ANALYSIS OF THE ROLE OF PrrA, PpsR and FnrL IN INTRACYTOPLASMIC MEMBRANE DIFFERENTIATION OF Rhodobacter sphaeroides 2.4.1 USING TRANSMISSION ELECTRON MICROSCOPY

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Oxygen dictates the catabolic "lifestyle" of *Rhodobacter sphaeroides*. When it is present, the bacteria are fully equipped for aerobic respiration. When it is absent, the cells outfit themselves to make use of energy-gathering options that do not require oxygen. This means that, even in the dark, the cells are fully enabled for phototrophy, so that if and when light is available they can take full advantage of its presence to obtain energy. Three global regulatory proteins have been identified that mediate oxygen control of gene expression in this organism, PrrA, PpsR, and FnrL. For each of these, regulon members include a subset of a cluster of genes known as the photosynthesis (PS) genes, which encode the structural proteins and the enzymes catalyzing biosynthesis of the pigments of the light-harvesting and reaction center complexes. The complexes are housed in a specialized structure called the intracytoplasmic membrane (ICM). Although details are emerging as to the differentiation process leading to fully formed ICM, other than transcription of the PS genes, the regulatory events are not known. As oxygen-responsive regulators of PS genes, PrrA, PpsR, and FnrL were evaluated with respect to their potential role in the formation of ICM using transmission electron microscopy. The major findings were as follows: (1) The absence of either PrrA or FnrL negatively affects ICM formation, (2) the lack of ICM in the absence of PrrA is partially, but not fully reversed by loss of PpsR function, (3) the FnrL requirement for ICM formation appears to be indirect, by directly activating transcription of a second regulatory protein that is necessary for normal ICM development.
DEDICATION

I dedicate this work to Anya and Oleh Fedotov. I am truly grateful to have you in my life.

Thank you for your unconditional love and support.
I would like to extend my deep appreciation to my advisor and mentor, Dr. Jill Zeilstra-Ryalls. During the last two years you have been an invaluable resource and guide in my research endeavors, always keeping me motivated with high expectations and quality work.
# TABLE OF CONTENTS

| Section                                                                 | Page |
|------------------------------------------------------------------------|------|
| ABSTRACT                                                               | i    |
| DEDICATION                                                             | ii   |
| ACKNOWLEDGEMENTS                                                       | iii  |
| LIST OF FIGURES/TABLES                                                 | vi   |
| LIST OF ABBREVIATIONS                                                 | viii |
| CHAPTER I: BACKGROUND AND SPECIFIC AIMS                                | 1    |
| Introduction                                                           | 1    |
| A. The cytoplasmic membrane and restructuring events                   | 1    |
| B. *Rhodobacter sphaeroides* membrane restructuring                     | 3    |
| C. Transcriptional regulators mediating oxygen control AIMS            | 5    |
| Specific Aims                                                          | 8    |
| CHAPTER II: ANALYSIS OF THE ROLE OF THE REDOX-RESPONSIVE REGULATORY PROTEIN PrrA IN ICM FORMATION | 9    |
| Introduction                                                           | 9    |
| Materials and Methods                                                  | 13   |
| Bacterial strains and growth conditions                                | 13   |
| Transmission Electron Microscopy specimen preparation                  | 14   |
| Absorption spectroscopy of membrane fractions and quantitation of pigments | 16   |
| Protein concentrating determinations                                  | 17   |
| Results                                                                | 17   |
| Ultrastructure of *R. sphaeroides* wild type 2.4.1 and Prr mutant bacteria | 17   |
| Absorption spectroscopy of *R. sphaeroides* wild type 2.4.1 and Prr mutant bacteria | 21   |
CHAPTER III: ANALYSIS OF THE ROLE OF THE OXYGEN-RESPONSIVE REPRESSOR PROTEIN PpsR IN ICM FORMATION

Introduction.................................................................................................................. 24
Materials and Methods................................................................................................ 25
  Bacterial strains and growth conditions................................................................. 25
  TEM Specimen Preparation.................................................................................... 26
Results....................................................................................................................... 26
Discussion.................................................................................................................. 29

CHAPTER IV: ANALYSIS OF THE ROLE OF THE ANAEROBIC REGULATORY PROTEIN FnrL IN ICM FORMATION

Introduction.................................................................................................................. 31
Materials and Methods................................................................................................ 33
  Bacterial strains and growth conditions................................................................. 33
  TEM Specimen Preparation.................................................................................... 34
  Absorption spectroscopy of membrane fractions and quantitation of pigments.... 34
  Protein Assays........................................................................................................ 36
Results....................................................................................................................... 36
  Ultrastructure of \textit{R. sphaeroides} wild type 2.4.1 and FnrL\textsuperscript{−} mutant bacteria...... 36
  Ultrastructure of \textit{R. sphaeroides} wild type 2.4.1 and Rsp2573\textsuperscript{−} mutant bacteria.... 37
  Absorption spectroscopy of \textit{R. sphaeroides} wild type strain 2.4.1, FnrL\textsuperscript{−} mutant strain JZ1678 and Rsp2573\textsuperscript{−} mutant strain JZ5210......................................................... 38
  Ultrastructure of \textit{Rhodobacter capsulatus} FnrL\textsuperscript{+} and FnrL\textsuperscript{−} mutant bacteria...... 38
Discussion.................................................................................................................. 39

CHAPTER V: SUMMARY AND FUTURE DIRECTIONS.................................................. 41
REFERENCES.......................................................................................................... 43
## LIST OF FIGURES AND TABLES

| Figure/Table | Description                                                                 | Page |
|--------------|------------------------------------------------------------------------------|------|
| Figure 1     | A schematic diagram of the Intracytoplasmic Membrane                        | 4    |
| Figure 2     | Photosynthesis gene cluster and transcriptional thermal diagram of PS genes  | 6    |
| Figure 3     | Differential transcription of PS genes of wild type versus mutant strains    | 7    |
| Figure 4     | Proposed model of gene regulation mediated by the PrrBA two-component system | 10   |
| Figure 5     | Arrangement of the prr gene cluster, and the transcripts detected from the   | 11   |
| Figure 6     | TEM micrographs of *Rhodobacter sphaeroides* bacteria that had been          | 19   |
| Figure 7     | TEM micrographs of *Rhodobacter sphaeroides* bacteria that had been          | 20   |
| Figure 8     | Absorption spectroscopy of membrane extracts from *Rhodobacter sphaeroides* | 21   |
| Figure 9     | Hypothetical model of PrrB cross-talk or branched regulation with a non-     | 23   |
| Figure 10    | Model of transcription regulation by the AppA-PpsR repressor-antirepressor   | 25   |
| Figure 11    | TEM micrographs of *Rhodobacter sphaeroides* bacteria that had been          | 28   |
| Figure 12    | Model of oxygen-responsive regulation of genes by FnrL during anaerobic      | 33   |
| Figure 13    | TEM micrographs of *Rhodobacter sphaeroides* bacteria that had been          | 36   |
| Figure 14    | TEM micrographs of *Rhodobacter sphaeroides* bacteria that had been          | 37   |
Figure 15. Absorption spectroscopy and bacteriochlorophyll concentrations of membrane extracts of *Rhodobacter sphaeroides* wild type 2.4.1 and mutant strains JZ1678 and JZ5210 grown under low-oxygen conditions........................................ 38

Figure 16. TEM micrographs of *Rhodobacter capsulatus* bacteria that had been cultured under low-oxygen conditions........................................................................ 39

Table 1. *Rhodobacter sphaeroides* strains used in this study........................................ 13

Table 2. Quantitative analysis of micrograph fieldview of cells containing ICM and their distribution in *Rhodobacter sphaeroides prrA* and *prrBCA* strains........ 18

Table 3. *Rhodobacter sphaeroides* strains used in this study........................................ 26

