The Function of PufQ in the Regulation of Bacteriochlorophyll Biosynthesis in *Rhodobacter capsulatus*

by

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Abstract

The *pufQ* gene of *Rhodobacter capsulatus* is essential for the synthesis of functional amounts of the photosynthetic apparatus. Here, we present evidence for the involvement of the *pufQ* gene product in the regulation of two enzymes in the bacteriochlorophyll biosynthesis pathway in response to changing oxygen tensions. A strain (HUP35A) bearing a chromosomal point mutation in the *pufQ* gene synthesizes more bacteriochlorophyll than JS01 and JS02, but still considerably less than wildtype *R. capsulatus*. Normally, the activities of HemB and HemZ in the wildtype strain were increased by about two-fold upon transfer from aerobic to semiaerobic growth conditions. Disruption of the *pufQ* gene, either by chromosomal knockout or point mutation, leads to an incomplete induction of the enzyme specific activities of both HemZ and HemB upon transfer to semiaerobic growth conditions. Therefore, it was concluded that the proline35alanine substitution appears to have an effect on the functionality of PufQ under photosynthetic growth conditions. We also present evidence that ORF227 is an aerobic repressor of *hemZ* transcription and a hypothetical model is proposed for the function of PufQ in the inactivation of the ORF227 repressor on *hemZ* transcription under photosynthetic conditions.

The conversion of ALA acid to PBG is the second step in the bacteriochlorophyll biosynthesis pathway, and is catalyzed by HemB. A complete kinetic analysis of HemB was undertaken and the effect of two porphyrins and two porphyrinogens on HemB specific activity was also determined. Here, we present evidence that the two porphyrinogens, Coprogen and Urogen, successive intermediates in the early pathway of bacteriochlorophyll biosynthesis, act in the manner of a potent two-fold intermediate feedback inhibition on HemB activity. Both of these porphyrinogens exhibit a mixed-type inhibition of HemB. Coprogen demonstrated K_i and K_I values of 3.05 μM and 13.6 μM, respectively, while Urogen demonstrated K_i and K_I values of 8.2 μM and 7.0 μM, respectively.

To Mom and Baba

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List of Abbreviations

ALA	δ-Aminolevulinic acid
Amp	ampicillin
Bchl	bacteriochlorophyll
BPh	bacteriopheophytin
Copro	coproporphyrin III
Coprogen	coproporphyrinogen III
ddH_2O	double-distilled water
DMSO	dimethyl sulphoxide
GGPP	geranyl-geranyl pyrophosphate
HMB	hydroxymethylbilane
ICM	intracytoplasmic membrane
Gm	gentamycin
kb	kilobases
kDa	kilodalton
Km	kanamycin
LHI	light harvesting I (B875)
LHII	light harvesting II (B800-850)
Mg Proto	magnesium protoporphyrin IX
mRNA	messenger RNA
ORF	open reading-frame
PBG	porphobilinogen
Proto	protoporphyrin IX
Protogen	protoporphyrinogen IX
RC	reaction centre
rpm	revolutions per minute
SDS	sodium dodecyl suphate
Tc	tetracycline
TCA	trichloroactetic acid
Uro	uroporphyrin III
Urogen	uroporphyrinogen III

1.0 Introduction

1.1 Bacterial Photosynthesis

Oxygenic photosynthesis, which uses chlorophyll *a* as the primary pigment, is shared by higher plant and algal chloroplasts, cyanobacteria and prochlorophytes. Anoxygenic photosynthesis of eubacteria uses a variety of similar pigments, termed bacteriochlorophyll *a-g*. Anoxygenic (or bacterial) photosynthesis involves the direct conversion of light energy to cellular energy in the form of ATP. In general, light-harvesting pigments embedded in membranes capture light energy and transfer that energy to the reaction center (RC). Here, the energy is converted into excited, low potential electrons. These low potential electrons are fed into an electron transport chain, where they "fall" through a series of electron carriers, generating a proton-motive force. Membrane-bound ATP synthases then use the proton-motive force to make ATP. Although purple bacterial RC's are related to the photosystem II RC of higher plants, they cannot oxidize water, and thus do not produce molecular oxygen. They are also not able to reduce $NADP^+$ to NADPH using electrons derived from water, and maintain their metabolic redox balance by oxidation of organic or reduced sulfur compounds.

