CONSTRUCTION AND CHARACTERIZATION OF A CARDIOLIPIN-DEFICIENT MUTANT IN *RHODOBACTER SPHAEROIDES*

By

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ABSTRACT

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The anionic phospholipid, cardiolipin, is found in the mitochondrial membrane of eukaryotes and cytoplasmic membrane of bacteria. To elucidate the role of CL in the purple non-sulfur bacteria, *Rhodobacter sphaeroides*, a cardiolipin-deficient mutant was derived from the wild type strain 2.4.1 by the disruption of the *cls* gene which encodes the enzyme cardiolipin synthase. The gene disruption resulted into a significant reduction in the level of CL compared to the wild type which corresponded with the impaired cardiolipin synthase activity in the mutant. However, traces of CL were still present in the mutant which was detected by using the radioisotope labeling method. The analysis of the effect of the reduction in CL level on growth showed a more pronounced effect on the respiratory growth of the mutant than the photosynthetic growth which indicated a specific requirement of CL during the respiratory growth. The growth of the mutant and the wild type under osmotic and heat stress was also compared but no significant difference was observed. Interestingly, under those conditions, the mutant formed more CL. The presence of the minimal amount of CL in the mutant and the synthesis of a significant level of CL in the mutant during stress indicated the possible involvement of a cardiolipin synthase independent mechanism for producing CL in *Rb. sphaeroides* which could be up-regulated under certain physiological conditions.

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PE	phosphatidylethanolamine
PC	phosphatidylcholine
PG	phosphatidylglycerol
CL	cardiolipin
OL	ornithine lipid
PS	phosphatidylserine
SQDG	sulfoquinovosyldiacylglycerol
DGTS	diacylglyceryl-*N,N,N*-trimethylhomoserine
GGD	glucosylgalactosyl diacylglycerol
MHD	monohexosyl diacylglycerol
ICM	intracellular membrane
CM	cytoplasmic membrane
C*c*O	cytochrome *c* oxidase
ACP	acyl carrier protein
PA	phosphatidic acid
CDP-DG	CDP-diacylglycerol
TLC	Thin layer chromatography
PLD	Phospholipase D
BSA	Bovine serum albumin
Tris	tris hydroxymethyaminomethane

Chapter I

Introduction

Bacterial membrane lipids

Lipids are the structural and functional components of all biological membranes, including bacterial membranes. The amphipathic lipid molecules are packed together to form a lipid bilayer which forms a barrier for the separation of cellular components from the extra-cellular environment and creates compartmentalization within a cell. The lipid bilayer provides a matrix for the insertion of membrane proteins, the function of which in turn is affected by the nature of the lipids that interact with the protein. Though the detailed molecular mechanism of regulation of protein function by the lipids is still not clearly understood, there is enough evidence to support the fact that the charges of the lipid head groups and non-bilayer forming property of lipids are crucial for the proper functioning of membrane proteins (van Voorst and de Kruijff, 2000).

Considerable amount of work has been done on the lipid protein interactions in the *E. coli* membrane, which has been made possible by the availability of lipid biosynthetic mutants. *E.coli* membranes have a very simple lipid composition, with phosphatidylethanolamine (PE) as the only zwitterionic lipid and two anionic lipids, phosphatidylglycerol (PG) and cardiolipin (CL). Anionic phospholipids are important for the function of different membrane proteins like preprotein translocases (Kusters *et al.*, 1991; Hendrick and Wickner, 1991). The *E. coli* mutant deficient in PG showed impaired preprotein translocation across the E. coli inner membrane, which could be fixed by supplying the other anionic phospholipids (Kusters *et al.*, 1991). Similar dependence on

anionic phospholipids for the protein function has been shown by another group of *E. coli* enzymes, permeases (Hendrick and Wickner, 1991). This indicates that it is the charge of the anionic lipids that is important for the translocation across the inner membrane of *E. coli*.

The interaction of anionic phospholipids and positively charged residues of the membrane proteins has been shown to be important for the topology of membrane proteins (van Klompenburg *et al.*, 1997). Thus, one point of regulation of protein function by a lipid molecule is at the insertion of protein into the membrane, because the correct orientation of a protein in a membrane is crucial for the proper functioning of the protein (van Klompenburg *et al.*, 1997; van Dalen and de Kruijiff, 2004).

Regulation of membrane protein function is not limited to the interaction of proteins and anionic lipids of the membrane. Non-bilayer forming lipids are equally important for protein topology (Zhang *et al.*, 2005) as well as maintaining the proteins in their fully functional forms (de Kruijff, 1997). It has been shown that an *E. coli* mutant lacking the non-bilayer forming lipid, PE, is dependent upon divalent cations like calcium and magnesium in the medium for growth, which would interact with the anionic lipid, CL, forming a derivative that has a non-bilayer forming property (Rietveld *et al.*, 1993; Rietveld *et al.*, 1994).

Though it is clear that lipids do influence the physiology of bacterial cells through interaction with the different membrane proteins, a direct role of the lipid in bacterial growth is still not well documented. However, the fact that a bacterium changes its lipid composition according to the growth condition indicates that the lipids might have some indirect role in the growth of the organism through interaction with the different

membrane proteins. The change in the lipid composition according to the growth conditions has been studied in purple non-sulfur bacteria, which will be discussed next.

Lipids in purple non-sulfur bacteria

Purple non-sulfur bacteria show a great variation in the lipid composition among species. Ornithine lipid (OL) is present in almost all species of purple non-sulfur bacteria (Imhoff *et al.*, 1982). Phosphatidylcholine (PC) is present in some species along with other phospholipids, PE, PG, CL and OL, whereas there is another group that lacks PC but contain the remaining lipids (Imhoff *et al.*, 1982). Sulfolipid (SQDG) is present as a minor lipid in some PC containing species (Imhoff *et al.*, 1982).

Effect of growth condition on the lipid composition

Light and oxygen are the two factors that govern the mode of growth in the purple non-sulfur bacteria. The bacteria can grow photoheterotropically under anaerobic condition in presence of light and chemoheterotropically under aerobic condition in the absence of light (Russell and Harwood, 1979). Due to the capability of the bacteria to grow under different conditions, it has been considered as a good system for studying the changes in the membrane structure, as well as lipid composition, under photosynthetic and non-photosynthetic growth.

Growth under photosynthetic condition involves the formation of intracellular membrane system (ICM), which contains the photosynthetic apparatus (Tai and Kaplan, 1985). The ICM is formed as an extension of the cytoplasmic membrane (Crook *et al.*, 1986) and its formation is regulated by the intensity of light (Holt and Marr, 1965).

Photosynthetic growth under low light condition results in more extensive formation of ICM compared to that under high light growth (Tai and Kaplan, 1985). Due to the presence of this extra membrane system in photosynthetically grown cells, their total phospholipid content per cell is higher compared to those grown under non-photosynthetic conditions (Imhoff *et al.*, 1982).

The phospholipids biosynthetic enzymes are localized in the cytoplasmic membrane (CM). Lipids are synthesized on the specific regions of CM and transferred to the ICM (Radcliffe *et al.*, 1985; Tai and Kaplan, 1985). Studies on synchronous cultures of *Rhodobacter sphaeroides* showed that the lipid transfer activity from CM to the ICM is highest just before cell division (Tai *et al.*, 1986). Explaining the reason for the higher phospholipids transfer activity, a possible role of the lipids in cell division has been proposed (Knacker *et al.*, 1985).

The formation ICM and lipid transfer activity seen in the photosynthetically grown cells raises the question whether lipid composition of CM changes under different growth conditions and whether there is a preferential increase of any phospholipid in the ICM, resulting from the transfer of specific lipids from the CM to the ICM. Several studies have been carried out to address this question about growth condition induced changes in lipid composition which has produced some mixed results. Comparison of phospholipid content in photosynthetically and non-photosynthetically grown cells showed more PE and PG in cells grown under the former condition (Steiner *et al.*, 1970). Later studies on the lipid composition of three members of Rhodospirilliaceae grown under photosynthetic and non-photosynthetic conditions showed an increase in the level of PG with decrease in the PE level in both the whole cell extract as well as in the ICM of

cells grown photosynthetically (Russell and Harwood, 1979). In contrast to that result, another study showed no such difference in the lipid composition of photosynthetically and non-photosynthetically grown cells (Onishi and Niederman, 1982). However, in photosynthetically grown cells, variation in light intensity did show an effect on lipid composition (Onishi and Niederman, 1982). Cells grown under low light condition showed an increase in the level of PE compared to those grown under high light condition (Onishi and Niederman, 1982). When the cell cultures growing under high light condition were moved to low light condition, an inhibition of phospholipid biosynthesis was observed (Campbell and Lueking, 1983).

Effect of media composition

Purple non-sulfur bacteria show the ability to form new lipids in response to the changes in the media composition as well as alter the level of existing lipids so as to compensate for the loss or decrease in the level of one lipid by another. In *Rhodopseudomonas sphaeroides*, presence of Tris in the medium resulted in the accumulation of a new lipid (Donohue *et al.*, 1982). Structural analysis of the lipid later showed it to be phosphatidyl-Tris, a xenobiotic compound (Schmid *et al.*, 1991). The accumulation of the lipid didn't show any effect on the growth of the bacteria but since it was formed at the expense of the major lipid, PE, (Donohue *et al.*, 1982) it can be speculated that phosphatidyl-Tris could help the bacteria to cope with the changes in the media, resulting from the addition of Tris.

Similarly, in *Rb. sphaeroides* grown under phosphate limitation, betaine lipid (DGTS) was formed along with two other new glycolipids, glucosylgalactosyl

diacylglycerol (GGD) and monohexosyl diacylglycerol (MHD) (Benning *et al.*, 1995). It was proposed that those non-phosphorous lipids substituted for the phospholipids (Benning *et al.*, 1995) and thus helped the bacteria survive the phosphate deprivation. An additional evidence for the substitution of phospholipids by non-phosphorous lipids is provided by sulfolipid deficient mutant of *Rhodoabcter sphaeroides* (Benning *et al.*, 1993). The sulfolipid deficient mutant showed a growth defect only under phosphate limited growth condition, thus indicating the compensation for the loss of sulfolipid by phospholoipid in the wild type cells grown under the same conditions (Benning *et al.*, 1993). On the other hand, the level of sulfolipid increased along with another non-phosphorous lipid, OL, under phosphate deprived growth (Benning *et al.*, 1995).

Osmolarity of the media is another factor that affects the lipid composition. In *Rhodobacter sphaeroides* grown under high salt condition, an increase in the level of cardiolipin with the decrease in PG was observed (Catucci *et al.*, 2004). It was proposed that the accumulation of cardiolipin in the membrane could prevent the cells from lysis and thus helping the bacteria survive the osmotic stress (Catucci *et al.*, 2004).

The capacity of purple non-sulfur bacteria to change the lipid composition depending upon the growth condition provides greater adaptability to a changing natural environment. Since lipids are the building blocks of the membranes, such changes in the lipid compositions are important for maintaining the membrane integrity. Besides, different lipids have also been found to be associated with several membrane proteins and the purpose of such changes in the lipids could be crucial for the proper functioning of various membrane proteins that are active under different growth conditions.

Protein lipid interaction in purple non-sulfur bacteria

Photosynthesis and respiration are two important membrane associated processes (Fyfe *et al.*, 2001). In purple non-sulfur bacteria structural analysis of the proteins involved in these processes, such as the photosynthetic reaction center and cytochrome *c* oxidase, have shown the association of specific lipid molecules with the proteins. Several studies were carried out to understand the details of those interactions and attempts were made towards the elucidation of possible functions of those lipids.

Lipids in the photosynthetic reaction center

The photosynthetic reaction center is involved in process of conversion of light energy to chemical energy through light driven electron transfer. Some photosynthetic organisms can possess both type I and type II reaction centers while others have one or the other. Purple bacteria have only type II reaction centers. The crystal structure of the reaction center from *Rb. sphaeroides* showed a molecule of cardiolipin bound to the intramembrane surface of the protein (McAuley *et al.*, 1999). In a detailed study on the cardiolipin-reaction center interaction it was observed that the cardiolipin molecule associates with the intramembrane region of the protein in such a way that the acyl chains of the lipid fit into the grooves formed by the alpha helices of different subunits of the protein, establishing a hydrophobic interactions with apolar residues of the protein (Fyfe *et al.*, 2001). The sequence comparison of the *Rb. sphaeroides* reaction center with that of the other photosynthetic bacteria with the similar reaction centers showed that the residues of the protein that interact with cardiolipin are conserved among those bacteria

(Wakeham *et al.*, 2001). That suggested the possible role of cardiolipin in the function of the reaction center.

In the process of analyzing of the function of cardiolipin, a mutant reaction center was constructed by changing one of the conserved residues, which resulted into the disruption of the interaction of cardiolipin with the protein (Fyfe *et al.*, 2004). Such disruption didn't affect the protein function but rather affected the thermal stability of the protein, thus showing the role of cardiolipin in stabilizing interaction among the subunits of the reaction center (Fyfe *et al.*, 2004).

Structural analysis of the reaction center from *Rb. sphaeroides* later revealed two additional lipids which had not been reported in the previous studies. A molecule of phosphatidylcholine (PC) and glucogalactosyldiacylglycerol (GGD) were also found associated with the hydrophobic transmembrane region of the protein (Camara-Artigas *et al.*, 2002). From the fact that PC and GGD molecules interact with the cofactors of the protein and those lipids are absent in the nonfunctional reaction center mutant (Camara-Artigas *et al.*, 2002), it can be derived that those lipids could possibly play some role in the function of the reaction center.

