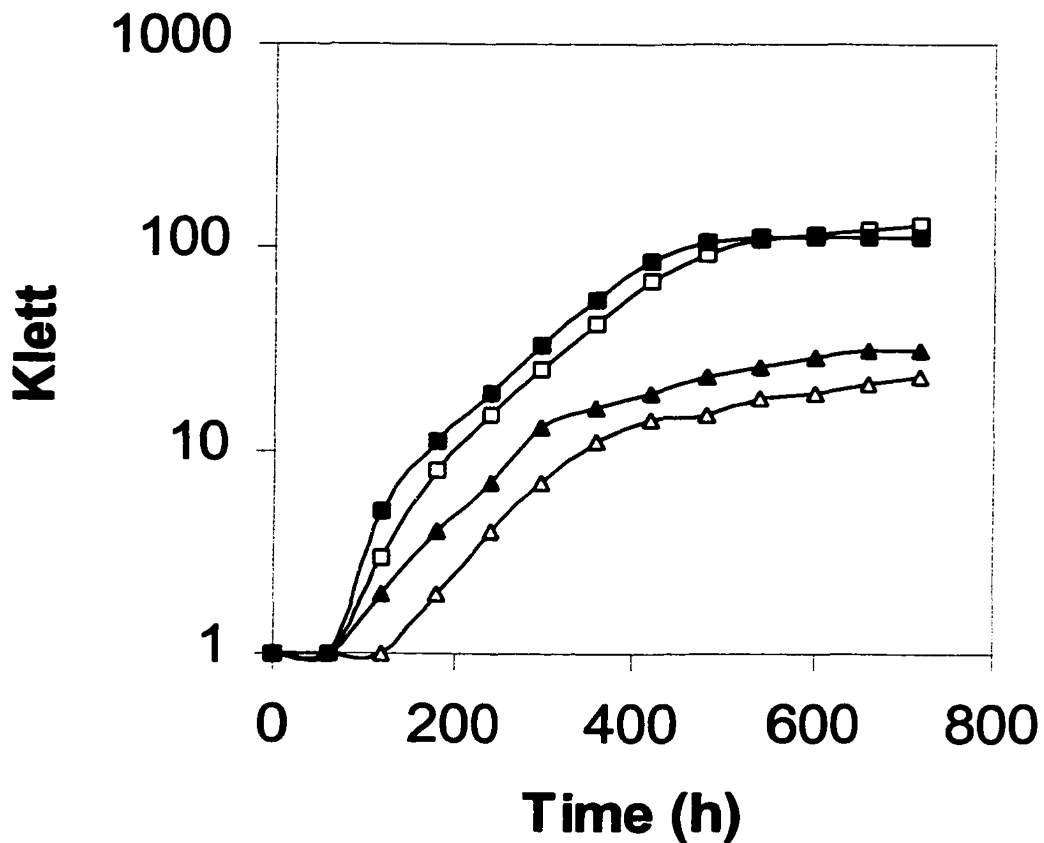

Fig. 2.4. Effect of pH on growth in medium B. Strains *B. firmus* OF4 811M (□, pH7.5 and ■, pH10.5) and DG301 (△, pH7.5 and ▲, pH10.5) were grown in media B, shaking at 30°C and 250 rpm. Cell growth was measured using a Klett-Summerson photometer (660-nm filter). One Klett unit corresponds to approximately 5 x 10^6 cells/mL.

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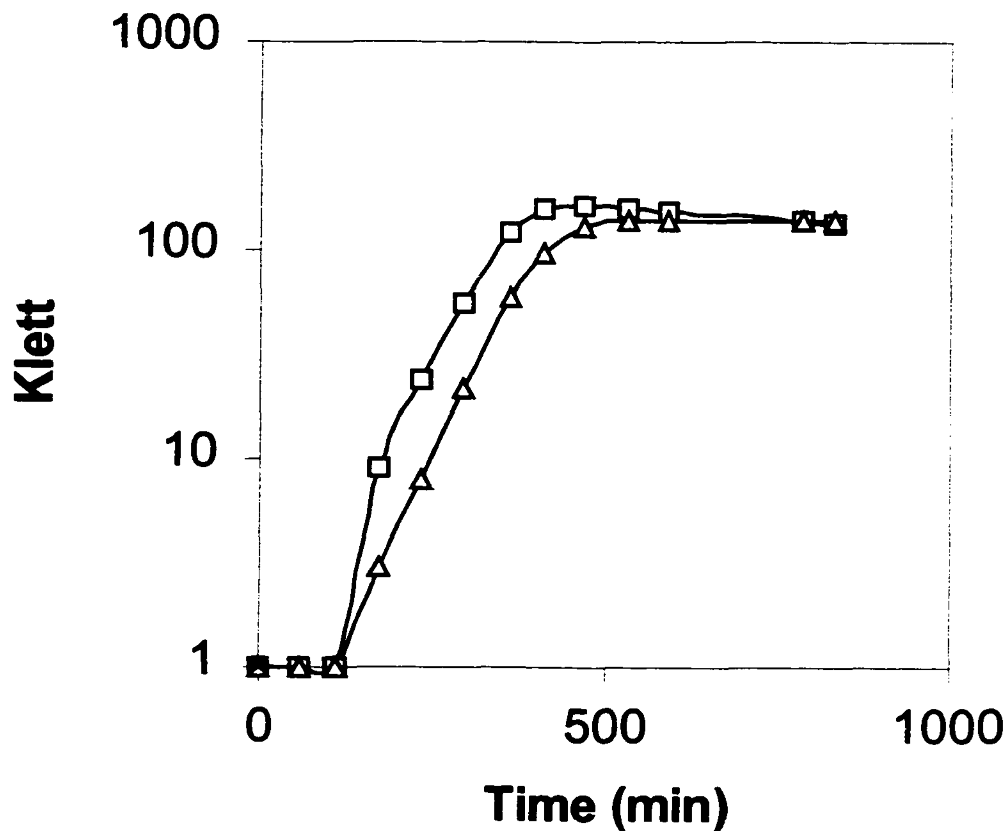

Fig. 2.5A. Growth in medium A with glucose as the carbon source. Strains *B. firmus* OF4 811M (□) and DG301 (△) were grown in medium A (pH 7.5) with glucose as the carbon source, shaking at 30°C and 250 rpm. Cell growth was measured using a Klett-Summerson photometer (660-nm filter). One Klett unit corresponds to approximately 5 x 10^6 cells/mL.

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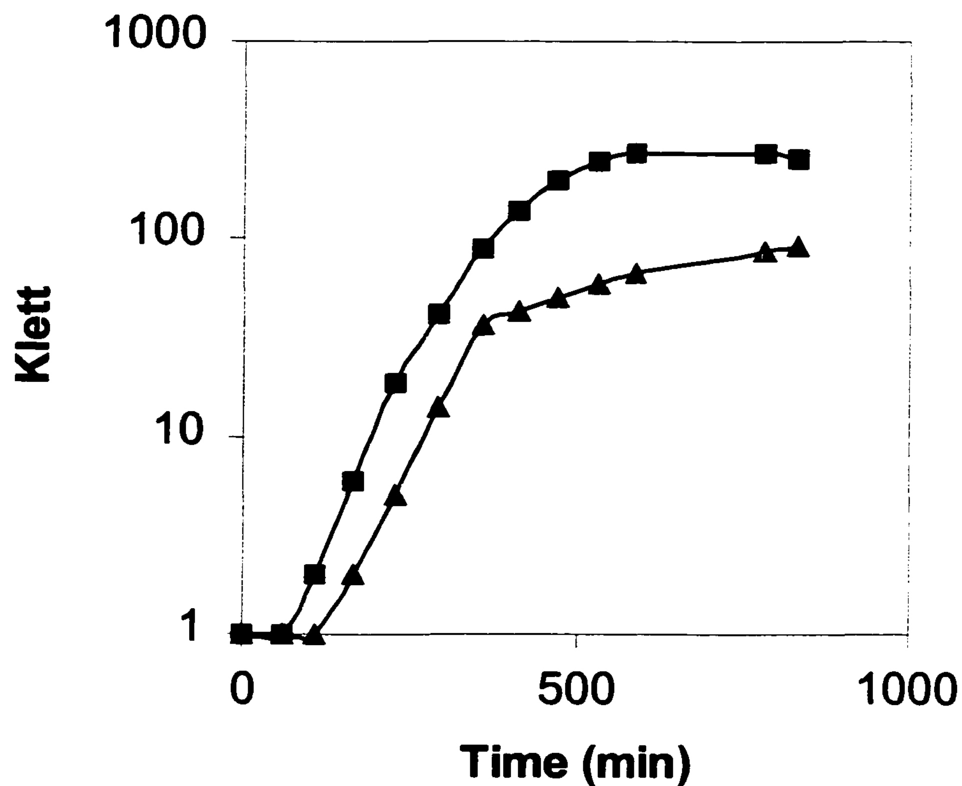

Fig. 2.5B. Growth in medium A with malate as the carbon source. Strains *B. firmus* OF4 811M (■) and DG301 (▲) were grown in medium A (pH 7.5) with malate as the carbon source, shaking at 30°C and 250 rpm. Cell growth was measured using a Klett-Summerson photometer (660-nm filter). One Klett unit corresponds to approximately 5 x 10^6 cells/mL.

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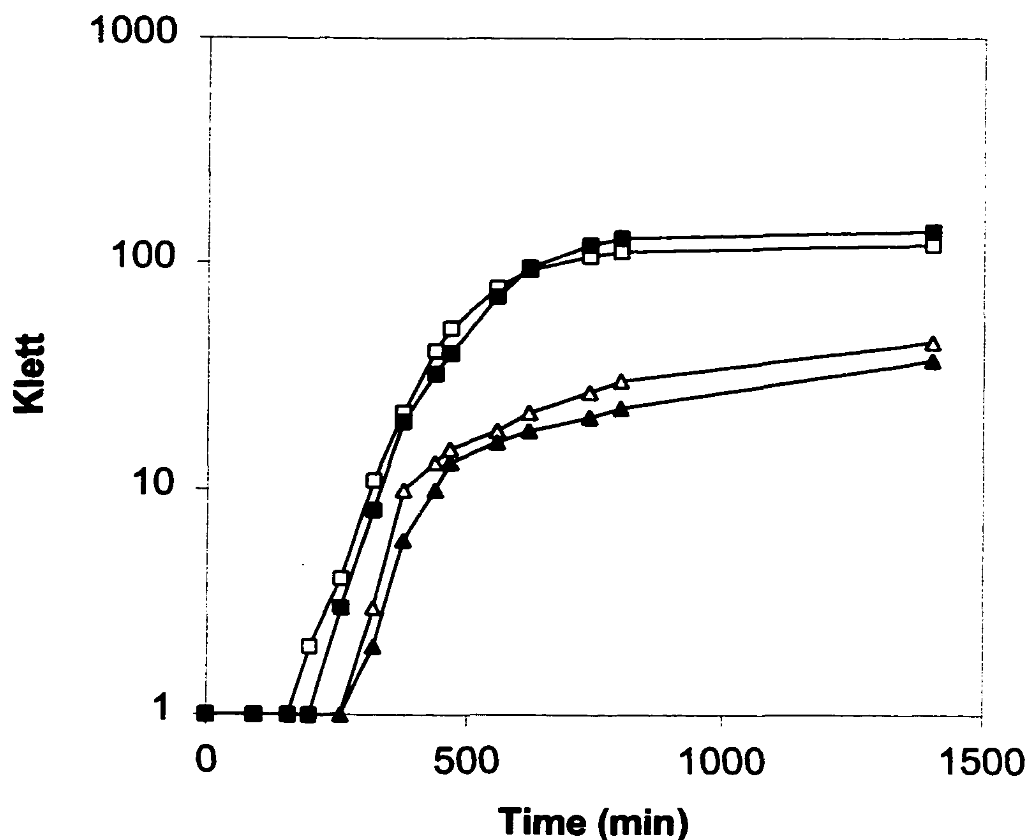

Fig. 2.6. Effect of carbon source on growth in medium B. Strains *B. firmus* OF4 811M (□, glucose and ■, malate) and DG301 (△, glucose and ▲, malate) were grown in medium B (pH 7.5), shaking at 30°C and 250 rpm. Cell growth was measured using a Klett-Summerson photometer (660-nm filter). One Klett unit corresponds to approximately 5 x 10^6 cells/mL.