Rhodobacter capsulatus, which contains bacteriochlorophyll *a* (Bchl), is a typical gram-negative facultative photosynthetic bacterium which offers a simple and attractive model for the study of the regulation of photosynthetic gene expression by light and oxygen. This purple non-sulfur bacterium benefits from a diverse metabolism allowing it

to respond and grow under a considerable range of environmental conditions. In the presence on an oxidizable carbon source such as malate (Weaver *et al.*, 1975) and sufficient oxygen levels, this bacterium can obtain its energy from aerobic respiration and oxidative phosphorylation. The ability to grow non-photosythetically demonstrates the importance of *R. capsulatus* as a model organism. For example, it enables researchers to study photosythetically defective mutants, a difficult task in higher plants.

When external oxygen levels decrease, a number of physiological changes at the cellular level allow for a change in metabolism from aerobic respiration to photosynthesis. Bacterial photosynthetic growth is dependent on the synthesis of new membrane components (Chory *et al.*, 1984), along with the pigment cofactors and apoproteins required for the photosynthetic apparatus. Upon reduction of the oxygen partial pressure below 2.5%, a series of morphological and physiological changes are induced, resulting in the formation of the intracytoplasmic membrane (ICM). The amount of ICM synthesized is, within measurable limits, inversely proportional to light intensity.

Photosynthetic capability in *R. capsulatus* is dependent upon the appropriate synthesis of a large number of polypeptides, both structural apoproteins that comprise the actual photosynthetic machinery, and enzymes that synthesize pigment cofactors. The photosynthetic apparatus is localized within the ICM of *R. capsulatus* and contains three distinct pigment-protein complexes: the RC, which is the site of primary photochemical charge separation, and the two Bchl-containing light-harvesting complexes (LHI and LHII) that function to absorb visible and infrared radiation and to funnel this energy with

high efficiency to the photochemical reaction center. The various pigment-binding apoproteins act primarily as structural elements for the proper orientation within the membrane of the pigments, consisting exclusively of Bchl, carotenoids, and bacteriopheophytin (BPh). In all wildtype photosynthetic bacteria, the RC is surrounded by at least one type of light-harvesting antenna complex. In *R. capsulatus*, a core supercomplex of the RC surrounded by LHI (B870) which is in turn surrounded by a variable amount of LHII (B800-850). Other species of photosynthetic bacteria have only one (LHI), or a third antenna complex in addition to LHI and LHII. Since the light-harvesting complexes act as antennae to harvest and funnel light energy to the RC, the light-harvesting complexes are thought of as accessories to photosynthesis. Mutants lacking light-harvesting complexes can grow photosynthetically, but only if provided with very high intensity incident light (Jones *et al.*, 1992).

1.1.1 Light-Harvesting Complex I

The crystal structure of the LHI complex has not been solved for *R. capsulatus*, although the structure of LHI complexes have recently been elucidated in two related species, *Rhodopseudomonas viridis* (Ikeda-Yamasaki *et al*, 1998) and *Rhodospirillum rubrum* (Walz *et al.*, 1998). The LHI complex is always found in intimate contact with the RC, and in a fixed stoichiometry range of 12-16 LHI subunits per RC (Karrasch *et al.*, 1995). In *R. rubrum,* LHI complex subunits placed in a ring of 16 were proposed to surround the reaction center. Such a ring has just sufficient diameter to allow the free rotation of the RC within the ring. As such, the LHI-RC supercomplex can be considered to form a minimally efficient photosynthetic unit (Karrasch *et al.*, 1995).

