MUTATIONAL ANALYSIS OF THE
RHODOBACTER CAPSULATUS
ORF162B GENE

by

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to the required standard

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Date **April 30, 1998**
There are several open reading frames (orfs) of unknown function in the photosynthesis gene cluster of *Rhodobacter capsulatus*. It is possible that these orfs, including *orf162b*, have a function in bacterial photosynthesis. When *R. capsulatus* RNA was probed for the presence of an *orf162b* transcript, a 4 kb polycistronic mRNA was detected. Strains were then created which possessed either a polar or non-polar deletion mutation of *orf162b*. These mutant strains were evaluated on the basis of their light harvesting (LH) and reaction center (RC) complex levels, chromatophore protein band intensities and their ability to grow photosynthetically. Mutants were complemented with *orf162b* in *trans*. In a non-polar mutant *orf162b* strain, decreases in LH and RC complex levels as well as photosynthetic growth rate were restored by *trans* complementation. When a polar mutation was inserted into *orf162b* such that any downstream orfs cotranscribed with *162b* would no longer be expressed, a reduction in LH and RC complex levels as well as photosynthetic growth rate was observed. As with the non-polar mutation, the LH and RC complex levels were increased upon *trans* complementation with *orf162b*, whereas the wild type generation time was not restored. It is concluded that *orf162b* and at least one orf found downstream of, and cotranscribed with, *orf162b* are genes that encode proteins which are involved in bacterial photosynthesis. A hypothesis that *orf162b* functions in the assembly or interaction of complexes is proposed.
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ABBREVIATIONS

a a  amino acid
A p r  ampicillin resistance
ATP  adenosine 5'-triphosphate
ATPase  adenosine 5'-triphosphatase
Bchl  bacteriochlorophyll
Ble r  bleomycin resistance
b p  base pair
BSA  bovine serum albumin
CFU  colony forming unit
Cyt  cytochrome
DNA  deoxyribonucleic acid
dUTP  2'-deoxyxynucleoside uridine-5'-triphosphate
EDTA  ethylenediaminetetra-acetic acid
Gly  glycine
G m r  gentamycin resistance
GTA  gene transfer agent
ICM  intracytoplastic membrane
k b  kilobase
KDa  kilodalton
Km r  kanamycin resistance
KU  klett unit
LH  light harvesting
MOPS  3-(N-morpholino)propanesulfonate
or f  open reading frame
<table>
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<tr>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PNS</td>
<td>purple non-sulfur</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>RC</td>
<td>reaction center</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Spr</td>
<td>spectinomycin resistance</td>
</tr>
<tr>
<td>TBE</td>
<td>tris borate EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>tris EDTA</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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1 INTRODUCTION

1.1 Photosynthesis in *Rhodobacter capsulatus*

The purple non-sulfur (PNS) bacteria, designated as such due to the inability of most species to oxidize elemental sulfur to sulfate, vary considerably in their metabolic and growth capabilities (Imhoff 1995). *Rhodobacter capsulatus* is a PNS bacterium capable of aerobic respiration as well as anaerobic photosynthetic growth, and so the effects of mutations that prevent photosynthetic growth can be readily evaluated in this organism by aerobic cultivation of mutant strains. These properties, coupled with the simplicity of the PNS bacterial photosystem, the availability of considerable amounts of *R. capsulatus* genomic DNA sequences, and the wealth of information about the structure and function of the bacterial photosynthetic apparatus, make *R. capsulatus* an ideal system to study the function and regulation of genes involved in bacterial photosynthesis (Bauer 1995).

Photosynthesis is widely thought of as the process by which light energy is used in plants, algae and photosynthetic bacteria to assimilate carbon dioxide for carbohydrate synthesis. However, a more general definition has been set forth, defining photosynthesis as a process by which light energy is transformed into chemical energy, in the form of phosphodiester bonds within ATP molecules. In PNS bacteria, the ATP formed in this process may be the sole source of energy during anaerobic photoheterotrophic growth, in which organic compounds are oxidized to
generate reducing power [NAD(P)H] and biosynthetic intermediates (Gest 1993).

Photosynthetic energy conversion in *R. capsulatus* takes place in the intracytoplasmic membranes (ICM). These invaginations of the cytoplasmic membrane generate spherical as well as tube-like structures that contain the pigment-protein complexes of the photosynthetic apparatus (Drews and Golecki 1995). Under low concentrations of oxygen, photosynthetic pigments are synthesized which associate with the reaction center (RC) and light harvesting (LH) complex proteins in the ICM (Fig. 1) (Drews 1986). The process of photosynthetic energy conversion begins with the absorption of a photon by a LH complex, followed by the transfer of this absorbed energy to a reaction center where it is utilized to initiate a sequence of electron transfers. The electron transfers result in the formation of a proton gradient which is used to drive ATP synthesis (Bauer *et al.*, 1993).

1.2 The photosynthetic apparatus of *R. capsulatus*

LH complexes contain light absorbing pigment molecules that function to increase the photon capturing area of the photosynthetic apparatus. The majority of light energy harvested by the RC is received from these complexes. Pigments involved in energy transfer include bacteriochlorophyll (Bchl) and, for shorter wavelengths, carotenoid molecules (Miroslav *et al.*, 1996). The LHI complex of purple bacteria is thought to contain about 32 Bchl molecules, based on a structure obtained
**Figure 1.** The photosynthetic apparatus of *R. capsulatus*. Light energy (depicted as wavy lines) is absorbed by bacteriochlorophyll molecules (ringed structures) of the LHI and LHII complexes before being transferred to the RC. A charge separation in the RC initiates a series of electron transfers, which in turn generates a proton gradient utilized in ATP synthesis (Fig. taken from Wellington *et al.*, 1991).
with the *Rhodospirillum rubrum* LHI (Karrasch *et al.*, 1995). These molecules can be distinguished by their long wavelength absorption maximum, resulting from protein-pigment interactions within the complex, of 870 to 875 nm. Protein-free Bchl molecules absorb light at about 770 nm, and it is through poorly understood interactions with amino acid side chains in proteins that the long wavelength absorbance maxima are red-shifted (Zuber and Cogdell 1995). In some photosynthetic bacteria (including *R. capsulatus*) there is an additional antenna called the LHII or B800-850 complex. According to crystal structure analyses in *Rhodopseudomonas acidophila* strain 10050, each LHII is thought to possess 18 Bchl molecules which absorb at 850 nm, and 9 Bchl which absorb at 800 nm for a total of 27 Bchl molecules (McDermott *et al.*, 1995).

Both LHI and LHII antennae are composed of two hydrophobic, low molecular mass proteins, designated α and β, which are organized in heterodimer pairs to form subunit structures. The light absorbing pigments are noncovalently attached to these dimers. The intact antenna complexes are oligomers of αβ protein subunits and their associated pigments (Miroslav *et al.*, 1996).

Three protein subunits designated heavy (H), medium (M) and light (L) according to their SDS-PAGE mobilities make up the RC complex which spans the ICM. A special pair of Bchl molecules within the RC captures energy from the LHI, which seems to be arranged as a ring of 16 αβ dimers that encircles the RC. The LHI-RC structure is called the core complex (Miroslav *et al.*, 1996).
The LHII antenna, when present, is arranged as a collection of separate, excentric rings around the concentric LHI/RC core structure. The number of LHII complexes associated with each core complex varies depending on the growth conditions of the bacteria. Cells grown under low light conditions synthesize more LHII structures relative to LHI/RC cores (Garcia et al., 1987). Between 8 to 10 LHII rings, each composed of 9 αβ pairs and their associated pigments, are believed to surround the core complex such that they make close contact with the LHI as well as neighboring LHII structures (Miroslav et al., 1996). The core complex together with peripheral LHII is called the photosynthetic unit. In purple bacteria, photosynthetic units, which are arrayed parallel to the plane of the membrane, are densely packed in the ICM and appear to form extended regions of complexes that are functionally connected for light energy transfer (Sundstrom and van Grondelle 1995).

1.3 The photosynthesis gene cluster

Many of the genes that code for the polypeptides that comprise the pigment biosynthesis enzymes, and structural proteins of the photosynthetic apparatus are grouped together in a 46 kilobase region of the _R. capsulatus_ genome termed the photosynthesis gene cluster (PGC) (Fig. 2) (Alberti et al., 1995). This gene cluster is organized into overlapping operons or "superoperons" which result in the concurrent transcription of specific photosynthesis genes from more than one promoter. This feature of the PGC is thought to allow for the efficient shift from aerobic respiratory to anaerobic photosynthetic growth in response to
Figure 2. Genes located in the *R. capsulatus* photosynthesis gene cluster. Bacteriochlorophyll biosynthesis genes (*bch*) are designated by diagonal lines, carotenoid biosynthesis genes (*crt*) are shown as cross-hatched boxes, LH and RC genes (*puh* and *puf*) are represented by lightly shaded boxes, and open reading frames of uncertain function (orfs) are shown as darkly shaded regions. The arrows represent transcripts of the *puhA* region that were reported in (Bauer *et al.*, 1991).
changes in environmental conditions (Beatty 1995). Located in the PGC are: the *puf* genes which encode the RCL and M polypeptides and the LHI α and β protein subunits; the *crt* and *bch* genes, which encode enzymes functioning in carotenoid and bacteriochlorophyll biosynthesis; and the *puhA* gene encoding the RC H subunit. Five open reading frames (orfs) are located downstream of and in the same transcriptional orientation as *puhA*. It is possible that at least some of these orfs, termed *orf214*, *orf162b*, *orf55*, *orf274* and *orf162a*, are genes transcribed as a unit along with *puhA* (Wong et al., 1996), although *puhA* transcript mapping experiments revealed only the two molecules shown in Fig. 2, both of which have 3' ends shortly after the *puhA* gene (Bauer et al., 1991).

Since the orfs downstream of *puhA* were first revealed by DNA sequence analysis, it was not clear if they were expressed (i.e., were genes) and, if so, had a function in photosynthetic growth. Recent gene disruption and complementation experiments carried out on *R. capsulatus* strains containing either transcriptionally polar disruptions or translationally in-frame (non-polar) deletion mutations within *puhA* and *orf214* suggested that *orf214*, and at least one additional gene downstream of *orf214*, were required to obtain wild type levels of LH1 and RC complexes. For example, when a polar antibiotic resistance cartridge was placed in *orf214*, the orf directly downstream of *puhA* and upstream of *orf162b* (Fig. 2), this mutant was incapable of photosynthetic growth. When this polar mutant strain was complemented with a plasmid containing *orf214* the organism did not fully regain the ability to grow photosynthetically. In contrast, a strain containing a translationally in-frame deletion of *orf214* was also photosynthetically impaired but when it was complemented in *trans* with
orf214, it was capable of photosynthetic growth at a rate similar to that of the parental strain (Wong et al., 1996). These data indicated that at least one gene downstream of orf214 (and within the same transcription unit) has an affect on the ability of R. capsulatus to photosynthesize.