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CHAPTER 3

A Second *Escherichia coli* Protein with CL Synthase Activity

1. Introduction

In the study reported in Chapter 1, a comparison of predicted amino acid sequences among CL synthases from *E. coli, B. firmus* and putative CL synthases from other prokaryotes showed extended homology along the whole sequences. Most notably, the enzymes all have two $HXK(X)_4D(X)_6G(X)_2N$ motifs (X represents any amino acid residue), also known as HKD motifs, a characteristic motif of the PLD superfamily, which includes phospholipase D, *E. coli* phosphatidylserine synthase, poxvirus envelope proteins [37, 38, 60], endonucleases [61], and *Yersinia pestis* murine toxin (Ymt) [62]. These proteins all have the ability to bind a phosphodiester moiety in their active site. The conserved HKD motifs form the active site. Determined crystal structure of phospholipase D from *Streptomyces* sp. strain PMF shows a single active site is formed by the two conserved HKD domains [63]. This feature should be common for PLD superfamily members since most of these proteins, including CL synthase, contain two copies of the conserved motif. One of the exceptions is nuc, an endonuclease from *Salmonella typhimurium*. It has only one copy of the motif. However, the crystal structure of nuc reveals that it forms a dimer and a single active site is formed within the dimer [64]. The two histidine residues in the HKD motifs play the leading roles in catalyzing the reaction. One of the histidines acts as a nucleophile, attacking the phosphodiester bond to form a phospho-

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enzyme intermediate. Kinetic analysis of nuc protein indicates the existence of such an intermediate [61]. The second histidine probably activates a water or primary alcohol molecule being a second nucleophile to complete the reaction.

E. coli CL synthase, specified by the *cls* gene located at min 28.02 of the *E. coli* genetic map [65], is likely to have a similar catalytic mechanism. CL synthase is a membrane enzyme. Kinetic studies reveal that CL synthase is stimulated by phosphate and inhibited by CL, the product of the reaction, and by phosphatidate [19]. It is interesting to note that unlike CL synthase, phospholipase D from *Streptomyces* sp. strain PMF is inhibited by phosphate [63] while activated by phosphatidate and Ca^{2+} [66]. *E. coli* CL synthase activity doesn't need Ca^{2+} [19].

E. coli has two genes that are homologous to *cls*, *f413* (*ybh*O) and *o493* (*ymd*C). A comparison of the products encoded by the *cls* and *f413* genes indicates extended regions of homology with 101 identities and 65 similarities (Figure 3.1). Both predicted amino acid sequences have the two HKD motifs characteristic of the PLD superfamily but the polypeptide specified by *cls* has about 100 residues at the N-terminus that are missing from the polypeptide specified by *f413*. The study reported here shows that the f413 protein catalyzes CL formation in vitro but not in vivo and that amino acid residues specified by the first 60 codons in *cls* are not essential for catalytic activity.

2. Materials and methods

2.1. Chemicals

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Ampicillin; kanamycin; tetracycline; chloramphenicol; isopropyl β-D-isopropylthiogalactopyranoside (IPTG); agarose; Folin & Ciocalteu's phenol reagent; Triton X-100; bovine serum albumin (fraction V); DEAE-cellulose; glycerokinase (from *E. coli*); high-molecular mass standard mixture for SDS gel electrophoresis; Brilliant Blue G-perchloric acid solution; gel fixing solution; L-α-phosphatidyl-DL-glycerol, dipalmitoyl; L-α-phosphatidate, dipalmitoyl; *E. coli* cardiolipin; cabbage phospholipase D Type I; 1, 3-dihydroxyacetone; polyethylene glycol (PEG)-8000 and acrylamide (ultra pure) were purchased from Sigma (St. Louis, MO). 1-Palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (NBD-PG) and L-α-phosphatidylcholine (egg lecithin) were from Avanti Polar Lipids (Alabaster, AL). NBD-PG was purified by thin-layer chromatography to remove impurities and concentrations were determined with a Dynatech MR 5000 Microplate Reader (Dynatech Laboratories, Chantilly, VA). Restriction endonucleases, T4 DNA ligase and calf-intestine alkaline phosphatase were products of GIBCO BRL (Gaithersburg, MD). Cytidine 5'-diphosphate-DL-diacylglycerol, dipalmitoyl was obtained from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ). Polygram® Sil G thin-layer chromatography plates were purchased from Brinkmann Instruments (Westbury, NY). UNISIL® activated silicic acid (200-325 mesh), a product of Clarkson Chemical Company (Williamsport, PA), was heated to 110°C for 2 h before use. [2-^{3}H]Glycerol was purchased from New England Nuclear (Wilmington, DE). [^{3}H]Sodium borohydride was from NEN Life Science

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Products (Boston, MA). [^{32}P]Phosphate was a product of ICN (Irvine, CA). QuikChange™ Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA). Oligonucleotide primers for site-directed mutagenesis were synthesized by CyberSyn, Inc. (Aston, PA).

2.2. Strains, plasmids and growth conditions

E. coli strains used in this study are listed in Table 3.1 and plasmids are listed in Table 3.2. Standard P1 transduction techniques [26] were used to construct new bacterial strains. Unless otherwise indicated, cells were cultured in LB broth (1.0% bactotryptone, 0.5% yeast extract and 0.5% sodium chloride). M9ZB broth was prepared as described by Studier and Moffatt [23]. Where indicated, IPTG was added to 0.8 mM and ampicillin, tetracycline, kanamycin, and chloramphenicol were used at 125, 34, 50 and 20 μg/mL, respectively. Cell turbidity was monitored with a Klett-Summerson photometer (660-nm filter). One Klett unit corresponds to approximately 5×10^6 cells/mL.

2.3. DNA isolation, manipulation and plasmid constructions

As described in Chapter 1, plasmids were routinely prepared by the rapid SDS-alkaline lysis method [25] and the PEG-$MgCl_2$ method [24] was used to purify plasmid DNA for sequence studies. Restriction endonucleases, calf intestine alkaline phosphatase, and T4 DNA ligase were used according to the manufacturers' instructions. DNA fragments were separated by agarose gel electrophoresis following restriction enzyme digestion and purified by the

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electroelution method as described in Chapter 1 [25]. Transformations were performed by the procedure of Brian Seed as previously described [16].

Plasmid pLC12-48 of the Clark and Carbon collection [67], which contains the *f413* gene, was obtained from the *E. coli* Genetic Stock Center and used to construct plasmid pDG3 (Figure 3.2). The 2.5-kb *Pst*I-*Hinc*II fragment of pLC12-48, containing the intact *f413* gene, was joined to plasmid pBluescript II KS(-) that had been digested with *Pst*I and *Eco*RV so that the *f413* gene was placed under the control of a T7 promoter. Plasmid pDG4 was obtained in a similar fashion except that pBluescript II KS(-) was digested with *Pst*I and *Hinc*II.

The cloning of *f413* gene was confirmed by DNA sequencing, performed by the DNA Sequencing Core Laboratory at the University of Florida (Gainesville, FL), using automated fluorescent cycle sequencing protocols and the ABI 373 Stretch DNA sequencer (Perkin-Elmer/ABI Division, Forster City, CA).

Two plasmids, pDG5 and pDG7, were constructed to study the effect that N-terminal deletions have on CL synthase activity. Constructions of plasmid pDG5 and pDG7 are shown in Fig. 3.3 and 3.4, respectively. Plasmid pLR3 was digested at two *Sac*I sites; one is inside the *cls* gene (182 nucleotides downstream from the translation start site) and the other is outside the 3'-end. The resulting 1.4-kb fragment, which specifies a CL synthase fragment extending from amino acid residue 61 to slightly beyond the carboxyl terminus, was ligated with plasmid pET23a that was digested with *Sac*I and treated with calf intestine phosphatase. Because the 1.4-kb fragment has a *Sac*I site at each end, two

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orientations are possible and both were obtained. In plasmid pDG5 the truncated *cls* gene is fused in frame to the T7 tag from the pET23a vector and the fused protein is just downstream from the T7 promoter and the ribosome-binding site supplied by the pET23a vector. Therefore plasmid pDG5 codes for a fusion protein with a T7 tag at the N-terminus that is linked to a CL synthase fragment, which extends from residue 61 to the C-terminus.

Plasmid pDG7 was constructed from plasmid pDG5 as shown in Figure 3.4. Plasmid pDG5 was digested with *Hind*III. The 1.35-kb fragment, which specifies a CL synthase fragment extending from amino acid residue 105 to the carboxyl terminus, was inserted into the *Hind*III site of pBluescript II KS(-) (digested with *Hind*III and treated with calf intestine phosphatase). Two orientations are possible. Plasmid pDG6 with the orientation shown in Figure 3.3 was digested with *Eco*RI and the resulting 1.35-kb fragment was inserted into the *Eco*RI site of pET23a (digested with *Eco*RI and treated with calf intestine phosphatase) so that the reading frame of the *cls* fragment is in frame with the T7 tag. Therefore plasmid pDG7 codes for a fusion protein with a T7 tag at the N-terminus that is linked to a CL synthase fragment, which extends from residue 105 to the C-terminus.

2.4. Construction of an E. coli *f413 null mutant*

An *E. coli* f413 null mutant was constructed as described by Winans *et al.* [68]. A kanamycin resistant cassette from plasmid pDG782 was inserted into the f413 gene at the *Cla*I site of plasmid pDG4. The resulting plasmid, pDG8,

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was linearized with *ScaI* and introduced into competent strain JC7623. Cells from thirty kanamycin-resistant recombinant colonies were purified and subsequently shown to be ampicillin-sensitive. One of these recombinants, designated DG1, was used for further study.

2.5. Amplification of gene products in E. coli

Induction studies were performed in M9ZB media supplemented with ampicillin. Chloramphenicol was also included when plasmid pLysS was present. IPTG was added when the turbidity reached 120 Klett units and cells were harvested 3 hours later. Crude membrane and aqueous fractions were isolated and stored as previously described [19]. Protein concentrations in these fractions were determined by a modified Lowry procedure [69].

2.6. SDS-polyacrylamide gel electrophoresis (PAGE)

Crude membrane and aqueous fractions were analyzed by SDS-PAGE as described by Laemmli [34]. The reagents and procedures were as described in Chapter 1. Bands were visualized with Brilliant Blue G-perchloric acid solution according to the manufacturer's instructions. Molecular masses of the overexpressed proteins were estimated by comparing their migrations on the SDS gel with protein standards. Relative protein concentrations were obtained from densitometer tracings using a GS300 Transmittance/Reflectance Scanning Densitometer purchased from Hoefer Scientific Instruments, San Francisco, CA.

2.7. Thin layer chromatography

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Thin layer chromatography of lipids was performed using silica gel G plates with one of the following solvent systems: (1) chloroform : methanol : acetic acid (65:25:8) [4], (2) tetrahydrofuran : methylal : methanol : 4 M ammonia (50:25:25:5) [4], (3) chloroform : methanol : water (40:10:1) [4], or (4) chloroform : methanol : ammonia (65:25:5) [70].

2.8. Preparation of phosphatidyl[2-^{3}H]glycerol.

Phosphatidyl[2-^{3}H]glycerol used in most experiments was prepared as previously described [19]. However, that used in the enzyme-temperature sensitivity studies was prepared by a new method. A 4 mL aqueous solution containing 300 mM sodium acetate (pH 5.6), 120 mM $CaCl_2$, 4 g dihydroxyacetone and 200 units cabbage phospholipase D was mixed with 31 mL of diethyl ether containing 300 mg of phosphatidylcholine. After vigorous stirring for 10 h at 26°C in a closed flask, diethyl ether was removed by evaporation and the aqueous phase extracted with 30 mL chloroform. After two washes with 10 mL water, the chloroform solution was dried. Then the lipid product was re-suspended in 1 mL chloroform and placed on a 1.2 x 20 cm column containing activated silicic acid, which then was washed with 30 mL of chloroform followed by 30 mL of chloroform:methanol (9:1). The desired product, phosphatidyldihydroxyacetone, was eluted with 30 mL of chloroform:methanol (3:1) and produced a single spot when analyzed by thin-layer chromatography using solvent system 1. As expected for a ketone derivative, this spot reacted with phenylhydrazine spray reagent [70]. Additional structural confirmation was

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provided by product characterization after sodium borohydride reduction. Approximately 50 mg of dry phosphatidyldihydroxyacetone was dissolved in 1 mL 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). After overnight storage at room temperature, 1 mL of water was added and the lipid product, phosphatidyl[2-^{3}H]glycerol, was extracted with chloroform. The radioactive lipid co-chromatographed with authentic phosphatidylglycerol when analyzed in solvent systems 1, 3, and 4. Phosphatidyl[2-^{3}H]glycerol prepared by the two methods were equally active when used as substrate for *E. coli* CL synthase.

2.9. CL synthase assay and preparation of fluorescent labeled CL.

Standard CL synthase assays using phosphatidyl[2-^{3}H]glycerol were performed as previously described [19]. One unit of enzyme activity is defined as 1 nmol of [2-^{3}H]glycerol released per min [19]. Crude membrane samples containing 0.05 μg of protein were used to assay wild type CL synthase but the protein concentrations were increased to 0.5-2.0 μg for all other enzyme preparations. To analyze the lipid products of the CL synthase assay, the standard assay was scaled up 20-fold and the lipids were extracted and chromatographed by thin-layer chromatography in solvent system 1 as described previously [19].

A new assay procedure using NBD-PG, a fluorescent lipid, was devised to follow the fate of the phosphatidyl group in reactions catalyzed by CL synthase

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and f413 protein. The standard fluorescence assay mixture consisted of 40 μM NBD-PG, 0.03% Triton X-100, 10 mM β-mercaptoethanol, 320 mM potassium phosphate (pH7.0) and 0.5-2.0 μg of crude enzymes in a total volume of 100 μL. Lipids were extracted as previously described [28] and analyzed by thin-layer chromatography using solvent system 1. TLC plates were scanned with a FluorImager Storm 560 (Molecular Dynamics, Sunnyvale, CA, USA). Relative amounts of each lipid were determined by fluorescence imaging with excitation at 460 nm and emission at 540-560 nm.