Lipids in cytochrome *c* oxidase

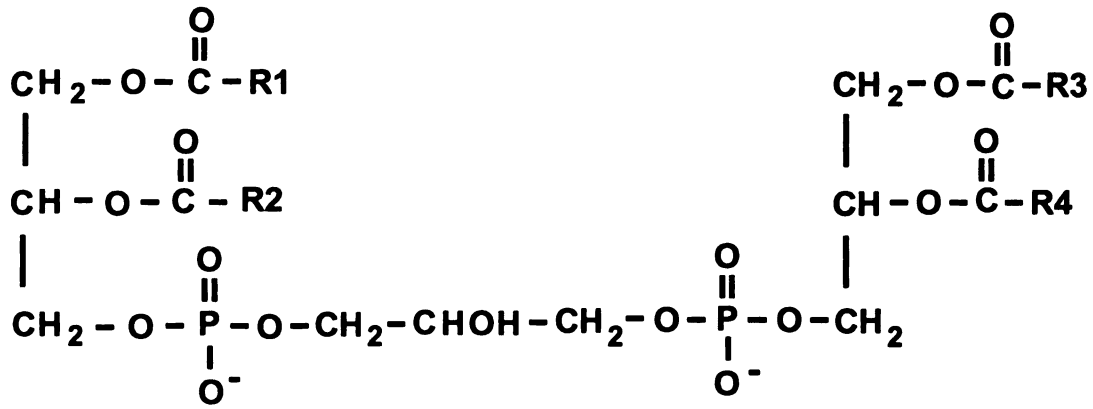
Cytochrome *c* oxidase (C*c*O) is the terminal enzyme of the respiratory electron transport chain that is involved in the reduction of molecular oxygen to water. C*c*O interacts with cytochrome *c* in the process of electron transfer and through the utilization of the energy derived from this process it acts as a proton pump. In the X-ray crystal structure of C*c*O from *Rb. sphaeroides* several lipid molecules were found associated with different

subunits of the protein and those lipids were identified as phosphatidylethanolamine (Svensson-Ek *et al.*, 2002). Later studies on the lipid component of the purified enzyme by mass spectrometry identified several other lipids: PE, PG, CL and SQDG (Hilmi, Y., 2002). Those lipid molecules remained attached to the enzyme even after several steps of the protein purification process (Hilmi, 2002). Though the importance of the lipid molecules for the enzyme in *Rb. sphaeroides* C*c*O has not yet been determined, the strong association of the lipid molecules with the enzyme raises the possibility that such interaction could be critical for the protein structure and function. Among the different lipids identified in the purified C*c*O complex, interaction of cardiolipin with the protein has been a subject of interest since the role of this lipid in the stability of the reaction center of *R. sphaeroides* has already been established. It would be interesting to know if cardiolipin could have similar function in C*c*O of the bacteria.

Cardiolipin: Structure and biosynthesis

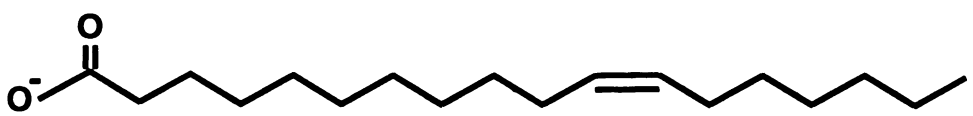
Cardiolipin is an anionic phospholipid found in the inner mitochondrial membrane of eukaryotes and cytoplasmic membrane of bacteria. This lipid consists of two phosphatidyl groups that are linked together by a glycerol molecule (Fig. 1.1). The presence of two phosphate groups in cardiolipin makes the lipid anionic. The lipid has four fatty acyl chains and depending upon the type of fatty acids occupying these positions there could be a variety of molecular species of cardiolipin (Schlame *et al.*, 2000). An organism may contain more than one species of cardiolipin. In *Rb. sphaeroides* cells grown under osmotic stress, two types of cardiolipin have been identified; tetravaccenylcardiolipin with vaccenic acid at all four positions and

(A)



(B)

(i)



(ii)

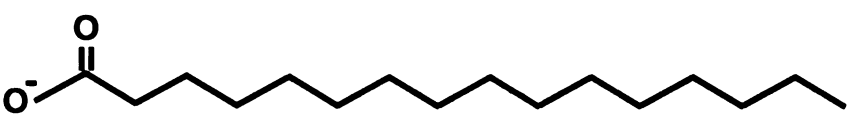


Figure 1.1 Structure of cardiolipin. (A) A cardiolipin molecule. RX= hydrocarbon chain of the fatty acid. (B) Fatty acids found in the different molecules of cardiolipin in *Rhodobacter sphaeroides*. (i) 18:1 (Δ^{11}), cis - Vaccenic acid and (ii) 16:0, Palmitic acid.

trivaccenylmonopalmitoylcardiolipin with vaccenic acid at three positions and palmitic acid at one position (Catucci *et al.*, 2004).

Cardiolipin is synthesized by different reactions in eukaryotes and prokaryotes, both catalyzed by the enzyme cardiolipin synthase. The eukaryotic cardiolipin synthase differ from the prokaryotic enzyme since they act upon different substrates. In eukaryotes, CDP-diacylglycerol (CDP-DG) and PG are used as substrates for the synthesis of cardiolipin while in prokaryotes two PG molecules are used as substrates. The polar lipid biosynthetic pathway that leads to cardiolipin synthesis in prokaryotes will be discussed with reference to *E. coli* since this pathway has been well illustrated in this bacterium (Raetz, 1978). The pathway is also shared by other bacteria. This holds true for the purple non-sulfur bacteria as supported by the results of the pulse-chase-labeling studies in *Rb. sphaeroides* (Cain *et al.*, 1983). In the prokaryotic pathway, acyl chain from acyl-ACP is transferred to sn-1 and 2 positions of glycerol 3-phosphate to form phosphatidic acid (PA), the reactions catalyzed by glycerol 3-phosphate 1-*O*-acyltransferase (PlsB) and 1-*O*-acyl glycerol 3-phosphate 2-*O*-acyltransferase (PlsC), respectively (Figure 1.2). PA is the precursor for the biosynthesis of PE, PG and CL. The first step is the activation of PA by CTP to form CDP-DG catalyzed by the enzyme phosphatidyl cyltidyltransferase (CdsA). The pathway branches after this step. The activated CDP-DG can either react with serine involving the enzyme phosphatidylserine synthase (Pss) to form phosphatidylserine (PS). PS then undergoes decarboxylation to form PE catalyzed by phosphatidylserine decarboxylase (Psd). In the other set of reactions, CDP-DG reacts with glycerol-3-phosphate to form phosphatidylglycerol 3-phosphate catalyzed by the enzyme phosphatidylglycerol 3-phoshate synthase (PgsA).

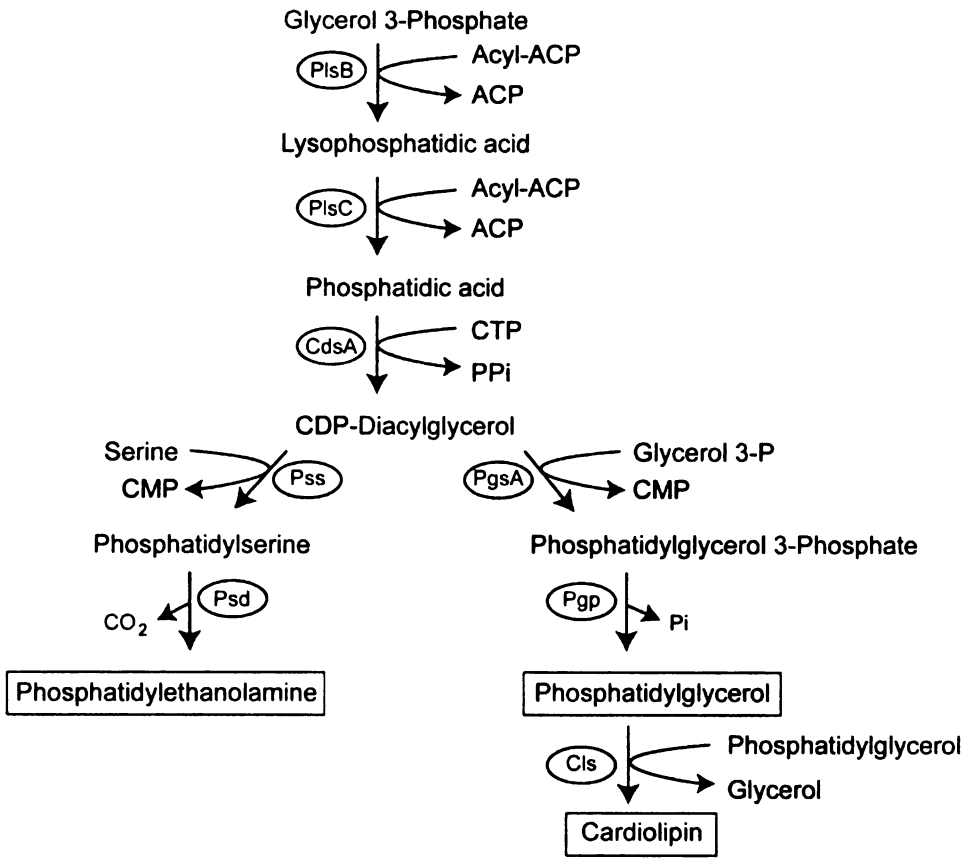


Figure 1.2 Prokaryotic pathway for phospholipid biosynthesis. Enzymes: PlsB, glycerol 3-phosphate 1-*O*-acyltransferase; PlsC, 1-*O*-acylglycerol 3-phosphate 2-*O*-acyltransferase ; CdsA, phosphatidyl cytidyltransferase; Pss, phosphatidylserine synthase; Psd, phosphatidylserine decarboxylase; PgsA, phosphatidylglycerol 3-phosphate synthase; Pgp, phosphatidylglycerol 3-phosphate phosphatase and Cls, cardiolipin synthase.

Phosphatidylglycerol 3-phosphate then gives off the phosphate to form the second lipid phosphatidylglycerol (PG), which is catalyzed by phosphatidylglycerol 3-phosphate phosphatase (PgpA). Cardiolipin synthase (Cls) then catalyzes the condensation of two PG molecules to form cardiolipin.

About the presented work

Lipids interact with the membrane proteins by either forming an annulus around the protein or through their incorporation within the transmembrane region of the protein (Lee, 2003). Protein-lipid interactions have received greater attention, not only due to the effect of lipids on protein function and stability as discussed earlier, but also because the retention of the lipid molecules on the protein has been considered to be an important factor for membrane protein crystallization (Garavito and FergusonMiller, 2001; Qin, 2005). With the increasing number of studies underway on the crystallization of membrane proteins to determine the enzyme mechanism, knowledge of the function of the individual lipid molecules associated with the proteins is a requirement. This study is part of a project that is aimed at determining the role of individual lipids in the structure and assembly of cytochrome *c* oxidase. After recognizing the role of the lipid molecules, their level in the enzyme could be manipulated so as to obtain a high resolution crystal structure of the protein.

Among the various membrane lipids found in *Rb. sphaeroides*, cardiolipin is one of the lipid that was detected by the mass spectrometric analysis of the purified cytochrome *c* oxidase of the bacteria (Hilmi, 2002). Though the function of this lipid in other membrane proteins from various sources has already been determined, the role of

cardiolipin in C*c*O of *Rb. sphaeroides* still needs to be investigated. The objective of this work was to construct a cardiolipin-deficient mutant strain of *Rb. sphaeroides* for the elucidation of the role CL in the bacterium.

The first part of the work describes about the identification of *cls* gene encoding cardiolipin synthase enzyme in *Rb. sphaeroides* and the genetic approaches used for the construction of the cardiolipin-deficient mutant. The second part involves the characterization of the mutant for obtaining the basic idea about its behavior in the absence of cardiolipin.

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Chapter II

Construction of a cardiolipin-deficient mutant in *Rhodobacter sphaeroides*

Abstract

In bacteria, cardiolipin is synthesized by the condensation of two phosphatidylglycerol molecules and this reaction is catalyzed by the enzyme cardiolipin synthase. By comparison with the amino acid sequence of the *E. coli* cardiolipin synthase, the putative cardiolipin synthase of *Rb. sphaeroides* was identified. The identity of the protein as cardiolipin synthase was confirmed by functional complementation of the *E. coli* SD9 strain, which contains no detectable level of cardiolipin due the combined defect in cardiolipin synthase and phosphatidylserine synthase. For the construction of a mutant deficient in cardiolipin in *Rb. sphaeroides*, the *cls* gene was cloned and disrupted by the insertion of a kanamycin resistance cassette following the deletion of the middle region of the gene. The disrupted copy was then forced to recombine with the homologous region in the chromosome, thus replacing the normal copy of the gene. The gene disruption in the mutant was verified by PCR analysis. Lipid analysis showed a reduction in the amount of cardiolipin in the *cls* knock out mutant (BC-m3) which demonstrated that the protein function had been affected by the gene disruption. Furthermore, expression of the wild type *cls* gene in BC-m3 resulted in CL synthesis in the mutant again, which confirmed that the *cls* gene product is involved and is sufficient for the synthesis of cardiolipin in *Rb. sphaeroides* and that the lipid phenotype in the mutant resulted from the inactivation of the gene.

Introduction

Cardiolipin (CL) is synthesized in different ways in bacteria and in eukaryotes. In eukaryotes, a phosphatidyl group is transferred from CDP-diacylglycerol (CDP-DG) to phosphatidylglycerol (PG) to form cardiolipin (CL). Previously it was thought that bacterial cardiolipin synthesis occurs through the same reaction (Stanacev *et al.*, 1967). Later, labeling studies carried out by Hirschberg and Kennedy showed that in bacteria, CL is synthesized from two PG molecules (Hirschberg and Kennedy, 1972).

Several experimental evidences support the hypothesis that in bacteria CL can be synthesized by reactions independent of cardiolipin synthase. In *E. coli*, an increase in cardiolipin synthesis was observed at the stationary growth phase and also upon the exposure to stress conditions, like treatment with phenethyl alcohol and infection with λ lysogen, without a corresponding increase in cardiolipin synthase activity (Tunaitis and Cronan, 1973). The explanation given to that phenomenon stated that under stressed conditions CL synthesis could have occurred by rearrangements of PG molecules without the involvement of cardiolipin synthase (Tunaitis and Cronan, 1973).