In addition, Rhodospirillum rubrum contains two orfs with sequence similarity to R. capsulatus orf214 and orf162b, and these R. rubrum orfs are located at the same positions relative to the puhA gene as the R. capsulatus homologues. Although R. rubrum orf13087 encodes an amino acid sequence with only 25% identity to R. capsulatus orf162b in an alignment (Fig. 3), hydropathy plots of the putative gene products of R. capsulatus orf162b and R. rubrum orf13087 are qualitatively similar and show the presence of a possible transmembrane sequence near the amino terminus of both proteins, followed by similar hydropathy profiles of less hydrophobic sequences (Fig. 4). These similarities suggest that R. capsulatus orf162b and R. rubrum orf13087 encode homologous proteins with an analogous function. It is conceivable that the amino termini of orf162b and orf13087 serve as anchors which localize these polypeptides to the intracytoplasmic membrane, while the rest of the proteins, which possess a cluster of highly conserved amino acids from Gly129 to Gly139 (R. capsulatus sequence; Fig. 3) provide a functional sequence to these putative polypeptides.

1.4 Thesis objectives

It would be useful to learn more information about the possible
Figure 3. An alignment of the *R. rubrum* orf13087 amino acid sequence with that of *R. capsulatus* orf162b shows 25% identity between the sequences. Identical amino acid residues are shaded.
Figure 4. Hydropathy plots of the putative gene products of *R. capsulatus orf162b* and *R. rubrum* I3087. Hydrophobic sequences of residues are given positive values on the hydropathy index shown in the y-axis. Amino acid positions are shown in the x-axis. The hydropathy index was calculated using a window of 11 amino acid residues. The certain and putative boundaries on the y-axis represent the probability of a transmembrane segment based on the Goldman, Engelman and Steitz (GES) algorithm included in the TopPred computer software program (Claros and von Heijne 1995).
**R. rubrum orf13087**

**R. capsulatus orf162b**

**R. rubrum orf13087**
function of the orfs located downstream of *puhA* to see if they are genuine genes, to see if they are required for normal photosynthetic growth, and if so, to use this information to design experiments to improve our understanding of the process of bacterial photosynthesis. The research described in this thesis is centered on the orfs of unknown function located downstream of *puhA* on the PGC. The explicit aim of this thesis is to ascertain if *orf162b* encodes a protein and, if so, to determine if this gene product is involved in the process of photosynthesis in *R. capsulatus*. 
2 MATERIALS AND METHODS

2.1 Bacterial strains

The *R. capsulatus* parental strains used in this thesis were SB1003 (Solioz and Marrs 1977), a derivative of the natural isolate B10 (Weaver *et al.*, 1975), and MW442 (Scolnik and Marrs 1987), an LHII− derivative of SB1003. *R. capsulatus* DE442, a gene transfer agent (GTA) overproducer that produces phage-like gene transducing particles (Yen *et al.*, 1979), was used as a GTA phage donor in transductions. The plasmid mobilizing strains *Escherichia coli* TEC5 (Taylor *et al.*, 1983) and S17-1 (Simon *et al.*, 1983) were used in conjugation experiments with *R. capsulatus*. *E. coli* C600 r-m+ (Bibb and Cohen 1982) and DH5α (Life Technologies, GIBCO BRL) were used as host strains for the construction and maintenance of plasmids.

2.2 *In-vitro* recombinant DNA techniques

Plasmid DNA was isolated from *E. coli* cultures by the alkaline lysis technique (Sambrook *et al.*, 1989) and purified with the use of Qiagen DNA affinity columns. DNA was purified from agarose gels by adsorption to silica beads, using a QIAEX DNA Extraction Kit (QIAGEN Inc.).

General DNA manipulations such as restriction endonuclease digestion, DNA dephosphorylation and ligation, agarose gel electrophoresis
and calcium chloride transformation were performed as described (Sambrook et al., 1989). New restriction enzyme sites were inserted by the linker tailing technique (Lathe et al., 1984).

A Gene Pulser apparatus was used as per the manufacturer's instructions to electro-transform *E. coli* cells (BIO-RAD Laboratories). DNA sequencing was performed by the Nucleic Acid Protein Service Unit at the University of British Columbia using an ABI automated DNA sequencer.

### 2.3 Growth conditions

*R. capsulatus* strains were grown at 30 to 34°C in either RCV, a minimal medium containing malate as the sole carbon source, or YPS, a rich medium containing yeast extract and peptone (Weaver et al., 1975). Strains grown on plates were grown on media containing 15 g/l agar. Photosynthetically grown plate cultures were grown in BBL GasPak anaerobic jars (Becton, Dickinson and Co.). *E. coli* strains were grown in Luria-Bertani (LB) medium (Sambrook et al., 1989).

Antibiotics, when present in *R. capsulatus* cultures, were added to the following concentrations: kanamycin sulfate 10 μg/ml, spectinomycin 10 μg/ml, streptomycin 10 μg/ml and gentamycin sulfate 2 μg/ml. For *E. coli* cultures, higher concentrations of antibiotics were used: ampicillin 200 μg/ml, kanamycin sulfate 50 μg/ml, spectinomycin 50 μg/ml and gentamycin sulfate 10 μg/ml.
In preparation for photosynthetic growth, *R. capsulatus* cells were grown under oxygen-limited conditions in RCV medium in Erlenmeyer flasks filled to 80% of their nominal capacity. These cultures were shaken at 150 RPM in a gyratory water bath shaker at 34°C for 2-3 days until the cultures reached a density of approximately 300 Klett units (KU; 1 KU is about 3.3 x 10^6 CFU/ml). These low-oxygen cultures were highly pigmented. For anaerobic photosynthetic growth, liquid cultures were inoculated to a turbidity of approximately 30 KU from the oxygen-limited cultures. These cultures were placed in screw-cap Pyrex tubes filled to capacity with liquid growth medium and incubated at 34°C in a water filled aquarium. The water bath was illuminated with tungsten filament incandescent lamps at various intensities. Light intensity was measured with a LI-COR photometer equipped with the LI-190SB quantum sensor (LI-COR Inc.). Water temperature and circulation in the water bath were maintained using a Haake D3-V circulator (Haake Mess-Technik GmbH Co.). Growth rate was followed by measuring the culture turbidity using a Klett-Summerson Photometer with a red (No. 66) filter.

2.4 Spectral analysis of light harvesting and reaction center complexes

*R. capsulatus* cultures were grown under oxygen-limited conditions to early stationary phase (150 KU). About 1.3 ml (6.5 x 10^8 CFU) from each culture was harvested and the cells were resuspended in a solution of 1 ml of 22.5% bovine serum albumin (BSA) in RCV medium. The presence of BSA reduces light scattering by cells within the sample and thus results in
a better measure of absolute absorption. Each sample was scanned for light absorption over a range of wavelengths (350 to 1000 nm) using a Hitachi U-2000 spectrophotometer. Data were collected using SpectraCalc software and spectra were analyzed with the Grams 386 software package (Galactic Industries Corporation). Pigments do not absorb light of 650 nm. Therefore, in order to compare spectra, scans were normalized by multiplication of spectra by a factor to yield a light scattering $A_{650}$ of 0.2.

2.5 Bacterial conjugation

Conjugation of plasmid DNA into *R. capsulatus* cells (with the exception of strain DE442) was usually accomplished using *E. coli* S17-1 as the plasmid donor strain. Overnight cultures of donor cells and *R. capsulatus* recipient cells grown to stationary phase were mixed in a 1:2 ratio by volume, pelleted (1 minute, 15,000 g) in a bench top microcentrifuge and resuspended in an equal volume of antibiotic-free RCV medium. A 10 μl volume of the mixture was deposited onto a dry RCV agar plate. After the liquid had absorbed, the plates were incubated overnight at 30°C allowing conjugation to take place. Negative controls consisted of equal concentrations of recipient cells in the absence of donor cells.

Cells from each spot were streaked onto RCV minimal medium agar plates containing the appropriate antibiotic in order to purify *R. capsulatus* transconjugants. After 3-5 days of growth at 30°C, isolated colonies were restreaked onto YPS medium containing antibiotics. Although the growth of
auxotrophic *E. coli* donor cells is not possible on RCV medium, the YPS plate which permits growth of the donor cells, ensures that the exconjugant has not been contaminated with *E. coli*.

### 2.6 Gene transfer agent transduction

The GTA over-producer, *R. capsulatus* DE442, was used as the donor strain in GTA transductions. Recombinant pUC plasmids of interest from *E. coli* hosts were first transformed into the plasmid donor strain, *E. coli* TEC5. This strain possesses a mobilizable vector (pDPT51) that contains a region of homology to the pUC plasmids (Taylor *et al.*, 1983). Thus a recombination event between the recombinant plasmid and the TEC5 plasmid is thought to allow for both the transfer and maintenance of the recombinant plasmid in DE442. Mobilizable recombinant plasmids transferred to DE442 by conjugation were selected for by the acquisition of antibiotic resistance on RCV agar plates. The exconjugants were grown photosynthetically in rich YPS medium to stationary phase in order to induce GTA production. The DE442 culture was filtered through a 0.45 μm Gelman Supor membrane filter in order to remove the bacterial cells and obtain the GTA-containing filtrate.

Recipient *R. capsulatus* cells were grown aerobically in YPS medium to stationary phase and 0.1 ml of culture was pelleted by microcentrifugation and resuspended in 0.1 ml of filter-sterilized G buffer (10 mM Tris-HCl, pH 7.8, 1.0 mM CaCl₂, 1.0 mM NaCl, 500 μg/ml BSA). A volume of 0.2 ml of GTA filtrate was mixed with 0.4 ml of GTA buffer and
0.1 ml of resuspended recipient cells and incubated for 60 minutes at 35°C. During this period, GTA particles adhere to the recipient cells. After phage adsorption, 0.9 ml of YPS broth were added to the culture and the recipient cells were incubated aerobically at 30°C for 4 hours to allow genetic recombination and expression of the antibiotic resistance gene. Portions of this culture were spread on YPS plates, with the appropriate antibiotic, for the selection of transductants. Negative controls consisted of the GTA suspension without recipient cells and recipient cells in the absence of the GTA-containing filtrate.