E. coli CL synthase was used to convert NBD-PG to fluorescent labeled CL. The standard fluorescence assay described above, scaled-up 40-fold and containing 4 μg of the crude membrane from DG10/pLR3, was incubated at 37°C for 1 hr. Then lipids were extracted and separated by TLC using solvent system 1. Fluorescent labeled CL, NBD-CL, was eluted from the TLC plate with chloroform:methanol:water (5:10:4). The fluorescent product co-chromatographed with authentic CL in solvent systems 1-3.

2.10. Site-directed mutagenesis

The Stratagene QuikChange™ Site-Directed Mutagenesis Kit was used to modify the *E.coli* CL synthase by substituting specific amino acid residues. The experiments were performed following the manufacturers' instruction. The resulting mutants were subjected to SDS-PAGE assay and enzyme activity assay.

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For each amino acid substitution, two oligonucleotide primers containing the desired mutation were synthesized. Each of the oligonucleotide primers is complementary to opposite strands of the parental plasmid DNA. The commercially synthesized desalted primers were purified with acrylamide gel purification method (see Appendix 2).

After purification, the primers were diluted to 10 ng/μL. About 100 ng of each primer was mixed with the same amount of the pairing primer, 5 ng of plasmid pLR3 DNA, 5 μL of 10 x reaction buffer, 1 μL of dNTP mix, 1 μL of *pfu* DNA polymerase (2.5 units/μl). Distilled water was added to bring the volume up to 50 μL. The tube was tapped and centrifuged briefly to force all contents to the bottom of the tube. About 50 μL of mineral oil was added to the top of the tube to prevent evaporation. The reaction mixtures were applied to a DNA Thermal Cycler (Perkin Elmer). The settings were as follow: (1) denature the template at 94°C for 1 min; (2) anneal primers at 55°C for 2 min; (3) extend at 72°C for 3 min. Amplify 25 cycles; (4) soak at 4°C. After the amplification was finished, 1 μL of *DpnI* restriction enzyme (10 units/μl) was added to each reaction mixture below the mineral oil overlay using a small, pointed pipet tip. The mixtures were gently and thoroughly mixed by pipeting the solutions up and down several times. After centrifugation in a microcentrifuge (12, 000 xg) for 1 min, the reaction mixtures were incubated at 37°C for 1 h to digest the parental supercoiled double-strand DNA. *E. coli* XL1-Blue Supercompetent cells were gently thawed on ice. An aliquot of 50 μL of the cells was transferred to prechilled Falcon 2059 tube for the

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transformation of each reaction mixture. One microliter of each reaction mixture was added to the cells. The contents were mixed by swirling and the tubes were incubated on ice for 30 min. After a 45-sec heatshock at 42°C, the tubes were placed on ice for 2 min. A half milliliter of NZY+ broth (contains 1% Casein hydrolysate, 0.5% yeast extract, 0.5% NaCl, 12.5 mM $MgCl_2$, 12.5 mM $MgSO_4$ and 0.4% glucose) preheated at 42°C was added to each tube. After incubating at 37°C for 1 h with shaking (250 rpm), the entire volume of each transformation reaction sample was plated on LB agar containing 125 μg/mL ampicillin. Up to several hundreds of transformants were observed after overnight incubation at 37°C on each plate. Six colonies from each transformation plate were purified for further study.

3. Results

3.1. Construction of plasmid pDG3

Plasmid pLC12-48 of the Clark and Carbon collection contains the *f413* gene. The 2.5-kb *PstI-HincII* fragment of pLC12-48, containing the intact *f413* gene, was inserted into plasmid pBluescript II KS to produce plasmid pDG3 in which the *f413* is under T7 promoter control (Figure 3.2).

3.2. The f413 protein as a CL synthase

IPTG was added to cultures of DG10/pDG3, DG10/pBluescript II KS(-), and DG10/pLR3 in M9ZB medium to induce *f413* and *cls* gene expression. After 3 h, crude membrane and water-soluble fractions were isolated from each culture and their proteins analyzed by SDS-PAGE. The membrane fractions from

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DG10/pDG3 (Fig. 3.5, lane 3) and DG10/pLR3 (Fig. 3.5, lane 4) each has a unique protein band that is not present in the membrane fraction from DG10/pBluescript II KS(-) or any of the water-soluble fractions. The molecular mass of the f413 protein is about 48 kDa and accounts for approximately 11% of the crude membrane protein obtained from DG10/pDG3, whereas that of CL synthase is about 46 kDa and accounts for approximately 16% of the crude membrane protein obtained from DG10/pLR3. The observed molecular mass for the f413 protein agrees with the value predicted from translation of its open reading frame (ORF). In contrast, the observed molecular mass for CL synthase is about 8 kDa less than the predicted value, indicating that post-translational cleavage probably occurs [45].

Crude membranes from IPTG-induced DG10/pDG3 cultures were tested for the ability to catalyze CL formation. A scaled-up CL synthase standard assay mixture in a total volume of 1 ml was incubated for 15 min and then lipids were extracted and analyzed by thin layer chromatography in solvent system 1. Approximately 17% of the radioactive lipids co-chromatographed with CL and the remainder was the starting material, PG. No other radioactive lipid was detected. In a parallel experiment using crude membranes from DG10/pBluescript, all of the tritium labeled lipid co-chromatographed with PG.

Previous studies showed that *pss*A1 *cls* double mutants do not grow on LB agar at 42°C but do so when a plasmid with a functional *cls* gene is present [17]. These growth properties provide an opportunity to determine whether the

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f413 protein catalyzes sufficient CL synthesis to allow a *pss*A *cls* double mutant to grow at 42°C. Strains needed to test this possibility were constructed by introducing plasmids pDG3, pLR3, pBluescript II KS(-) or pET3 into DG9 (*pss*A1 *cls*::Tn*10*dCam). When DG9/pDG3, DG9/pLR3, DG9/pBluescript II KS(-) and DG9/pET3 were streaked on LB agar supplemented with ampicillin at 42°C, only DG9/pLR3 formed colonies. DG9/pDG3 did not form colonies at 42°C even when IPTG was present. This result, suggesting that overexpressed f413 protein does not catalyze CL synthesis in vivo, was confirmed by monitoring [^{32}P]phosphate incorporation into the phospholipids of growing cells. Strains DG10/pDG5, DG10/pLR3 and DG10/pBluescript II KS(-) were cultured in 5 ml of M9ZB broth supplemented with ampicillin at 37°C. When the turbidity reached 40 Klett units, IPTG was added to a final concentration of 0.8 mM and 25 μCi of [^{32}P]phosphate (carrier free) were added 30 min later. Labeled glycerophospholipids, extracted from harvested cells after a 2-h incubation, were analyzed by thin layer chromatography using solvent system 1. CL contained approximately 15% of the radioactive label that was incorporated in the lipids produced by DG10/pLR3. However, the quantity of labeled CL produced by DG10/pDG3 was at the background level observed in DG10/ pBluescript II KS(-). These results confirm that f413 protein catalyzes little, if any, CL synthesis in vivo, even when the expression level is very high. Furthermore, the *f413* gene appears to be dispensable because strain DG1, which has an *f413* null mutation, grows normally when cultured in LB broth under aerobic or anaerobic conditions (data

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not shown). Double mutants that also have a *cls*::Tn*10*dTet null mutation also grow normally under these conditions (data not shown).

3.4. Characterization of polypeptides expressed by cls *genes with 5'-terminal deletions.*

Two lines of evidence suggest that the first 100 residues specified by *cls* are not needed for catalytic activity. First, the molecular mass of CL synthase is approximately 8 kDa less than predicted from the *cls* sequence. The most likely explanation for this difference, post-translational cleavage of a segment consisting of the first 75-80 residues, has been difficult to prove because the N-terminus appears to be blocked [71]. The second line of evidence, which was presented above, is that the f413 protein can catalyze CL formation in vitro even though it lacks codons corresponding to about the first 100 codons in *cls*. It was therefore of interest to determine whether truncated CL synthases missing 60 or 104 residues from their N-termini could catalyze CL synthesis. To this end, two plasmids, pDG5 and pDG7, with deletions at the 5'-terminus of *cls* were constructed as described in Materials and Methods. The truncated *cls* gene in pDG5 is fused to a T7 tag protein so that residue 61 of CL synthase is attached to the T7 tag. The recombinant *cls* gene in plasmid pDG7 is similar to that in plasmid pDG5 but has a larger deletion so that the T7 tag is linked to residue 105 of CL synthase. Predicted molecular masses of the fused proteins specified by the recombinant genes in plasmids pDG5 and pDG7 are 49.9 and 45 kDa, respectively.

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When introduced into wild type *E. coli*, plasmids pDG5 and pDG7 had no effect on cell growth or viability. However, very low transformation efficiencies were observed when attempts were made to introduce the plasmids into BL21(DE3). Furthermore, BL21(DE3) transformants bearing the recombinant plasmids grow poorly and lose viability, suggesting that the fusion proteins are toxic to the cells. To lower background expression, DG11 was constructed by introducing plasmid pLysS into DG10 [72].

Strains DG11/pDG5, DG11/pDG7, and DG11/pET23a were cultured in M9ZB medium supplemented with both ampicillin and chloramphenicol. After a 3-h incubation with IPTG, cells were harvested and crude membranes and water-soluble fractions were isolated as described in Materials and Methods. When these fractions were analyzed by SDS-gel electrophoresis, a dark band corresponding to a molecular mass of about 48 kDa and accounting for approximately 18% of total protein was observed in the DG11/pDG5 membrane fraction (Fig. 3.5, lane 5). The fact that the molecular mass of this band is greater than that of mature wild type CL synthase indicates that the truncated protein does not undergo normal post-translational cleavage. However, the posobility of some type of post-translational cleavage cannot be ruled out completely because the molecular mass of the observed protein is about 2 kDa less than the predicted value. Another dark band corresponding to a molecular mass of about 43 kDa and accounting for approximately 28% of total protein was observed in the DG11/pDG7 membrane fraction (Fig. 3.5, lane 6). Once again,

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the post-tranlational cleavage cannot be completely ruled out because the molecular mass of the observed band is about 2 kDa less than the predicted value. Both dark bands were unique to the membrane fraction in which they were present and both were only observed in membranes obtained from IPTG induced cells.

Crude membranes obtained from strain DG11/pDG5 catalyzes [^{3}H]glycerol release from phosphatidyl[2-^{3}H]glycerol. However, no activity was detected in crude membranes from DG11/pDG7 (Table 3.3). IPTG-induced strain DG11/pDG5 was incubated in the presence of [^{32}P]phosphate and its incorporation into glycerophospholipids monitored as described in section 3.2. Overexpression of the truncated protein had no effect on labeled CL formation (data not shown).

3.5. *Kinetic comparisons*

The kinetic properties of CL synthase, f413 protein and truncated CL synthase missing residues 2-60 were compared by measuring [2-^{3}H]glycerol release from phosphatidyl[2-^{3}H]glycerol at 37°C for 5 min. Linear reaction rates are observed under these conditions (data not shown). CL and phosphatidate inhibit all three enzymes (Fig. 3.6). CL synthase is the most sensitive to CL, whereas f413 protein is the most sensitive to phosphatidate. All three enzymes are stimulated by inorganic phosphate although the concentration response curves are somewhat different (Fig. 3.7). In contrast, the pH profiles for all three of the enzyme activities are similar (Fig. 3.8). Specific activities of crude

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membranes from IPTG-induced DG10/pLR3, DG10/pDG3, DG10/pDG5 were determined to be 450, 50, and 30 units/mg protein, respectively (Table 3.3). However, concentrations of overexpressed enzymes in the crude membranes are different. Taking that into account, specific activities for CL synthase, f413 protein and the truncated CL synthase can be estimated as 2800, 450 and 170 units/mg enzyme, respectively. Although CL inhibits all three of the CL synthase activities, its concentration is not a significant factor when comparing the three enzyme activities because the membrane dilutions that were used resulted in approximately equal CL concentrations in the assay mixtures.

Temperature stabilities of crude membranes from DG10/pLR3, DG10/pDG3 and DG11/pDG5 were compared using the [2-^{3}H]glycerol release assay. After incubation at 55°C for the indicated time, crude membranes were added to otherwise complete assay mixtures and the reaction mixture incubated at 37°C for 5 min. As shown in Fig. 3.9, wild type CL synthase did not lose any activity after a 15-min incubation at 55°C. However, truncated CL synthase lost about 50% of its activity after a 15-min incubation and the f413 protein lost almost all of its activity after just 5 min at 55°C. Because crude membrane extracts were diluted to different extents to keep total enzyme activities similar, these experiments were repeated with the addition of crude membranes from DG11/pET23a to equalize total membrane protein concentrations. Identical temperature-sensitivity results were observed under these conditions (data not shown).