The idea about the existence of secondary pathway for cardiolipin biosynthesis developed later with the studies on mutants unable to synthesize cardiolipin due to a defect in the enzyme cardiolipin synthase. The cardiolipin biosynthetic mutant in *E. coli* was first isolated in the process of screening the population of chemically mutagenized cells for the mutants defective in phospholipid biosynthesis (Pluschke *et al.*, 1978). The mutation in the *cls* gene resulted in a defective cardiolipin synthase activity and a reduction in the level of CL (Pluschke *et al.*, 1978). The residual amount of cardiolipin in the *cls*$^-$ mutant was further decreased by a mutation in the second gene, *pss*, encoding the

enzyme phosphatidylserine synthase which is normally involved in PE biosynthesis (Shibuya *et al.*, 1985). The *E. coli* SD9 strain, with a double mutation in *cls* and *pss*, showed a significant reduction in the level of cardiolipin, to less than 0.1%, which was not achievable by *cls*$^-$ mutation alone (Shibuya *et al.*, 1985). The results indicate a possible role of phosphatidylserine synthase in the synthesis of the residual amount of cardiolipin observed in the *cls*$^-$ mutant of *E. coli*.

More supporting evidence for the possible involvement of phosphatidylserine synthase in CL synthesis came from the study of an *E. coli cls* null mutant. Despite the absence of cardiolipin synthase activity resulting from the disruption of the *cls* gene, the null mutant showed some residual CL (Nishijima *et al.*, 1988). The level of residual cardiolipin in the *cls* null mutant showed a five fold increase when the cells were grown up to the stationary growth phase compared to that in the exponential phase (Nishijima *et al.*, 1988). The involvement of phosphatidylserine synthase in the synthesis of CL was analyzed by varying the expression levels of the *pss* gene in the *cls* null mutant. Supporting the hypothesis about the CL synthesis in the mutant by a pathway catalyzed by phosphatidylserine synthase, it was observed that the level of CL formed in the mutant was dependent upon the level of expression of the enzyme (Nishijima *et al.*, 1988).

The attempt to verify the ability of phosphatidylserine synthase to make CL by using the purified enzyme for an *in vitro* enzyme assay was not successful (Shibuya *et al.*, 1985). However, the results from the mutant studies discussed above highlight the possibility of the presence of a secondary pathway for cardiolipin biosynthesis independent of cardiolipin synthase in *E. coli*, which is probably due to phosphatidylserine synthase.

In *E. coli*, two genes homologous to *cls* have been identified, *f413* (*ybhO*) and *o493* (*ymdC*) (Guo and Tropp, 2000). The product of the *f413* gene showed several points of identity at the amino acid level with the *E. coli* cardiolipin synthase. Despite the sequence similarity, the protein was unable to show the enzyme activity *in vivo* which was examined by its capability of functionally complementing E. coli SD9 strain with no detectable CL (Guo and Tropp, 2000). That renders the protein less likely to be involved in the CL synthesis in *cls* null mutant. Thus the evidence so far supports the phosphatidylserine synthase being responsible for catalyzing the alternative pathway for CL biosynthesis in *E. coli*.

The general phospholipid biosynthetic pathway found in *E. coli* is also shared by other bacteria and therefore it can be speculated that the existence of a secondary pathway for CL biosynthesis could be true for all the bacteria that contain cardiolipin. It would be interesting to see if the different pathways of CL biosynthesis are also present in the purple non-sulfur bacterium *Rb. sphaeroides* which contains CL as one of the membrane lipids. The objective of the present study is to construct a CL deficient mutant of *Rb. sphaeroides* through the disruption of the *cls* gene which would impair the cardiolipin synthase activity. The analysis of the mutant would definitely provide some clues regarding the possible existence of a secondary pathway for CL synthesis in this bacterium.

This chapter presents the identification of the *cls* gene in *Rb. sphaeroides* using bioinformatics and genetic approaches and the genetic techniques used for the construction of the *cls* knock out mutant.

Materials and Methods

Bacterial strains, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2.1. Sistrom's minimal media with succinate (Benning and Somerville, 1992) was used for the preparation of both solid media and liquid culture for growing *Rb. sphaeroides* cells. The cells on solid media were grown under anaerobic photoheterotrophic condition in a closed plexiglass box with a flow of nitrogen gas in the presence of light (approximately 30 $\mu molm^{-2}s^{-1}$) coming from a 60 watt incandescent bulb (Benning and Somerville, 1992). For liquid cultures, Erlenmeyer flasks with lids were used. The media was filled up to the brim of the flasks so as to remove as much air as possible. The cultures were grown in presence of light (approximately 30 $\mu molm^{-2}s^{-1}$) and were mixed by shaking a few times in a day. The temperature in the box was maintained at 30° C by adjusting the position of the lamp. For growing *E. coli* cells, LB medium was used for the preparation of solid media and liquid culture and the cells were grown at 37° C.

Search for the *cls* gene in *Rb. Sphaeroides.*

The putative *cls* gene for *Rb. sphaeroides* was identified by using the *E. coli* genome database. Protein sequence for cardiolipin synthase of *E. coli* was obtained from the *E. coli* K12 W3110 genome database at http://ecoli.naist.jp using the enzyme name as the keyword search. The sequence (id # JW1241) was used for the BLAST search against the microbial genomes (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi), selecting for *Rb. sphaeroides* 2.4.1 protein sequences. The best hit in the search result was selected as the putative cardiolipin synthase of *Rb. sphaeroides*.

Table 2.1 Bacterial strains and plasmids used in this work.

Strain or plasmid	Description	Source
Strains		
R. sphaeroides		
2.4.1	Wild type	Benning *et al.* (1993)
BC-m3	2.4.1 derivative; Kan^r	This work
E. coli		
S17-1	RP4-2-Tc::Mu-Km::Tn7$Tp^rSm^rPro^-$	Simon *et al.* (1983)
SD9	*cls pss*-1	Shibuya *et al.* (1985)
Plasmids		
pPICT-2	Cloning vector; Cm^r	Kawaguchi *et al.* (2001)
pGEM-T Easy	Cloning vector; Amp^r	Promega
pUC4K	Source of Kanamycin resistance gene	Amersham
pSUP202	pBR325 derivative; $Amp^rCm^rTc^r$	Simon *et al.* (1983)
pPICT-2-I	pPICT-2 with the 600 nt. segment I	This work Fig. 2.2
pGEM-T-II	pGEM-T-Easy with 591 nt. segment II	This work Fig. 2.2
pPICT-2-I/II	pPICT-2 with segments I and II	This work Fig. 2.2
pPICT-2-CK	pPICT-2 with construct Δ*cls*::*kan*R	This work Fig. 2.2
pSUP-CLD	pSUP202 with the construct for the disruption of *cls* gene; $Kan^rAmp^sCm^s$	This work Fig. 2.2
pQE-30	Expression vector; Amp^r	Qiagen
pQE-*cls*	pQE-30 with the amplified coding region of *cls* gene; Amp^r	This work
pRK415	Expression vector; Tet^r	Keen *et al.* (1998)
pRK-*cls*	pRK415 with the amplified coding region of the *cls* gene with upstream and downstream regions; Tet^r	This work

Expression of the *cls* gene of *Rb. sphaeroides* in *E. coli*.

An overnight culture of *Rb. sphaeroides* 2.4.1 was prepared in Sistrom's media. Genomic DNA was isolated from 1 ml of the culture by using the Wizard Genomic DNA Purification Kit (Promega). Using the genomic DNA as a template, the *cls* gene was PCR amplified by using the primers P1 and P6 (Table 2.2) as forward and reverse primers, respectively, which would introduce a *Bam*HI site at the 5' end and a *Hind*III site at the 3'end. The restriction site at the 5' end was selected in such a way that it would allow the PCR product to be in frame with the N-terminal 6XHis tag of the vector. The PCR product was cloned into the vector pPICT-2 (Table 2.1) and sequenced at the Michigan State University Research Technology Support Facility (RTSF). From the resulting plasmid, the inserted fragment was cut out with *Bam*HI and *Hind*III and was sub-cloned into the expression vector pQE-30, which had been linearized by the same enzymes, to obtain the plasmid pQE-*cls*. The insert in the plasmid was rechecked by the restriction digestion and sequencing using the gene specific and vector specific primers. Electro-competent SD9 cells were transformed with pQE-*cls* and with the vector pQE30 as a control and the transformants were selected by ampicillin resistance.

Single colonies of the SD9 transformant (SD9-T) and the control transformant (SD9- C) were inoculated to 6ml LB medium with ampicillin (50 μg/ml) and incubated at 30° C overnight with shaking. One ml of each of the cultures was inoculated to 50 ml of LB media containing ampicillin (50 μg/ml). The cultures were grown for 4-5 hours until the OD_{600} of the culture reached 0.4 – 0.6. After that, 0.4 mM IPTG was added to each culture and the cultures were incubated overnight at 30° C with shaking. The cells were harvested for lipid analysis.

Table 2.2 List of oligonucleotides used as PCR primers for the amplification of various regions of the *cls* gene and for the verification of the gene disruption. The restriction sites added by the primers during the amplification process are underlined.

Primers	Sequence
P1	5′- GCGGGATCCATGATCGACGACTGGCTGGGCGTCC - 3′ *Bam*HI
P2	5′- GCGAAGCTTGGATCCTCAGAGGTAGCTCTGGATCG - 3′ *Hind*III *Bam*HI
P3	5′- GCGGTCGACGTTGCGCCCGCCCACGATGGCC - 3′ *Sal*I
P4	5′- GCGGTCGACGGCGAGGCGATCACCGCGCTCC - 3′ *Sal*I
P5	5′ - CTGGCAGAGCATTACGCTGACTTG - 3′
P6	5′ - GCGAAGCTTTCAGAGGTAGCTCTGGATCGGCAGA - 3′ *Hind*III
P7	5′ - GATCTCGTGGCGCTGCAGGAGG - 3′ *Pst*I
P8	5′ - GGAAGCTTGAATTCGAGAGCCAC - 3′ *Hind*III

Lipid analysis by Thin layer chromatography

Cells from a 4.5 ml of culture were collected by centrifugation and suspended in 50µl water. For lipid extraction, 500µl of chloroform-methanol-formic acid (1:2:0.1, v/v/v) was added and mixed well using a vortex. To the sample, 250µl of 1M KCl-0.2M H_3PO_4 was added for phase separation followed by centrifugation. The lower organic phase was collected and loaded on Silica TLC plates (Si250; Baker) which had been activated by baking at 120° for 2 hours. The lipids were separated by two dimensional TLC using the solvent chloroform-methanol-water (65:25:4, v/v/v) for the first dimension and chloroform-methanol-acetic acid-water (170:25:25:4 v/v/v/v) for the second dimension. Lipids were visualized by staining with iodine and also by spraying with 50% H_2SO_4 followed by baking at 120° C for 20 minutes.

Construction of plasmid, pSUP-CLD, for disruption of *cls* gene

The strategy used for obtaining the plasmid construct for gene disruption is shown in Fig. 2.1. Using the genomic DNA from the wild type strain 2.4.1 as a template, a 600 nucleotide 5' region of the *cls* gene (segment I) was PCR amplified using the primers P1 and P3 (Table 2.2). Similarly, a 591 nucleotide 3' region of the gene (segment II) was amplified by using another set of primers, P2 and P4.The amplified products were cloned in the cloning vectors pPICT-2 and pGEM-T Easy, respectively, to generate the plasmids pPICT-2-I and pGEM-T-II. Segment II from the plasmid pGEM-T-II was cut out by double digestion with *Sal*I and *Hind*III and was ligated into pPICT2-I which had been digested with the same enzymes to generate the plasmid pPICT-2-I/II. From the pUC4K

Figure 2.1 Schematic diagram showing the strategy used for the construction of the plasmid pSUP-CLD for the disruption of the *cls* gene in *Rb. sphaeroides*. Grey boxes represent the *cls* gene and amplified regions of the gene. The black box represents the kanamycin resistance cassette (*kan*R). The orientation of the *kan*R in the gene construct is shown by the direction of the arrowhead. Restriction sites: B, *Bam*HI; S, *Sal*I; H, *Hind*III. Antibiotic resistance genes in the plasmids are shown as triangles: Cm, chloramphenicol reistance gene; Ap, ampicillin resistance gene and Tc, tetracycline resistance gene. *Bam*HI site located within Tc (*) gene in the plasmid pSUP202 was used for the insertion of the gene disruption construct (Δ*cls*::*kan*R) into the plasmid.