Table 4. Strains and plasmids used in this study.......................................................... 35
| Abbreviation | Description |
|--------------|-------------|
| AppaA        | activation of photo-pigment and *puc* expression protein A |
| ATP          | adenosine triphosphate |
| Bchl         | bacteriochlorophyll |
| bp           | base pair |
| CrpK         | cAMP receptor-type protein K |
| CM           | cytoplasmic membrane |
| DMSO         | dimethyl sulfoxide |
| FnrL         | fumarate-nitrate reductase regulator-type protein L |
| g            | gravitational force |
| ICM          | intracytoplasmic membrane |
| Kn           | kanamycin |
| kV           | kilovolt |
| LHI          | light harvesting antenna complex I |
| LHII         | light harvesting antenna complex II |
| µl           | microliter |
| mΩ           | milli-Ohm |
| PpsR         | photo-pigment suppressor protein R |
| PS           | photosynthesis |
| PrrA         | photosynthesis response regulatory protein A |
| PrrB         | photosynthesis response regulatory protein B |
| PrrC         | photosynthesis response regulatory protein C |
| psi          | pound per square inch |
| Abbreviation | Description |
|--------------|-------------|
| RC           | reaction center |
| Rif          | rifampicin |
| SCHIC        | sensor containing heme instead of cobalamin |
| BLUF         | sensor of blue light using flavin adenine dinucleotide |
| Sp           | spectinomycin |
| St           | streptomycin |
| Tp           | trimethoprim |
CHAPTER 1 BACKGROUND AND SPECIFIC AIMS

Introduction

A. The cytoplasmic membrane and restructuring events.

The cytoplasmic membrane (CM) plays a universal role in cells of all three domains of life. This semipermeable barrier isolates the cytoplasm from the external environment. The outer and inner leaflets of the phospholipid bilayer component of the CM are held together by the intermolecular interactions of hydrophilic head groups and hydrophobic fatty acid chains. The integrity of the CM is strengthened by a cytoskeleton in both eukaryotes and in bacteria (reviewed in Shih and Rothfield 2006), as well as by peripheral and integral proteins. The latter not only add strength to the phospholipid bilayer but they provide the cell with the means to import and export molecules. Further, while changes in environmental factors such as pH, temperature, radiation, and so on can directly affect the biomolecules of the cell, changes in these and other external factors can also be communicated from the outside to the inside of the cell by the membrane-localized proteins. The typical outcome of this communication is alterations in gene expression, which encompass changes at the transcription and post-transcription levels.

With respect to the CM, environmental changes result in changes in gene expression that lead to alterations in composition and concentration of both the lipids and the proteins. For example, in response to changes in temperature, the kinds of fatty acids present change so that proper membrane fluidity is maintained. The membrane can also undergo regulated restructurings that are critical to cell function. In eukaryotic cells, these events, such as are required for phagocytosis and cell motility, are commonplace (Lippencott and Li 2000). However, among bacteria, only a few such restructurings have been described, and are thus far
limited to the α-proteobacteria. As these bacteria are the closest living relatives of the endosymbiotic progenitor of the mitochondrion, it is intriguing to consider the possibility that the restructurings in the bacteria and the morphogenesis of cristae in mitochondria constitute phenologs, a concept recently described by McGary et al. 2010). Details of the two known bacterial membrane restructurings follow.

Species of *Magnetospirillum*, including *M. magneticum* AMB-1 and *M. gryphiswaldense* MSR-1, which are members of the Rhodospirillaceae family of α-proteobacteria, develop invaginations of the cytoplasmic membrane that enclose magnetite crystal particles (Taoka et al. 2006). These structures are called magnetosomes and are needed for magnetotaxis and aerotaxis (Smith et al. 2006). The *mam* and *mms* gene clusters that are important for magnetosome formation (Ullrich et al. 2005) are down-regulated under aerobic growth conditions in *M. gryphiswaldense* (Schübbe et al. 2006, Fukuda et al. 2006, Richter et al. 2007), and so magnetosome formation is thought to be oxygen-dependent. However, the regulatory proteins involved in altering transcription of the genes involved in magnetosome formation have not yet been elucidated.

The Rhodobacteraceae family of α-proteobacteria includes *Rhodobacter sphaeroides* whose CM undergoes a differentiation event leading to the formation of the intracytoplasmic membrane (ICM) which houses the photosynthetic apparatus (Chory et al. 1984). As is appropriate for anoxygenic photosynthesis, ICM formation is induced by lowering oxygen tensions. DNA binding proteins that mediate oxygen control of photosynthesis or PS genes (genes that code for the structural proteins and enzymes that synthesize the photopigments of the photosynthetic apparatus) are known. This makes it possible to consider whether or not they regulate other aspects of ICM formation.
B. *Rhodobacter sphaeroides* membrane restructuring.

*Rhodobacter sphaeroides* is an excellent model for examining how cells reconfigure their molecular composition to adapt to changing conditions. A dramatic example of these adaptations is the switch between aerobic respiration and anoxygenic phototrophy, precipitated by a change in oxygen availability. These metabolisms are mutually exclusive because genes encoding products that support these processes are under oxygen control, but in opposing fashion. Accompanying the change in the membrane-embedded molecular species from the electron transport chain to the photosynthetic apparatus is a complete morphological change of the membrane itself, to form the specialized photosynthetic ICM.

The precise sequence of events during ICM biogenesis is not yet known. However, past and recent sophisticated microscopic studies, coupled with biochemical investigations, have provided information regarding certain features of the process. (i) Immature ICM resembles disk-like vesicles with inner diameters of 70 nm that within 15 hours form into 50-70 nm sphere-like vesicles (Chory *et al.* 1984, Tucker *et al.* 2010). (ii) Towards forming fully developed ICM, the dimeric complexes of light-harvesting antenna (LHI) and reaction center (RC) line up in longitudinal rows and deform the surrounding lipids (Scheuring *et al.* 2004, Qian *et al.* 2005, Frese *et al.* 2004, Hsin *et al.* 2009). This introduces a $146^\circ$ curvature in the membrane (Qian *et al.* 2008). (iii) The peripheral light harvesting antenna complexes (LHII) are inserted next, and fill the membrane space between the dimeric complex chain rows (Bahatyrova *et al.* 2004a). (iv) The final proper positioning of the LHI-RC and LHII complexes is achieved once non-photosynthetic proteins dissociate from the membrane, allowing bending and further tight packing of the pigment-protein complexes (Chandler *et al.* 2008). A schematic diagram
depicting the fully formed ICM with the embedded photosynthetic pigment-protein complexes is shown in Figure 1.

Figure 1. A schematic diagram of the ICM based on work by Sener et al. (2007), Bahatyrova et al. (2004b), and Frese et al. (2004). The CM forms invaginations that house the photosynthetic apparatus composed of the proteins and pigments of the RC, LHI, and LHII complexes. RC form dimeric complexes with LHI in the presence of PufX protein, which together are assembled into rows. The space between these rows is filled with LHII complexes.

In addition to the photo-pigments and proteins comprising the LH and RC complexes, assembly factors are required for ICM formation (Chen et al. 2002, Tucker et al. 2010). Furthermore, several studies indicate that the kinds and amounts of phospholipids present in ICM versus CM are different (Lueking et al. 1978, Russell and Harwood 1979), and that they are important for stability and function of membrane-localized energy producing complexes. The studies suggest that the phospholipids in ICM stabilize photosystem complexes and are important for optimal light reaction chemistry and ATP production (Lee 2003), while the phospholipids in
CM stabilize electron transport complexes and are important for optimum electron flow, proton pumping, and ATP production (Giustini et al. 2005, Hunte 2005).

Collectively, this information tells us that membrane differentiation and ICM formation is a highly complex process that requires coordinated expression of many genes. Since a reduction in oxygen tensions is necessary and sufficient to initiate ICM formation, obvious candidates for transcription factors that could regulate this event are those that are known to mediate oxygen control of PS genes.