2.7 Construction of a translationally in-frame deletion of orf162b

Digestion of *R. capsulatus* chromosomal DNA with BamH I cleaves the PGC into a series of fragments termed Bam fragments, labelled alphabetically on the basis of decreasing size (Taylor *et al.*, 1983). A 2051 bp section of the *R. capsulatus* PGC, generated via BamH I digestion and called the BamK fragment contains orf214, orf162b, orf55, orf274 (Fig. 2) and has been cloned into the BamH I site of pUC13 (Messing 1983) to create pUC13::BamK (W. Collins, personal communication).

In order to make a translationally in-frame deletion in orf162b, pUC13::BamK was cleaved with PflM I and BsaB I (restriction enzyme sites located within orf162b). This double digest removed 63% of the coding region of orf162b (Fig. 5). Treatment of the PflM I to BsaB I fragment with T4 DNA polymerase removed the 3' protruding terminus created by PflM I
Figure 5. Outline of the construction of pAH1 which contains a translationally in-frame deletion of *R. capsulatus orf162b*. *R. capsulatus* orfs are shown as unshaded arrows. The *lacZ* (α allele) and kanamycin resistance genes are shown as lightly shaded arrows. Darkly shaded arrows represent the origin of replication.
2. Treat with T4 DNA polymerase to remove 3' overhangs
3. Ligate blunt ends together

1. *Sma* I digest
2. Isolate 1.3 kb KIXX cartridge

1. *Mlu* I, *Xba* I digest to remove *BamH I* site
2. Klenow fill-in 5' overhangs
3. Ligate KIXX cartridge into former *BamH I* site

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**pUC13::BamK**

- **4.7 kb**
- **orf155**
- **orf162b**
- **lacZ**
- **orf214**
- **Bsa I**
- **BamH I**

**pUC4::KIXX**

- **4.2 kb**
- **orf1274**
- **Sma I**
- **Knf**
- **ori**

**pUC13::BamKΔ162b**

- **4.4 kb**
- **orf155**
- **orf162b**
- **lacZ**
- **orf214**
- **Mlu I**
- **Xba I**
- **BamH I**

**pAH1**

- **5.7 kb**
- **orf1274**
- **orf155**
- **orf162b**
- **lacZ**
- **Knf**
- **ori**
- **Hind III**
digestion. When the blunt ends were joined together in a ligation reaction, an in-frame deletion of orf162b on pUC13::BamK was created. The reading frame across the deletion site was confirmed by DNA sequence analysis (data not shown), and the plasmid was designated pUC13::BamKΔ162b.

Digestion of pUC13::BamKΔ162b with Xba I and Mlu I removed 81 bp including one of the two BamHI sites flanking the R. capsulatus photosynthesis genes of the BamK fragment. The Klenow fragment of DNA polymerase I was used to fill in 3' recessed termini created by the restriction enzyme digest. A kanamycin resistance (Km\(^r\)) cartridge, KIXX, was cloned into the former BamHI site. The KIXX cartridge was removed from pUC4::KIXX as a 1.3 kb Sma I fragment containing the Km\(^r\) gene as well as the first 153 bp of the 380 bp bleomycin resistance (Ble\(^r\)) gene (Barany 1985). Thus, a Km\(^r\) marker was associated with the in-frame deletion of orf162b and the entire R. capsulatus DNA segment could be removed as a Hind III to BamHI fragment from this plasmid, designated pAH1 (Fig. 5).

2.8 Construction of the suicide plasmid pAH2

The R. capsulatus BamK fragment containing the KIXX cartridge flanking orf214 was removed from pAH1 as a 3 kb Hind III to BamHI fragment and cloned into pRR1 at the Hind III/BamHI site to create pAH2 (Fig. 6). The suicide plasmid pRR1 was derived from the mobilizable plasmid pSUP203 (Simon et al., 1983) but in addition to the pSUP203 genes, a 7.5 kb BamHI to Bgl II fragment containing the lacZ gene was
**Figure 6.** Construction of the mobilizable plasmid pAH2 which contains a translationally in-frame deletion of *orf162b*. *R. capsulatus* orfs are shown as unshaded arrows. The *lacZ* and *Km* genes are shown as lightly shaded arrows. Darkly shaded arrows represent the origin of replication.
1) *BamH I, Hind III* digest
2) Ligate Km\(^{r}\) *BamH I - Hind III* fragment into pRR1
cloned into the *Bam*H I site of pSUP203 to generate a 16.1 kb plasmid (R. Reyes, personal communication). The new plasmid, pAH2, is mobilizable and, although it may be transferred by conjugation from an *E. coli* host to an *R. capsulatus* recipient, it will not be replicated in the latter cell type unless it integrates into the *R. capsulatus* chromosome. The *lacZ* gene can be expressed in *R. capsulatus*, allowing blue/red screening of cells to screen for the presence of pAH2.

2.9 Ultraviolet light mutagenesis of *R. capsulatus* recombinants

*R. capsulatus* cells were grown aerobically at 34°C to a density of about 10⁹ cells per ml. A 5 ml volume of this culture was placed into each of 5 sterile petri dishes and exposed to either no UV light (dish #1) or UV light for 30, 45, 60, or 75 seconds (dish 2, 3, 4, and 5 respectively). Besides the negative control, only dish #2 (8% survival) had a survival rate greater than 0.5%. As it was feared that the *R. capsulatus* cultures exhibiting a low survival rate ran a high risk of acquiring other mutations during the UV light exposure, the culture exhibiting an 8% survival rate was chosen for further experimentation.

2.10 Construction of a deletion mutation in pAH4 by disruption of *orf162b* with a kanamycin resistance cartridge

In a second approach to create a non-polar mutation in *orf162b*, the KIXX cartridge was inserted into the deleted *orf162b* sequence. This
cartridge rarely has a polar effect when it is inserted into a gene which is cotranscribed with genes located downstream of the cartridge insertion site (Bollivar et al., 1994). A 10 bp dephosphorylated linker (5'-AGCGGCCGCT-3') containing a Not I restriction enzyme site was inserted into the (BsaB I to PflM I) deletion site of pUC13::BamKΔ162b. This plasmid was then linearized with Not I and treated with the Klenow fragment of DNA polymerase I to fill in the 5' overhangs created by Not I digestion. The KIXX cartridge, as a 1.3 kb Sma I fragment, was ligated into the filled in Not I site (Fig. 7). The resulting plasmid was termed pAH4.

2.11 Construction of a polar mutation in the orf162b BsaB I to PflM I deletion site

In order to create a polar mutation in orf162b that would prevent the expression of orf162b as well as downstream orfs that might be transcribed from the same promoter as orf162b, the Omega (Ω) cartridge was inserted into the deletion site of pUC13::BamKΔ162b. The Omega cartridge confers spectinomycin and streptomycin resistance and contains, in the flanking regions of the fragment, transcription and translation termination signals (Prentki and Krisch 1984). The Omega cartridge was removed from pH45Ω as a 2 kb Sma I fragment. The resulting plasmid, shown in Fig. 8, was named pAH3.

2.12 Construction of the complementation plasmid pAH8
Figure 7. Construction of pAH4 which contains a non-polar mutation in a deletion site located within orf162b. *R. capsulatus* orfs are shown as unshaded arrows. The lacZ (α allele) and Km\(^r\) genes are shown as lightly shaded arrows. Darkly shaded arrows represent the origin of replication.
1) Insert 10 bp Not I linker into orf162b deletion site by linker tailing
2) Not I digest
3) Klenow fill-in 5' protruding ends
4) Ligate KIXX cartridge into Not I site

1) Sma I digest
2) Purify 1.3 kb KIXX cartridge
Figure 8. Construction of pAH3 which contains a polar mutation in the deletion site of orf162b. R. capsulatus orfs are shown as unshaded arrows. The Omega cartridge and the lacZ (α) gene are shown as lightly shaded arrows.
1) \textit{Pfu} I, \textit{BsaB} I digest
2) Treatment with T4 DNA polymerase removes 3' overhangs from the deletion site
3) Ligate the Omega cartridge into the \textit{orf162b} deletion site
An expression vector was created so that orf162b could be expressed from a plasmid in R. capsulatus orf162b mutants. Plasmid pUC13::orf162b (W. Collins, personal communication) contains orf162b as a 797 bp Cla I to EcoR I fragment, including a small region of the orf214 sequence found directly upstream of orf162b. An EcoR I linker was inserted into the Pst I site located within the orf214 sequence, by the linker tailing method, yielding plasmid pUC13::orf162b2 (Fig. 9). This allowed the orf162b sequence to be removed as a 748 bp EcoR I fragment and reduced the size of the orf214 fragment by 49 bp to 212 bp.

The EcoRI fragment containing orf162b from pUC13::orf162b2 was ligated into the mobilizable plasmid pRR5C (R. Reyes, personal communication) at an EcoR I site located downstream of a R. capsulatus puf promoter (Fig. 9). The plasmid pRR5C is a derivative of the broad host range expression vector pPUFP1 (Bollivar et al., 1994) which is capable of being maintained in R. capsulatus cells. In addition to its broad host range capabilities, pRR5C confers gentamycin resistance (Gm\textsuperscript{r}), thus following conjugation of this construct, termed pAH8, into R. capsulatus orf162b mutants, the presence of the plasmid was selected for on the basis of Gm\textsuperscript{r}.

2.13 Southern blots

A 100 ml R. capsulatus culture was aerobically grown under low oxygen conditions to early log phase (150 KU), pelleted by centrifugation and resuspended in 2 ml of 25% sucrose solution in 50 mM Tris-HCl (pH 8). Added to this solution was 5 mg of lysozyme, 0.5 ml of 0.5 M EDTA and 2
Figure 9. Construction of the orf162b complementation plasmid, pAH8. The Gm\(^r\) and lacZ (α) genes are shown as lightly shaded arrows. The puf promoter of pRR5C and pAH8 is shown as an arrow located upstream of EcoR I. The origin of replication is represented as a darkly shaded arrow.
1) EcoR I digest

Remove 748 bp orf162b sequence as an EcoR I fragment
2) Ligate orf162b into the EcoR I site of pRR5C
ml of Triton-X lysis solution (50 mM Tris-HCl [pH 8], 10 mM EDTA, 2% Triton-X-100 [octyl phenoxy polyethoxyethanol]). This suspension was mixed and incubated at 50°C for 10 minutes to allow cell lysis. One gram of CsCl per ml of lysate was added, and portions of the solution were transferred to 5.5 ml ultracentrifuge tubes containing 200 µl of EtBr solution (10 mg/ml). The tubes were centrifuged at 297,805 x g for 16 hours at 15°C. The highly visible chromosomal DNA band was removed and extracted with isopropanol equilibrated with CsCl-saturated water to remove the ethidium bromide from the chromosomal DNA. The cesium chloride was removed by dialysis in 1.0x TE buffer (Sambrook et al., 1989).