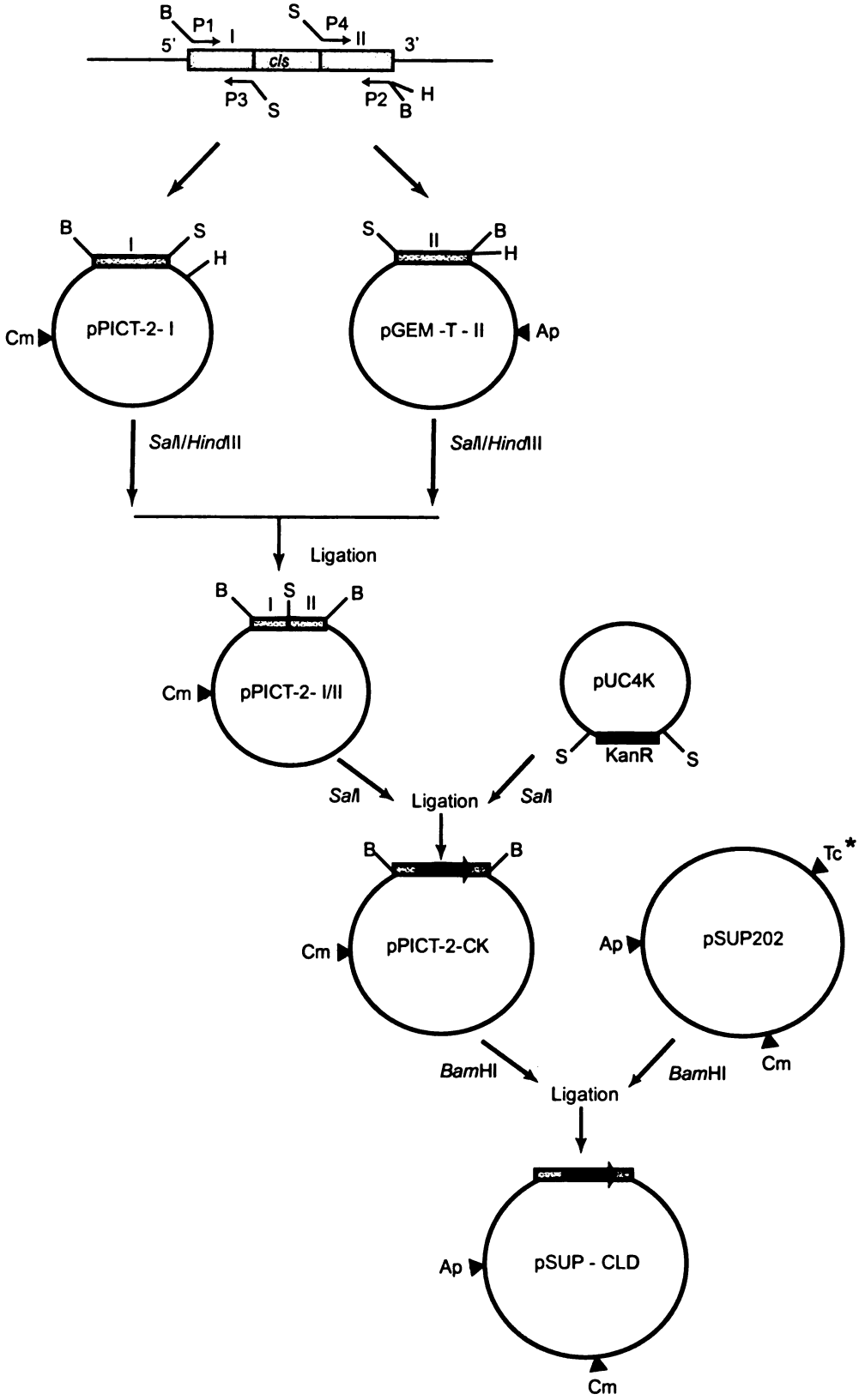
B
P1
I
S
P4
II
5'
3'
cls
P3
S
P2
B
H
pPICT-2- I
Cm
pGEM -T - II
Ap
SalI/HindIII
SalI/HindIII
Ligation
pPICT-2- I/II
pUC4K
KanR
SalI
Ligation
SalI
pPICT-2-CK
pSUP202
Tc*
Ap
Cm
BamHI
BamHI
Ligation
pSUP - CLD
Ap
Cm

Figure 2.1

plasmid, the kanamycin resistance cassette (*kan*R) was cut out by using *Sal*I sites placed on either side of the gene and was inserted into the plasmid pPICT-2-I/II which had been digested by the same enzyme. The resulting plasmid pPICT2-CK contained the *kan*R cassette inserted between segments I and II. The orientation of *kan*R was checked by digesting the plasmid pPICT2-CK with *Hind*III. Since a *Hind*III site is placed asymmetrically within *kan*R, digestion by the enzyme would produce fragments of different sizes based upon the orientation of the gene (Benning *et al.*, 1993). Using *Bam*HI, the gene construct was cut out from the plasmid pPICT2-CK and was ligated into the suicide plasmid pSUP202 by using the *Bam*HI site placed within the tetracycline resistance gene, to obtain the plasmid pSUP-CLD.

Biparental conjugation

E. coli strain S17-1 was used as a donor for mobilizing the plasmid pSUP-CLD into the *Rb. sphaeroides* wild type strain 2.4.1 (Simon *et al.*, 1983). Biparental conjugation was carried out following the protocol from Cao *et al.*, 1992. For this, S17-1 cells were transformed with pSUP-CLD and the transformants were selected by kanamycin resistance. Fifty ml of culture of wild type 2.4.1 strain was prepared in Sistrom's medium and 5 ml of overnight culture of S17-1 transformants harboring the plasmid pSUP-CLD was prepared in LB medium containing kanamycin (50 μg/ml). Cells were collected from 1 ml of 2.4.1 culture by centrifugation and suspended in 500 μl LB medium. Similarly, cells were collected from 1 ml of S17-1 transformant culture by centrifugation and suspended in 500 μl of LB medium. The two cell suspensions were mixed and cells were harvested by centrifugation and suspended in 100 μl LB medium. The 100 μl of the cell

mixture was placed on the nitrocellulose filter laid over LB plate. The plate was then incubated at 30° C for 6-10 hours in the dark. The nitrocellulose filter was then taken off the plate and placed into a 50 ml Corning tube and 3 ml of Sistrom's media was added to it. The tube was shaken a few times so as to allow the cells to come off the filter and get suspended in Sistrom's media. The cells were then collected by centrifugation and washed twice with cold Sistrom's media and finally suspended in 500 µl Sistrom's media. From the cell suspension, 25 µl of the cells were then plated on Sistrom's-agar plates with kanamycin (100 µg/ml). The high concentration of kanamycin has been recommended for selection of the conjugants following the conjugation process (Benning *et al.*, 1993). The plates were grown under photosynthetic conditions for 4-5 days until colonies of the conjugants started to appear on the plates. Individual colonies were streaked out on fresh Sistrom's-agar plates with kanamycin (25µg/ml). The colonies were selected for double crossovers by kanamycin resistance and ampicillin and chloramphenicol sensitivity (Simon *et al.*, 1983).

PCR verification for the disruption of the *cls* gene in the mutant

To verify the gene disruption in the mutant, genomic DNA was isolated from the wild type and the mutant strains and two PCR reactions were done using different sets of primers: (a) Primers P1 and P2 specific for the two ends of the *cls* gene were used to compare the length of the gene in the wild type and the mutant and (b) *cls* gene specific primer P2 and *kan*R specific primer P5 were used to confirm that the mutant had the kanamycin resistance cassette inserted into the genomic copy of the *cls* gene. The sizes of the PCR products were compared by gel electrophoresis.

Expression of the wild type *cls* gene in the mutant

Using the expression plasmid pRK415, the wild type *cls* gene was expressed in the mutant strain using the native promoter of the gene itself. For this, the *cls* gene including the 610 bp upstream region and the 344 bp downstream region of the gene was amplified so as to include the native promoter and terminator regions of the gene. Since those regions of the gene had not been precisely determined, a larger region upstream and downstream of the gene was included. Upstream primer P7, which included a *Pst*I site, and downstream primer P8, which introduced a *Hind*III site, were used for PCR amplification. The PCR product was cloned into pGEM-T Easy vector and was sequenced and then sub-cloned into the plasmid pRK415 by using the *Pst*I and *Hind*III sites. The resulting plasmid pRK-*cls* was then transformed into *E. coli* S17-1 for its mobilization. From the S17-1 transformant, the plasmid pRK-*cls* was transferred to the mutant by biparental conjugation by using the protocol described above. The conjugants were selected on Sistrom's-agar plates containing tetracycline (0.5 μg/ml). Individual tetracycline resistant transformant colonies were then streaked out on fresh Sistrom's-agar plates with tetracycline (0.5 μg/ml).

Results

Identification of the *cls* gene of *Rb. sphaeroides*

For the identification of the putative cardiolipin synthase in *Rb. sphaeroides*, the amino acid sequence of *E. coli* cardiolipin synthase was BLASTed against the protein sequences of *Rb. sphaeroides*. The search result showed the best hit for the Phospholipase-D family of proteins (protein accession number YP_353546.1). The protein sequence showed 27%

identity with that of the *E.coli* cardiolipin synthase (Fig 2.2) and the motif search result showed the presence of two HKD motifs (Fig 2.3). The presence of the HKD motifs (HxKxxxxDxxxxxxGxxN, where x is any amino acid) is the characteristic of all members of the phospholipase D (PLD) group of enzymes that includes bacterial and plant PLDs, bacterial cardiolipin synthase and eukaryotic and bacterial phosphatidylserine synthases (Ponting and Kerr, 1996).

To further confirm the identity of the protein as cardiolipin synthase, the protein was expressed in the SD9 strain using the plasmid pQE-30. The SD9 strain is the *E. coli cls pss*-1double mutant that has a significant reduction in the level of CL compared to the wild type strain K-12 W3110 (Fig. 2.4 A, B). After the induction of protein expression by adding 0.4 mM IPTG, the transformant with the plasmid pQE-*cls*, SD9-T, showed cardiolipin synthase activity which resulted in CL synthesis (Fig. 2.4 D) whereas no CL was synthesized in the control transformant carrying the plasmid pQE-30, SD9-C (Fig. 2.4 C). Parallel samples for the transformants SD9-T and SD9-C were prepared in which no IPTG was added. In both of those samples, no CL was formed (data not shown). It was observed that for the induction of protein expression 0.4 mM IPTG was sufficient. However, upon the addition of 1 mM IPTG, the transformants SD9-T showed an increase in the CL level whereas no CL was synthesized in the control transformant SD9-C (data not shown). The corresponding increase in the protein expression with the increasing concentration of IPTG in SD9-T further confirmed that the CL synthesis in those cells resulted from the *lac* promoter driven expression of the *cls* gene of *Rb. sphaeroides* carried by the plasmid pQE-*cls*.

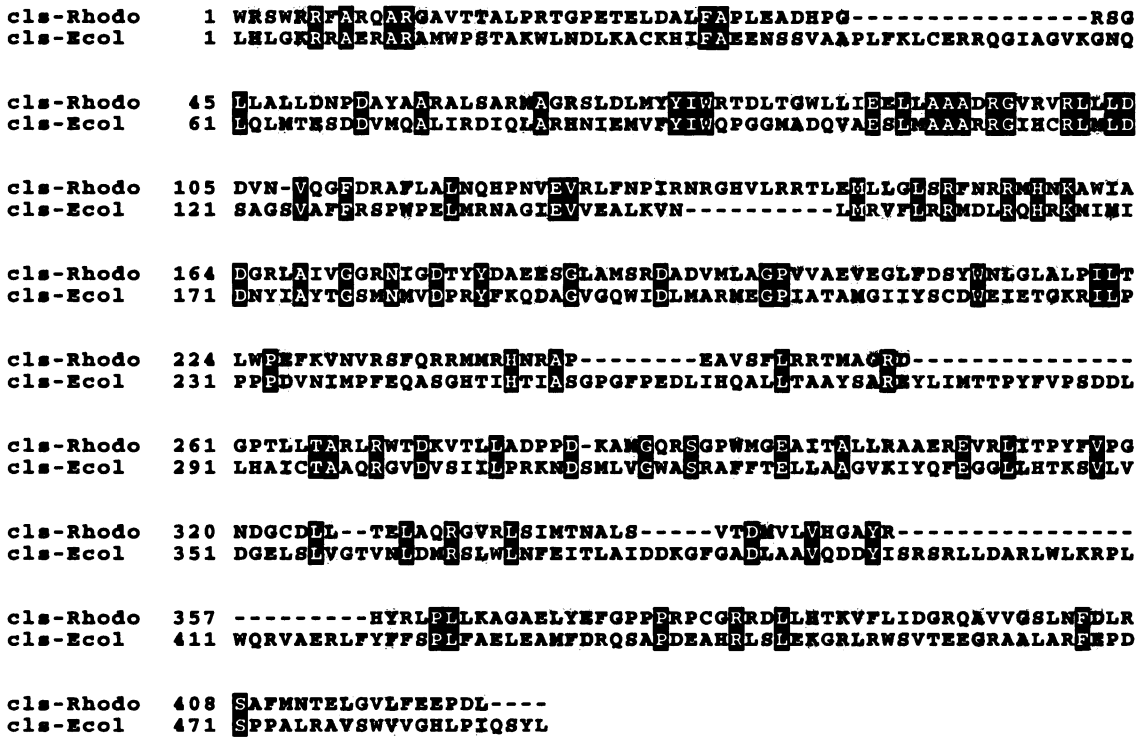

Figure 2.2 Multiple-sequence alignment of the cardiolipin synthase of *E. coli* and *Rb. sphaeroides*. Black shading indicates the identical residues and the grey shading indicates the conserved residues.

MIDDWLGVLAWVLAAVAAVAGLSAFTWRSWRRFARQARGAVTTALPRTGPETELDALFAP
LEADHPGRSGLLALLDNPDAYAARALSARMAGRSLDLMYYIWRTDLTGWLLIEELLAAADR
GVRVRLLLDDVNVQGFDRAFLALNQHPNVEVRLFNPIRNRGHVLRRTLEMLLGLSRFNRRM
HNKAWIADGRLAIVGGRNIGDTYYDAEESGLAMSRDADVMLAGPVVAEVEGLFDSYWNLG
LALPILTLWPEFKVNVRSFQRRMMRHNRAPEAVSFLRRTMAGRDGPTLLTARLRWTDKVT
LLADPPDKAMGQRSGPWMGEAITALLRAAEREVRLITPYFVPGNDGCDLLTELAQRGVRLSI
MTNALSVTDMVLVHGAYRHYRLPLLKAGAELYEFGPPPRPCGRRDLLHTKVFLIDGRQAVV
GSLNFDLRSAFMNTELGVLFEEPDLFAELEAMFDRQSAPDEAHRLSLEKGRLRWSVTEEGRA
ALARFEPDSPPALRAVSWVVGHLPIQSYL

Figure 2.3 Amino acid sequence for cardiolipin synthase of *Rb. sphaeroides*. The two HKD motifs present in the sequence are underlined.
(http://motif.genome.jp/)

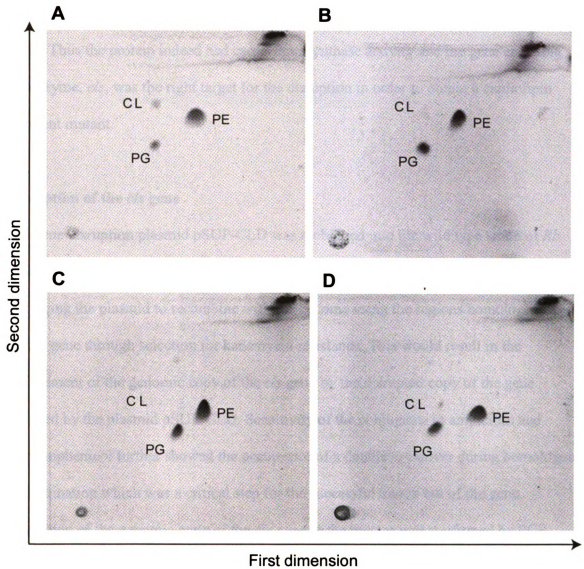

Figure 2.4 Two-dimensional chromatogram of lipids from *E. coli* strains. (A) K12 wild type strain W3110, (B) SD9, (C) SD9 transformant (SD9-C) carrying pQE30 vector and (D) SD9 transformant (SD9-T) carrying the *cls* expression plasmid construct pQE-*cls*. Phospholipids: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; and CL, cardiolipin.