LHI complex subunits contain two small hydrophobic polypeptides designated B875α and B875β in a 1:1 stiochiometry. The α and β polypeptides are small (50-60 amino acids) membrane spanning polypeptides which contain polar amino- and carboxy-termini, separated by a central hydrophobic stretch of amino acids (Tadros *et al.*, 1983; Tadros *et al.*, 1984). Various studies using proteolytic mapping, Western blots, and membrane-protein labeling reagents have revealed insight into the topology and orientation of these proteins within the bilayer (Hundle and Richards, 1990; Francis and Richards, 1980; Takemoto *et al.*, 1987). The carboxy- and amino-termini of both the α- and β-subunits have been localized on the cytoplasmic and periplasmic surfaces, respectively. Collectively, each αβ dimer binds a total of two carotenoid molecules, and two Bchl molecules. The Bchl cofactors are bound by conserved histidine residues contained within the membrane span (one on the α polypeptide, and one of the β polypeptide), which comprise the fifth ligand of the magnesium ion contained within Bchl (Drews, 1996).

1.1.2 Light-Harvesting Complex II

LHII is the most abundant light-harvesting species and is present at levels that are variable in relation of the reaction center complex and inversely proportional to the incident light intensity (Sturgis and Neidermann, 1996). LHII performs a similar function to that of LHI; these two complexes, together with the RC in *R. capsulatus* comprise the Photosynthetic unit. LHII exits as a dimer of two small hydrophobic polypeptides, designated B800-850α and B-800-850β. In *R. capsulatus*, each αβ dimer binds three Bchl molecules and two carotenoids. Two Bchl are bound to single conserved histidine

residues contained within the membrane span of the α- and β-subunits; the third Bchl is bound to the cytoplasmic side of the membrane by another conserved histidine located in the amino terminus of the β-subunit (Babst *et al.*, 1991; Hunter *et al.*, 1993). In *Rhodobacter sphaeroides*, these dimers are arranged in rings of nine, the central axis of which is tilted with respect to the membrane normal (Walz *et al.*, 1998).

1.1.3 Reaction Center Complex

The reaction center consists of three protein subunits, L (light), M (medium) and H (heavy), in a stiochiometry of 1:1:1. They have apparent molecular weights of 21, 24, and 28 kDa, respectively, as determined by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis analysis (Okamura *et al.*, 1982). The deduced molecular weights from DNA sequence analyses however have subsequently shown these proteins to have atypical electrophoretic mobilities. The H subunit is a fairly hydrophilic protein with an deduced molecular weight of 28 kDa, whereas the L and M subunits, which are both very hydrophobic proteins, have deduced molecular weights of 31 kDa and 34 kDa, respectively (Williams *et al.*, 1983; 1984; 1986).

The structure of the reaction center has not been solved for *R. capsulatus*, although X-ray crystal structures of *R. viridis* (Deisnhofer *et al.*, 1984, Lancaster and Michel, 1999) and the closely related species, *R. sphaeroides* (Allan *et al.*, 1986; Ermler *et al.*, 1994), have been obtained. These two structures show considerable similarity within these bacterial species, so there may also be conservation of this structure in *R. capsulatus*. In *R. sphaeroides*, the L and M subunits bind four molecules of Bchl, two