Ten µg of chromosomal DNA were digested with BamH I and electrophoresed in a 1% agarose mini-gel in 0.5x TBE buffer (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA) at room temperature. The DNA was denatured by soaking the gel in 500 ml of a 0.2 M NaOH and 0.5 M NaCl solution for 30 minutes at room temperature. To neutralize the gel, it was washed 2-3 times in 500 ml 5x TBE for 10 minutes at room temperature. The ionic composition of the gel was reduced by soaking it in 500 ml 0.5x TBE for 20 minutes.

A positively charged nylon membrane (Boehringer Mannheim GmbH) which had been cut to the size of the mini-gel was equilibrated in 0.5x TBE for 10 minutes at room temperature before being placed on the gel. DNA was transferred to the membrane by electro-blotting at 30 V in 0.5x TBE for 16 hours, followed by 80 V for 2 hours in a BIO-RAD Trans-Blot Electrophoretic Transfer Cell (BIO-RAD Laboratories). The membrane was rinsed in TBE and air dried. The transferred DNA was fixed to the nylon
membrane under vacuum in an 80°C oven for 2 hours.

The probe was created by random primed DNA labelling with digoxigenin-dUTP (DIG-dUTP) using a DNA labeling and detection kit (Boehringer Mannheim GmbH). The prehybridization, hybridization and detection procedures were carried out as per the manufacturer's instructions provided with the detection kit. Membranes were incubated with colour detection solution for 8 hours.

2.14 Northern blots

Total RNA was isolated from *R. capsulatus* cells grown under either oxygen-limited conditions or photosynthetically (anaerobically) to a density of 150 KU. A volume of 25 ml of *R. capsulatus* culture was added to a 50 ml centrifuge tube packed with ice and centrifuged for 10 minutes at 12,100 x g, and the cells were resuspended in 125 μl of 0.3 M sucrose, 0.01 M sodium acetate (pH 4.5). To lyse the cells and denature the cellular proteins, 125 μl of a 0.01 M sodium acetate (pH 4.5) and 2% SDS solution were added. The sample was immediately vortexed and heated to 65°C for 90 seconds and 250 μl of 65°C unbuffered, dH2O-equilibriated phenol were added. The sample was vortexed, cooled in an ethanol-dry ice bath for 15 seconds, centrifuged and the aqueous layer was retained. Phenol extractions were repeated on the aqueous layer 2-4 times, until the aqueous phase appeared clear. The RNA was precipitated by the addition of 30 μl of 3 M sodium acetate and 900 μl of 95% ethanol. The RNA was dissolved and stored at on ice in 180 μl of RNA storage buffer (20 mM
sodium phosphate, 1 mM EDTA [pH 6.6]).

Residual DNA was removed from the RNA preparation by addition of 30 units of DNase I in incubation buffer (100 mM sodium acetate, 5 mM MgSO₄, pH 5.0) followed by incubation for 30 minutes at room temperature. After phenol extraction and ethanol precipitation, the RNA was resuspended in 40 µl RNA storage buffer and stored at -80°C. Total RNA concentration was determined through absorbance values of the sample at 260 nm. One absorbance unit was taken to equal 40 µg RNA.

Twenty µg of total RNA were mixed with 2 volumes of sample mix (500 µl formamide, 120 µl formaldehyde and 10x MOPS buffer [0.2 M MOPS, 50 mM anhydrous sodium acetate, 10 mM EDTA, pH 6.5]) and heated to 68°C for 10 minutes to remove any secondary structure. One volume of loading buffer (5 ml glycerol, 4 ml dH₂O, 1 ml 10 mg/ml ethidium bromide was added to each sample before being loaded onto a 1.2%, 2 M formaldehyde gel and electrophoresed in 1x MOPS buffer at 30 V for 16 hours.

After electrophoresis, the gel was soaked in 0.05 N sodium hydroxide at room temperature for 20 minutes to fragment high molecular weight RNA molecules for improved transfer. The gel was then rinsed in sterile dH₂O and equilibrated 4 times in 0.5x TBE for 10 minutes. RNA was electro-blotted onto a Biotrans positively charged nylon membrane (ICN Biomedicals Inc.) in a BIO-RAD Trans-Blot Electrophoretic Transfer Cell. The transfer process was carried out in 0.5x TBE at 30 V overnight, followed by 80 V for 2 hours.
The nylon membrane was prehybridized for 3 hours in 10 ml of prehybridization buffer (12.5 ml formamide [50%], 5 ml 5x P buffer [1% BSA, 1% ficol, 250 mM Tris-HCl pH 7.5, 0.5% sodium pyrophosphate, 5% SDS, 1% polyvinylpyrrolidone], 5 ml of 50% dextran sulfate, 1.45 g of sodium chloride and denatured salmon sperm DNA to a concentration of 0.1 mg/ml) at 42°C. The probe was synthesized from the 424 bp BsaB I to EcoR I orf162b sequence removed from the BamK fragment of the R. capsulatus PGC (Fig. 2). The QIAEX agarose gel purified 424 bp fragment was used to create a $^{32}$P radio-labelled probe using a Rediprime Random Primer Labelling kit (Amersham International) in accordance with the manufacturer’s instructions. The prehybridization buffer was supplemented with approximately 50 ng of alkali denatured probe and the hybridization reaction was allowed to proceed overnight at 42°C. The hybridization of the DNA probe to its complementary mRNA sequence was visualized by autoradiography. The nylon membrane was incubated at -80°C on X-OMAT AR X-ray film (Eastman Kodak Company) with an intensifying screen for 23-72 hours.

2.15 Chromatophore isolation

Chromatophores were isolated from 100 ml cultures of R. capsulatus cells grown in RCV medium under low oxygen conditions to a density of 300 KU. These cells were disrupted by passage through a French press, (15,000 psi) to release and fragment the ICM into vesicles (chromatophores) which contain the photosynthetic apparatus. The cellular debris was removed from the suspension by centrifugation at 25,800 x g
for 8 minutes, and the supernatant was centrifuged at 412,000 x g for 14 minutes to pellet the chromatophores. This crude chromatophore preparation was resuspended in 1 ml of chromatophore buffer (20 mM 3-[N-morpholino]propanesulfonate [MOPS], 100 mM KCl, 1 mM MgCl₂ [pH 7.2]) (Jackson et al., 1986) and loaded onto a 3-layered (20%-40%-60%) sucrose step gradient in 50 mM Tris (pH 8) for further purification. The sucrose gradient was centrifuged at 68,600 x g for 17 hours at 4°C. The purified chromatophores were collected from the 20%-40% sucrose interface, diluted 1:1 in 50 mM Tris-HCl (pH 8), centrifuged at 450,000 x g for 15 minutes to pellet the chromatophores, and resuspended in 500 µl chromatophore buffer. A typical yield was 2 to 4 mg of total membrane protein.

The protein concentrations of the chromatophore preparations were determined using a modified Lowry assay (Peterson 1983). Protein absorbance values were measured at 660 nm with BSA as the standard.

2.16 Gel electrophoresis of chromatophore proteins

Chromatophore proteins were separated by electrophoresis on a sodium dodecyl sulfate (SDS) polyacrylamide gel by the Schägger and von Jagow method (Schägger and Jagow 1987). As it appeared that *R. capsulatus* SB1003 chromatophores contained a larger percentage of light harvesting complex proteins than *R. capsulatus* MW442 chromatophores, in order to visualize and compare levels of other chromatophore proteins between the 2 species, more SB1003 proteins had to be loaded on the gel.
It was empirically determined that when a total of 80 µg of chromatophore proteins from *R. capsulatus* MW442-derived strains and 110 µg of chromatophore proteins from *R. capsulatus* SB1003-derived strains were loaded per lane on the gel, most of the bands were similar in intensity. Each gel was run for 16 hours at a constant current of 18 mA and stained overnight in a 0.25% solution of Coomassie brilliant blue G in 10% methanol and 40% acetic acid.
3 RESULTS

3.1 Construction of R. capsulatus orf162b mutants

3.1.1 Construction of a non-polar mutation in orf162b

The mobilizable plasmid, pAH2, which contains a translationally in-frame deletion of orf162b (Δorf162b) within the BamK fragment as well as a Km\(^r\) cartridge inserted upstream of orf214, flanking the BamK orfs (Fig. 6), was used to attempt to create a non-polar mutation in strains SB1003 and MW442. Purified pAH2 DNA was electroporated into E. coli S17-1. The recipient E. coli cells were mated with R. capsulatus DW21, a strain which contains the Omega cartridge in the center of the otherwise intact orf162b sequence on the chromosome. Since the Omega cartridge confers spectinomycin resistance (Sp\(^r\)), the loss of orf162b can be detected by the concurrent loss of Sp\(^r\) in this strain. Because pAH2 is a suicide plasmid, recipient DW21 cells would only acquire Km\(^r\), in addition to Sp\(^r\), if pAH2 integrated into the R. capsulatus chromosome (Fig. 10).

Three recipients, DW21K1, DW21K2 and DW21K3, were selected on the basis of their ability to grow on a medium containing kanamycin. Southern blot analysis of BamH I digested chromosomal DNA using a BamK probe demonstrated that only one of these recipients, DW21K1, contained pAH2 integrated in the orf162b region (Fig. 11). Lane 1, a positive control, contained the 2 kb BamK fragment. Lane 2, contained chromosomal DNA from DW21K1. The 19 kb band shows that pAH2 had integrated into the
Figure 10. An example of how homologous recombination between strain DW21 and pAH2 would incorporate this plasmid into the host chromosome and confer Km⁺ to the recombinant organism. An alternative recombination in the orf162b to orf274 region is also possible.
Figure 11. Southern blot hybridization analysis of chromosomal DNA isolated from strain DW21K1. Lane 1 contains the BamK fragment. A strong hybridization signal at 2 kb represents the BamK fragment used in the synthesis of the probe. Lane 2 contains BamH I digested DW21K1 chromosomal DNA. The 19 kb band represents pAH2 that has been integrated into the DW21 chromosome upstream of orf162b. The 4 kb signal represents the Omega-disrupted BamK fragment.
chromosome of DW21 through a single crossover occurring upstream of orf162b (Fig. 10), while the 4 kb band represents the Omega-disrupted BamK fragment. A 4 kb hybridization signal was detected from DW21K2 DNA (data not shown), corresponding to the BamK::Omega fragment of DW21. The absence of a second band indicated that pAH2 did not integrate into this region of the chromosome, and so perhaps Km\textsuperscript{r} arose spontaneously in this strain. Similarly, when DW21K3 chromosomal DNA was probed with BamK, only one 2 kb signal was detected (data not shown). As the size of the wild type BamK fragment is 2 kb, this strain possesses neither pAH2 nor the Omega fragment, indicating once again that Km\textsuperscript{r} arose through means other than the KIXX cartridge.