C. Transcriptional regulators mediating oxygen control.

The PS genes of *R. sphaeroides* are clustered within a 138,748 bp region of chromosome I. Figure 2 is a transcriptional diagram of the genes, together with a "thermal diagram" indicating the differences in transcription of these genes in cells grown under aerobic *versus* phototrophic conditions, based on microarray data (Pappas et al. 2004). At least three DNA binding proteins mediate oxygen control of these genes, PrrA, PpsR, and FnrL (reviewed in Zeilstra-Ryalls and Kaplan 2004). The microarray data available for each of these regulators (Eraso et al. 2008, Moskvin, Zeilstra-Ryalls and Gomelsky unpublished; Moskvin et al. 2005) was used to generate Figure 3, which shows the fold-difference in transcription of each gene in wild type *versus* a corresponding mutant strain under conditions that are permissive for growth and indicative of their roles in PS gene expression.

PrrA (Photosynthesis response regulatory protein A) is the DNA binding regulatory protein of a redox-responsive two-component regulatory system (Eraso and Kaplan 1995). PrrA- mutants cannot grow photosynthetically, but the mutant bacteria are able to grow anaerobically in the dark using alternative electron acceptors such as dimethyl sulfoxide (DMSO) (Eraso and Kaplan 1994). The need for PrrA for phototrophic growth can already be explained by virtue of
the fact that it activates transcription of many PS genes in response to lowering oxygen tensions (Figure 3 and Eraso et al. 2008). PpsR (photopigment suppressor protein R) is a transcription repressor of certain PS genes under aerobic conditions (Figure 3 and Gomelsky et al. 2003), and so its most important role is thought to be preventing the coincidence of Bchl a in the presence of oxygen and light, which can create a lethal situation through the production of reactive oxygen species. FnrL (fumarate-nitrate reductase regulator-type protein L) is the R. sphaeroides homologue of the global anaerobic regulatory Fnr protein of E. coli. R. sphaeroides FnrL is essential for all anaerobic growth, which includes anaerobic growth in the dark with the alternate electron acceptor dimethyl sulfoxide (DMSO) and anaerobic growth in the light (Zeilstra-Ryalls and Kaplan 1995). However, the inability of FnrL− mutant bacteria to grow phototrophically is not yet understood, since the available evidence indicates that transcription of PS genes are not critically affected by the presence or absence of FnrL in the cell (Figure 3 and Ouchane et al. 2007). At present, there is no information available as to whether or not any of these regulatory proteins have a role beyond the regulation of PS genes in membrane restructuring. The goal of this study is to investigate each of these transcription factors with respect to oxygen control of ICM formation.

Figure 2. Photosynthesis gene cluster and transcriptional thermal diagram of PS genes based on the transcriptome data of Pappas et al. (2004). The color indicates fold-differences of PS gene transcripts in cells grown under high versus low-oxygen tensions.
Figure 3. Differential transcription of PS genes of wild type versus mutant strains under respective growth conditions based on microarray data (Eraso et al. 2008, Moskvin, Zeilstra-Ryalls and Gomelsky unpublished; Moskvin et al. 2005). Each bar represents the fold difference in the presence versus the absence of the transcription factor indicated for one PS gene. The genes are in reverse order from Figure 2.
Specific Aims

Fnrl, PrrA, and PpsR are three DNA binding proteins that mediate oxygen control of gene expression in *R. sphaeroides*. Each of these global regulators will be evaluated with respect to their role in ICM development. The specific aims of this study are as follows:

1. Use transmission electron microscopy (TEM) to determine the membrane morphology of the mutant strains that lack one or the other of the three known regulators of photosynthesis genes, Fnrl, PrrA and PpsR.

2. Consider the findings from Specific Aim 1 within the context of the current literature and databases.

3. Design and execute additional studies to address questions emanating from Specific Aim 2.

Chapters II-IV present the investigations and their outcomes directed towards each of the three regulatory proteins. Chapter V summarizes the findings from these studies and describes what future directions stem from them.
CHAPTER II: ANALYSIS OF THE ROLE OF THE REDOX-RESPONSIVE REGULATORY PROTEIN PrrA IN ICM FORMATION

Introduction

Communication between the external environment and the cytoplasm of the bacterial cell, or signal transduction, is often mediated by two-component regulatory systems. The prototypical two-component system consists of a sensor and response regulator. The sensor is an integral membrane protein that possesses both phosphatase and kinase activity towards itself and towards the response regulator (Eraso and Kaplan 1995). An activating signal external to the cytoplasm stimulates autophosphorylation and, in turn, the transfer of the phosphate to the response regulator. The phosphorylated cytoplasmic regulatory protein binds to DNA, thereby altering the transcription of target genes. In the absence of the signal the dephosphorylation activity of the sensor predominates.

The Prr (photosynthesis response regulator) redox-responsive two-component system is composed of the PrrB and A proteins. PrrB is the membrane-localized sensor protein and PrrA is the cytoplasmic DNA-binding regulatory protein. The current model of action of the Prr system has evolved from a number of studies, including an investigation of the kinase and phosphatase activities of PrrB (Eraso and Kaplan 1995). The signal sensed by this regulatory system is not defined at the molecular level. However, it is clear (i) that Prr responds to changes in cellular redox status, such as occurs when oxygen tensions are lowered, and (ii) the rate of electron flow through the high oxygen affinity \( cbb_3 \)-type cytochrome \( c \) oxidase. Thus, under aerobic conditions, the level of electron flow through the oxidase is high, yielding a signal that is inhibitory for the kinase activity of PrrB (O’Gara et al. 1998, Oh and Kaplan 1999). The signal
is thought to be communicated to PrrB via a third protein, PrrC (Eraso and Kaplan 2000) that is also membrane-localized. The outcome is that phosphatase activity of PrrB towards itself and PrrA predominates in the presence of oxygen. When oxygen is limiting, electron flow through the oxidase is low. Under those conditions, there is no inhibitory signal, and PrrB kinase activity predominates, so both PrrB and PrrA are phosphorylated (O’Gara et al. 1998, Oh and Kaplan 1999).

Figure 4. Proposed model of gene regulation mediated by the PrrBA two-component regulatory system. A change in cellular redox status generates a signal to which PrrB responds by autophosphorylation on a histidine residue. The phosphate group is then transferred from the histidine of PrrB to an aspartate residue of PrrA. Phosphorylation of PrrA alters its affinity for DNA target sequences.

It is known that, for certain target genes, transcription activation involves the phosphorylated form of PrrA. These include several PS genes (Eraso and Kaplan 1994, Eraso et al. 2008). However, several studies have shown that unphosphorylated PrrA can also bind to
DNA (Ranson-Olson and Zeilstra-Ryalls 2008, Gomelsky et al. 2008), and for some sequences unphosphorylated PrrA has higher affinity than the phosphorylated protein (Ranson-Olson and Zeilstra-Ryalls 2008). This makes the regulatory outcome for PrrA phosphorylation highly complex, and while there are data available from transcriptome profiling (Eraso et al. 2008) of wild type versus PrrA− cells, it is unlikely that those data describe the entire scope of regulatory events mediated by PrrA. Further, as is true of any such microarray analyses, they do not tell us whether the transcriptional differences are due to direct or indirect PrrA activity. Be that as it may, it is well-established that a functional prrA gene is required for phototrophic growth of *R. sphaeroides* (Eraso and Kaplan 1994), and it was this PS− phenotype that led to the identification of the prr genes (Eraso and Kaplan 1994). The arrangement of the prr genes is shown in Figure 5.

![Image](image.png)

**Figure 5.** Arrangement of the prr gene cluster, and the transcripts detected from the cluster, based on results reported by Eraso and Kaplan (Eraso and Kaplan 2000).