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3.6. Phospholipase D activity of the f413 protein

E. coli has been reported to have a CL-specific phospholipase D activity [73], raising the possibility that the f413 protein might account for this activity. Crude membranes containing either CL synthase or the f413 protein can convert NBD-PG to CL (Fig. 3.10). However, a comparison of the products formed in the two enzyme catalyzed reactions reveals a major difference. Only two fluorescent lipids, NBD-CL and NBD-PG are present after incubation with CL synthase, whereas NBD-phosphatidate is also present after incubation with f413 protein. Product identification is based upon thin-layer chromatographic comparisons of fluorescent lipids with authentic standards in solvent systems 1,3, and 4. Thus, the f413 protein appears to catalyze phosphatidyl group transfer to PG to form CL or to water to form phosphatidate. Additional experiments were performed to determine if the f413 protein could catalyze phosphatidyl group transfer to other primary alcohols. No additional products were observed when crude membranes containing the f413 protein were incubated with NBD-PG in the presence of 5 mM L-serine, ethanolamine, butanol, or ethanol (data not shown). Similar results were obtained when the primary alcohol receptor concentrations were increased to 0.5 M. Further experiments, performed to determine if either CL synthase or f413 protein could hydrolyze fluorescent labeled CL, indicated that neither crude membranes containing CL synthase nor those containing f413 protein could do so (data not shown).

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3.7. The conserved histidine residues in the HKD domains are essential for E. coli *CL synthase activity.*

The conserved HKD motifs form the active site for phospholipase D. The histidine residues in the motifs are essential for the enzyme activity. Mutation of either one causes the enzyme to lose activity completely [61, 74]. To investigate whether this is also true for CL synthase, site-directed mutagenesis of plasmid pLR3 was used to substitute the two histidine residues (His224 and His404) to glutamine in the two conserved HKD regions in *E. coli* CL synthase. The primers used to alter His224 are 5'-CGT ATG GAC CTG CGC CAA (CAT→CAA) CGC AAG ATG ATC ATG ATC G-3' and 3'-GCA TAC CTG GAC GCG GTT (GTA→GTT) GCG TTC TAC TAG TAC TAG C-5'. The primers used to alter His404 are 5'-G TTT GAA GGC GGG TTA CTG CAA (CAT→CAA) ACC AAG AGC GTG CTG GTC G-3' and 5'-C AAA CTT CCG CCC AAT GAC GTT (GTA→GTT) TGG TTC TCG CAC GAC CAG C-5'. As described in section 2.10, six transformants were selected for each mutation reaction. Plasmids were isolated from these strains and subsequently introduced into BL21(DE3). The transformed strains containing the mutated plasmids are designated H224Q and H404Q, respectively.

Crude membranes were isolated from IPTG-induced H224Q and H404Q strains. They were analyzed by SDS-PAGE as described in Materials and Methods to ensure the expression of the amplified CL synthase proteins. As shown in Figure 3.11, a dark band co-migrating with the overexpressed wild type

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CL synthase from BL21(DE3)/pLR3 was present in each of the mutant samples. The crude membranes from H224Q and H404Q were assayed by the standard [2-^{3}H]glycerol release assay. CL synthase activity of the crude membranes containing either altered enzyme was found to be less than 0.1% of that of their wild type counterpart. This result indicates histidines in the conserved HKD regions are essential for CL synthase activity.

4. Discussion

Sequence comparisons reveal a marked similarity between the *E. coli* proteins specified by *f413* and *cls*. Most notably, each of the predicted sequences has two HKD motifs, a characteristic feature of the PLD superfamily. Another noteworthy feature became apparent when the predicted sequences for the two *E. coli* proteins were compared to the predicted sequence for *Bacillus firmus* OF4 *cls* [57]. All three proteins have a conserved RXHRK sequence. The histidine residue within this sequence is part of the first HKD motif. A protein sequence search of the NCBI data base reveals that other bacterial proteins that have been predicted to act as CL synthases also have this RXHRK sequence. Despite the high degree of homology among the three predicted protein sequences, differences are also evident. One of the most important of these is that the protein specified by *f413* lacks an N-terminal segment encoded by *E. coli cls*.

Previous studies suggested that a segment consisting of approximately 75-80 residues is cleaved during post-translational processing. Since studies by

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Shibuya and colleagues demonstrated that *E. coli* CL synthase loses activity with the removal of 66 amino acid residues from the C-terminus [50], the most likely explanation is N-terminal cleavage. The present study shows that the f413 product and the truncated CL synthase encoded by a *cls* fragment that is missing codons corresponding to residues 2-60 can convert PG to CL. However, truncated CL synthase that is missing residues 2-104 cannot do so. The reason why the smaller protein does not catalyze CL synthesis is not clear. Perhaps the truncated protein is easily denatured. In this regard, it is interesting to note that the f413 protein loses nearly all of its activity after incubation at 55°C for 5 min, whereas wild type *E. coli* CL synthase remains fully activity after a 15 min incubation. Neither the f413 protein nor the truncated CL synthase that is missing residues 2-60 appears to catalyze CL formation within the cell. The reason for this inability is not evident. Perhaps proper post-translational processing is required for the enzyme to move to its proper location. Based upon the observation that *E. coli* mannitol transport mutants convert CL and mannitol to phosphatidylmannitol, Shibuya and coworkers propose that CL synthase's active site faces the periplasmic space [39]. If so, then perhaps the N-terminal segment provides a signal for proper CL synthase placement within the cell. CL synthase, f413 protein, and the two truncated forms of CL synthase all seem to be membrane-bound. If the proteins are indeed associated with the cell membrane, the nature of the association must be determined. The hydrophobic segment at the N-terminus of CL synthase appears to be cleaved after

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translation, leaving the mature protein without a segment that can be embedded in the membrane. The same problem exists for f413 protein and the two truncated proteins, which each lacks this region altogether. Perhaps, the proteins are linked to a lipid group that anchors them to the membrane. Although the possibility that f413 protein and the truncated proteins are present as inclusion bodies has not been rigorously ruled out, attempts at isolating these proteins as water-soluble molecules by growing cells at 25° C were unsuccessful (data not shown).

Gene *f413* belongs to a gene operon that includes two other genes. The functions of all three genes are unknown. The sequence of *f318*, the gene downstream from *f413*, suggests that it may have permease activity. The *f413* gene appears to be dispensable since cells with a null mutation in this gene grow normally under either aerobic or anaerobic conditions. Furthermore, cells that also have a *cls* null mutation also grow normally under both sets of conditions. Attempts to demonstrate that the f413 protein has phospholipase D activity were only partially successful. Small quantities of phosphatidate were observed when membranes containing the f413 protein were incubated with PG but none were observed when CL was used as the substrate. The f413 protein was unable to transfer a phosphatidyl group from PG to serine, ethanolamine, butanol, or ethanol nor could it transfer a phosphatidyl group from CL to any of these primary alcohols.

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Dixon and coworkers have proposed a general mechanism of action for members of the PLD superfamily [62]. A positively charged lysine side chain in an HKD active site motif appears to bind the negatively charged substrate. The histidine residue acts as a nucleophile, attacking the phosphodiester bond to form a phosphoenzyme intermediate. The histidine residue in the other HKD motif acts as a general acid, donating a proton to the leaving group. Both conserved histidines are essential for CL synthase activity since conversion of either one to glutamine causes CL synthase to lose activity. Consistent with the conservation of residues at the catalytic site, wild type CL synthase, f413 protein, and the truncated protein missing residues 2-60 all have similar pH profiles. Furthermore, all three of the enzyme activities are stimulated by phosphate and inhibited by CL and phosphatidate. The reason for phosphate stimulation is not known. Perhaps phosphate helps release the product, CL, from the enzyme. Phosphate stimulation may be unique to enzymes with CL synthase activity because it has not been reported for any other enzymes in the PLD superfamily. However, it should be noted that Nuc protein (an endonuclease and PLD member) catalyzes phosphate (oxygen)-water exchange and forms a covalent phosphohistidine intermediate [61]. Both CL synthase (wild type or truncated form) and f413 protein are inhibited by CL and phosphatidate. Phosphatidate may compete with the substrate for binding at the active site to form a substrate-enzyme intermediate. In this regard, it is interesting to note that phosphatidate

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activates bacterial and plant phospholipase D enzymes by interacting with an allosteric site [66].

The other *E. coli* gene that is homologous to *cls, o493* (or *ymdC*) has been cloned in the attempt to study the gene product (Wang, Z., Guo, D. and Tropp, B.E., unpublished data). A 10-kb DNA fragment containing gene *o493* and flanking regions was obtained by digesting lambda EMBL4 clone 232 (kindly provided by Dr. Kenneth E. Rudd) DNA with *Kpn*I. It was inserted into plasmid pBluescript II KS(-) at the site of *Kpn*I under the T7 promoter to form a recombinant plasmid, pBlue-O493. A 4-kb DNA fragment containing mainly gene *o493* was obtained by digesting pBlue-O493 with *Sca*I and *Hind*II (both are blunt end cutters). The DNA fragment was then inserted into pBluescript II KS(-) at a *Hind*II site so that gene o493 is under the control of the T7 promoter. Both the resulting plasmid, pZW-O493, and plasmid pBlue-O493 were transformed into BL21(DE3) in order to amplify the o493 gene product. However, when applied to SDS-PAGE analysis, no identifiable bands corresponding to amplified proteins were observed in either the membrane fraction or the water-soluble fraction from the IPTG-induced cells of strain BL21(DE3)/pZW-O493 or BL21(DE3)/pBlue-O493. Moreover, neither fraction showed significant CL synthase activity. The reason for these results is not clear. One possibility is the gene wasn't expressed. Sequence analysis revealed the gene itself doesn't have a good ribosome binding site. It may belong to a multi-gene operon and the expression is somehow regulated. It is also possible that mutations were introduced into the

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gene during the course of the cloning, causing the deactivation of the gene. Nucleotide sequencing should be able to examine this possibility.

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TABLE 3.1

Bacterial Strains

Strain	Parent	Genotype or description	Source or ref.
HB101		F' *recA13 supE44 rpsL20* (sm^r) *hsd20* (r_B^-, m_B^-) *ara14 galK2 lacY1 proA2 xyl15 leu mtl1* λ^-	[32]
JC7623		*RecB21 recC22 sbcB15*	[17]
LK30	JC7623	*cls*::miniCam*10*	[29]
SOH142		*HfrKL16 thi-1 relA1 spo77 pss-1 zee*::miniTet*10*	[15]
SOH9	MC4100	*GlpR glpD cls*::Tn*10*dTet3	[15]
BL21(DE3)		F^- *ompT hsdSB* ($r_B^- m_B^-$), *dcm gal* (DE3)	[12]
DG1	JC7623	F413::kan	This study
DG6	BL21(DE3)	*cls*::Tn*10*dTet3	(P1)SOH9 x BL21(DE3)
DG7	BL21(DE3)	*pss-1 zee*::miniTet*10*	(P1)SOH142 x BL21(DE3)
DG9	DG7	*cls*::miniCam*10*	(P1)LK30 x DG7
DG10	DG6	F413::kan	(P1)DG1 x DG6
DG11	DG10	Plasmid pLysS was introduced into DG10	This work
XL1-Blue MRF'		*Δ(mcrA) 183 Δ(mcrCB-hsd SMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*[F' *proAB lacI^qZΔM15* Tn*10*(Tet^r)]	Product of Stratagene®, La Jolla, CA, USA

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TABLE 3.2

Plasmids

Plasmid	Description	Ref.
pLC12-48	Plasmid from Clarke and Carbon collection containing *f413*	[16]
pDG782	Plasmid containing kanamycin-resistance cassettes	[33]
pBluescript II KS(-)	Cloning vector	---[a]
pET23a	pET system vector containing a T7-tag	[12][b]
PLysS	Provides T7 lysozyme that inhibits T7 RNA polymerase	[26]
pLR3	Plasmid bearing *E. coli cls*	[9]
pDG3	*f413* inserted within pBluescript II KS(-)	This study
pDG4	*f413* inserted within pBluescript II KS(-)	This Study
pDG5	Truncated *cls* gene inserted within pET23a	This study
pDG7	Truncated *cls* gene inserted within pET23a	This study
pDG8	Disrupted *f413* inserted within pBluescript II KS(-)	This study

[a] Product of Stratagene® (La Jolla, CA, USA)

[b] Product of Novagene, Inc. (Madison, WI, USA)

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TABLE 3.3

CL Synthase Activity

Strains	Enzyme specified	Lipid products	Sp. activity (units/mg protein)
DG10/pLR3	CL synthase	CL	450
DG10/pDG3	f413 protein	CL and PA	50
DG10/pDG5	Truncated CL synthase missing residues 2-60	CL	30
DG11/pDG7	Truncated CL synthase missing residues 2-104	Not detected	---
DG11/pBluescript	None	Not detected	---

The strains were induced with 0.8 mM IPTG and crude membranes were obtained and assayed as described in section 2. Specific activities were calculated using total protein concentrations in the crude membranes. One unit of enzyme activity is defined as 1 nmol of [2-^{3}H]glycerol released per min [9]. PA: phosphatidate.