In strain DW21K1, a second internal recombination event, occurring downstream of orf162b on the chromosome and within the duplicated region of the PGC, would lead to the excision of the original orf162b containing the Omega cartridge as well as the surrounding duplicated orfs and non-homologous plasmid DNA (Fig. 12). This event would result in the replacement of the original chromosomal Omega-disrupted orf162b with the deletion construct from pAH2 and the concurrent loss of both Sp\textsuperscript{r} and Km\textsuperscript{r}. However, after approximately 10,000 DW21K1 colonies were screened for the loss of Km\textsuperscript{r} and Sp\textsuperscript{r}, no such colonies were found.

Since ultraviolet (UV) light exposure stimulates RecA-dependent recombination in some species of bacteria, the R. capsulatus DW21K1 strain was exposed to UV light for 30 seconds in an attempt to induce a recombination event which would remove the suicide plasmid from the chromosome (see Materials and Methods). This culture was grown under
Figure 12. Homologous recombination between the duplicated orfs located downstream of the Omega cartridge in the PGC of strain DW21K1 would remove the plasmid DNA as well as the KIXX and Omega cartridges from the chromosome, while the Δorf162b sequence would remain.
non-selective conditions, in rich YPS medium, for several days during which time the culture was serially transferred. Samples from the third sub-culture were plated onto RCV agar plates and screened for kanamycin and spectinomycin sensitivity. Although 10 plates of 100 colonies each were tested, no such colonies were found.

Since it seemed extraordinarily difficult to obtain the above described second recombination, an alternative approach was taken to create a non-polar mutation in orf162b. It has been shown that the KIXX cartridge which contains a Km\(^r\) gene followed by the first 153 bp of the 380 bp Ble\(^r\) gene rarely has a polar effect when used in gene disruptions in which the transcriptional polarity of the Km\(^r\) gene is the same as the disrupted gene (Bollivar et al., 1994). Therefore, a KIXX disruption of Δorf162b, provided by pAH4, was used in gene replacement experiments. On pAH4, the KIXX cartridge was inserted into the orf162b deletion site (Fig. 7). If orf162b::KIXX were transcribed and translated in R. capsulatus, two fusion proteins would be produced. One of these proteins would possess 38 amino acids from the N-terminus of orf162b and 14 amino acids from the beginning of the KIXX cartridge (Fig. 13A). The second protein would have 51 amino acids from the N-terminus of the Ble\(^r\) gene followed by 24 amino acids, translated out of frame, from the 3' region of orf162b (Fig. 13B).

Plasmid pAH4 was transformed into the mobilizing strain E. coli TEC5. Plasmids such as pAH4 which are derived from pUC plasmids are able to fuse with the mobilizable plasmid in E. coli TEC5, pDPT51, by homologous recombination, and thus are transferred at a low frequency to
Figure 13. Fusion proteins created by the insertion of the KIXX cartridge into orf162b at the BsaB I to PflM I deletion site. (A) The upstream fusion protein is composed of 38 amino acids (aa) from the N-terminus of orf162b (DNA codons underlined, aa residues in bold type) and 14 aa from the beginning of the KIXX cartridge. (B) The downstream fusion protein possesses 51 aa from the N-terminus of the BleR gene (DNA codons underlined, aa residues in bold type) and 24 aa translated out-of-frame from the 3' region of orf162b. Arrows indicate the fusion sites.
A

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
atg  gca  cag  ctt  ccc  ctc  gcc  cca  cag  cgc  ccc  gag  acc  ccc  gcc  aa
M A Q L P L S P A P Q R P E T K T P G K

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
ccc  gag  gcc  gag  ctg  atc  ccc  cca  tca  cta  cca  gag  ttc  gat  ttc
P E A E L I P K P L L R A M I E R P G S

41 42 43 44 45 46 47 48 49 50 51 52
ttc  tca pga  ttc  tca  tgt  ttc  gag  gct  tat  cat  cga  taa
F S R F S C L T A Y H R *

B

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
atg  acc  gac  cca  gca  cgg  ccc  acc  ctc  cca  tca  cga  gag  ttc  gat  ttc
M T D Q A T P N L P S R D F D S T A A F

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
tat  gaa  aag  tgg  gcc  ttc  gaa  atc  gtt  ttc  cga  gac  gcc  gcc  tgg  atc  atc  ctc  cag  cg
Y E R L G F G I V F R D A G W M I L Q R

41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60
agg  gat  ctc  atg  atg  gag  ttc  ttc  gcc  cac  ccc  gcc  cgc  tct  ggt  cca  cgc  acc  ttt  ac
G D L M L E F F A H P G R S G P P N F T

61 62 63 64 65 66 67 68 69 70 71 72 73 74 75
cct  ttg  gcg  cca  caa  gca  agg  ccc  cct  tgc  aac  gta  ttg  tgc  aca  tga
P L A P T A R P P S S V F S T *
R. capsulatus cells.

The E. coli TEC5 (pAH4) cells were conjugated with R. capsulatus DE442, a GTA overproducer strain. GTA particles resemble a generalized transducing phage which randomly packages host cell DNA in 4 kb linear fragments (Yen et al., 1979). These particles are routinely used for R. capsulatus strain construction by transduction. Kanamycin resistant R. capsulatus DE442 exconjugants were assumed to contain the pAH4 plasmid, as the pDPT51 segment of the recombinant plasmid allows the plasmid to replicate in an R. capsulatus host while the pAH4 segment confers Km\(^r\).

When R. capsulatus DE442 cells are grown photosynthetically, GTA phage particles are produced which package 4 kb host cell DNA fragments which, in this case, would include segments of the TEC5::pAH4 plasmid. The GTA particles were used to transduce the LHII\(^+\) strain, R. capsulatus SB1003, and the LHII\(^-\) strain, R. capsulatus MW442. A double crossover of the homologous regions flanking \(orf162b\) on the bacterial chromosome and the KIXX-disrupted \(\Delta orf162b\) gene (from pAH4) supplied by some GTA particles, would result in the replacement of the wild type gene with the mutated version, thus conferring Km\(^r\), due to the KIXX cartridge located in the \(orf162b\) deletion site.

Four independent Km\(^r\) \(orf162b\) KIXX-disrupted mutants were selected for study and called SB1003Kan1 (SBK1), SB1003Kan2 (SBK2), MW442Kan1 (MWK1) and MW442Kan2 (MWK2). My studies of these mutants are presented in sections 3.2 through 3.4.
3.1.2 Construction of a polar mutation in orf162b

In order to construct a polar mutant of orf162b at the same site as in the non-polar KIXX-disrupted mutants, pAH3 was transformed into the mobilizing strain, E. coli TEC5. Plasmid pAH3 (Fig. 8) contains an Omega-disrupted orf162b deletion construct (∆orf162b::Omega). After an E. coli TEC5 (pAH3) and R. capsulatus DE442 conjugation, the GTA particles produced by DE442 were used to transduce the ∆orf162b::Omega segment into R. capsulatus strains SB1003 and MW442. As the Omega fragment contains both transcriptional and translational stop signals within the sequences flanking the streptomycin and spectinomycin resistance genes, a polar effect on the expression of downstream genes that are cotranscribed with orf162b would be seen when the Omega cartridge is inserted into the chromosome. If a double crossover replaced the intact chromosomal orf162b sequence with the ∆orf162b::Omega fragment, a Sp\textsuperscript{r} strain would be generated. Two Sp\textsuperscript{r} polar mutants were chosen from the transduced SB1003 and MW442 cultures for further study (see section 3.3) and called SB1003Spec (SBSpec) and MW442Spec (MWSpec).

3.2 Analysis of deletion mutants MWK1 and SBK1

3.2.1 Southern blot analyses of chromosomal DNA

The chromosomal organization of the presumably non-polar orf162b mutants was confirmed by Southern blot analysis of BamH I digested chromosomal DNA (Fig. 14). The 2 kb BamK fragment was used as a probe
Figure 14. Southern blot analysis of BamH I digested chromosomal DNA from orf162b mutants. Lane 1 contains the BamK fragment which generates a strong 2 kb signal. Lanes 2 and 6 contain DNA from the parental strains SB1003 and MW442. Lanes 5 and 9 contain SBSpec and MWSpec DNA respectively. The 1.0 and 0.7 kb fragments flanking the Omega cartridge are detected by the probe. Lanes 3 (SBK1), 4 (SBK2), 7 (MWK1), and 8 (MWK2) contain DNA from the non-polar mutants. All show a strong hybridization signal at 3.0 kb representing the BamH I fragment containing Δorf162b with the Km\textsuperscript{r} cartridge in the deletion site.
to detect the BamK or other homologous fragments in the chromosomal digests. A 2 kb hybridization signal in the control lane 1 corresponds to the BamH I restriction fragment (BamK) obtained from pUC13::BamK. Although the BamK sequence is 2 kb in length in the parental SB1003 and MW442 strains, shown in lanes 2 and 6, SBK1/SBK2 and MWK1/MWK2 should possess a 0.3 kb deletion in orf162b coupled with the insertion of a 1.3 kb Km⁰ cartridge in the deletion site, to generate a 3 kb BamH I fragment. As bands of 3 kb were seen with these strains, (Fig. 14, lanes 3, 4, 7, and 8), it was concluded from this experiment that the gene replacements had occurred as planned.

3.2.2 Northern blot analysis of R. capsulatus RNA

To determine if orf162b is transcribed in R. capsulatus and is therefore an expressed gene, a Northern blot hybridization experiment was carried out on SB1003 RNA (Fig. 15). RNA from strain DW1 was also included in this experiment so that the contribution of transcription read-through from puhA (to orf162b expression) could be determined. As DW1 possesses a polar mutation in puhA (upstream of orf162b), in this strain transcription read-through would not occur from any promoters located upstream of puhA.