The result demonstrated that the expression of the putative cardiolipin synthase of *Rb. sphaeroides* was able to functionally complement the cardiolipin deficient *E. coli* strain. Thus the protein indeed had cardiolipin synthase activity and the gene encoding the enzyme, *cls*, was the right target for the disruption in order to obtain a cardiolipin deficient mutant.

Disruption of the *cls* gene

The gene disruption plasmid pSUP-CLD was mobilized into the wild type strain of *Rb. sphaeroides* by using *E. coli* S17-1 as a donor. Disruption of the *cls* gene was carried out by forcing the plasmid to recombine with the genome using the regions homologous to the *cls* gene through selection for kanamycin resistance. This would result in the replacement of the genomic copy of the *cls* gene by the disrupted copy of the gene supplied by the plasmid pSUP-CLD. Sensitivity of the conjugants to ampicillin and chloramphenicol further showed the occurrence of a double crossover during homologous recombination which was a critical step for the successful knock out of the gene. Disruption of the genomic copy of the *cls* gene in the mutant was confirmed by PCR analysis by using two different sets of primers (Fig. 2.5 A) and comparing the sizes of the PCR products from the wild type and the mutant by gel electrophoresis. The PCR amplification of the *cls* gene in the wild type and the mutant using the primers specific for the two ends of the gene (P1 and P2) resulted into a larger product in the mutant due to an insertion (Fig. 2.5B). The size of the PCR amplified product in the mutant, approximately 2.6 Kb, was equal to the sum of the sizes of the 5'region (0.6 Kb) and 3' region (0.59 Kb) of the *cls* gene that had been amplified and used for obtaining the gene

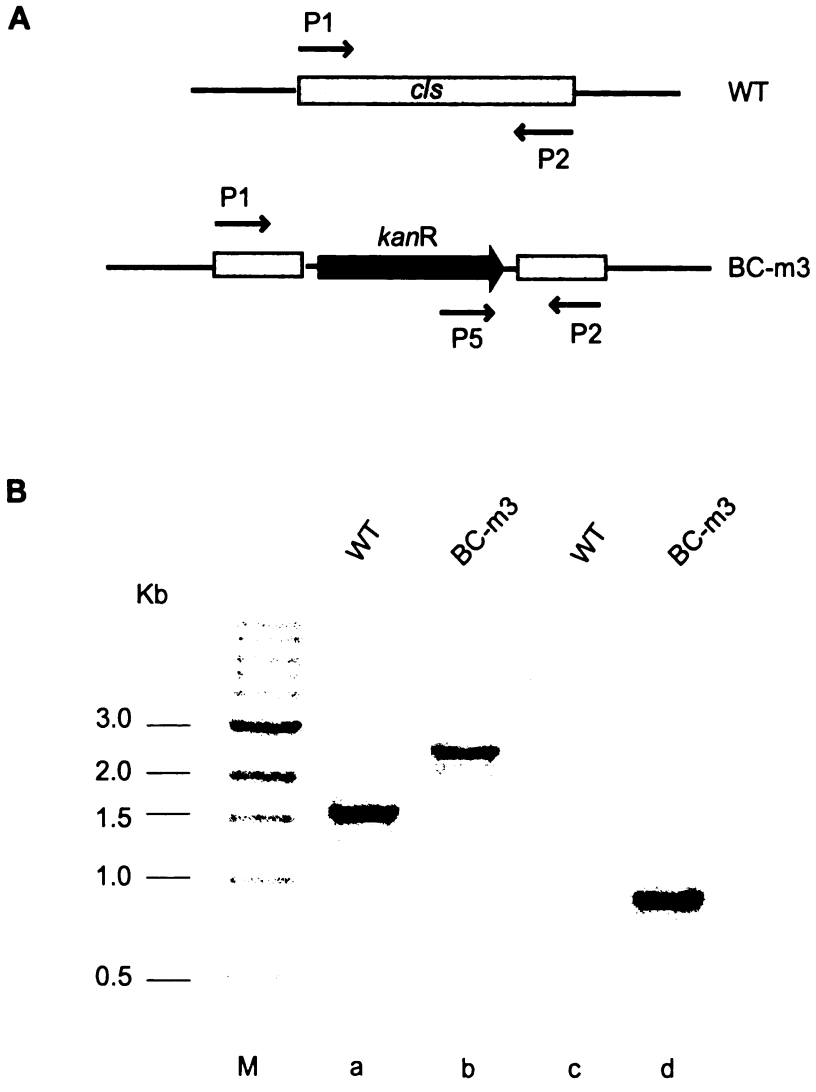

Figure 2.5 PCR verification for the disruption of the *cls* gene. (A) Schematic diagram showing the positions for the PCR primers P1, P2 and P5. (B) Comparison of the sizes of the PCR products by gel electrophoresis. Lane a and b show the products resulting from the amplification of the *cls* gene using the primers P1 and P2 from WT and BC-m3, respectively. Lane c and d show the products resulting from the amplification using the primers P2 and P5 from WT and BC-m3, respectively.

disruption construct plus the size of the kanamycin resistant cassette (1.2 Kb) used for the same. Similarly, PCR amplifications using the *kan*R specific primer P5 and the *cls* gene specific primer P2 resulted in a small fragment of the predicted size (0.96 Kb) in the mutant while no amplification was seen in wild type since *kanR* is present only in the mutant.

Lipid analysis by thin layer chromatography was carried out to measure the effect of gene disruption in the mutant. The mutant showed a significant reduction in the level of cardiolipin compared to the wild type (Fig. 2.6 A, B). Out of the five mutant lines obtained, the single mutant line BC-m3 was selected for further experiments based on the results from PCR verification and lipid analysis.

Complementation of the mutant by the wild type *cls* gene

To demonstrate that the lipid phenotype of the mutant BC-m3 resulted from the disruption of the *cls* gene, the wild type gene was reintroduced in BC-m3 using the plasmid pRK-*cls*, which resulted in the formation of the cardiolipin in the mutant (Fig. 2.6 C). Thus the expression of the wild type *cls* gene functionally complemented the CL deficient mutant, BC-m3, indicating that the gene disruption was responsible for the lipid phenotype.

Figure 2.6 Two dimensional chromatogram showing the lipids from the *Rb. sphaeroides* strains. (A) wild type strain 2.4.1, (B) *cls* knock out mutant BC-m3, and (C) BC-m3 harboring the plasmid for the expression of wild type *cls* gene, pRK-*cls*. Lipids: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; SL, sulfolipid; OL, ornithine lipid; CL, cardiolipin.

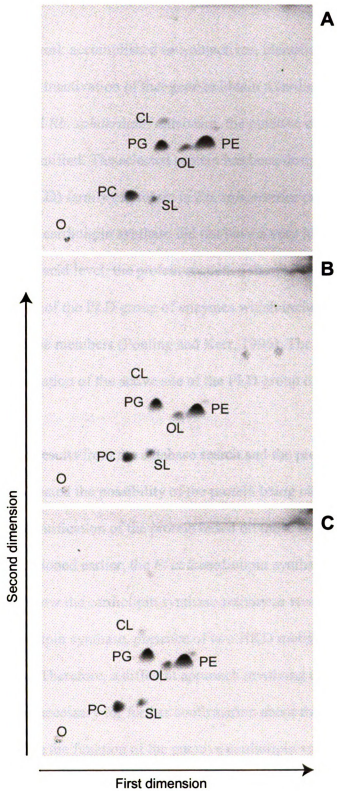
A
CL
PG
PE
OL
PC
SL
O
B
CL
PG
PE
OL
PC
SL
O
C
CL
PG
PE
OL
PC
SL
O
Second dimension
First dimension

Figure 2.6

Discussion

This section of the work accomplished two objectives, identification of the *cls* gene in *Rb. sphaeroides* and inactivation of that gene to obtain a cardiolipin deficient mutant. Using the *E. coli* and *Rb. sphaeroides* databases, the putative cardiolipin synthase of *Rb. sphaeroides* was identified. The selected protein has been designated as a member of phospholipase D (PLD) family of protein in *Rb. sphaeroides* protein sequence database. Though the putative cardiolipin synthase did not have a very high identity with the *E. coli* protein at the amino acid level, the protein sequence showed the presence of two HKD motifs characteristic of the PLD group of enzymes which includes bacterial cardiolipin synthase as one of the members (Ponting and Kerr, 1996). The two HKD motifs are involved in the formation of the active site of the PLD group of enzymes (Stuckey and Dixon, 1999).

Though the results from the database search and the presence of a characteristic sequence motif indicated the possibility of the protein being cardiolipin synthase of *Rb. sphaeroides*, the identification of the protein based on those two criteria was not conclusive. As mentioned earlier, the *E. coli* cardiolipin synthase homolog encoded by *f413* gene did not show the cardiolipin synthase activity *in vivo* despite the high sequence identity with cardiolipin synthase, presence of two HKD motifs and ability to form cardiolipin *in vitro*. Therefore, a different approach involving the functional analysis of the protein became necessary for further confirmation about the identity of the protein.

To determine the function of the putative cardiolipin synthase of *Rb. sphaeroides*, the ability of the protein to functionally complement *E. coil* SD9 strain was analyzed. Due to its much reduced level of cardiolipin, the strain has been used as a good system

for identifying cardiolipin synthase in other organisms like *Arabidopsis* (Katayama *et al.*, 2004) and *Bacillus subtilis* (Kawai *et al.*, 2004) as well as for confirming the identity of *E. coli* proteins which have been designated as cardiolipin synthase homologues based upon the amino acid sequence identity (Guo and Tropp, 2000). Expression of the putative cardiolipin synthase of *Rb. sphaeroides* in SD9 strain resulted in the formation of CL in the bacterium which demonstrated the ability of the protein to functionally complement the CL deficient mutant. Thus the result provided i*n vivo* evidence that confirmed the identification of the protein as cardiolipin synthase of *Rb. sphaeroides*.

In order to obtain a CL-deficient mutant, the *cls* gene in the wild type strain was inactivated by the replacement of the normal copy of the gene by the disrupted copy supplied by the gene disruption plasmid construct pSUP-CLD. The pure mutant line was selected based upon the following three criteria:

1. Antibiotic resistance and sensitivity: For knocking out the gene by homologous recombination, a double crossover event was necessary since the single crossover would result in the incorporation of the plasmid pSUP202 along with the construct for gene disruption which would leave the genomic copy of the *cls* gene uninterrupted. In order to ensure the occurrence of double crossovers, the conjugants were selected not only for kanamycin resistance but also for ampicillin and chloramphenicol sensitivity indicating the loss of the plasmid but incorporation of the disrupted copy of the *cls* gene into the genome. By this method the single crossovers could be separated out.

2. Segregation of the wild type and the mutant chromosome: For obtaining the pure mutant line, it was necessary to confirm that the mutant was clear from the contamination from the wild type chromosome. This could be achieved by PCR verification for the gene

disruption. During PCR amplification using the primers specific for the two ends of the gene, the mutant lines with improperly segregated chromosomes would give a mixed result showing products corresponding to both the wild type and the mutant whereas the pure mutant line would show a single product larger than that in the wild type.

3. Reduced level of cardiolipin: The mutant line with disrupted *cls* gene and free from the contamination of the wild type chromosome would show reduction in the level of cardiolipin compared to that in the wild type. The lipid phenotype in the mutant could be checked by lipid analysis and comparison of the CL level in the mutant with that in the wild type.

Out of the five mutant lines obtained, only one mutant line (BC-m3) was selected for further analysis since it agreed to all the above criteria. All the data presented in this work is from BC-m3 only.

The lipid data obtained from the *cls* knock out mutant showed that CL is absent in the mutant suggesting that cardiolipin synthase catalyzed pathway could presumably be the sole CL biosynthetic pathway in *Rb. sphaeroides*. Therefore, unlike in *E. coli* in which the *cls* null mutant showed residual amount of cardiolipin due to possible existence of an alternative pathway for cardiolipin synthesis, disruption of the *cls* gene in *Rb. sphaeroides* lead to the absence of CL. But it was too early to make any conclusion regarding the complete absence of CL in the mutant based on the qualitative data provided by TLC analysis. Therefore, a more sensitive and quantitative approach was taken for the lipid analysis which will be discussed in the following chapter. However, reduction in the level of cardiolipin in the mutant compared to the wild type clearly indicated that the cardiolipin synthase activity had been impaired by the gene disruption.

Expression of the wild type *cls* into BC-m3 resulted into the formation of cardiolipin in the mutant which indicates that the product of *cls* gene is sufficient for synthesizing cardiolipin. The data supports the result of the functional complementation of SD9 strain.

In summary, the gene encoding the enzyme cardiolipin synthase in *Rb. sphaeroides* was identified and the mutant deficient in cardiolipin was constructed by the inactivation of that gene. The following chapter discusses the analysis of the phenotypic consequences of the gene disruption in the mutant.