In broad terms, the role of PrrB in photosynthesis gene expression is described by the phenotypes of two PrrB mutants. If the membrane-spanning sensor domain is absent, the regulation mediated by oxygen flow through the *cbb*$_3$-type cytochrome *c* oxidase is absent, and so the protein remains in the kinase-active state regardless of the presence or absence of oxygen (Oh et al. 2001). Thus, such mutants have photosynthetic pigment-protein complexes even under aerobic conditions (Oh et al. 2001). Deletion of the entire PrrB gene results in a loss of phototrophic growth under low to medium light intensities (Gomelsky and Kaplan 1995b), and in
anaerobic dark (with DMSO) cells the levels of pigment-protein complexes are reduced relative to wild type (O’Gara et al. 1998); i.e., the phosphorelay system by which the low-oxygen signal is transduced to PrrA is absent. However, it is noteworthy that, unlike PrrA\(^{-}\) mutants, the PrrB\(^{-}\) mutant bacteria are capable of growth under high light (Gomelsky and Kaplan 1995b). This suggests there may be other ways in which phosphorylation/dephosphorylation of PrrA can be achieved. Such a possibility was indicated in a study by Gomelsky and Kaplan, who reported that multiple copies of \(hupT\), encoding a histidine kinase for hydrogen uptake, restored phototrophic growth to a PrrB\(^{-}\) mutant of \(R.\) sphaeroides (Gomelsky and Kaplan 1995a). Since the \(prrB\(^{-}\)\) mutation in that study creates a frame-shift after amino acid residue 103, both the sensing and the catalytic domains of the protein are absent. PrrA was presumably promiscuously phosphorylated by HupT. However, it is unclear whether there is any physiologically significant phosphorylation of PrrA by other two-component sensor proteins.

In contrast to PrrB\(^{-}\) and PrrA\(^{-}\) mutants, PrrC\(^{-}\) mutants have no phototrophic defect. Rather, consistent with its proposed role in communicating the inhibitory signal generated by oxygen via the \(cbb_3\)-type cytochrome \(c\) oxidase to PrrB, PrrC\(^{-}\) mutants have elevated levels of pigment-protein complexes under aerobic conditions (Eraso and Kaplan 2000). This is the same phenotype as is observed for the PrrB\(^{-}\) mutants lacking the sensor domain, for which the kinase activity is constitutive.

Although PrrA\(^{-}\) mutants cannot grow photosynthetically, their respiratory capacity is apparently unaffected, and they can grow in the dark both aerobically and anaerobically using DMSO as alternate electron acceptor (Moskvin et al. 2005, Eraso et al. 2008). Since the absence of oxygen is necessary and sufficient to induce transcription of photosynthesis genes, it was possible to establish that cells lacking \(prrA\) have no detectable photosynthesis pigment-protein
complexes by growing mutant bacteria anaerobically in the dark with DMSO. Whether or not the process of ICM formation is also compromised has not previously been determined. The goal of this investigation is to examine the role of the prr genes in membrane differentiation.

Materials and Methods

**Bacterial strains and growth conditions.** *Rhodobacter sphaeroides* strains used in this study are listed in Table 1, together with their relevant characteristics and source. In all cases, Sistrom’s succinate minimal medium A (Sistrom 1960) was used for growth of the bacteria. Low-oxygen growth was conducted by inoculation of *R. sphaeroides* into 100 ml of medium in 250 ml Erlenmeyer flasks that were incubated at 30°C in a New Brunswick gyratory shaking water bath (model G76) at a speed setting of 2.3. Anaerobic growth was performed by inoculation of screw-capped tubes completely filled with medium that was supplemented with 0.1% yeast extract and 60 mM DMSO (Fales *et al.* 2001).

| Strain   | Relevant characteristics                  | Reference or source               |
|----------|-------------------------------------------|-----------------------------------|
| 2.4.1    | Wild type                                 | W. Sistrom                        |
| BR107    | ΔprrA::loxP                               | Ranson-Olson and Zeilstra-Ryalls 2008 |
| PRRBCA2  | $\Delta$(BspEII-Tth111I)prrBAC::Tp$^R$    | Oh *et al.* 2000                  |
| PRRA1    | prrA(PstI)::ΩSp$^R$/St$^R$               | Eraso and Kaplan 1995             |
| PRRA2    | $\Delta$(BstBI-PstI)prrA::ΩSp$^R$/St$^R$ | Eraso and Kaplan 1995             |
**TEM specimen preparation.** Cells from 40 ml of mid- to late-log phase liquid cultures were collected in 50 ml conical tubes by centrifugation at 2,688 x g for 20 minutes at room temperature. The cells were resuspended in 2 ml Karnovsky’s fixative solution (Karnovsky 1965), composed of 2% paraformaldehyde (aqueous 12% paraformaldehyde stock solution), 2.5% glutaraldehyde, 10% w/v sucrose with additives 0.05% (w/v) tannic acid and 10% (w/v) sucrose in 0.1 M cacodylate buffer with final pH of 7. Glutaraldehyde forms cross-linking covalent adducts with secondary amines in proteins, thereby initiating cell preservation (Sabatini et al. 1963). The solution was transferred to 2 ml microfuge tubes which were then incubated 2 hours at room temperature while mixing on a rotator. The fixed cells were collected by centrifugation at 15,306 x g for 1 minute at room temperature, resuspended in an equal volume of 10% sucrose (w/v) in 0.1 M cacodylate buffer, pH 7, incubated 15 minutes at room temperature while mixing on a rotator, and collected by centrifugation at 15,306 x g for 1 minute. This washing step was repeated three times, then the washed cells were collected in a pellet and resuspended in 1 ml of a 2% (stock solution) aqueous solution of osmium tetroxide (OsO$_4$) (Electron Microscopy Sciences, Inc., Hatfield, PA) and incubated for 1 hour at room temperature. OsO$_4$ acts on lipids in the membrane by cross-linking and preserving the membrane further. The cells are washed three times as described previously, using 1 ml of 18 mΩ water.

The treated and washed cells were partially dehydrated by resuspending them first in 75% and then in 95% ethanol for 15 minutes each, and finally in 100% ethanol for 30 minutes, pelleting after each step by centrifugation at 15,306 x g for 1 minute. The cells were then further dehydrated by resuspending them in propylene oxide (CH$_3$CHCH$_2$O, a transition dehydrant) for 15 minutes at room temperature all while mixing on a rotator; after collecting cells by
centrifugation at 15,306 x g for 1 minute at room temperature, the pellet was resuspended for a second time in the same solution.

The dehydrated cell pellets were infiltrated with 1 ml 50/50 mixture of Spurr’s low viscosity embedding medium (Spurr 1969) purchased from Electron Microscopy Sciences, Inc., and propylene oxide solution overnight on the rotator. The cells were collected by centrifugation at 15,306 x g for 1 minute and infiltration continued by resuspending them in 1 ml of fresh 100% Spurr’s embedding medium. The resuspended cells were then incubated overnight while mixing on rotator. The infiltrated cells were collected the next day at 15,306 x g for 20 minutes at room temperature and resuspended in 0.5 ml of fresh 100% Spurr’s embedding medium. The solution was transferred to bottlenecked polymerization tubes, which were centrifuged for 20 minutes at 15,306 x g to pellet the cells. The Spurr’s embedding medium was then polymerized overnight at 70°C in a dry oven.