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```
CLS     1 MTTVYTLVSWLAILGYWLLIAGVTLRILMKRRAVPSAMAWLLIIYILPLVGIIAYLAVGE
f413    1 ------------------------------------------------------------

CLS    61 LHLGKRRAERARAMWPSTAKWLNDLKACKHIFAEENSSVAAPLFKL ER QGIAGVK  Q
f413    1 ------------------------------------------MK SW E------  K

CLS   121    TES DVMQ  I D  QL RHN E VF    QPGG  D   AES         R    HCR
f413   11    ENG QYYP   F A  GE QER  I ET    FEDD   K  HAA         Q    AE

CLS   181 SA  VAFFRSPWP  MRN  IE  EALKVNLM   LR MD --  Q          NY   I
f413   71 GY   PDLSDEFVN   T-A  --  FRYYDPRP   GM  TN  FR  M          AR   FI
                                                        *  *    *

CLS   239 S   MVDP  YFKQDAGVG  WI  LMA         ATA  GIIYSCDWEIE   K ---ILPPPP V
f413  128 G   YSAE  MSSYGPEAK  --  YAV         VED  LQFELENLPGQ   A  RWWRRHHKA E

CLS   296  IM  F  QASGHTI  TIASGPGFPE  L  HQAL  TAAYS   EY   TTP   V  SDDL    C
f413  186  RQ  G  AQVLLVW  DNEE----HR  D  ERHY  KMLTQ   RE   ANA   F  GYRF    R

CLS   356 T  Q    D  S   PRKN  SM  GW  S  AF  TE   A   K   Q EGGL   T  SV    GELS
f413  242 K  R    R  K   QGEP  MP  RV  A  LL  NY   K   Q  E RRRP   G  VA    DHWA
                                                                   *  *    *

CLS   416 L   V    MR   W   F  IT  A  D  G  GAD  AAVQDDY  SRS-RL  AR  WL  PL  RV
f413  302 T   S    PL   S   L  AN  I  H  H  NQT  RDNLNGI  AADCQQ  ET  LP  TW  LT