An orf162b-specific DNA probe was created from a 424 bp BsaB I-EcoR I agarose gel-purified DNA fragment removed from pUC13::BamK. This sequence extended from 101 nt into the orf162b coding sequence to 32 nt into orf55 located downstream of orf162b in the PGC (see Fig. 2).
Figure 15. Northern blot analysis of \textit{R. capsulatus} RNA using an \textit{orf162b} probe. Lane 1 contains RNA harvested from SB1003 cells grown photosynthetically, while lane 2 contains SB1003 RNA purified from cells grown under oxygen-limited conditions. Lane 3 contains DW1 RNA harvested from cells grown under conditions of reduced aeration. A total of 20 \mu g of RNA was loaded per lane. The hybridization signal in lanes 1 and 2 indicates the presence of an unstable \textit{orf162b} species of approximately 4 kb. In lane 3, \textit{orf162b} mRNA was not detected.
Strong hybridization signals appeared in lanes 1 and 2 of Fig. 15, which contained SB1003 RNA harvested from cells grown either photosynthetically (lane 1) or under oxygen-limited conditions (lane 2). An apparently unstable species of approximately 3.5 to 4 kb gave the largest signal and a smear of smaller species extended down to the 0.2 kb range. Lane 3 contained DW1 RNA harvested from cells grown under oxygen-limited conditions (this strain is incapable of photosynthetic growth). When RNA from strain DW1 was hybridized with the same orf162b probe, little or no mRNA was detected, indicating that orf162b transcription originates from the puhA promoter and/or the bch promoter located approximately 10 kb upstream of puhA on the PGC (Alberti et al., 1995).

3.2.3 Photosynthetic growth studies

Photosynthetic growth experiments with the orf162b mutants were conducted using several light intensities. The growth curves presented in this thesis utilized a high light intensity of 150 μE/m$^2$/s and a low light intensity of 30 μE/m$^2$/s and were representative of the results from three separate experiments.

During photosynthetic growth under conditions of high light intensity, the LHII–Δorf162b::KIXX strain MWK1 had approximately the same doubling time as the LHII– parental strain, MW442 (Fig. 16A). However, a reduced rate of MWK1 growth compared to MW442 was observed when photosynthetic growth was measured under conditions of low light intensity (Fig. 16B).
Figure 16. Photosynthetic growth of *R. capsulatus* strains grown under variable light intensities. (A) The growth rate of the LHII- mutant MWK1 is compared to that of the parental strain, MW442, during high light and (B) low light growth (C) The growth rate of the LHII+ mutant SBK1 is compared to that of the wild type strain SB1003 during high light and (D) low light growth.
A) Growth of Nonpolar Mutant in an LHII- Background Under High Light Intensity

B) Growth of Nonpolar Mutant in an LHII- Background Under Low Light Intensity

C) Growth of Nonpolar Mutant in an LHII+ Background Under High Light Intensity

D) Growth of Nonpolar Mutant in an LHII+ Background Under Low Light Intensity
Table I. Doubling time of photosynthetically grown *R. capsulatus* cells during log-phase growth.

<table>
<thead>
<tr>
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<th>Doubling Time (g)</th>
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<tbody>
<tr>
<td></td>
<td>High Light</td>
</tr>
<tr>
<td>MW442</td>
<td>5.5</td>
</tr>
<tr>
<td>MWK1</td>
<td>5.3</td>
</tr>
<tr>
<td>SB1003</td>
<td>3.0</td>
</tr>
<tr>
<td>SBK1</td>
<td>8.2</td>
</tr>
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</table>

The photosynthetic growth rate of the LHII+ Δ*orf162b::KIXX* strain SBK1 differed from that of the parental strain under conditions of high and low light intensity. Under both conditions, strain SBK1 had a longer doubling time during photosynthetic growth than SB1003 (Figs. 16C and 16D). These data are summarized in Table I. It is interesting to note that, although *R. capsulatus* cultures usually enter the stationary phase during photosynthetic growth when the culture reaches a cell density of approximately 350-400 KU, the LHII+ SBK1 strain entered stationary phase at a culture density of 100-150 KU during high light photosynthetic growth (Fig. 16C). After 40-60 hours, exponential growth resumed until a cell density of approximately 400 KU was reached. SBK1 colonies isolated from this culture and regrown under photosynthetic conditions did not exhibit a transient early stationary phase, suggesting that cells from the original culture containing a secondary mutation overgrew the primary mutant and allowed the SBK1 culture to eventually surpass a density of
150 KU during high light photosynthetic growth.

To further characterize the photosynthetic growth of SBK1, photosynthetic growth of individual cells was evaluated on solidified RCV medium by enumeration of the number and sizes of colonies that formed. When the SBK1 strain was compared to the wild type strain SB1003 after 3 days of high light photosynthetic growth, the SBK1 colonies had a diameter of approximately 0.1 mm, whereas the wild type colonies measured 2 mm across. After an additional 3 days of photosynthetic growth, it was discovered that 1% of the SBK1 colonies were capable of continued photosynthetic growth, which was interpreted to indicate a very high frequency of a secondary mutation that suppressed the effects of the original mutation. When individual colonies of presumed suppressor mutants were isolated and cultivated aerobically on YPS agar plates, a change in pigmentation was observed for several of the colonies examined. While colonies of strain SBK1 are normally dark red when grown aerobically on agar plates, the double mutants varied in pigmentation from pale pink to brownish-red as well as yellowish-green. Cell pigmentation can be attributed to the concentration of bacteriochlorophyll and carotenoid molecules. Since different operons within the PGC encode carotenoid and bacteriochlorophyll biosynthesis genes (Fig. 2), the variation in phenotypes of the suppressor mutants and the high frequency at which they arise suggests that numerous types of mutations at several loci give rise to the suppressor mutants (see Discussion).

3.2.4 Absorption spectroscopy analysis of LH and RC complex levels
As described earlier (section 3.1.1), non-polar \textit{orf}162b deletion mutants were created in both LHII\textsuperscript{+} and LHII\textsuperscript{-} strains. The LHII\textsuperscript{+} strain, SB1003 is the wild type strain whereas the LHII\textsuperscript{-} strain has a mutation that prevents LHII formation. Spectral analysis of \textit{R. capsulatus} cells, grown under oxygen-limited conditions to induce the production of proteins involved in photosynthesis, allows quantitative comparisons of LHII, LHII and, in an LHII\textsuperscript{-} background, RC levels. The LHI complex generates an absorbance peak at 870 nm whereas LHII yields two peaks at 800 and 850 nm, and one of the RC peaks is at approximately 800 nm. Thus, in a wild type strain the LHII 800 and 850 nm peaks dominate the spectrum, while the less abundant RC 800 nm peak is obscured by the LHII peak at this wavelength. Similarly, in a wild type (LHII\textsuperscript{+}) background the less abundant LHI 870 nm peak appears as a shoulder on the LHII 850 nm peak. However, in a LHII\textsuperscript{-} background, in which the LHII 800 nm and 850 nm peaks are absent, the RC 800 nm and the LHI 870 nm peaks are visible. Since LHII absorbance can mask the LHI and RC absorbance peaks, its presence is undesirable when comparing \textit{in vivo} LHI and RC levels in different strains.

When the LHII\textsuperscript{-} \textit{orf}162b::KIXX strain MWK1 was compared to MW442, MWK1 clearly possessed lower LHI (870 nm) and RC (800 nm) levels (Fig. 17). Similarly, the LHII\textsuperscript{+} SBK1 mutant possessed less LHI in comparison to the wild type strain (evident from the decrease in the 870 nm shoulder of the LHII 850 nm peak; Fig. 18). Although it seemed that this strain also possessed less LHII, the apparent reductions in the LHII peaks could be due to reductions in RC and LHI, which contribute to these peaks (see above).
Figure 17. Whole cell absorption spectra of *R. capsulatus* LHII− strains grown under low aeration conditions. The parental strain MW442 (blue) is compared with the mutant strains MWK1 (green) and MWSpec (red). Scans were normalized by multiplication of spectra by a factor to yield a light scattering \( A_{650} \) value of 0.2.
Figure 18. Whole cell absorption spectra of *R. capsulatus* LHII⁺ strains grown under low aeration conditions. The parental strain SB1003 (blue) is compared with the mutant strains SBK1 (green) and SBSpec (red). Scans were normalized by multiplication of spectra by a factor to yield a light scattering $A_{650}$ of 0.2.
3.2.5 SDS-PAGE analysis of chromatophore proteins

In order to evaluate any changes in concentration of RC or LH complex proteins in membranes of the *R. capsulatus* mutants, SDS-PAGE analyses were carried out on chromatophore proteins purified from cells grown under conditions of low aeration. As it appeared that SB1003 chromatophores contained a higher total protein count (perhaps due to the presence of LHII) than MW442 chromatophores, a greater amount of SB1003 protein was loaded per lane on the gel. A total of 80 μg of chromatophore proteins from the LHII⁻ MW442-derived strains and 110 μg of chromatophore proteins from the LHII⁺ SB1003-derived strains were loaded per lane. Relative amounts of LH and RC proteins were compared between either LHII⁻ or LHII⁺ strains and not between the two species. The RC and LH bands were identified on the basis of their electrophoretic mobilities.

As seen in Fig. 19, there were no major changes in RC H (28 kDa), RC M (24 kDa) and RC L (21 kDa) polypeptide levels in the LHII⁺ mutant SBK1 in lane 3 compared to the wild type SB1003 strain in lane 2. Although the intensities of bands seemed to be slightly reduced in the mutant. Similarly, the levels of the three RC subunits in the LHII⁻ strain MWK1, shown in lane 6, were about the same as in the parental strain, MW442 shown in lane 5. In regard to the LH subunit levels, there was a reduction in LHI α and LHI β polypeptides in the LHII⁻ strain MWK1 (lane 6) compared to MW442 (lane 5). However, a reduction in LHI α and LHI β was not observed by this method in the LHII⁺ strain SBK1 (lane 3) when this strain was compared to the wild type strain SB1003.
Figure 19. SDS-PAGE analysis of chromatophore proteins isolated from *R. capsulatus* SB1003, MW442 and related strains grown under low aeration conditions. A total of 80 µg of chromatophore proteins from the MW442-derived strains and 110 µg of chromatophore proteins from the SB1003-derived strains were loaded per lane. Lane 1, low molecular weight standards; lane 2, SB1003; lane 3, SBK1; lane 4, SBSpec; lane 5, MW442; lane 6, MWK1; lane 7, MWSpec.
3.3 Analyses of deletion mutants MWSpec and SBSpec

3.3.1 Southern blot analysis of chromosomal DNA

The chromosomal organization of MWSpec and SBSpec was analyzed by Southern blotting using a BamK probe (Fig. 14). The presence of the 2 kb Omega cartridge within the deletion site in orf162b should have generated 3 fragments after BamH I digestion: the 2 kb Omega cartridge (which was not detected by the probe), and the 1 kb and 0.7 kb fragments, which flank the Omega cartridge on the chromosome and thus were detected by the BamK probe (Fig. 14, lanes 5 and 9). These results show that the correct chromosomal organization was obtained, consistent with the goal to replace the wild type orf162b allele with the deletion construct Δorf162b::Omega.