The polymerization tubes were broken, releasing the polymerized blocks containing the cells. These blocks were trimmed with a razor blade and then sectioned with a Sorvall Porter-Blum MT-2 Ultra Microtome using a diamond knife (Delaware Diamond Knives, Inc., Wilmington, DE) to sections with thicknesses of approximately 70 nm, as determined by their silver interference. These thin sections were collected with a loop and transferred onto copper-coated 300 mesh square carbon grids and allowed to dry. The grids were stained by a sinking method (with the section side up) in alcoholic solution of 2% (w/v) uranyl acetate for 10 minutes. Then, holding the grids with forceps, they were rinsed with 50% ethanol (v/v) followed by a brief rinse with distilled water. The grids were stained with Reynolds lead citrate stain (Pb+2(C6H5O7)−3) for 5 minutes. This stain was prepared by dissolving 2.7% (w/v) lead nitrate and 3.5% (w/v) hydrated sodium citrate in distilled water (Reynolds 1963). The residual stain
was removed by rinsing the grids with 0.01 M NaOH and then distilled water. The grids were placed (section side up) on filter paper and air-dried.

The thinly sectioned cells were visualized using a Zeiss EM-10 transmission electron microscope at 60 kV accelerating potential. Images were captured onto Kodak 4489 film (Eastman Kodak Co., Rochester, NY) using 2.5 second exposures. The exposed negatives were developed for 8 minutes in Kodak D-19 developer, followed by a 30 second rinse with running water, and then fixed with Kodac Ektaflow fixer solution for 8 minutes. Excess fixer solution was removed by a 30-minute rinse in running water. The negatives were then air-dried in a drying fan chamber. These micrographs were scanned with an Epson 750 PRO scanner (Epson America Inc., Long Beach, California). For size reference, a micron bar was added to the digitized images using the Quartz PCI software (Quartz Imaging Co., Vancouver, BC, Canada).

Absorption spectroscopy of membrane fractions and quantitation of pigments. Protein synthesis was halted by the addition of chloramphenicol solution (20 mg/ml in 95% ethanol) to a final concentration of 1.5% (v/v) to the cultures, which were then chilled on ice. The cells were pelleted at 2,688 x g for 10 minutes at 4˚C. The cell pellet was resuspended in 5 ml of 0.1 M sodium phosphate buffer, pH 7.7. Just before cell lysis, protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO) was added (100 µl/50 ml of culture). The cells were lysed by passaging them through a French pressure cell at 700 psi. Insoluble debris was pelleted by centrifugation for 20 minutes at 21,952 x g at 4˚C. Spectra were recorded between wavelengths of 950-350 nm using a Hitachi U-2010 spectrophotometer (Hitachi America Ltd., Chula Vista, CA). The Bchl a levels in the photosynthetic pigment-protein complexes were calculated from the spectral data using the method of Meinhardt (Meinhardt et al. 1985).
Protein concentration determinations. Protein concentrations were determined using the bicinchoninic acid protein assay reagent (Pierce Biotechnology, Inc., Rockford, IL). Bovine serum albumin was used as a standard.

Results

Ultrastructure of R. sphaeroides wild type and Prr mutant bacteria. Thin sections of cells cultured under both low-oxygen and anaerobic-dark with DMSO conditions were examined using TEM. For those mutants in which only the prrA gene is defective, strains PRRA1, PRRA2 and BR107 (Table 1), a low number, on average 5-10, of polarized invaginations of the membrane were present (Figure 6, Table 2) in the thin sections of cells cultured under low-oxygen (30-40% of cells seen in a micrograph fieldview). However, no such invaginations were observed in sections of PRRBCA2 (Table 1) cells, in which all three prr genes have been deleted. Likewise, no invaginations were observed in any of the thin sections prepared from any of the mutant strains that had been grown anaerobically in the dark with DMSO. Fully developed ICM was observed in thin sections of the wild type 2.4.1 cells that had been cultured under either condition (Figure 7).
Table 2. Quantitative analysis of micrograph fieldview of cells containing ICM and their distribution in *Rhodobacter sphaeroides prrA* and *prrBCA* strains.

| Strain    | Total number of cells in the fields | Number of cells with visible ICM | Distribution of ICM-type structures |
|-----------|-------------------------------------|----------------------------------|-------------------------------------|
| BR107     | 68                                  | 28                               | polar                               |
| PRRA1     | 77                                  | 28                               | polar                               |
| PRRA2     | 66                                  | 20                               | polar                               |
| PRRBCA2   | 79                                  | 9                                | sporadic                            |
Figure 6. TEM micrographs of *Rhodobacter sphaeroides* bacteria that had been cultured under low-oxygen conditions. Micrographs are of (A) wild type 2.4.1 cells, (B) PRRA1 mutant cells, (C) PRRA2 mutant cells, (D) BR107 mutant cells, and (E) PRRBCA2 mutant cells. The red arrowheads indicate ICM, blue arrow indicate sporadic invaginations. The micron bar equals 1 µm.
Figure 7. TEM micrographs of *Rhodobacter sphaeroides* bacteria that had been cultured under anaerobic-dark conditions, with DMSO as alternate electron acceptor. Micrographs are of (A) wild type 2.4.1 cells, (B) PRRA1 mutant cells, (C) PRRA2 mutant cells, (D) BR107 mutant cells, and (E) PRRBCA2 mutant cells. The red arrowhead is indicating ICM. The micron bar equals 1 µm.
Absorption spectroscopy of *R. sphaeroides* wild type and Prr\(^{-}\) mutant bacterial membranes. Figure 8 shows absorption spectroscopy of extracts of wild type and mutant bacteria cultured under low-oxygen conditions. Using this method, the amounts of the pigment-protein complexes were below detectable levels in all the prr\(^{-}\) mutants, and no differences between PrrA\(^{-}\) *versus* PrrBCA\(^{-}\) mutant bacteria were observed. Therefore, phenotypic differences between the PrrA\(^{-}\) mutants *versus* the PrrBCA\(^{-}\) mutant only became apparent from the physical examination performed here using TEM.

![Absorption spectrum](image)

**Figure 8.** Absorption spectroscopic analysis of membrane extracts from *Rhodobacter sphaeroides* cells grown under low-oxygen conditions. Equivalent amounts of total protein from each sample were analyzed.

Discussion

Based on the TEM results, the prr genes are required for normal ICM formation. This outcome suggests that the Prr regulatory system regulates not only genes involved in the synthesis of pigment-protein complex components, but that it also directly or indirectly dictates
other transcriptional events associated with ICM development. An unanticipated and novel
discovery made during these studies was the observation of ultrastructural differences of low-
oxygen cells with defective prrA genes versus those in which the entire prr gene cluster is
absent. This suggests that PrrB and/or PrrC may participate in regulation of events associated
with ICM formation that do not involve PrrA.

While transcriptomic and proteomic data are available that could be used as a guide to
direct us to potentially important genes regulated by PrrA that are involved in ICM formation,
there are currently no similar data available at the genome wide level for PrrB or C. Before this
investigation, the need to perform such experiments was not evident, since other methods used to
evaluate the physiological status of R. sphaeroides, such as comparisons of growth rates or even
spectral analyses, gave no indication that there were any differences between cells lacking prrA
alone versus those lacking all three prr genes.