CLS   475 AER -  Y  F------------   L  -------------------------
f413  362 KSV  A  H  LRHFPALVGWLPAH  R  AQVDPPAQPTMETQDRVETENTGVKP
```

Fig. 3.1. Sequence alignments of *E. coli* CL synthase (CLS) and f413 protein. Histidine (H), lysine (K) and aspartic acid (D) residues in the conserved HKD regions are asterisked underneath.

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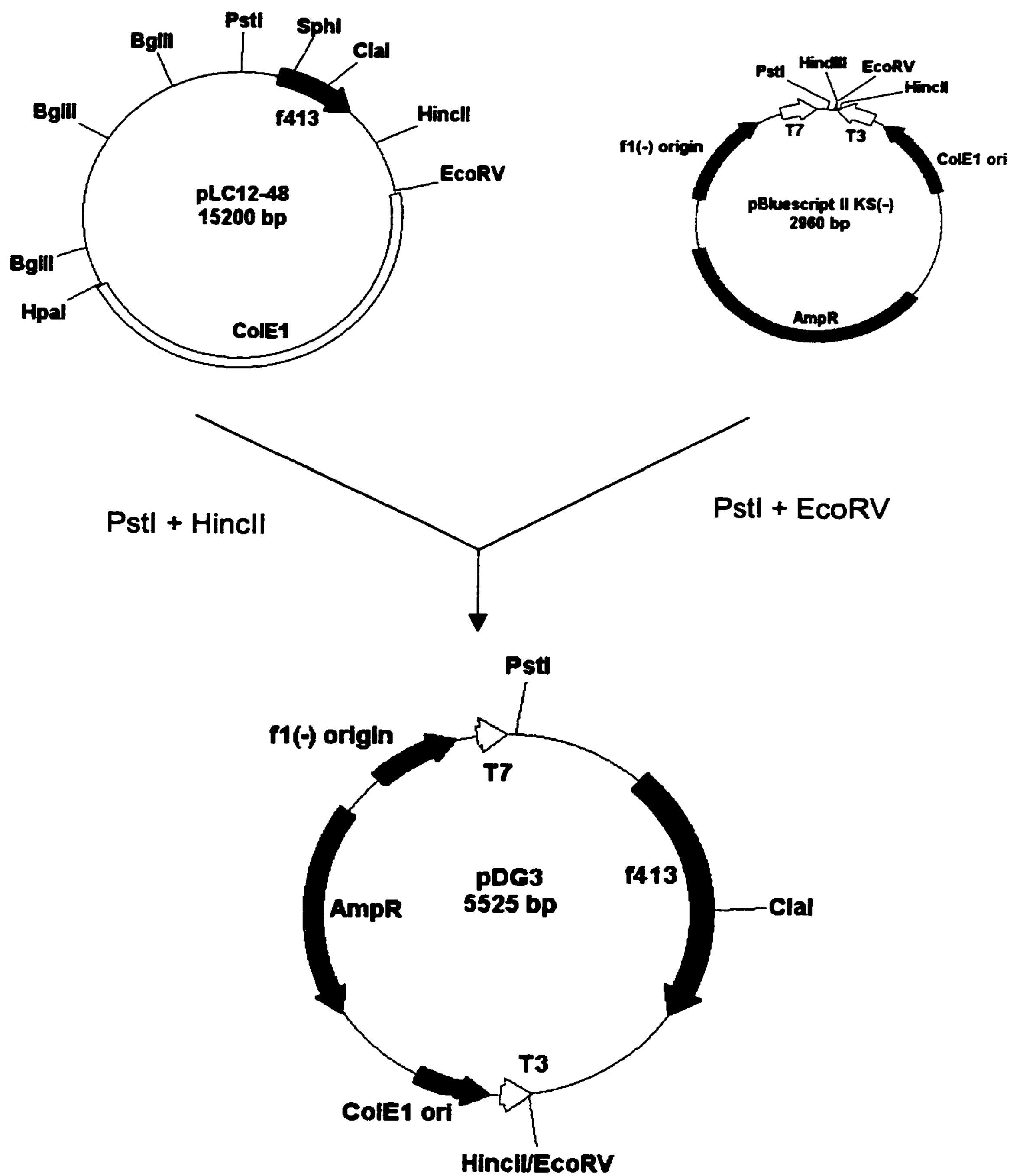

Fig. 3.2. Construction of plasmid pDG3.

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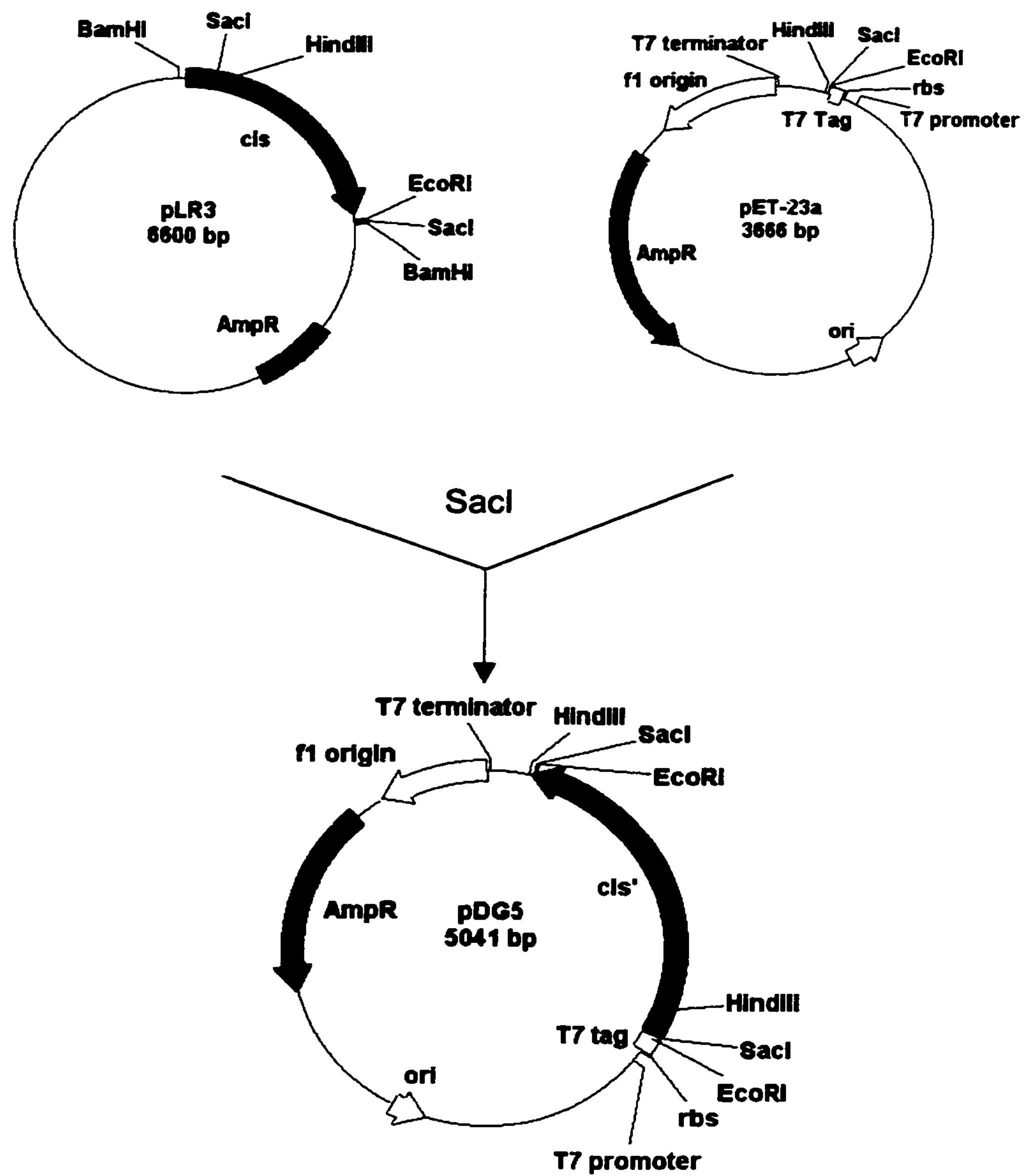

Fig. 3.3. Construction of plasmid pDG5.

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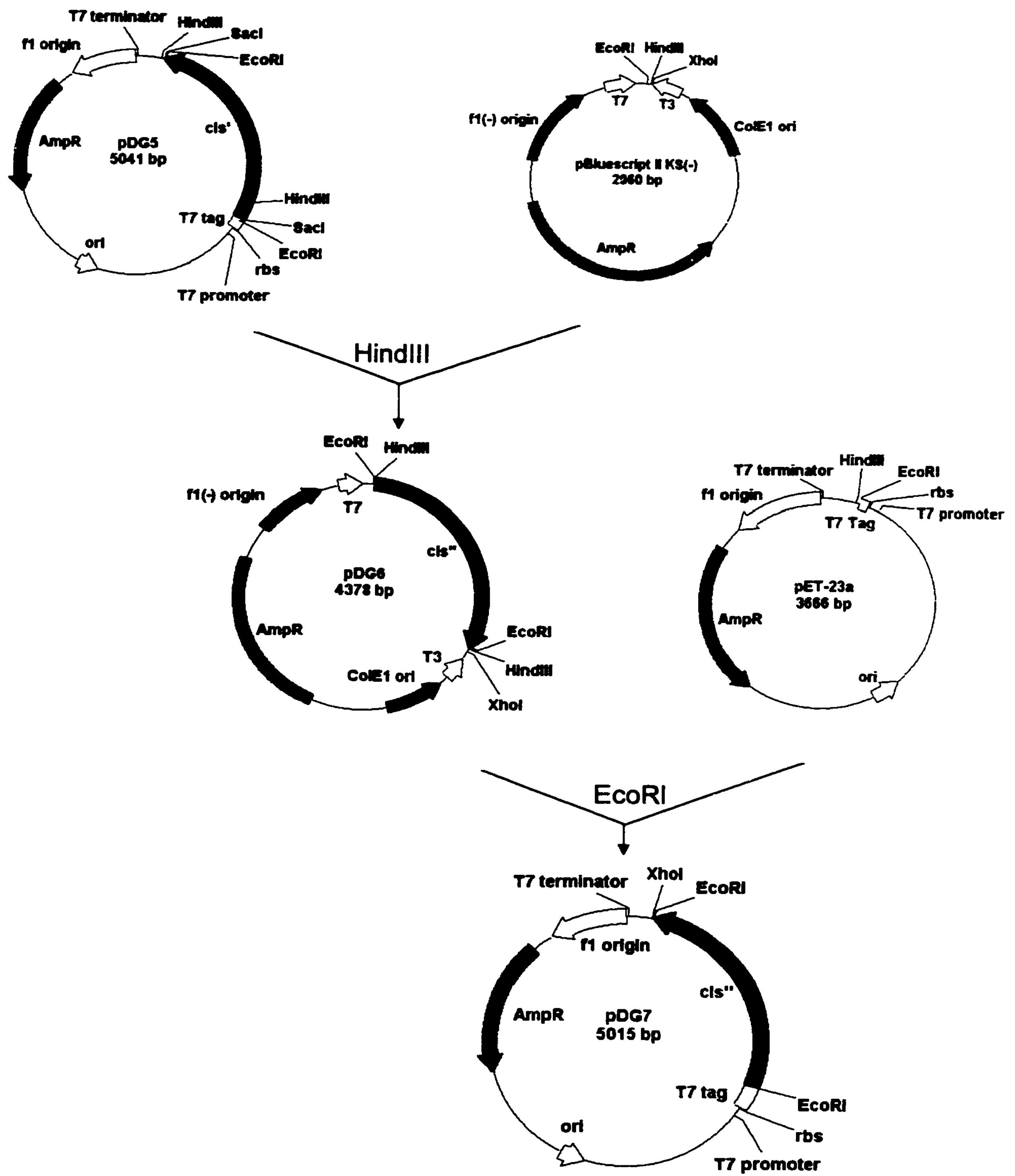

Fig. 3.4. Construction of plasmid pDG7.

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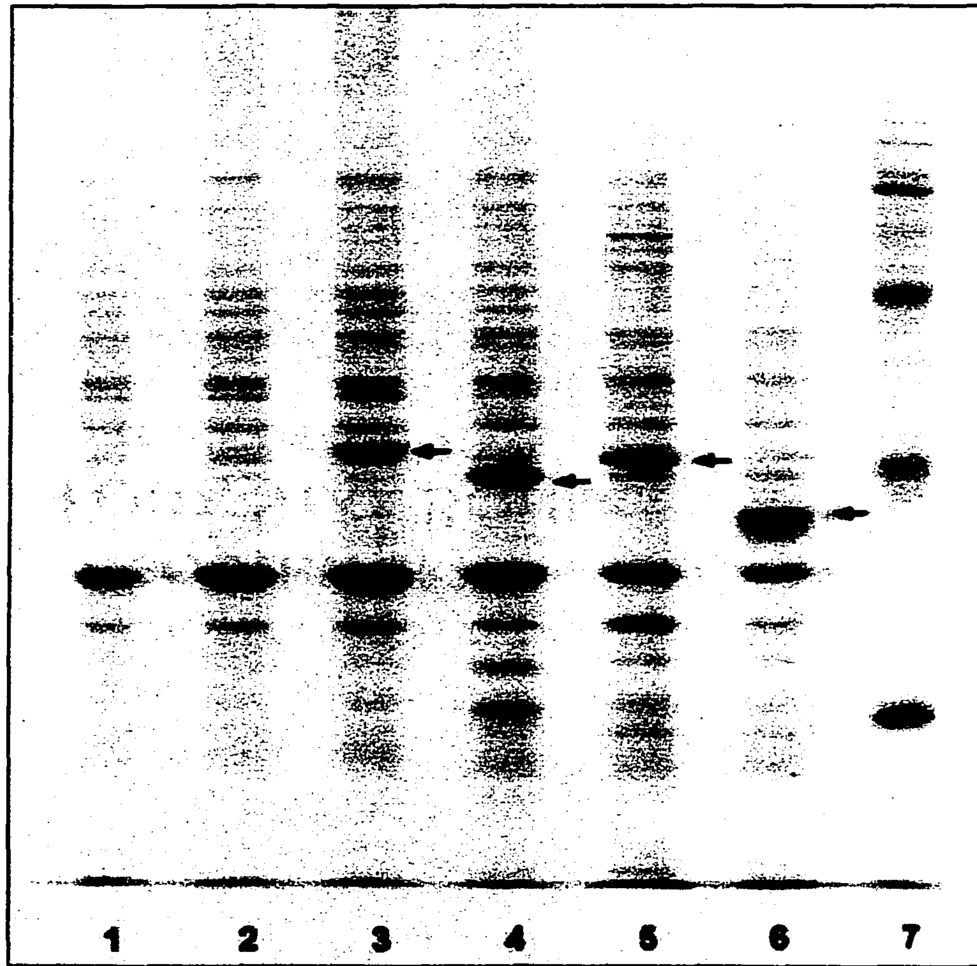

Fig. 3.5. SDS-PAGE analysis of overexpressed proteins. Crude membranes from IPTG-induced cells, prepared as described in Materials and Methods, containing about 40 μg proteins were analyzed. Lane1, DG11/pET23a; Lane 2, DG10/pBluescript; Lane 3, DG10/pDG3; Lane 4, DG10/pLR3; Lane 5, DG11/pDG5; Lane 6, DG11/pDG7. Overexpressed proteins are indicated by arrows. Lane 7 is the high molecular mass protein standard mixture.

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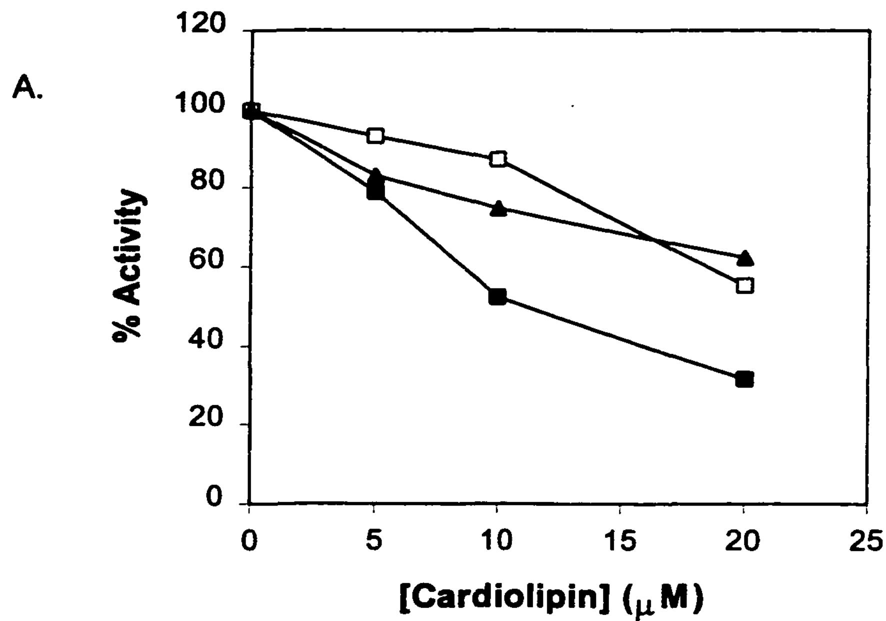

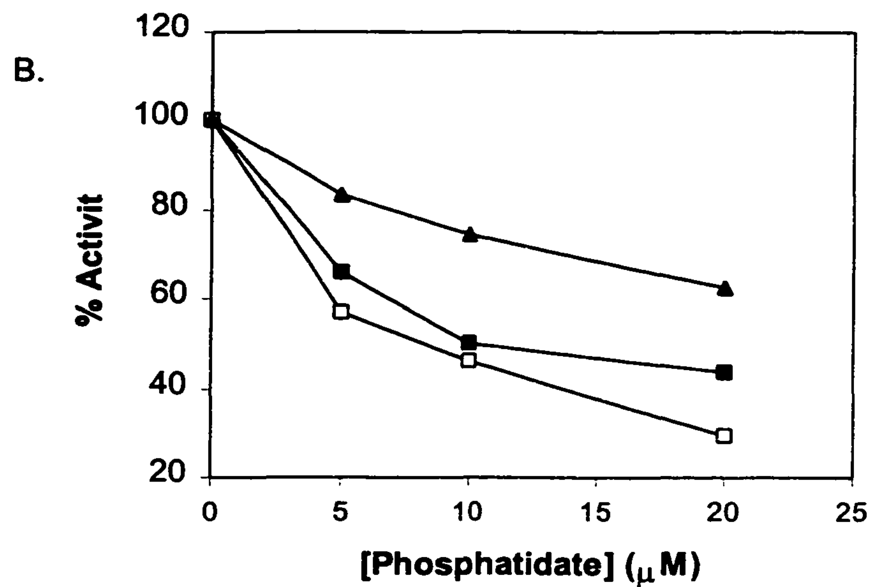

Fig. 3.6. Effect of (A) CL and (B) phosphatidate on CL synthase activities of crude membranes from DG10/pDG3 (□), DG10/pLR3 (■), and DG11/pDG5 (▲). CL and PA were added at the indicated concentrations. CL synthase activities were measured after 5 min at 37°C as described in Materials and Methods.

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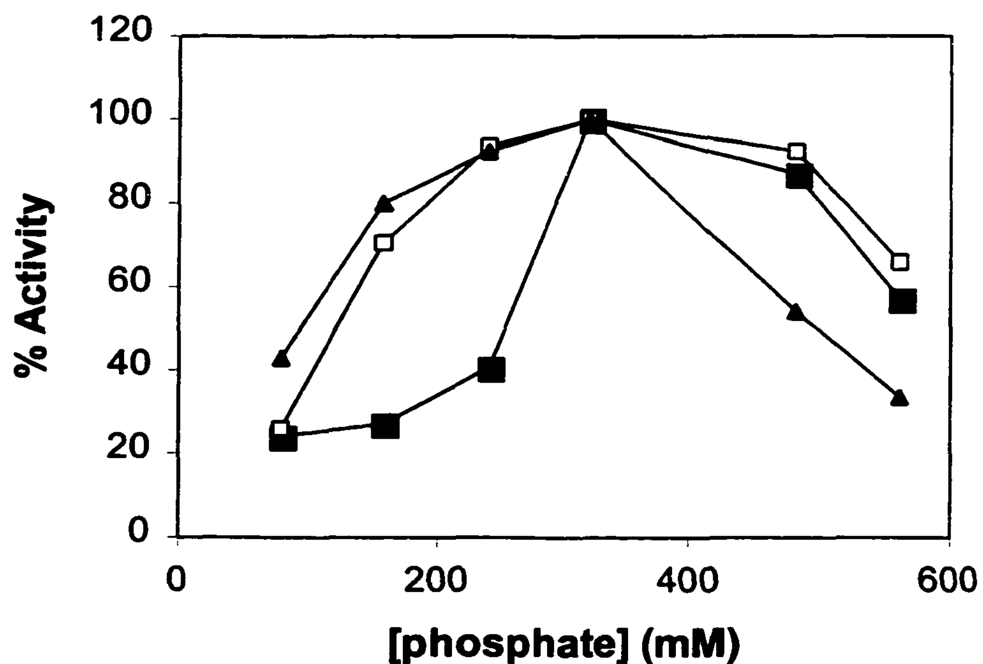

Fig. 3.7. Effect of phosphate concentrations on CL synthase activities of crude membranes from DG10/pDG3 (□), DG10/pLR3 (■) and DG11/pDG5 (▲). Phosphate buffers (pH7.0) were used at the indicated concentrations. CL synthase assays were performed as described in Materials and Methods. Glycerol release was measured after 5 min at 37°C.

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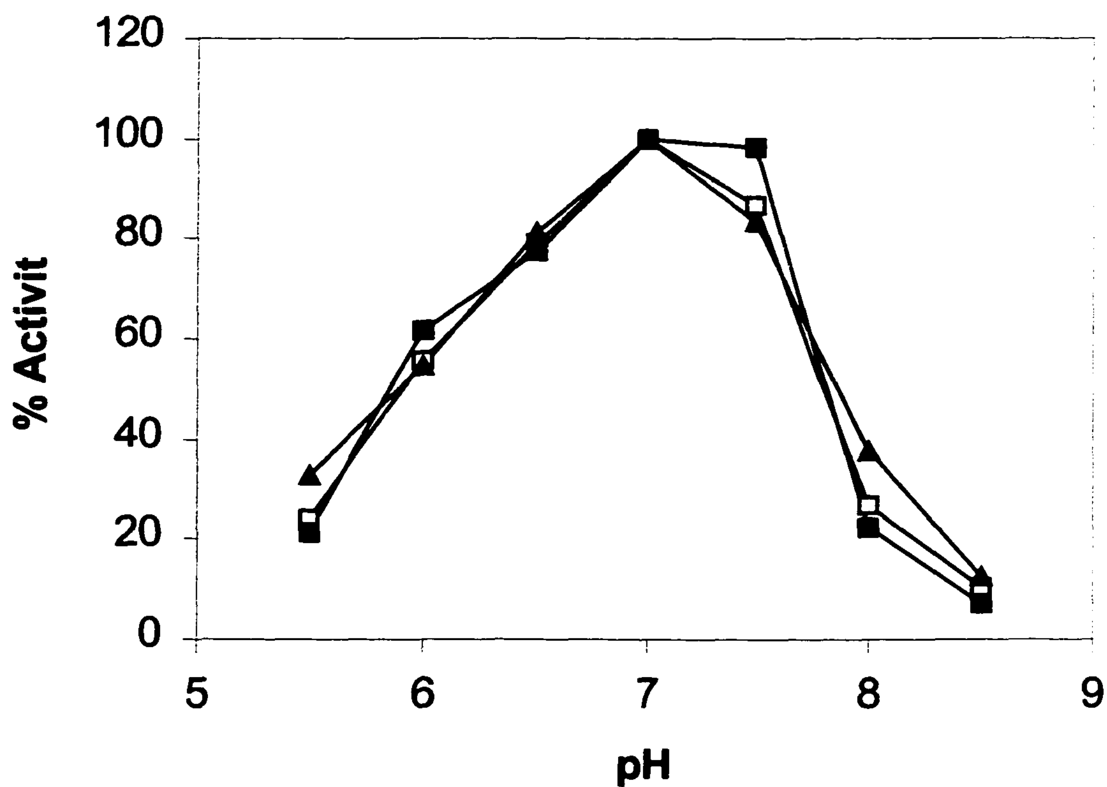

Fig. 3.8. pH profiles of the CL synthase activities of crude membranes from DG10/pDG3 (□), DG10/pLR3 (■) and DG11/pDG5 (▲). pHs were adjusted to the indicated values with phosphate buffer at a final concentration of 320 mM. CL synthase assays were performed as described in Materials and Methods. Glycerol release was measured after 5 min at 37°C.

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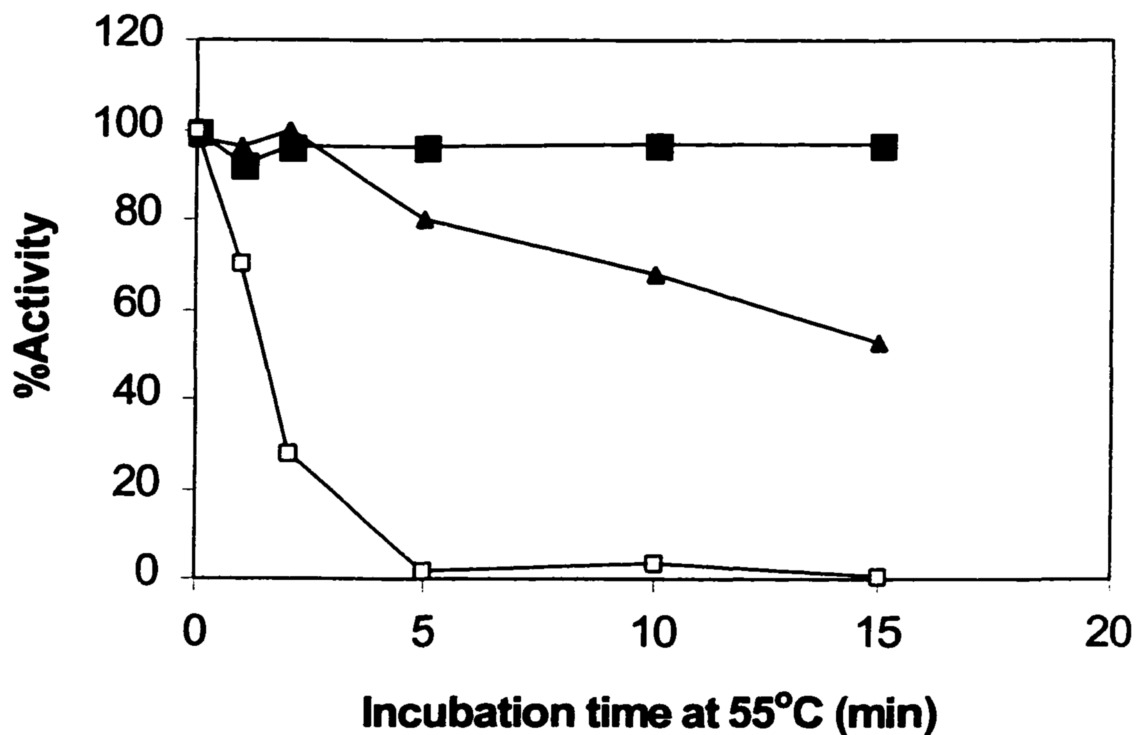

Fig. 3.9. Temperature profiles of crude membranes from DG10/pLR3 (■), DG10/pDG3 (□) and DG11/pDG5 (▲). CL synthase assays were performed as described in Materials and Methods. [2-^{3}H]glycerol release was measured after 5 min at 37°C.

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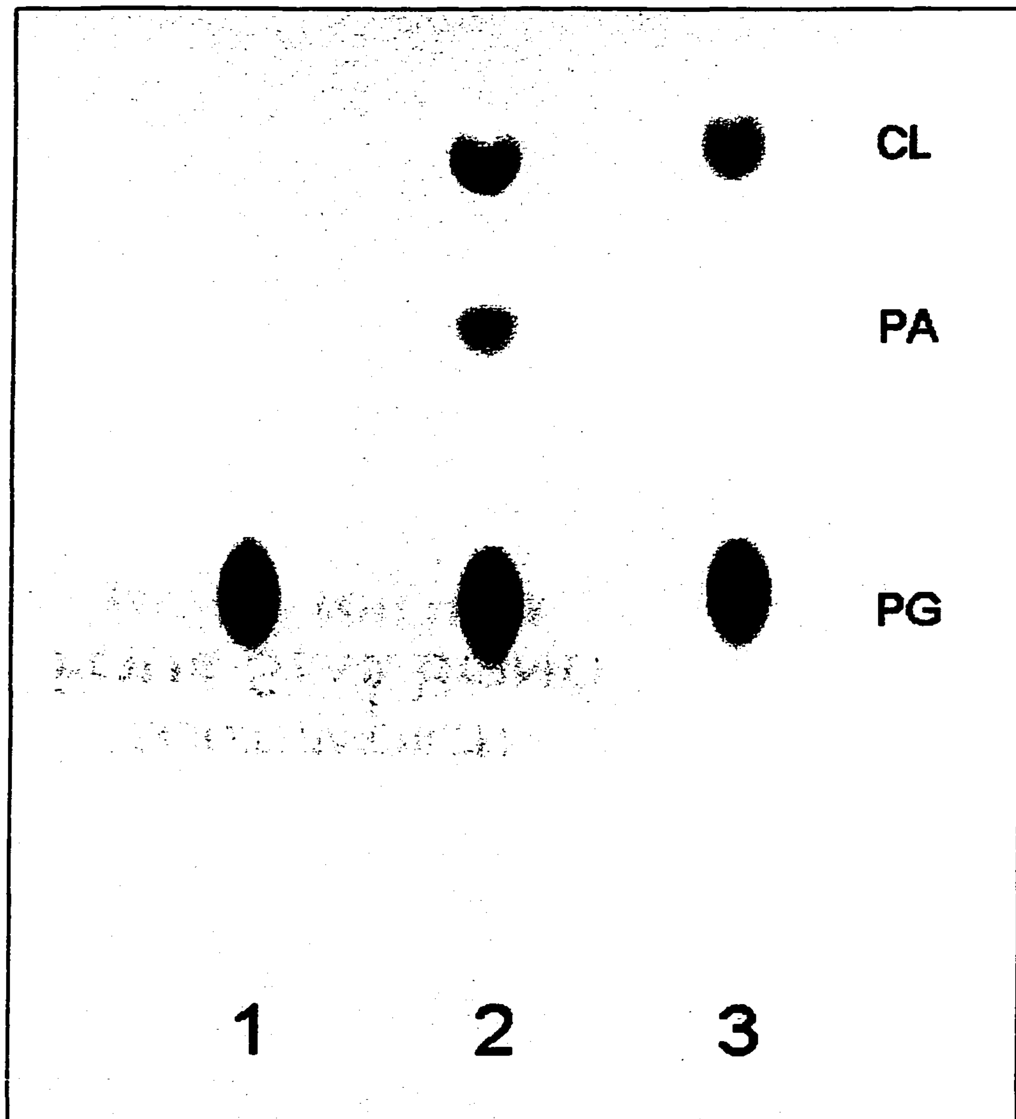

Fig. 3.10. Fluorescence imaging of NBD-PG assays. Enzyme assay and product analysis were performed as described in Materials and Methods. Assays were stopped after 15-min incubation at 37°C. Lane1, DG10/pBluescript; Lane 2, DG10/pDG3; Lane 3, DG10/pLR3. PA: phosphatidate.

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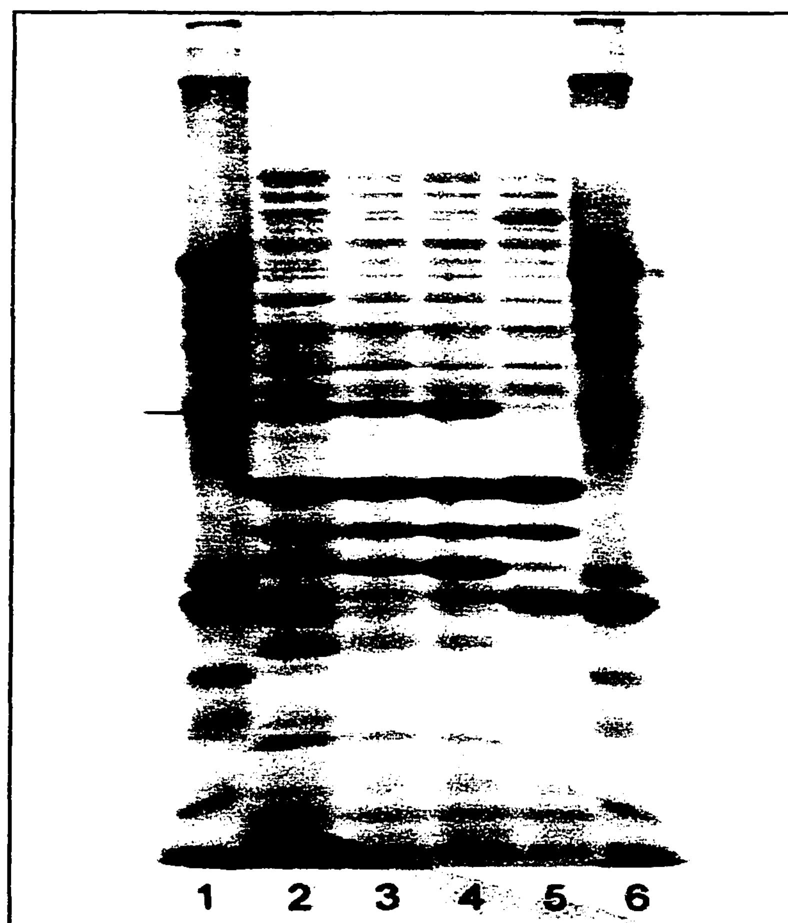

Fig. 3.11. SDS-PAGE analysis of mutant proteins. Crude membranes from IPTG-induced cells, containing about 40 μg proteins, were analyzed. Lane 1 and 6 are the high molecular mass protein standard mixture. Lane 2, H224Q; Lane 3, H404Q; Lane 4, BL21(DE3)/pLR3; Lane 5, BL21(DE3)/pET3. Overexpressed proteins are indicated by the arrow.

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APPENDIX 1

Preparation of Reagents and Media for *B. firmus* Growth and Protoplast Transformation

Medium A (Complex medium)

Medium A was prepared by mixing two solutions that were prepared and autoclaved separately. Solution 1 contains 0.2 M K_2HPO_4 (pH7.7~7.8) or 0.2 M Na_2CO_3-$NaHCO_3$ buffer (pH10.8), 3.2 mM citric acid, 0.4 mM $MgSO_4$, 1% Bacto-Peptone, 0.4% Bacto-yeast extract, and 0.4 M NaCl (for pH7.5 medium) or 0.4 M KCl (for pH10.5 medium). Solution 2 contains 1% D-glucose (3% of Bacto-Agar is also included when necessary). After autoclaving, equal volumes of the two solutions were mixed at 60°C to get medium A.

Metals 44 solution

In 800 mL of distilled water, the following reagents were added and stirred to dissolve in the following order: 2.5 g of EDTA (free acid, may need heating and addition of NaOH to dissolve), 10.95 g of $ZnSO_4$, 6.0 g of $FeSO_4$, 1.54 g of $MnSO_4$, 0.39 g of $CuSO_4$, 0.25 g of $Co(NO_3)_2 \bullet 6H_2O$ and 0.18 g of $NaB_4O_7 \bullet 10H_2O$. Then a few drops of concentrated H_2SO_4 were added to retard precipitation and the final volume was brought up to 1 L.

Trace salts solution (STS)

In 800 mL of water, 20 g of nitrilotriacetic acid (NTA, free acid) was dissolved and neutralized with 14.5 g of KOH. Then the following reagents were added in order: 6.7 g of $CaCl_2 \bullet 2H_2O$, 0.02 g of ammonium molybdate, 0.2 g of $FeSO_4$ and 100 mL of Metal 44 solution. The solution was adjusted to pH6.8 and the volume was brought up to 1 L. The solution was sterilized by autoclaving.

Medium B (Malate-containing medium)