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Chapter III

Characterization of the cardiolipin-deficient mutant of *Rhodobacter sphaeroides*

Abstract

Comparison of the lipid compositions of the *Rb. sphaeroides* wild type 2.4.1 and the *cls* knock out mutant BC-m3 showed a reduction in the level of CL in the mutant which corresponded with the impaired cardiolipin synthase activity. The effect of the reduced level of CL on growth was analyzed by comparing the growth of the mutant and the wild type under both photosynthetic and respiratory growth conditions. The respiratory growth of the mutant was more affected than the photosynthetic growth as indicated by the lowered final cell density in the mutant compared to the wild type. In order to define the role of cardiolipin for the growth of the bacteria under stress conditions, the growth of the wild type and the mutant in high salt media and under high temperature conditions was measured but no significant difference in the growth was observed under those conditions. Interestingly, the lipid analysis of the wild type and the mutant exposed to high osmotic and temperature stress showed the formation of CL in the mutant. Thus the result supported the requirement of CL by the bacteria for the growth under stressed conditions and the possible involvement of a secondary pathway independent of cardiolipin synthase for the biosynthesis of cardiolipin in *Rb. sphaeroides* like in *E. coli*.

Introduction

The biochemical study of the various lipid biosynthetic mutants is a common approach used for the elucidation of the function of different membrane lipids (Cronan, 2003). The analysis of the cardiolipin-deficient mutants in bacteria and yeast has provided an insight into some specific role of cardiolipin in the bacterial and the mitochondrial membranes.

The studies on the *cls* knock out mutant in *Saccharomyces cerevisiae*, *crd1*Δ, have established the requirement of CL for the proper functioning of mitochondria. The mutant *crd1*Δ showed temperature sensitivity in growth and also reduced respiratory growth due to the lack of stabilization of the respiratory chain complexs, both of which could be complemented by the expression of wild type *CRD1* in the mutant (Zhong *et al.*, 2004; Zhang *et al.*, 2002). The mitochondrial membrane functions like ATPase activity, C*c*O activity and protein import were also impaired in the mutant (Jiang *et al.*, 2000), which demonstrated the indispensable role of CL in mitochondria.

In bacteria, analyzing the *cls* knock-out mutants for the elucidation of role of CL is more complicated due to other possible mechanisms for CL biosynthesis as discussed in the previous chapter. The growth analysis of the *E. coli cls* null mutant and the wild type did not show a significant difference (Nishijima *et al.*, 1988). From that observation, it appeared that CL is not required for the bacterial growth; but since the mutant retained traces of CL, another possibility for the apparent lack of growth differences could be because the residual CL present in the mutant could be sufficient to allow the near normal growth of the mutant. In the latter case, linking a phenotype to CL deficiency becomes more difficult.

Moreover, the *E. coli cls* null mutant showed an increase in the level of the

residual CL at the stationary growth phase, in which the wild type cells have been shown to increase the CL content at the expense of PG (Hiraoka *et al.*, 1993). A similar observation was made in the triple knock-out mutant of *Bacillis subtilis,* with disruption in the *cls* gene and the *cls* homologues. Though no detectable level of CL was present in the mutant at the exponential growth phase, CL was seen in the mutant after the initiation of sporulation (Kawai *et al.*, 2004).

In bacteria, the ability of the *cls* knock-out mutant to retain a minimal amount of CL and synthesize CL according to the physiological requirements makes it difficult to determine the effect of CL deficiency. However, a more detailed analysis of the mutant could identify a more specific role of CL in the bacteria. As an example, although the *B. subtilis* mutant disrupted in the *cls* gene and the *cls* homologues synthesized CL during sporulation, it was still unable to meet the requirement for the germination of the spores at the wild type rates (Kawai *et al.*, 2006).

The complexities mentioned above could arise during the analysis of the *Rb. sphaeroides cls* knock-out mutant BC-m3. Under those circumstances, the previous studies carried out on the *E. coli* and *B. subtilis cls* null mutants serve as references for the better understanding of the behavior of the *Rb. sphaeroides* mutant. This chapter discusses the basic characterization of the *cls* knock-out mutant BC-m3 which includes the comparative analysis of the lipid composition of the wild type and the mutant, cardiolipin synthase activity analysis and the growth measurements under normal and stressed conditions.

Materials and Methods

Quantitative lipid analysis

Lipid compositions of the wild type and the cardiolipin-deficient mutant BC-m3 were determined by *in vivo* labeling of the lipids by [^{14}C]acetate followed by quantification of the lipids by using liquid scintillation counting analysis as described (Weissenmayer *et al.*, 2000). Overnight cultures of the wild type and the mutant were grown in Sistrom's media. For growing the mutant, kanamycin (25 μg/ml) was added to the media. In 15 ml Falcon tubes, 3ml of each of the cultures was taken and [^{14}C]acetate was added to the final concentration of 0.4 μCi/ml and incubated for 28 hours at 30° with shaking. Cells were collected by centrifugation and washed with 1.5 ml water twice and suspended in 50 μl water. From the cells, lipids were extracted by the addition of 500 μl of chloroform-methanol-formic acid (1:2:0.1, v/v/v), mixing well by using a vortex, followed by the addition of 250 μl of 1M KCl-0.2M H_3PO_4. The lower organic phase was collected by centrifugation and loaded on TLC plates which had been activated by baking at 120° for 2 hours. The lipids were separated by two dimensional TLC using the solvent chloroform-methanol-water (65:25:4, v/v/v) for the first dimension and chloroform-methanol-acetic acid-water (170:25:25:4, v/v/v/v) for the second dimension. The plates were exposed to phosphoimager for obtaining the image of the labeled lipids. The lipids on the TLC plates were then visualized by iodine staining. Individual spots were scraped off from the TLC plate and radioactivity was measured by using a scintillation counter. The relative amount of each lipid was calculated as the percentage of total radioactivity present in the lipid samples from the wild type and the mutant.

Cardiolipin synthase activity assay

In vitro cardiolipin synthase activity assay in the wild type and the CL-deficient mutant BC-m3 was carried out by following the protocol of Pluschke *et al.*(1978).

(A) Preparation of membrane fractions: Cultures of the wild type and BC-m3 strains were grown in 250 ml of Sistrom's media in a capped 250 ml Erlenmeyer flasks under photosynthetic conditions as described in the previous chapter for 36 hours. The cells were harvested by centrifugation and suspended in 20 ml of 50 mM Tris-HCl (pH 7.5) and broken by using a French Press at 18,000 p.s.i. The cell debris was removed by centrifugation for 15 minutes at 4° C at 15,000 x *g*. The supernatant was centrifuged again for 2 hours at 4° C at 100,000 x *g*. The pellet was then suspended in 1 ml of 50 mM Tris-HCl and the protein concentration was determined by using Bradford's method (Bradford, 1976) with BSA as a standard.

(B) Preparation of ^{14}C labeled PG: An overnight culture of the wild type strain was prepared in Sistrom's media. Lipids were labeled by adding [1-^{14}C]acetate to the 3 ml of the culture by using the same protocol as mentioned above. Then the ^{14}C-labeled lipid was extracted and separated by two dimensional TLC as described above, followed by visualization with iodine staining. The PG spot on the silica plate was scraped off and collected in a 1.5 ml tube. Lipids were extracted from it by adding chloroform-methanol-formic acid (1:2:0.1, v/v/v) and 1M KCl-0.2 M H_3PO_4 followed by centrifugation. The lower phase of the sample was transferred to a fresh 1.5 ml tube and the extract was dried along with 2 μg of cold PG under the stream of nitrogen. The residue was dispersed in 100 μl of 0.5% Triton X-100 by sonication for 20 minutes and used as a substrate for the enzyme activity assay.

For the reaction, 100 µl of the substrate was added to the membrane (500 µg of protein) preparation from the wild type and the mutant. The samples were then incubated at 30° for 90 minutes. After that, the reaction was stopped by adding cold chloroform-methanol (1:2, v/v) and 10 µg of unlabeled CL. To the sample, 500 µl of chloroform and 500 µl of water were added. The organic phase from each sample was collected by centrifugation and was loaded on pre-activated TLC plates. The lipids were separated by two dimensional TLC and visualized by iodine. The silica from the PG and CL spots on the plate was collected by scraping. The silica from the SL spot was also collected as a background radioactivity measurement. Radioactivity in each spot was measured by scintillation counting.

Phosphatidylserine synthase activity assay

In vitro phosphatidylserine synthase activity assay in the wild type and the CL-deficient mutant BC-m3 was carried out by following the protocol for cardiolipin synthase activity assay as described above with the following changes:

(A) Preparation of membrane fractions: Membrane fractions from the wild type and the mutant cells were prepared in the same way as for the cardiolipin synthase activity assay except that 34 hours cell cultures were used, assuming that phosphatidylserine synthase activity is the higher during the exponential phase than stationary phase as in *E. coli* (Hiraoka *et al.*, 1993).

(B) Preparation of ^{14}C labeled PG: Using the method described above, ^{14}C labeled PG was prepared. For preparing the substrate for the reaction, 10 µg of CDP-DG was added to the labeled PG.

(C) For the reaction, 100 µl of the substrate was added to the membrane (500 µg of protein) preparation from the wild type and the mutant. The samples were then incubated at 30° for 60 minutes. To the reaction 0.1 M magnesium chloride was added since the magnesium requirement has been shown for the activity of the phosphatidylserine synthase enzyme in *Rhodobacter sphaeroides* (Radcliffe *et al.*, 1989).

Lipid analysis of the cells grown under stressed conditions

The wild type and BC-m3 cells were exposed to osmotic stress following the protocol described by Catucci *et al.*, 2004. For this, 6 ml of overnight cultures of the wild type and BC-m3 were prepared in Sistrom's media. For growing BC-m3 cells, kanamycin (25 µg/ml) was added to the media. From the 6 ml of cultures, 3 ml of each culture was added to 100 ml of Sistrom's media and grown overnight under respiratory condition at 30° C. The cells were then harvested by centrifugation and resuspended in 1 ml of water. Out of the 1 ml of the cell suspension, 500 µl of each of the wild type and the mutant cell suspensions were added to 50 ml polypropylene tubes, each containing 40 ml of buffer composed of 20 mM Tris-HCl (pH 7.5) and 500 mM NaCl. The remaining 500 µl of the wild type and BC-m3 cell suspensions were added to the tubes containing 40 ml of control buffer composed of 20 mM Tris-HCl without NaCl. The cells were incubated at room temperature with a slow and constant shaking for 180 minutes. Then cells were harvested and resuspended in 1.5 ml of 0.5 M Tris-HCl. From that, 500 µl of cell suspension was used for lipid analysis.

Lipids were extracted from the wild type and the mutant cells exposed to osmotic stress and also from the control cells using Bligh and Dyer's method (Bligh and Dyer,

1959). To the cells collected from 500 µl of the cell suspension, 750 µl of chloroform-methanol (1:2, v/v) was added and the sample was left on the bench for 30 minutes. The sample was mixed by vortexing a few times. After that, the samples were centrifuged at 11,000 x *g* for 10 minutes and the supernatant was transferred to 1.5 ml tube. To the sample, 250 µl of water and 250 µl of chloroform was added and the mixture was left at the bench for 30 minutes. The sample was mixed a few times by vortexing. The lower chloroform phase was collected by centrifugation and transferred to a fresh 1.5 ml tube and dried under the steam of nitrogen. The residue was redissolved in 100 µl of chloroform-methanol (1:1, v/v) and lipids were separated by TLC and visualized by spraying with 50% H_2SO_4 followed by baking at 120° C for 20-45 minutes. By using the same method, lipid analysis was also carried out for the wild type and the mutant grown in the normal Sistrom's media at 37° C.

Growth analysis

The growth of the wild type and the CL-deficient mutant BC-m3 was analyzed under both photosynthetic and respiratory growth conditions. The wild type and BC-m3 cells were grown on Sistrom's-agar plates without and with kanamycin (25 µg/ml), respectively. For photosynthetic growth, the cells were grown by the method described in the previous chapter and for the respiratory growth the cells were grown at 30° C in the absence of light. Five ml of overnight cultures of the wild type and the mutant were prepared in Sistrom's media. From that, 3 ml of culture were added to the 50 ml of Sistrom's media in a 50 ml Corning tube. The tube was filled up to the brim and capped. The cells were then grown photosynthetically. Calculated volumes of the wild type and

the mutant pre-cultures were then added to 250 ml Sistrom's media in Klett flasks so as to start from the same initial Klett reading. The cells were grown photosynthetically and the growth of the cells was analyzed by measuring the turbidity of the culture by using the Klett photometer. For respiratory growth, 50 ml of culture was grown in the 250 ml Klett flask with a foam stopper without light and with vigorous swirling.

Growth of the wild type and the mutant under stressed conditions was also analyzed. For the high salt growth measurements, 50 ml pre-cultures for the wild type and BC-m3 were prepared in Sistrom's media. The pre-cultures were used to inoculate 250 ml Sistrom's media containing different concentrations of NaCl and grown photosynthetically in Klett flasks. In order to find out the maximum concentration of NaCl that would exert maximum stress to the cells without killing them, the wild type and the mutant cells were grown in Sistrom's media containing 0.75 M, 0.80 M, 0.85 M and 1.25 M NaCl. The cells were unable to grow in the NaCl concentrations of 0.8 M or higher. Therefore, 0.75 M NaCl was used for the high salt growth measurements. For the high temperature growth, the wild type and BC-m3 pre-cultures were inoculated to 50 ml Sistrom's media and the cells were grown at 37° C in Klett flasks in the absence of light.

Results

Lipid composition of the wild type and the mutant

The lipids in the wild type and the CL-deficient mutant BC-m3 were quantified by growing the cells with [1-^{14}C]acetate so as to homogenously label the lipids followed by measuring the radioactivity incorporated by the individual lipids (Fig. 3.1). Comparison of lipid compositions of the wild type and the mutant showed a reduction in the level of CL in BC-m3 (Table 3.1). An increase in the level of PG, which is a precursor of CL, was observed in the mutant. Similarly, a small increase in the level of PE was observed in the mutant compared to the wild type. No difference in the levels of SL, OL and PC was observed in the wild type and the mutant.