The difference in phenotypes between the prrA\textsuperscript{−} mutant bacteria versus those lacking the
entire prr gene cluster might be explained by cross-talk or branched regulation between PrrB
(Figure 9) and a non-cognate response regulatory protein. Such a regulator must be able to
recognize at least a subset of the PrrA DNA targets, and to be consistent with the results
presented here, it must be present in cells grown under low-oxygen conditions but absent in cells
grown anaerobically in the dark with addition of DMSO.
Figure 9. Hypothetical model of PrrB cross-talk or branched regulation with a non-cognate response regulator in a prrA background. PrrB is proposed to phosphorylate an alternative response regulator present in cells grown under low-oxygen conditions but not under anaerobic conditions, that can transcribe a set of genes regulated by PrrA responsible for ICM formation.
CHAPTER III: ANALYSIS OF THE ROLE OF THE OXYGEN-RESPONSIVE REPRESSOR PROTEIN PpsR IN ICM FORMATION

Introduction

Oxygen and light are environmental parameters that can directly interact with molecules inside the cell. In addition to proteins in the membrane that are capable of perceiving changes in such parameters and mediating changes in gene expression, e.g. the PrrB sensor kinase protein, *R. sphaeroides* has cytoplasmic proteins having analogous functions. The AppA protein (activation of photopigment and *puc* expression protein A) senses each of these effectors, and they induce a conformational change that alters its ability to interact with the DNA binding protein PpsR (photopigment suppressor protein R). AppA senses oxygen via a SCHIC (sensor containing heme instead of cobalamine) domain and blue light via a BLUF (sensor of blue light using flavin adenine dinucleotide) domain. In the presence of oxygen and blue light the AppA-PpsR protein-protein interaction is thought to be disrupted. PpsR is then available for DNA binding, and acts as a "master" repressor of PS genes. Disabling *ppsR* leads to the expression of photosynthesis genes in the presence of oxygen, and cells lacking PpsR are genetically extremely unstable. Suppressor mutations are rapidly acquired (Gomelsky and Kaplan 1995c), and several among these suppressor mutations result in reduced Bchl *a* production; *i.e.*, PS genes (Zeilstra-Ryalls and Kaplan 1998). It is thought that this genetic instability is due to the dangerous situation in which Bchl is produced when oxygen and light are also present, leading to the formation of lethal reactive oxygen species (reviewed in Zeilstra-Ryalls and Kaplan, 2004).

Recent studies have shown that *appA* transcription is PrrA-dependent (Gomelsky *et al.* 2008). They also indicate that PrrA affects interactions between AppA and PpsR, which in turn
influences the regulatory activity of PpsR. As discussed previously (Chapter II), PrrA regulatory targets include several PS genes that are also regulated by PpsR (Figure 3). The consequences of this regulatory complexity are made apparent by virtue of the fact that, although PrrA mutant bacteria are unable to grow phototrophically, bacteria lacking both PrrA and PpsR can grow phototrophically (Gomelsky et al. 2008). The status of either \textit{ppsR} or \textit{ppsR} \textit{prrA} mutant bacteria with respect to ICM formation has not been determined. In order to do so, TEM was used to examine the ultrastructure of cells grown under inducing anaerobic conditions.

![Diagram of transcription regulation by the AppA-PpsR repressor-antirepressor system.](image)

**Figure 10.** Model of transcription regulation by the AppA-PpsR repressor-antirepressor system. AppA, in response to oxygen or blue light, binds to PpsR, thereby releasing PpsR from the DNA (based on Gomelsky et al. 2008).

**Materials and Methods**

**Bacterial strains and growth conditions.** The strains used in this study, together with their relevant characteristics and source are listed in Table 3. \textit{R. sphaeroides} wild type strain 2.4.1, \textit{ppsR} mutant strain PPS1, and \textit{ppsR} \textit{prrA} mutant strain RPS1 were grown at 30°C under
anaerobic-dark conditions in screw-capped tubes completely filled with Sistrom’s succinate minimal medium A (Sistrom 1960) supplemented with 60 mM DMSO and 1% yeast extract (Fales et al. 2001).

Table 3. *Rhodobacter sphaeroides* strains used in this study.

| Strain | Relevant characteristics | Reference or source |
|--------|--------------------------|---------------------|
| 2.4.1  | Wild type                | W. Sistrom          |
| PPS1   | *ppsR::ΩKn^R*            | Gomelsky and Kaplan 1997 |
| RPS1   | *ppsR::ΩKn^R prrA::ΩTp^R* | Gomelsky and Kaplan 1997 |

**TEM specimen preparation.** Cells from 40 ml of mid- to late-log phase liquid cultures were collected in 50 ml conical tubes by centrifugation at 2,688 x g for 20 minutes at room temperature. Specimens were prepared for TEM according to the protocol described in Chapter II.

**Results**

PpsR is an aerobic repressor of photosynthesis genes. Thus, PpsR⁻ mutant bacteria can grow and are stable under phototrophic conditions (Gomelsky *et al.* 2000). Gomelsky *et al.* have found that, unlike PrrA⁻ mutant bacteria, *R. sphaeroides* PpsR⁻ PrrA⁻ mutants can grow phototrophically (Moskvin *et al.* 2005). Therefore, cells could be grown under anaerobic-light conditions for TEM analysis. However, since light intensity influences the amount of ICM in the cell, in order to achieve greater uniformity and reproducibility in terms of ICM content, the cells were grown under anaerobic-dark with DMSO conditions.
ICM formation was apparently not affected by the absence of PpsR, as the ultrastructure of the PPS1 mutant cell membrane is similar to that of the wild type. This was to be expected, since PpsR functions as a repressor of PS genes under aerobic conditions. The structure of the membrane is very different in the double mutant RPS1, in which long, tubular-shaped ICM is a clearly visible feature of the cells (Figure 11). Evidently, despite the abnormal appearance of the ICM, the photosynthesis machinery is nevertheless operational as the cells can grow phototrophically (Moskvin et al. 2005), although their growth is considerably slower than wild type (Moskvin et al. 2005).
Figure 11. TEM micrographs of *Rhodobacter sphaeroides* bacteria that had been cultured under anaerobic-dark conditions with DMSO as alternate electron acceptor. Micrographs are of (A) 2.4.1 wild type cells, (B) RPS1 mutant cells, and (C) PPS1 mutant cells. Red arrowheads indicate ICM, the tubular ICM present in RPS1 cells are indicated by the green arrowhead. The micron bar equals 1 µm.
Discussion

PpsR is thought to regulate transcription only in the presence of oxygen. Therefore, the normal appearance of ICM in the PPS1 mutant bacteria grown under anaerobic conditions was not unexpected. However, this in no way explains the morphology of the membrane in RPS1. Gomelsky et al. have proposed that the inability of PrrA\(^{-}\) mutant bacteria to grow phototrophically is not due to the lack of PrrA-mediated activation of PS genes. Rather, they suggest that it is the inability to antirepress PpsR-regulated genes (Gomelsky et al. 2008). Therefore, phototrophic growth is possible when PpsR function is lost by mutation in a \(\text{prrA}^{-}\) background. This idea is strongly supported by the fact that suppressor mutations that restore phototrophic growth to PrrA\(^{-}\) mutant bacteria were mapped to the \(\text{ppsR}\) gene (Gomelsky et al. 2008). The present study demonstrates that, although the PpsR\(^{-}\) PrrA\(^{-}\) mutant bacteria can grow by photosynthesis, the ICM is not normal. This key observation suggests that there may be genes regulated by PrrA and not by PpsR that are required for ICM development.

The ultrastructure of RPS1 cells is reminiscent of that previously reported by Kiley et al. for mutant strain RS104, which was isolated as a pale reddish brown mutant following N-methyl-N-nitro-N-nitrosoguanidine (MNNG) treatment of the wild type strain RS2 (Meinhardt et al. 1985). While the nature of the mutation is not known, the phenotype of the mutant is LHII and (colored) carotenoid-minus. A tubular arrangement of the inner membrane has also been reported by other groups for other strains of \(R.\ sphaeroides\) (Hunter et al. 1988, Kiley et al. 1988, Sabaty and Kaplan 1996, Siebert et al. 2004). The one known common feature among all of them is that they lack the pigment-protein LHII complex. Similarly, absorption spectroscopy of RPS1 lysates indicates diminished LHII complex levels (Gomelsky et al. 2008). Whether this is due to an absence of the LHII polypeptides and/or associated assembly factors or a reduction
in Bchl production is not known. If Bchl is limiting, it indicates that it is preferentially incorporated into RC and LHI, which makes sense physiologically since LHII is not essential for phototrophic growth, provided sufficient light is available. An investigation of the genes involved in producing the LHII proteins and pigments that are modified such that their transcription is uncoupled from PrrA regulation should make it possible to examine these possibilities. Should the outcome implicate involvement of additional genes, *i.e.*, ICM morphology remains abnormal, candidate genes to consider would be other PrrA-regulated genes. It is conceivable that this analysis could identify new genes required for ICM formation.
CHAPTER IV: ANALYSIS OF THE ROLE OF THE ANAEROBIC REGULATORY PROTEIN FnrL IN ICM FORMATION