Medium B was prepared at pH7.5 or pH10.5. It contains 0.1 M Na_2HPO_4 buffer (pH7.5) or 0.1 M Na_2CO3-$NaHCO_3$ buffer (pH10.5), 1 mM K_2HPO_4, 0.1 mM $MgSO_4$, 50 mM D,L-malate (or 0.5% D-glucose), 1% STS (trace salts solution) and 0.1% yeast extract. Buffers were prepared at twice the concentrations indicated above and with pH values about 0.2-0.3 higher than the desired values. After autoclaving, they were mixed with equal volumes of 0.2% yeast extract solution. A stock solution of malate was made in 1 M concentration and autoclaved. It was then added to each mixture along with the STS solution.

Media for protoplasting and transformation

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2x SMM	1 M sucrose, 0.04 M sodium malate, 0.04 M $MgCl_2$, pH7.5. Autoclave.
4x PAB	7% Bacto penassay broth (Antibiotics Medium No. 3, Difco), pH7.5. Autoclave.
SMMP	Mix equal volumes of 2x SMM and 4x PAB. Filter sterilize and keep at 4°C.
PEG	30 g of PEG 8000 in 100 ml of 1x SMM. Autoclave.

Modified DM3 medium

The modified medium was prepared as described in the following table.

No.	Reagents	Amount used	Final Concentration	Volume (mL)
1	Sodium succinate	70 g	0.5 M	500
	Trizma-base	3.65 g	30 mM	
2	Yeast Extract	5 g	0.5%	100
	Casamino acid	5 g	0.5%	
3	Purified agar	10 g	1%	250
	Glucose	20 g	2%	
4	$MgCl_2 \bullet 6H_2O$	6.1 g	30 mM	50
5	$CaCl_2$ (1M stock)	1.25 mL	1.25 mM	1.25
6	BSA	0.4 g	0.04%	100
7	Antibiotics	Added from stock solutions when necessary.		

All reagents were sterilized by autoclaving except 6 and 7. They were filter sterilized. Reagents 1 and 2 were adjusted to pH7.5 with NaOH solution prior to autoclave. The modified DM3 medium was prepared by mixing all seven reagents at 60°C. The final volume was 1 L. Plates were poured immediately after mixing. Fresh plates of the modified DM3 medium were used in this study.

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APPENDIX 2

Acrylamide Gel Purification of Oligonucleotides

1. Stock solutions

1) Acrylamide stock

acrylamide	38 g
bis-acrylamide	2 g
dH_2O	to 100 mL

Filter the solution through a 0.45 micron filter and store in a dark bottle at 4°C.

2) 5xTBE

Tris base	54 g
boric acid	27.5 g
0.5 M EDTA (pH8)	20 mL
dH_2O	to 1.0 L

2. Prepare a polyacrylamide/urea gel

For 12% gel

urea	31.5 g
acrylamide stock	23.5 mL
5xTBE	15 mL
dH_2O	14 mL

Warm to 37°C to dissolve urea and then filter through a 0.45 micron filter. Cool to room temperature.

10% ammonium persulfate	0.45 mL
TEMED	35 μL

Mix well and pipet into the gel mode.

3. Sample preparation

1) Resuspend oligonucleotide samples in TE (10 mM Tris, pH7.5, 1 mM EDTA) to make the final concentration 0.2 O.D. units/μL (1 O.D. unit~30 μg).
2) Take out 20 μL of each sample, add 20 μL of 1 mM EDTA, 0.04%(w/v) bromophenol blue made in formamide.
3) Heat in boiling water bath for 3 min.

4. Electrophoresis

1) Before loading sample, run the gel at 30 mA for 20 min.
2) Load samples with a 50 μL syringe.
3) Run bromophenol blue to bottom at 30 mA. Turn off the power.

5. Recovery of oligonucleotides from the gel.

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1) Remove the gel from the glass plates and place it on a piece of saran wrap.
2) Place the plastic wrap with the gel on a fluorescence plate.
3) Visualize the oligonucleotide bands under a hand-held UV lamp. The upper-most dark band is the desired band.
4) Cut the band off with a razor blade and put it into 5 mL of 0.3 M NH_4OAc and 0.01 M $MgCl_2$.
5) Rock overnight to elute the oligonucleotide.
6) Filter the fluid through a 0.2 micron filter.
7) Extract the fluid repeatedly with anhydrous 2-butanol until aqueous volume is reduced to 100 μL.
8) Transfer to eppendorf tube and add 900 μL of cold ethanol.
9) Chill 30 min in dry-ice ethanol bath.
10) Spin 30 min in microfuge. Remove ethanol and vacuum dry.
11) Resuspend the pellet in 100 μL of TE.

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REFERENCES

1. De Siervo, A. J. and Salton, M. R. J. (1971) Biochim. Biophys. Acta 239, 280-292.

2. Hirschberg, C. B. and Kennedy, E. P. (1972) Proc. Natl. Acad. Sci. USA 69, 648-651.

3. Hostetler, K. Y., Van Den Boshch, H. and Van Deenan, L. L. M. (1972) Biochim. Biophys. Acta 260, 507-513.

4. Tunaitis, E. and Cronan, J. E., Jr. (1973) Arch. Biochem. Biophys. 155, 420-427.

5. van den Bosch, H. (1974) Annu. Rev. Biochem. 43, 243-277.

6. Hoch, F. L. (1992) Biochim. Biophys. Acta 1113, 71-133.

7. Allegrini, P. R., Pluschke, G. and Seelig, J. (1984) Biochemistry 23, 6452-6458.

8. Haines, T. H. (1983) Proc. Natl. Acad. Sci. USA 80, 160-164.

9. Kates, M., J.-Y. Syz, D. Gosser, and T.H. Haines (1993) Lipids 28, 877-882.

10. Seddon, J. M., Kaye, R. D., and Marsh, D. (1983) Biochim. Biophys. Acta 734: 347-352.

11. Rietveld, A. G., Killian, J. A., Dowhan, W. and De Kruijff, B. (1993) J. Biol. Chem. 268, 12427-12433.

12. Powell, G. L., Knowles, P. F. and Marsh, D. (1990) Biochemistry 29, 5127-5132.

13. De Kruijff, B. and Cullis, P. R. (1980) Biochim. Biophys. Acta 602, 477-490.

14. Eble, K. S., Coleman, W. B., Hantgan, R. R., and Cunningham, C. C. (1990) J. Biol. Chem. 265, 19434-19440.

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15. Robinson, N. C., Zborowski, J. , and Talbert, L. H. (1990) Biochemistry 29, 8962-8969.

16. Heber, S. and Tropp, B. E. (1991) Biochim. Biophys. Acta 1129, 1-12.

17. Ohta, A., Obara, T., Asami, Y. and Shibuya, I. (1985) J. Bacteriol. 163, 506-514.

18. Hiraoka, S., Nukui, K., Uetake, N., Ohta, A., and Shibuya, I. (1991) J. Biochem. 110, 443-449.