Cardiolipin synthase activity

The cardiolipin synthase activity in the wild type and the mutant BC-m3 was measured by the formation of labeled CL *in vitro* from the condensation of ^{14}C-labeled PG and unlabeled PG molecules by the crude protein extract from the wild type and the mutant. Compared to the protein from the wild type, the same amount of protein from the mutant formed a significantly reduced level of CL (< 0.1). The result indicated a decreased activity of cardiolipin synthase in the mutant (Table 3.2).

Phosphatidylserine synthase activity

The phosphatidylserine synthase activity in the wild type and the mutant BC-m3 was measured by the formation of labeled CL *in vitro* from ^{14}C-labeled PG and CDP-DG by the crude protein extract from the wild type and the mutant. Compared to the wild type

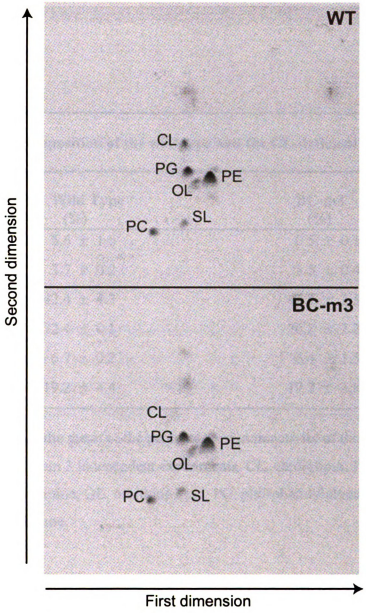


Figure 3.1 Two-dimensional chromatogram of ^{14}C-labeled lipids from the wild type and the mutant BC-m3. Lipids: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; SL, sulfolipid; OL, ornithine lipid; CL, cardiolipin. The ^{14}C-labeled lipids were visualized by using a phosphoimager.

Table 3.1 Lipid composition of the wild type and the CL-deficient mutant BC-m3

Lipid	Wild Type (%)	BC-m3 (%)
CL	5.6 ± 1.6	0.5 ± 0.1
OL	3.7 ± 0.2	3.5 ± 0.4
PE	42.4 ± 4.3	44.1 ± 2.5
PG	22.4 ± 0.1	26.2 ± 2.2
SL	6.7 ± 0.2	6.4 ± 1.3
PC	19.2 ± 4.4	19.2 ± 3.8

The values given are the mean of the percentage of radioactivity of the individual lipids ± the standard errors from 3 independent experiments. CL, cardiolipin; PE, phosphatidylethanolamine; OL, ornithine lipid; PG, phosphatidylglycerol; SL, sulfolipid; PC, phosphatidylcholine.

Table 3.2 Analysis of *in vitro* cardiolipin synthase activity.

Strain	PG (%)	CL (%)
Wild type (2.4.1)	97.36 ± 0.51	2.64 ± 0.51
BC-m3	99.91 ± 0.15	0.09 ± 0.15

Cardiolipin synthase activity was measured as the labeled CL synthesized from the ^{14}C-labeled PG by the wild type and the mutant crude protein extract. The reaction was incubated at 30° C for 90 minutes. The values given are the mean of the percentage of radioactivity in phosphatidylglycerol (PG) and cardiolipin (CL) ± standard errors from three independent cardiolipin synthase activity assays.

protein, the same amount of the mutant protein formed higher amount of labeled CL (Table 3.3). Though the amount of labeled CL formed by the wild type protein was reduced compared to the mutant but the level was still significant. The result indicated the possible involvement of phosphatidylserine synthase activity for the formation of residual CL in the mutant. The enzyme activity is somehow reduced in the wild type. It needs to be mentioned that a higher standard error was seen in the result obtained from the mutant compared to the wild type due to the variability in the data from the experimental repeats.

Response of the mutant to osmotic and heat stress

Analysis of lipid from the wild type cells exposed to osmotic stress for 180 minutes showed an increase in the level of CL (Fig. 3.2 B) compared to the cells grown in control buffer (Fig 3.2 A). No CL was seen in the mutant cells suspended in the control buffer (Fig.3.2 C) whereas detectable level of CL was present in the cells exposed to osmotic stress (Fig. 3.2 D). The CL spot was confirmed by using a CL standard in the lipid extract from the mutant and co-migration of the CL standard with the CL spot seen on the TLC plate. Two new lipid spots were also seen on the TLC plates. Since those new lipids were present in both the osmotically stressed cells as well as those suspended in control buffer, the lipids could have probably come from the Tris buffer used for the experiment. Previous studies have shown that certain strains of *Rb. sphaeroides* are capable of forming a xenobiotic lipid when grown in media containing Tris (Donohue *et al.*, 1982; Schmid *et al.*, 1991).

Table 3.3 Analysis of *in vitro* phosphatidylserine synthase activity.

Strain	PG (%)	CL (%)
Wild type (2.4.1)	99.64 ± 0.26	0.36 ± 0.24
BC-m3	98.89 ± 1.36	1.11 ± 1.36

Phosphatidylserine synthase activity was measured as the labeled CL synthesized from the ^{14}C- labeled PG and CDP-DG by the wild type and the mutant crude protein extract. The reaction was incubated at 30° C for 60 minutes. The values given are the mean of the percentage of radioactivity in phosphatidylglycerol (PG) and cardiolipin (CL) ± standard errors from three independent phosphatidylserine synthase activity assays.

Figure 3.2 Two-dimensional chromatogram of lipids from the wild type and the mutant exposed to osmotic stress. Lipids from (A) the wild type cells suspended in the control buffer; (B) the wild type cells suspended in buffer with 500 mM NaCl; (C) BC-m3 cells suspended in the control buffer; (D) BC-m3 cells suspended in the buffer with 500 mM NaCl. Lipids: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; SL, sulfolipid; OL, ornithine lipid; CL, cardiolipin. The unidentified lipids are shown by arrows.

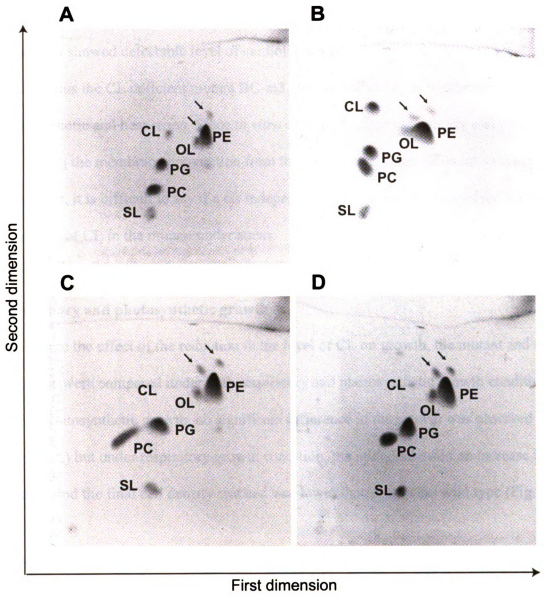


Figure 3.2

Similar observations were made in the mutant cells grown in regular Sistrom's media at high temperature. The mutant cells grown at 37° C up to the stationary growth phase also showed detectable level of cardiolipin (Fig. 3.3 B).

Thus the CL deficient mutant BC-m3 showed the ability to synthesize cardiolipin under osmotic and heat stress. Since *in vitro* cardiolipin synthase activity assay was not done using the membrane preparation from the cells grown under different stress conditions, it is difficult to say if a *cls* independent pathway could be involved for the formation of CL in the mutant under stress.

Respiratory and photosynthetic growth

To analyze the effect of the reduction in the level of CL on growth, the mutant and the wild type were compared under both respiratory and photosynthetic growth conditions. Under photosynthetic growth, no significant difference in the growth was observed (Fig.3.4 A) but under respiratory growth condition, the mutant showed an increase in the lag time and the final cell density reached was lower than that in the wild type (Fig. 3.4 B).

Growth under high salt and high temperature conditions

In order to check if CL is essential for the growth under high salt conditions, the growth of the wild type and the mutant was compared under different concentration of NaCl. In the media with 0.75 M NaCl, both the wild type and the mutant cells were able to grow after an elongated lag time but no significant difference in the growth was observed

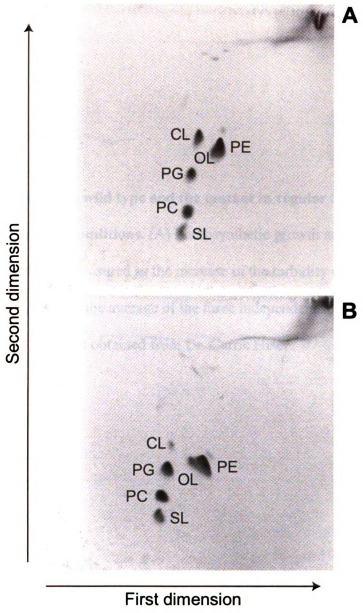
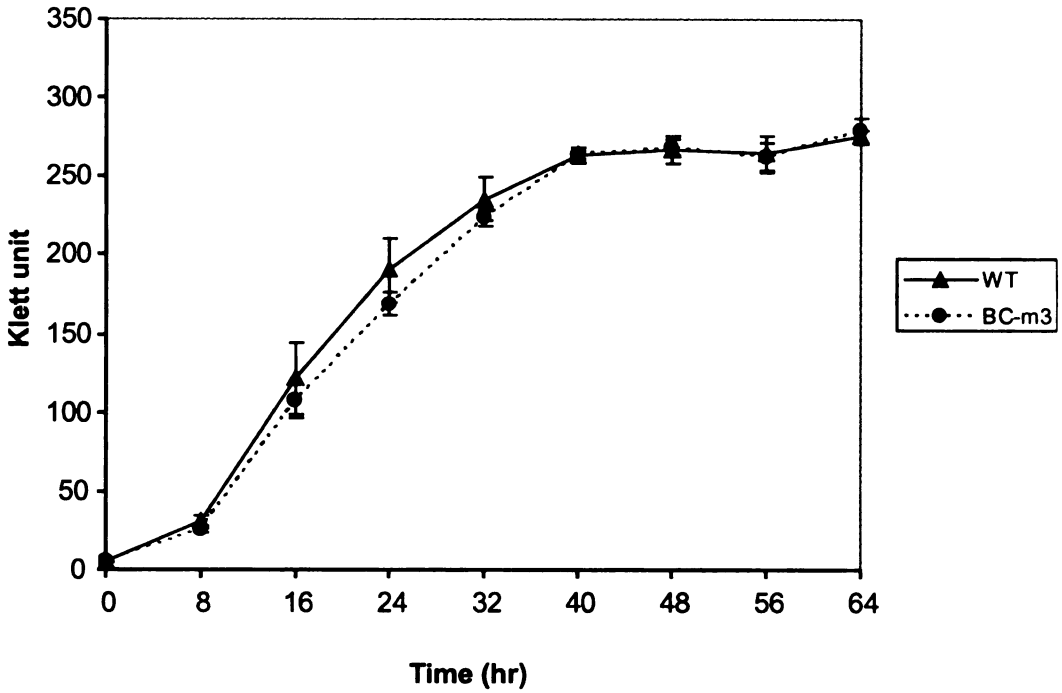


Figure 3.3 Two-dimensional chromatogram of lipids from the wild type and the mutant exposed to temperature stress. Lipids from the (A) the wild type cells and (B) the BC-m3 mutant cells, grown at 37°C. Lipids: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; SL, sulfolipid; OL, ornithine lipid; CL, cardiolipin.

Figure 3.4 Growth of the wild type and the mutant in regular Sistrom's media under different growth conditions. (A) Photosynthetic growth and (B) Respiratory growth. The growth was measured as the increase in the turbidity of the cultures in Klett units. The values given are the average of the three independent measurements. The respiratory growth data was obtained from Dr. Carrie Hiser.

A


350
300
250
200
150
100
50
0
Klett unit
0
8
16
24
32
40
48
56
64
Time (hr)
WT
BC-m3

B

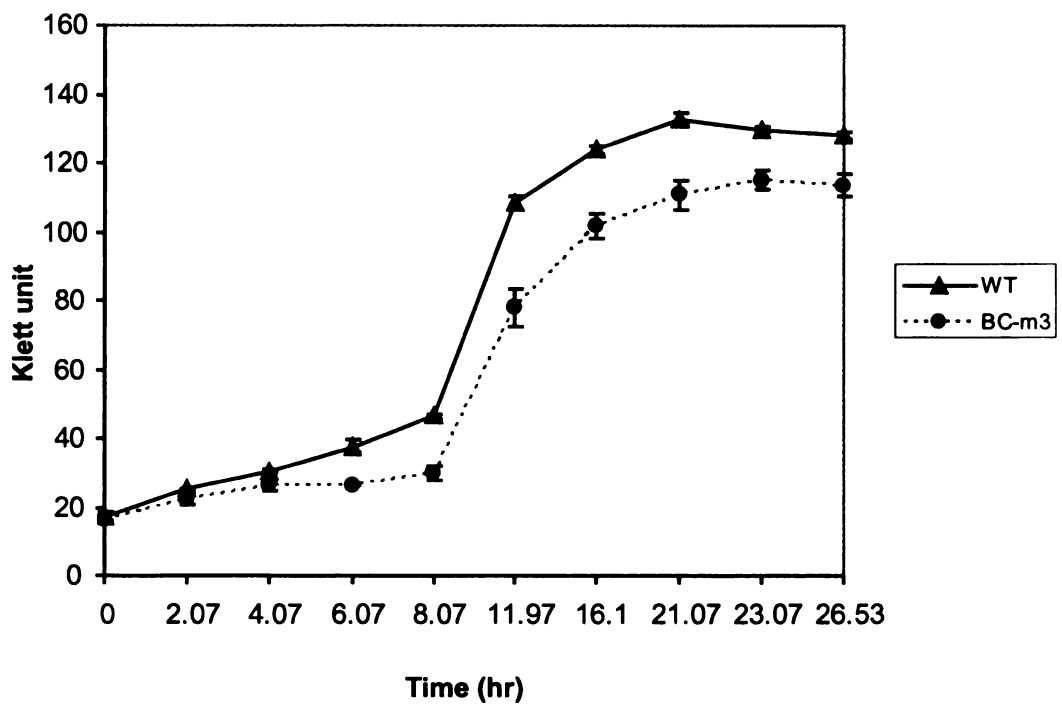
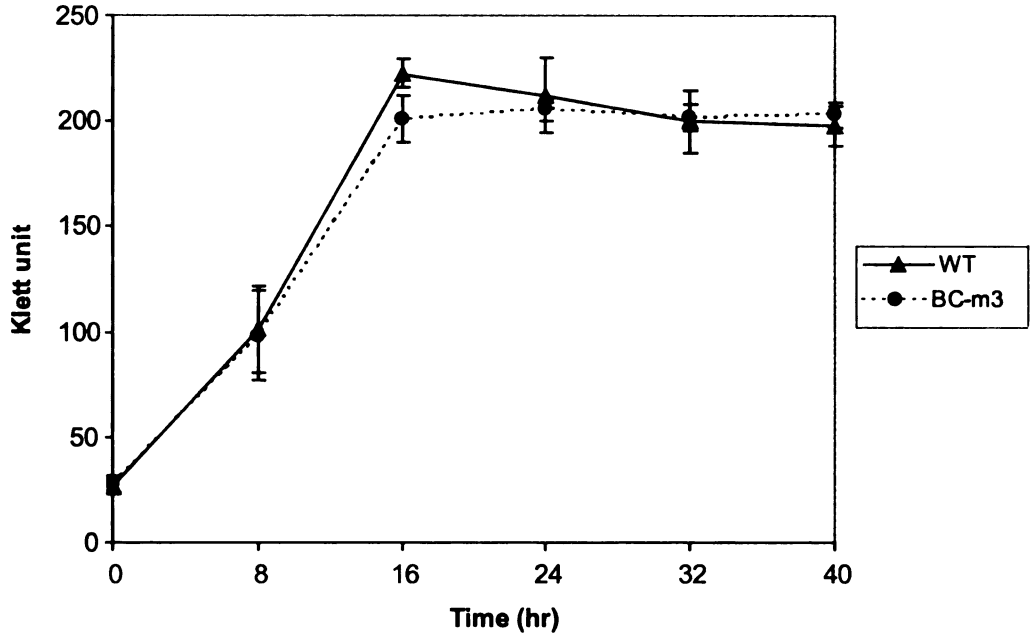