3.3.2 Photosynthetic growth studies

The results of photosynthetic growth experiments with the polar mutants differed from those of the non-polar mutants. In the LHII- strain MWSpec, regardless of whether photosynthetic growth occurred under conditions of high or low light intensity, the absence of orf162b (as well as the possible decrease in transcription of downstream orfs) had a negative effect on growth rate (Figs. 20A and B). Similarly, the LHII+ strain SBSpec exhibited a reduced rate of photosynthetic growth under both conditions, although these cultures, at both light intensities, grew at a faster rate than the LHII+ non-polar orf162b mutants and an early stationary phase was not observed (Figs. 20C and D). These data are summarized in Table II.
Figure 20. Photosynthetic growth of *R. capsulatus* strains grown under variable light intensities. (A) The growth rate of the LHII- polar *orf162b* mutant, MWSpec, is compared to that of the parental strain, MW442, during high light and (B) low light growth. (C) The LHII+ polar *orf162b* mutant, SBSpec, is compared to that of the wild type, SB1003, during high light and (D) low light growth.
Growth of Polar Mutant in an LHII- Background Under High Light Intensity

Growth of Polar Mutant in an LHII+ Background Under High Light Intensity

Growth of Polar Mutant in an LHII- Background Under Low Light Intensity

Growth of Polar Mutant in an LHII+ Background Under Low Light Intensity

[Graphical representations of growth curves for different conditions and strains]
<table>
<thead>
<tr>
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<th>Doubling Time (g)</th>
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<tr>
<td></td>
<td>High Light</td>
<td>Low light</td>
</tr>
<tr>
<td>MW442</td>
<td>5.5</td>
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<tr>
<td>MWSpec</td>
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<td>4.1</td>
</tr>
<tr>
<td>SBSpec</td>
<td>4.5</td>
<td>6.2</td>
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</table>

**Table II.** Doubling time of photosynthetically grown *R. capsulatus* cells during log-phase growth.

### 3.3.3 Absorption spectroscopy analysis of LH and RC complex levels

When the LHII− polar mutant, MWSpec, was compared to the parental type strain MW442, the absorbance data indicated a reduction in LHI levels as well as RC levels (Fig. 17). In the LHII+ polar mutant, SBSpec, there was a small reduction in LHI evident from the decrease in the 870 nm shoulder of the LHII 850 nm peak (Fig. 18).

### 3.3.4 SDS-PAGE analysis of chromatophore proteins

Differences in the concentrations of chromatophore proteins in the polar mutant strains were compared by SDS-PAGE analysis (Fig. 19). There was no obvious change in RC H, M and L polypeptides in the LHII+ mutant SBSpec (lane 4) compared to the wild type SB1003 strain (lane 2) and the
LHII\textsuperscript{−} mutant MWSpec in lane 7 compared to the parental strain, MW442 (lane 5). Although the intensities of these bands seemed to be slightly reduced in the SBSpec mutant. Furthermore, LH levels in the LHII\textsuperscript{+} strain SBSpec (lane 4) were about the same (or, perhaps, slightly increased) as compared to the parental strain SB1003. However, there was a small reduction in LHI \( \alpha \) and LHI \( \beta \) band intensities in the LHII\textsuperscript{−} MWSpec mutant shown in lane 7.

3.4 \textit{Trans} complementation of \textit{orf}162\textit{b} mutants

3.4.1 Photosynthetic growth studies

An expression vector, pAH8, was created so that \textit{orf}162\textit{b} could be expressed in \textit{trans} in \textit{R. capsulatus orf}162\textit{b} mutants (Fig. 9). When polar and non-polar \textit{orf}162\textit{b} deletion mutations in LHII\textsuperscript{−} and LHII\textsuperscript{+} strains were complemented with pAH8, the low light photosynthetic doubling times of the complemented mutants (Table III; doubling times for non-complemented mutants are shown in brackets) decreased to approximately the value of the parental strains in the non-polar mutants but not in the polar mutants, MWSpec and SBSpec. (As the differences in growth rates were subtle under high light conditions, this data was not included.) Therefore, complete complementation did not occur in the MWSpec and SBSpec strains. I conclude that this difference is due to the absence of expression of genes located downstream of \textit{orf}162\textit{b} in the PGC. The early stationary phase seen previously in SBK1 was eliminated by complementation.
<table>
<thead>
<tr>
<th>Doubling Time (g)</th>
<th>Low light (hr)</th>
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<tbody>
<tr>
<td>MW442</td>
<td>10.0</td>
</tr>
<tr>
<td>MWK1(pAH8)</td>
<td>12.0 (19.1)</td>
</tr>
<tr>
<td>MWSpec(pAH8)</td>
<td>19.1 (25.1)</td>
</tr>
<tr>
<td>SB1003</td>
<td>4.1</td>
</tr>
<tr>
<td>SBK1(pAH8)</td>
<td>4.2 (16.5)</td>
</tr>
<tr>
<td>SBSpec(pAH8)</td>
<td>5.2 (6.2)</td>
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</table>

**Table III.** Doubling time of orf162b-complemented *R. capsulatus* mutants during log-phase photosynthetic growth under low light intensity. Doubling times for non-complemented mutants are shown in brackets.

3.4.2 Absorption spectra

When the non-polar deletion mutant MWK1 was complemented with pAH8, spectral analysis showed that both LHI and RC levels increased to approximately the level of complexes found in the parental strain, MW442 (Fig. 21). Similarly, *trans* complementation in the polar LHII\(^{-}\) strain MWSpec(pAH8) increased LHI and RC levels (Fig. 22). However, there was a broad range of absorbance in the 770 to 800 nm range in the polar complemented strain MWSpec(pAH8) that remains unexplained.
Figure 21. Whole cell absorption spectra of LHII\textsuperscript{+} strains grown under low aeration conditions. The parental strain MW442 (blue) is compared with strains MWK1 (green) and MWK1(pAH8) (red). Scans were normalized by multiplication of spectra by a factor to yield a light scattering $A_{650}$ of 0.2.
Figure 22. Whole cell absorption spectra of LHII- strains grown under low aeration conditions. The parental strain MW442 (blue) is compared with strains MWSpec (green) and MWSpec(pAH8) (red). Scans were normalized by multiplication of spectra by a factor to yield a light scattering $A_{650}$ of 0.2.
When strain SBK1 was complemented with pAH8, LHI, LHII and possibly RC complex levels increased to levels associated with the wild type strain (Fig. 23). Trans complementation in the polar LHII⁺ strain SBSpec(pAH8) had no affect on spectral absorbance (Fig. 24). This result is not surprising since the original replacement of orf162b with Δorf162b::Omega in SB1003 had a minimal affect on absorbance values in SBSpec. It should be noted that it is possible that the dominant LHII 800 and 850 nm absorbance peaks are concealing small changes in LHI and RC complex levels in this strain.
Figure 23. Whole cell absorption spectra of LHII+ strains grown under low aeration conditions. The parental strain SB1003 (blue) is compared to strains SBK1 (green) and SBK1(pAH8) (red). Scans were normalized by multiplication of spectra by a factor to yield a light scattering $A_{650}$ of 0.2.
Figure 24. Whole cell absorption spectra of LHII+ strains grown under low aeration conditions. The parental strain SB1003 (blue) is compared to strains SBSpec (green) and SBSpec(pAH8) (red). Scans were normalized by multiplication of spectra by a factor to yield a light scattering $A_{650}$ of 0.2.
4 DISCUSSION

In order to determine if orf162b is transcribed in R. capsulatus, RNA from the photosynthetically wild type strain SB1003 was hybridized with a radioactively labelled orf162b DNA probe. This northern blot analysis, presented in Fig. 15, shows directly that a transcript containing orf162b sequence information is present in SB1003 cells grown under oxygen-limited (lane 2) as well as anaerobic photosynthetic conditions (lane 1). RNA from strain DW1 was also included in this experiment (lane 3). DW1 cells were grown under low oxygen conditions, as this strain is incapable of photosynthetic growth because the polar Omega cartridge has been inserted into the puhA gene (1.5 kb upstream of orf162b; see Fig. 2) preventing synthesis of the RC H subunit. Since strain DW1 did not contain a transcript that hybridized to the orf162b probe, the promoter of orf162b, and perhaps downstream genes cotranscribed with this orf, must be located upstream of the polar mutation in puhA. In fact, based on the size (approximately 4 kb), of the largest transcripts from SB1003 RNA detected by the orf162b probe, it seems that the orf162b transcript arises from the promoter located near the 5' end of the puhA gene (Bauer et al., 1991). However, the smear of transcripts detected in this northern blot indicates that the orf162b transcript is unstable and prone to degradation by cellular RNases. It has been shown that very large photosynthesis gene messages are so unstable that the primary transcript is not detectable in a northern blot, although segments resulting from post transcriptional cleavages may still be detected (Wellington et al., 1991). Thus, it is possible that orf162b is also part of a larger transcript originating from the bch
promoter located 10 kb upstream of *puhA* on the PGC, and that these large molecules are cleaved into smaller segments as fast as they are synthesized.

Non-polar mutations were created in the *R. capsulatus orf162b* to determine if disruption of this sequence had an effect on photosynthetic growth and synthesis of the photosynthetic apparatus. Polar mutations were also generated for two reasons. Firstly, a phenotypic difference between the two classes of mutations would indicate that the Km\textsuperscript{r} cartridge, used to create the non-polar mutants, did not have a polar effect on the expression of orfs located downstream of *orf162b*. Secondly, if the photosynthetic phenotype of the polar mutants differed from that of the non-polar mutants this would indicate that *orf162b* was transcribed along with at least one other gene situated downstream of *orf162b* in the PGC. Moreover, these genes would provide a function in the process of photosynthesis in *R. capsulatus*. To facilitate the interpretation of spectral absorbance data, polar and non-polar *orf162b* mutations were created in a LHII\textsuperscript{-} as well as a LHII\textsuperscript{+} (wild type) background.