19. Ragolia, L., Tropp, B. E. (1994) Biochim. Biophys. Acta 1214, 323-332.

20. Guffanti, A. A., Finkelthal, O., Hicks, D. B., Falk, L., Sidhu, A., Garro, A., and Krulwich, T. A. (1986) J. Bacteriol. 167, 766-773.

21. Clejan, S., Krulwich, T. A., Mondrus, K. R., and Seto-Young, D. (1986) J. Bacteriol. 168, 334-340.

22. Maloy, S. R. and Nunn, W. D. (1981) J. Bacteriol. 145, 1110-1112.

23. Studier, F. W. and Moffatt, B. A. (1986) J. Mol. Biol. 189, 113-130.

24. Nicoletti, V.G. and Condorelli, D.F. (1992) BioTechniques 14, 534-536.

25. Sambrook, J., Fritsch, E. F., and Maniatis, T.(1989) Molecular cloning, a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

26. Miller, J. H. (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY.

27. Ivey, D. M., Guffanti, A. Bossewitch, S., Padan, E. and Krulwich, T. A. (1991) J. Biol. Chem. 266, 23483-23489.

28. Hwang, Y.-W., Engel, R., Tropp, B. E. (1984) J. Bacteriol. 157, 846-856.

29. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) J. Mol. Biol. 215, 403-410.

30. Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J. (1997) Nucleic Acids Res. 25, 3389-3402.

31. Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673-4680.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

32. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105-132.

33. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254.

34. Laemmli, U.K. (1970) Nature 227, 680-685.

35. Kong, L., Siranosian, K. J., Grossman, A. D. and Dubnau, D. (1993) Mol. Microbiol. 9, 365-373.

36. Ivaniševic, R., Milic, M., Ajdic, D., Rakonjac, J. and Savic, D. J. (1995) J. Bacteriol. 177, 1766-1771.

37. Koonin, E.V. (1996) Trends Biochem. Sci. 21, 242-243.

38. Ponting, C.P. and Kerr, I.D. (1996) Protein Science 5, 914-922.

39. Shibuya, I., Yamagoe, S., Miyazaki, C., Matsuzaki, H. and Ohta, A. (1985) J. Bacteriol. 161, 473-477.

40. Iuchi, S., Matsuda,Z., Fujiwara,T. and Lin, E. C. C. (1990) Molecular Microbiology 4 (5), 715-727.

41. Weisemann, J. M. and Weinstock, G. M. (1988) DNA 7, 389-398

42. Hanahan, D. (1985) in DNA cloning Vol. 1, a practical approach (Glover, D. M., ed.), pp. 109-135, IRL Press, Oxford.

43. Winans, S. C., Elledge, S. J., Krueger, J. H. and Walker, G. C. (1985) J. Bacteriol. 161, 1219-1221.

44. Casabadan, M. J. (1976) J. Bacteriol. 104, 541-555.

45. Tropp, B. E., Ragolia, L., Xia, W., Dowhan, W., Milkman, R., Rudd, K. E., Ivaniševic, R. and Savic, D. J. (1995) J. Bacteriol. 177, 5155-5157.

46. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L. and Boyer, H. W. (1977) Gene 2, 95-113.

47. Sutcliff, J. G. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 77-90.

48. Jiang, F., H.S. Rizavi, and M.L. Greenberg (1997) Molecular Microbiology 26, 481-489.

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49. Jiang, F., M.T. Ryan, M. Schlame, M. Zhao, Z. Gu, M. Klingenberg, N. Pfanner, and M.L. Greenberg (2000) J. Biol. Chem. 275 (29) 22387-22394.

50. Nishijima, S., Asami, Y., Uetake, N., Yamagoe, S., Ohta, A. and Shibuya, I. (1988) J. Bacteriol. 170, 775-780.

51. Shibuya, I., Miyazaki, C. and Ohta, A. (1985) J. Bacteriol. 161, 1086-1092.

52. Krulwich, T.A. (1995) Molecular Microbiology 15 (3), 403-410.

53. Ivey, D.M. et al (1994) J. Bacteriol. 176, 5167-5170.

54. Clejan, S., A.A. Guffanti, M.A. Cohen, and T.A. Krulwich (1989) J. Bacteriol. 171, 1744-1746.

55. Gilmour, R. and T.A. Krulwich (1997) J. Bacteriol. 179, 863-870.

56. Ito, M., A.A. Guffanti, J. Zemsky, D.M. Ivey, and T.A. Krulwich (1997) J. Bacteriol. 179, 3851-3857.

57. D. Guo, B.E. Tropp, Biochim. Biophys. Acta 1389 (1998) 34-42.

58. Biswas, I., A. Gruss, S.D. Ehrlich, and E. Maguin (1993) J. Bacteriol.175, 3628-3635.

59. Quirk, P.G., D.B. Hicks and T.A. Krulwich (1991) J. Biol. Chem. 268,678-685.

60. Exton, J. H. (1997) J. Biol. Chem. 272, 15579-15582.

61. Gottlin, E.B., Rudolph, A.E., Zhao, Y., Matthews, H.R., Dixon, J.E. (1998) Proc. Natl. Acad. Sci. USA 95, 9202-9207.

62. Rudolph, A.E., Stuckey, J.A., Zhao, Y., Matthews, H.R., Patton, W.A., Moss, J., Dixon, J.E. (1999) J. Biol. Chem. 274, 11824-11831.

63. Leiros, I., Secundo, F., Zambonelli, C., Servi, S. and Hough, E. (2000) Structure 8, 655-667

64. Stuckey, J.A. and Dixon, J.E. (1999) Nat. Struct. Biol. 6, 278-284

65. Hiraoka, S., Matsuzaki, H., Shibuya, I. (1993) FEBS Lett. 336, 221-224.

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66. Geng, D., Chura, J., Roberts, M.F. (1998) J. Biol. Chem. 273 (20) 12195-12202.

67. Nishimura, A., Okiyama, K., Kohara, Y., Horiuchi, K. (1992) Microbiol. Rev. 56, 137-151.

68. Winans, S.C., Elledge, S.J., Krueger, J.H., Walker, G.C. (1985) J. Bacteriol. 161, 1219-1221.

69. Peterson, G.L. (1977) Anal. Biochem. 83, 346-356.

70. Kates, M. Techniques of Lipidology, 2nd Edition, in: R.H. Burton and P.H. van Knippenberg (ed.), Laboratory Techniques in Biochemistry and Molecular Biology, volume 3, part 2, Elsevier, Amsterdam, 1986.

71. Tropp, B.E. (1997) Biochim. Biophys. Acta 1348, 192-200.

72. Studier, F.W. (1991) J. Mol. Biol. 219, 37-44.

73. Cole, R., Proulx, P. (1977) Can. J. Biochem. 55, 1228-1232.

74. Sung, T.-C., Roper, R.L., Zhang, Y., Rudge, S.A., Temel, R., Hammond, S.M., Morris, A.J., Moss, B., Engebrecht, J. and Frohman, M.A. (1997) EMBO J. 16, 4519-4530.

75. Guérout-Fleury, A.-M., Shazand, K., Frandsen, N., Stragier, P. (1995) Gene 167, 335-336.

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