160
140
120
100
80
60
40
20
0
Klett unit
0
2.07
4.07
6.07
8.07
11.97
16.1
21.07
23.07
26.53
Time (hr)
WT
BC-m3

Figure 3.4

Figure 3.5 Growth of the wild type and the mutant under osmotic and heat stress. The wild type and the mutant were grown in (A) regular Sistrom's media at 37°C and (B) modified Sistrom's media containing 0.75 M NaCl. The growth was measured as the increase in the turbidity of the cultures in Klett units. The values given are the average of the three independent measurements.

A


250
200
150
100
50
0
Klett unit
0
8
16
24
32
40
Time (hr)
WT
BC-m3

B

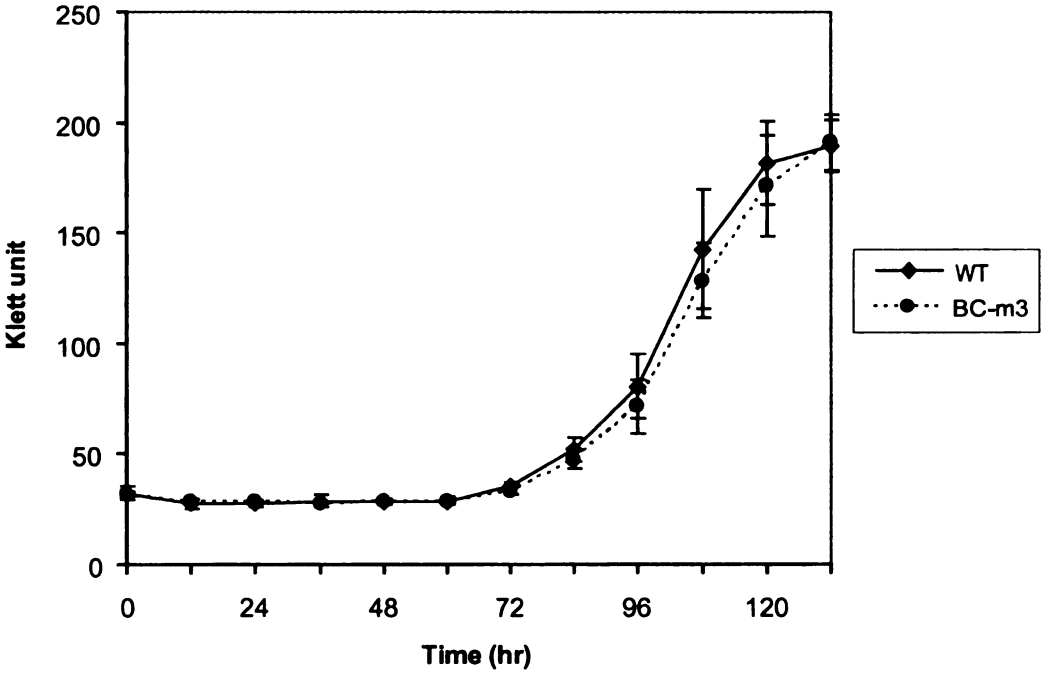

250
200
150
100
50
0
Klett unit
0
24
48
72
96
120
Time (hr)
WT
BC-m3

Figure 3.5

(Fig. 3.5 B). Similarly, no significant difference in the growth of the wild type and the mutant was observed at 37° C (Fig. 3.5 A).

Discussion

Before starting the physiological analysis of the *cls* knock-out mutant BC-m3, the first essential step was to compare the level of CL in the mutant and the wild type. Though the initial lipid analysis by TLC had shown the absence of CL in the mutant, a more quantitative approach was necessary before reaching the conclusion that CL is completely absent in the mutant. Since radioactive labeling of the lipids is a more sensitive technique for detecting the lipids that are present in a small amount, the approach was applied for the quantitative analysis of the lipids. The result showed the presence of traces of CL in the mutant under the growth condition used for the experiment. A similar observation had been made in the E. coli *cls* null mutant (Nashijima *et al.*, 1988). As discussed in the previous chapter, the *E. coli cls* null mutant showed a reduced but significant amount of CL and the level of CL increased at the stationary growth phase (Nashijima *et al.*, 1988). No growth phase specific lipid analysis was carried out in this study, which opens the possibility that the level of the residual CL present in the mutant BC-m3 could be different at different growth phases.

The reduced level of CL in the mutant showed a more pronounced effect on the respiratory growth than the photosynthetic growth. Dependence of respiratory growth on CL level has been already shown in *Saccharomyces cerevisiae* (Zhong *et al.*, 2004). Similar to what was observed in the *Saccharomyces cerevisiae crd1*Δ mutant (Zhang *et al.*, 2002; Pfeiffer *et al.*, 2003) the reduced respiratory growth of the mutant BC-m3 could

be due to the unstable respiratory chain complexes resulting from the reduced level of CL. Such difference in the growth of the wild type and the mutant was not observed during the photosynthetic growth which could be due to the substitution of CL by another anionic lipid, PG. One study on the lipid analysis of the three members of Rhodospirilliaceae grown under different growth conditions had reported that the level of PG increased upon the photosynthetic growth (Russell and Harwood, 1979) which suggested that the bacteria preferentially increase the level of PG for the photosynthetic growth. Another possible reason for the lack of stronger effect on the photosynthetic growth of the mutant BC-m3 could be because the traces of CL could be sufficient for the photosynthetic growth, whereas the CL requirement could be higher for the respiratory growth.

Studies on *Rb. sphaeroides* and *B. subtilis* had shown that bacteria require CL for osmotic adaptation (Catucci *et al.*, 2004; López *et al.*, 2006). Based on those observations, it was postulated that the mutant BC-m3, due to the reduced level of CL, would be unable to grow in the media with high osmolarity. But the growth of the mutant and the wild type in the media containing 0.75 M NaCl did not show a significant difference. Unlike the *Saccharomyces cerevisiae* CL deficient mutant *crd1*Δ, which was unable to grow at temperature of 37° C and above, the mutant BC-m3 showed near wild type growth at 37° C. The ability of the mutant BC-m3 to grow at high osmolarity and high temperature conditions was quite surprising but an explanation for that observation could be derived from the result of the lipid analysis of the cells exposed to the high salt and grown under the high temperature conditions. The lipid analysis of those cells showed a detectable level of CL in the mutant. The result demonstrated that CL is in fact

required for the growth under osmotic and temperature stress and therefore the mutant with defective cardiolipin synthase is probably able to use an alternative mechanism for CL biosynthesis which has not yet been identified.

The presence of traces of CL in the mutant raised the possibility that, as in *E. coli*, an alternative pathway for CL biosynthesis could be present in *Rb. sphaeroides*. Exploring that possibility, it was necessary to confirm that cardiolipin synthase activity was absent in the mutant. The *in vitro* cardiolipin synthase activity assay showed that the mutant was unable to synthesize CL by the condensation of two PG molecules. That showed that cardiolipin synthase activity was absent in the mutant and the residual CL present in the mutant could therefore be coming from a cardiolipin synthase independent pathway. However it needs to be mentioned that the enzyme activity assay was carried out using the established protocol for the *E. coli* enzyme assuming that cardiolipin synthase of *Rb. sphaeroides* also have the same kinetic properties. Therefore, at this point, this assay can be considered only as a plus-minus type of experiment which shows whether or not the mutant protein is able to make CL by the condensation of two PG molecules under the particular assay condition used for this experiment.

In the *cls* disrupted mutant of *E. coli*, several experimental observations support the involvement of phosphatidylserine synthase in catalyzing the synthesis of CL by using the CDP-DG and PG as the substrates (Nishijima *et al.*, 1988; Shibuya *et al.*, 1985). The possibility of the involvement of phosphatidylserine synthase in the synthesis of the residual CL in the *cls* knock-out mutant BC-m3 was checked by *in vitro* synthesis of CL by using the protein extracts from the wild type and the mutant and CDP-DG and ^{14}C-labeled PG as the substrates. Though the result showed some labeled CL was formed by

the mutant protein, no conclusions could be drawn from the results of that experiment due to the high standard error of the results. Thus at this point, more experiments would be required in order to define the source of the residual CL in the mutant.

In summary, the mutant BC-m3 showed a significant reduction in CL levels, which corresponded to the undetectable cardiolipin synthase activity in the mutant. For the traces of CL in the mutant, a cardiolipin synthase independent pathway could be responsible. Interestingly, the alternative pathway for CL biosynthesis appeared to be up-regulated under stress conditions which could be responsible for the accumulation of CL in the wild type and increase in the CL level in the *cls* null mutant. Thus it can be speculated that the accumulation of CL observed in the *Rb. sphaeroides* wild type cells following the exposure to osmotic stress (Cattuci *et al.*, 2004) could be due to the up-regulation of the alternative pathway rather than cardiolipin synthase catalyzed pathway.

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Chapter IV

Summary and conclusions

The anionic phospholipid, CL, has been found to be associated with different membrane proteins, providing structural stability to the protein, as well as influencing the protein function (Fyfe et al., 2004; Robinson, 1982; Gomez and Robinson, 1999). CL is one of the lipids that were found in cytochrome *c* oxidase (C*c*O) of *Rb. sphaeroides* (Hilmi, 2000). Based on the established role of CL in different membrane proteins it can be speculated that CL could be essential for the structure and function of C*c*O of *Rb. sphaeroides*. The best approach to determine the role of CL in C*c*O would be by the analysis of the protein activity and the structure in the absence of CL. The objective of the work was to construct a CL-deficient mutant in *Rb. sphaeroides* and characterize the mutant, which could later be used as a tool for the elucidation of the role of CL in C*c*O.

The gene encoding the enzyme cardiolipin synthase in *Rb. sphaeroides*, which was identified using the bioinformatics and genetic approaches, was targeted for the construction of the CL-deficient mutant, BC-m3. Disruption of the *cls* gene resulted into a significant reduction in the level of CL and the impaired the cardiolipin synthase activity, but traces of CL were still present in the mutant. Interestingly, the *cls* knock-out mutant BC-m3 synthesized a detectable amount of CL during osmotic and heat stress. The retention of the minimal amount of CL by the mutant and the synthesis of CL during stress indicated the possible involvement of an alternative pathway for CL biosynthesis as in *E. coli* (Nishijima *et al.*, 1988). The existence of the alternative pathway for CL

biosynthesis has also been proposed in *B. subtilis* in which the *cls* null mutant still synthesized CL after the initiation of the sporulation phase (Kawai *et al.*, 2004).

The existence of the alternative mechanism for CL biosynthesis could provide selective advantage to the bacteria in order to fulfill the CL requirement during certain physiological conditions like heat and osmotic stress as observed in this study. In support of that hypothesis, a previous study on *Rb. sphaeroides* had shown that the wild type cells 2.4.1 accumulated more CL upon the exposure to osmotic stress (Catucci *et al.*, 2004). There could be other stress conditions which still need to be investigated, that would induce CL synthesis in the mutant.

Based on the result of the analysis of the mutant BC-m3, it can be speculated that if there had been no cardiolipin synthase independent pathway in *Rb. sphaeroides*, then the disruption of the *cls* gene would have resulted in a stronger phenotype under both the normal and stress growth conditions. However, even with the minimal level of CL present in the mutant, a significant effect was seen in the respiratory growth. It would be interesting to find out the specific effect of CL deficiency in respiratory growth. The analysis of the effect of reduced level of CL in the activity of C*c*O and other respiratory pathway enzymes could provide some clues for defining the more specific roles of CL.

This work has generated the first CL deficient mutant in Gram-negative bacteria other than *E. coli* and the first in a photosynthetic organism, thus it serves as an alternative system for carrying out CL related studies. Since *Rb. sphaeroides* shows both photosynthetic and respiratory growth, unlike the *E. coli*, the mutant would be useful for determining the role of CL in different enzymes involved in photosynthesis and respiration.

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