Introduction

Since oxygen freely diffuses across the cytoplasmic membrane, the concentration of oxygen inside of the cell depends upon its concentration outside of the cell. FnrL (fumarate-nitrate reductase regulator-type protein L) is a cytoplasmic regulatory protein whose activity is thought to respond directly to changes in oxygen tensions (Zeilstra-Ryalls and Kaplan 1995). The protein belongs to a family of such proteins, the Fnr-Crp family. All members are characterized by the presence of an effector domain located within the N-terminal region and a DNA binding domain located within the C-terminal region. For FnrL, the effector domain contains an oxygen-labile 4Fe-4S cluster whose presence is required for the protein to be properly configured for DNA binding. Thus, the protein regulates genes when oxygen is limiting. FnrL is essential for all anaerobic growth, both in the light and in the dark with DMSO (Zeilstra-Ryalls and Kaplan 1995). Yet the FnrL− mutant transcriptome profile (Figure 3) indicates that PS genes are little altered in their expression relative to wild type cells, and phototrophic growth is not restored by expressing those genes that are appreciably regulated by FnrL and participate in photo-pigment and structural protein production (Ouchane et al. 2007). By contrast, the inability of the mutants to grow anaerobically in the dark using the alternate electron acceptor DMSO has an easy explanation. The dorA gene encodes DMSO reductase, which is responsible for completing the anaerobic respiratory chain. This gene requires FnrL for its transcription (Zeilstra-Ryalls et al. 1997).
The inability of \textit{fnrL} \textsuperscript{\textminus} mutants to grow under any anaerobic condition makes difficult the identification of those FnrL-dependent genes that are specific to phototrophic growth. However, a recent finding has provided a fresh perspective on the question as to why FnrL \textsuperscript{\textminus} mutants cannot grow phototrophically. Namely, phototrophic growth is restored to FnrL \textsuperscript{\textminus} mutants by the addition of multiple copies of the \textit{R. sphaeroides} \textit{crpK} gene, coding for another member of the Fnr-Crp protein family. The CrpK protein (\textit{cAMP \text{receptor-type protein \text{K}}}) lacks cysteines that could participate in formation of a 4Fe-4S cluster, and so its effector is likely something other than oxygen. That phototrophic growth is restored implies that it binds to DNA sequences that are similar to the FnrL targets. Since anaerobic-dark growth is not restored, the DNA target sequences must be a subset of those genes regulated by FnrL, or CrpK is only expressed or active even in multicopy under phototrophic growth conditions. Inactivation of \textit{crpK} has no effect on phototrophic growth under standard anaerobic-light conditions. Using Affymetrix oligonucleotide microarray genechips, a list of genes that are potentially differently expressed in the presence and absence of \textit{fnrL}, with and without multiple copies of \textit{crpK} has been generated. Among the genes is \textit{rsp2573}, which codes for yet another Fnr-Crp type protein. Deletion of \textit{rsp2573} confers a loss of the ability to grow phototrophically to the cell. Collectively, these findings suggest that \textit{rsp2573} is a component of a regulatory cascade also involving \textit{fnrL} and \textit{crpK}, and that the inability of the \textit{fnrL} \textsuperscript{\textminus} mutants to grow phototrophically is a consequence of the absence of expression of \textit{rsp2573}.

The gene targets of \textit{rsp2573} regulation are not known. However, given that the expression of the PS genes are little affected by the absence of FnrL, it is likely that \textit{rsp2573} regulates genes other than PS genes that are nevertheless required for phototrophic growth. These may include genes whose products are necessary for proper ICM development.
investigate this possibility, TEM was used to examine and compare the ultrastructures of FnrL’
mutant bacteria, FnrL’ mutant bacteria with multiple copies of crpK, and Rsp2573’ mutant
bacteria. All were grown under low-oxygen conditions, which are permissive for growth of
FnrL’ mutants and also are inducing for ICM formation.

![Figure 12. Model of oxygen-responsive regulation of genes by FnrL during anaerobic growth. An oxygen-labile 4Fe-4S cluster promotes the proper conformation of the dimeric protein for binding to DNA.](image)

**Materials and Methods**

**Bacterial strains and growth conditions.** Table 4 lists the bacterial strains and plasmids
used in this study, together with their relevant characteristics and sources. *R. sphaeroides* was
grown in Sistrom’s succinate minimal medium A (Sistrom 1960). Low-oxygen growth was
conducted by inoculation of *R. sphaeroides* into 100 ml of medium in 250 ml Erlenmeyer flasks
that were incubated at 30°C in a New Brunswick gyratory shaking water bath (model G76) at a
speed setting of 2.3. *Rhodobacter capsulatus* strains were grown under the low-oxygen
conditions in Sistrom's succinate minimal medium A (Sistrom 1960) supplemented with fructose. As necessary, for plasmid maintenance Kn was added to bacterial cultures to a final concentration of 50 µg/ml.

**TEM specimen preparation.** Cells from 40 ml of mid- to late-log phase liquid cultures were collected in 50 ml conical tubes by centrifugation at 2,688 x g for 20 minutes at room temperature. Specimens were prepared for TEM according to the protocol described in Chapter II.

**Absorption spectroscopy of membrane fractions and quantitation of pigments.** Protein synthesis was halted by the addition of chloramphenicol solution (20 mg/ml in 95% ethanol) to a final concentration of 1.5% (v/v) to the cultures, which were then chilled on ice. The cells were pelleted at 2,688 x g for 10 minutes at 4°C. Preparation of crude lysates, absorption spectroscopy, and quantitation of photo-pigments were performed as described in Chapter II.

**Protein concentration determinations.** Protein concentrations were determined as described in Chapter II.
Table 4. Strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Reference or source |
|-------------------|--------------------------|---------------------|
| **Rhodobacter sphaeroides** | | |
| 2.4.1 | Wild type | W. Sistrom |
| JZ1678 | $\Delta fnrL::\Omega$Kn$^R$ | Zeilstra-Ryalls and Kaplan 1995 |
| JZ1691 | $\Delta fnrL::\Omega$Sp$^R$/St$^R$ | Zeilstra-Ryalls and Kaplan 1995 |
| JZ5210 | $\Delta rsp2573::loxP$ | Lab collection |
| **Rhodobacter capsulatus** | | |
| 2.3.1 | Wild type | American Type Culture Collection |
| SB1003 | Spontaneous Rif$^R$ prototrophic derivative of 2.3.1 | Yen and Marrs 1976 |
| RGK295 | $\Delta fnrL::Kn^R$ derivative of SB1003 | Zeilstra-Ryalls *et al.* 1997 |
| RGK296 | $\Delta fnrL::Kn^R$ derivative of SB1003; Kn$^R$ in opposite direction to RGK295 | Zeilstra-Ryalls *et al.* 1997 |
| **Plasmids** | | |
| pRK2013 | ColE1 replicon, Tra$^+$ of RK2, Kn$^R$ | Ditta *et al.* 1980. |
| pBBR1-MCS2 | Kn$^R$ | Kovach *et al.* 1994 |
| pCrpK | $rsp2572$ sequences amplified from *R. sphaeroides* 2.4.1 inserted into the EcoRV site of pBBR1-MCS2 | Lab collection |
Results

Ultrastructure of *R. sphaeroides* wild type 2.4.1 and FnrL\(^{-}\) mutant bacteria. Thin sections of cells cultured under low-oxygen conditions were examined using TEM (Figure 13). In contrast to the typical ICM observed in the thin sections of wild type cells having the plasmid pBBR1-MCS2 (Figure 13A), only a few membrane invaginations were seen in the sections of the *fnrL* null mutant JZ1691 with pBBR1-MCS2 (Figure 13B). However, ICM that is normal in appearance were observed in FnrL\(^{-}\) mutant cells with plasmid *pcrPK* (Figure 13C).