Southern blot analyses of chromosomal DNA from the *orf162b* mutants confirmed that each strain contained a deletion in which 63% of the central coding region of *orf162b* had been removed and replaced with a KIXX cartridge (conferring Km\textsuperscript{r}) in the non-polar mutants, and an Omega cartridge (providing Sp\textsuperscript{r}) in the case of the polar mutants.

Photosynthetic growth rate comparisons of non-polar mutants to the parental strain showed that the absence of *orf162b* increases the
generation time in both LHII− and LHII+ strains of *R. capsulatus* (Fig. 16; Table I). This is pronounced under conditions of low light intensity, suggesting that there may be a deficiency in the mutants' ability to harvest light energy during photosynthetic growth. In addition, an unusual growth pattern was observed when the SB1003-derived strain SBK1, thought to be more efficient in regard to photosynthetic light harvesting than the LHII− MW442-derived strains (due to the presence of the LHII complex), was cultivated under high light conditions (Fig. 16C), whereas no such effect was seen for its LHII− counterpart, MWK1. It appears that, although the SBK1 strain initially grew quickly under high light conditions, the generation time of these cells continuously increased until cell growth was barely perceptible. After 2-4 days of photosynthetic incubation, cells that apparently contained secondary mutations which allowed normal photosynthetic growth, increased in number sufficiently to result in an increase in culture turbidity. However, the frequency at which these cells arose (10−2) is too high to be attributed solely to a random secondary mutational event. One possibility is that under conditions of stress, the expression of genes, whose protein products are involved in genetic recombination, are induced. Thus, when SBK1 cultures ceased to grow photosynthetically, a genetic pathway may have been induced which led to a high rate of genetic recombination and therefore mutation.

The growth kinetics of SBK1 resemble those of a culture that has depleted the medium of an essential substance. However, under low light conditions the premature stationary phase did not occur (Fig. 16D), and so one would have to imagine a substance that is required for rapid growth under high light but not for slow growth under low light. Furthermore,
high light cultures of the LHII\textsuperscript{−} non-polar mutant, MWK1, did not exhibit a premature stationary phase (Fig. 16A). Therefore, one would have to also imagine a substance that is required by \textit{orf162b} mutants that contain LHII but is not required by equivalent mutants that lack LHII. Since the only components of LHII that differ from LHI are the two protein subunits, the idea that the premature stationary phase of high light SBK1 cultures is caused by depletion of a substance seems unlikely.

Spectral analyses of LH and RC complex levels showed that the LHI and RC levels dropped in the LHII\textsuperscript{−} mutant MWK1 in the absence of \textit{orf162b}. Similarly, in strain SBK1 the absence of \textit{orf162b} led to decreases in absorption peaks, presumably due to decreases in the LHI and RC complexes (as in MWK1), although it is possible that there was a decrease in the amount of the LHII complex. These data suggest that \textit{orf162b} may act \textit{in vivo} to facilitate the production of a functional photosynthetic unit. That is, \textit{orf162b} alone or in conjunction with other gene products may aid in the correct assembly of the photosynthetic unit, which contains a core complex of LHI and RC in LHII\textsuperscript{−} strains, or this core complex surrounded by LHII in LHII\textsuperscript{+} strains. Perhaps in the SB1003-derived strains (which possess LHII), the assembly of the photosynthetic unit is more complex and photosynthesis is affected to a greater degree in the absence of \textit{orf162b} than in the MW442 (LHII\textsuperscript{−}) derived strains. That is, perhaps the LHII complex somehow interferes with the assembly or proper function of the core complex, conceivably by competing for membrane sites or other factors that become rate-limiting for assembly, or for electron transport, in the absence of \textit{orf162b}.
As photosynthetic units are present even in the absence of *orf162b*, I propose that assembly can occur in its absence, although the assembly of the constituent complexes occurs at a slower pace. This explains the more severe effects of *orf162b* mutation on the SB1003-derived strains, such as SBK1, since they possess more complex photosynthetic units that presumably require a greater number of protein interactions for assembly. In the 2-3 days that *R. capsulatus* cells are grown under oxygen-limited conditions in preparation for photosynthetic growth, the subunits of the photosynthetic unit are synthesized and able to gradually assemble. As these units are not utilized in aerobic respiration, cell division and growth is not affected by their concentration. When cultures of SBK1 are transferred to anaerobic, high light conditions allowing for growth only by means of photosynthesis, this strain initially grows fairly quickly by means of the pre-assembled complexes. However, with each cell division, the quantity of photosynthetic units per cell decreases because cell division occurs more rapidly than new units are assembled. As time progresses, each cell contains less and less of the photosynthetic complexes, until the level per cell drops to the point that a lack of chemical energy causes the cells to become dormant. This unusual growth pattern does not occur when SBK1 is grown photosynthetically under low light conditions because cells divide less frequently. With a doubling time of 16.5 hours, there is enough time between cell divisions for sufficient quantities of photosynthetic units to accumulate to allow for photosynthetic growth. In the MW442-derived strain MWK1, its less complex photosynthetic units assemble quickly enough in the absence of *orf162b* to allow photosynthesis to occur under low or high light conditions. In these LHII⁻ strains, the doubling time during photosynthetic growth was greater than in the parental strain only
under low light growth conditions.

The hypothesis described above has the drawback that one would think that growth of SBK1 would not completely cease upon depletion of the RC and LHI complexes by rapid cell division during high light growth, but that growth would simply slow to a rate that was governed by the rate of orf162b-independent assembly. However, this hypothesis has the advantage that it could be tested in experiments in which cells grown under high aeration were shifted to low aeration and the rates of de novo assembly of LHI and RC complexes were determined. Assembly rates would be lower in orf162b mutants if my model is correct. It would also be interesting to grow SBK1 cultures under photosynthetic high light conditions until cells entered the premature stationary phase, and harvest such cells for spectral analysis of photosynthetic complexes. Under the proposed hypothesis, such cells would be expected to contain lower amounts of RC and LHI complexes than the cultures grown in conditions of reduced aeration.

The evaluation of chromatophore proteins in SDS-PAGE showed a small decrease in LHI α and β polypeptides in the LHII− mutants but not in the LHII+ mutants (Fig. 19). Moreover, there was no detectable decrease in RC polypeptides. The absence of a large decrease in polypeptide levels is consistent with the proposed function of orf162b in assembly of a functional photosynthetic unit but not in altering the expression of genes themselves. That is, if the decreases in RC and LHI complex levels is not due to a decrease in the amount of RC and LHI polypeptides, then a post-translational process such as complex assembly must be affected by the
mutation of orf162b.

The analyses of polar mutants revealed that these mutants possess a different phenotype from the non-polar mutants. This indicates that at least one orf located downstream of orf162b has a function in photosynthesis, and is cotranscribed with orf162b. Under both high and low light conditions, the LHII+ and LHII- polar mutants exhibited a decreased rate of photosynthetic growth compared to the parental strains (Fig. 20; Table II). As the generation time of MWSpec low light photosynthetic cultures was 25.1 hours whereas the MWK1 generation time was 19.1 hours, the orfs found downstream of orf162b probably play a role in enhancing photosynthetic growth. However, strain SBSpec, with a generation time of 6.2 hours under the same conditions of low light intensity, grew better photosynthetically than SBK1 at 16.5 hours, and so in this instance the lack of transcription read-through into downstream orfs had a positive effect on photosynthesis. It is conceivable that the absence of functional gene products encoded by the orfs found downstream of orf162b slow the rate of photosynthetic growth to an extent that allows the assembly of enough photosynthetic units to support photosynthetic growth after each cell division. Regardless of the exact mechanism, it seems that one or more genes located downstream of orf162b has a different effect on photosynthesis.

When the orf162b mutants were complemented in trans with pAH8, the non-polar mutants increased in photosynthetic growth rate to a value comparable to the respective parental strains whereas the polar mutants did not (Table III). The absence of complete complementation in strains
MWSpec(pAH8) and SBSpec(pAH8) is attributed to the absence of expression of one or more genes located downstream of orf162b, and thus supports the idea that orf162b transcription continues through and is required for expression of at least one additional gene.

Spectral analysis of the complemented mutants showed that in regard to photosynthetic complex levels, approximately wild type levels of RC 800 nm and LHI 870 nm peaks were obtained in MWK1(pAH8)(Fig. 21). In MWSpec(pAH8), the LHI peak was restored to approximately the wild type level, but the RC peak was disrupted by a broad region of absorbance from about 760 nm to 800 nm (Fig. 22). A similar spectrum was obtained by Conan Young with a polar mutation in orf1696, which is located upstream of the puhA gene (Fig. 2). The basis of this unusual spectrum is not yet known, although it seems to originate from bacteriochlorophyll that is abnormally associated with proteins (Young 1997). In SBK1(pAH8) the absorption peaks closely resemble the parental strain, SB1003 (Fig. 23). In contrast to MWSpec(pAH8), the SBSpec(pAH8) spectrum is superimposable on the mutant SBSpec spectrum, both of which are deficient in the long-wavelength LHI shoulder on the 850 nm LHII peak (Fig. 24).

All of the above data indicate that not only the orf162b gene product, but also some factor derived from transcription of 3' sequences is important for photosynthetic physiology. Alterations in the level of expression of these genes have complex effects, which are manifested differently depending on the presence or absence of the LHII complex. The importance of the work described in this thesis is not so much in the provision of an exact, mechanistic explanation for how these gene products
function as it is in the discovery of these new genes, and the revelation of
the unsuspected complexity surrounding photosynthesis in this simple
bacterial system.
5 CONCLUSIONS

Very little information other than DNA sequence data was available about the orfs found downstream of puha on the PGC when I began my research, although recent studies had suggested that orf214 and orf162b are required for optimal LHI and RC levels and hence optimal photosynthetic growth (Wong et al., 1996). The northern blot analyses described in this thesis demonstrate that these orfs, and possibly others, are cotranscribed from a promoter situated upstream of puha. The additional experiments outlined in this thesis revealed that there were phenotypic differences between the orf162b mutants and the parental strains, and support the hypothesis that these orfs are genes involved in the process of photosynthesis. From the data presented in this thesis, I propose that the absence of orf162b affects the levels of functional LH and RC complexes in such a way that photosynthetic growth is inhibited. Since there is no indication that orf162b has a direct effect on LH or RC polypeptide levels, a reasonable interpretation is that this gene is involved in the assembly or interaction of components of the photosynthetic apparatus. It is also probable that at least one orf located downstream of orf162b is required for optimal photosynthetic growth. Further experimentation is necessary to elucidate the exact mode of action of the orf162b and downstream gene products in R. capsulatus. It will be interesting to see what future experiments will tell us about how these genes function in bacterial photosynthesis.
6 REFERENCES