![TEM micrographs of *Rhodobacter sphaeroides* bacteria that had been cultured under low-oxygen conditions. Micrographs are of (A) 2.4.1(pBBR1-MCS2) cells, (B) JZ1691(pBBR1-MCS2) cells, and (C) JZ1691(*pcrPK*) cells. Red arrowheads indicate ICM. The micron bar indicates 1 µm.](image-url)
Ultrastructure of *R. sphaeroides* wild type 2.4.1 and Rsp2573' mutant bacteria. Thin sections of cells cultured under low-oxygen conditions were examined using TEM (Figure 14). Again, typical ICM structures were apparent in thin sections of the wild type 2.4.1 cells (Figure 14A). However, the ultrastructure of mutant strain JZ5210 (Table 4), in which the coding sequences of *rsp2573* have been deleted, resembles that of the FnrL’ mutant strain JZ1691 (Figure 13), with only a few membrane invaginations (Figure 14B).

Figure 14. TEM micrographs of *Rhodobacter sphaeroides* bacteria that had been cultured under low-oxygen conditions. Micrographs are of (A) 2.4.1 cells and (B) JZ5210 mutant bacteria. Red arrowheads indicate ICM. Micron bar equals 1 μm.
Absorption spectroscopy of *Rhodobacter sphaeroides* wild type strain 2.4.1, FnrL⁻ mutant strain JZ1678 and Rsp2573⁻ mutant strain JZ5210. The levels of pigment-protein complexes were measured in crude lysate preparations of wild type and mutant bacteria grown under low-oxygen conditions (Figure 15). As has been reported previously, complex levels are reduced in the absence of FnrL (Zeilstra-Ryalls et al. 1997) and the levels are similarly reduced in the absence of Rsp2573. Thus, the levels of pigment-protein complexes correlated with the presence and absence of ICM in the cells (Figures 13 and 14).

![Figure 15](image.png)

Figure 15. (A) Absorption spectroscopy and (B) bacteriochlorophyll concentrations of membrane extracts of *Rhodobacter sphaeroides* wild type 2.4.1 and mutant strains JZ1678 and JZ5210 grown under low-oxygen conditions. Equivalent amounts of total protein from each sample were analyzed.

Ultrastructure of *Rhodobacter capsulatus* FnrL⁺ and FnrL⁻ mutant bacteria. Although both species of *Rhodobacter* require FnrL for anaerobic dark growth with DMSO, unlike *R. sphaeroides* FnrL⁻ mutants, *R. capsulatus* FnrL⁻ mutant bacteria are capable of phototrophic growth. Further, spectral complex levels are unaffected by the absence of FnrL in *R. capsulatus* (Zeilstra-Ryalls et al. 1997). The ultrastructure of the *R. capsulatus* FnrL⁻ mutant bacteria, strains RGK295 and 296 (Table 4), were examined by preparing thin sections of cells
cultured under low-oxygen conditions and examining them using TEM (Figure 16). In contrast to the abnormal appearance of *R. sphaeroides* FnrL− mutant bacterial cell membranes (Figure 13), the membrane morphology of *R. capsulatus* FnrL− mutant bacteria appeared similar to the wild type strain SB1003. Therefore, for *R. capsulatus*, the absence of FnrL did not seemingly affect ICM formation.

![Figure 16](image)

**Figure 16.** TEM micrographs of *Rhodobacter capsulatus* bacteria that had been cultured under low-oxygen conditions. Micrographs are of (A) SB1003 cells, (B) RGK295 mutant cells, and (C) RGK296 mutant cells. Red arrowheads indicate ICM. The micron bar corresponds to 1 µm.

**Discussion**

While the PS genes of *R. sphaeroides* are little changed in their transcription levels by the presence *versus* the absence of FnrL (Figure 3), FnrL− mutant bacteria are nevertheless unable to form normal ICM. The ability of multiple copies of crpK to restore phototrophic growth to the FnrL− mutant is paralleled by the fact that normal ICM is apparently also restored. The inability
of Rsp2573– bacteria to grow phototrophically is potentially explained by the absence of normal ICM. Expression of \textit{rsp2573} requires FnrL (Zeilstra-Ryalls, unpublished results), and this requirement can be bypassed by extra copies of \textit{crpK} (Zeilstra-Ryalls, unpublished results). Since PS gene expression is largely unaffected by the absence of FnrL, it follows that Rsp2573 regulates genes other than PS genes that are required for phototrophic growth, possibly at the level of ICM formation. It also suggests that, if Rsp2753 acts directly, it is an activator of transcription of those genes.

It has been established previously that, unlike \textit{R. sphaeroides} FnrL– mutants, \textit{Rhodobacter capsulatus} FnrL– mutants are unaltered in their ability to grow phototrophically (Zeilstra-Ryalls \textit{et al.} 1997). Consistent with that finding, the ultrastructure of the \textit{R. capsulatus} ICM appeared normal. The difference in FnrL requirements with respect to phototrophic growth and ICM development between the two species of \textit{Rhodobacter} could be explained by the fact that, based on an inspection of the \textit{R. capsulatus} SB1003 genome, both \textit{crpK} and \textit{rsp2573} are absent. Therefore, \textit{rsp2573}-mediated regulation does not occur in \textit{R. capsulatus}. Thus, the findings presented here not only provide a plausible explanation for previous observations but also suggest a direction for future investigation that may lead to the identification of new insights into what genes are required for ICM formation, and phototrophic growth.
CHAPTER V: SUMMARY AND FUTURE DIRECTIONS

Transcriptomic and proteomic investigations have provided new insights into regulatory events that are mediated by PrrA, PpsR, and FnrL as *R. sphaeroides* responds to changes in oxygen availability. Absorption spectroscopy has also been a useful tool in evaluating the role of these proteins in photosynthetic membrane development. However, the participation of these global regulators in ICM formation has not previously been examined at the ultrastructural level. This investigation has added to our understanding of the significance of each transcriptional regulator in normal development of ICM. With respect to the Prr redox-responsive regulatory system, evidence was presented that certain regulatory events might take place that are independent of PrrA. The hierarchical regulation mediated by PpsR and PrrA (Gomelsky *et al.* 2008) is supported by the findings here. But the absence of PpsR does not completely restore PS in a *prrA* background since the membranes are not normal. It suggests that limited availability of Bchl leads to preferential formation of RC and LHI, and so the membrane structure is the same as in cells lacking LHII. This predicts that increasing Bchl availability, which could be achieved by restructuring the genes to be *prrA* activation independent, should lead to normal ICM. With respect to FnrL, the findings presented here add to evidence that FnrL participates in a regulatory cascade with *rsp2573* as the next step in the switch to phototrophic growth, which is initiated by lowering oxygen tensions.

Having made these visual observations, a logical next step is to obtain quantitative data. This would involve isolation of chromatophores using methods such as those described by Fraker and Kaplan (Fraker and Kaplan 1971). The molecular composition of the isolated chromatophores could also be examined to determine whether or not all of the usual components are present, and at what levels. These studies also provide impetus for new investigations of
gene expression, including the use of Affymetrix oligonucleotide array genechips to examine transcriptional differences between PrrA<sup>−</sup> versus PrrBCA<sup>−</sup> cells, the differences in transcription of genes in PpsR<sup>−</sup> versus PpsR<sup>−</sup> PrrA<sup>−</sup> bacteria, and transcriptional differences of cells with and without the newly identified regulatory protein <i>rsp2573</i>. 
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