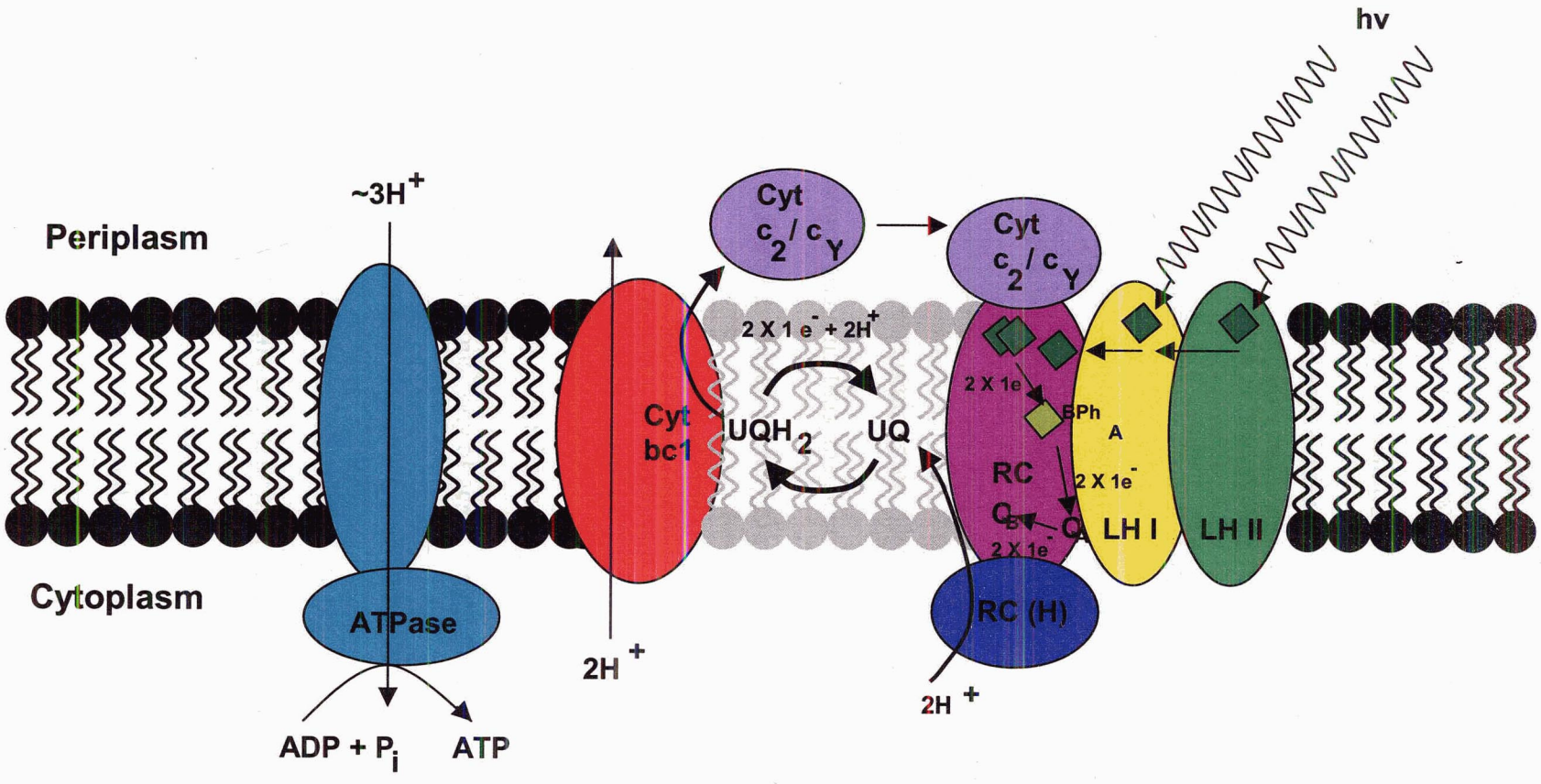
molecules of BPh *a*, two ubiquinones (Q_A and Q_B), a non-heme iron atom, and a single carotenoid. The L and M subunits of the reaction center are approximately symmetric about a plane normal to the membrane surface. Except for the single carotenoid molecule, the pigment cofactors are also structurally symmetric.

The L and M subunits are essential for reaction center activity. The H subunit binds no pigments and is not required for electron transfer *in vitro*, but is required for *in vivo* photosynthetic growth (Feher and Okamura, 1978). The H subunit may serve as a structural anchor protein for maintaining the proper association of L and M subunits within the membrane (Debus *et al.*, 1985; 1986). Of the four molecules of Bchl that the L and M subunits bind, two molecules, called the special pair, is where photo-oxidation occurs. The other two molecules are called the voyeur Bchl molecules since their participation in electron transfer has not yet been conclusively demonstrated.

1.1.4 The Mechanism of Bacterial Photosynthesis

The events leading to charge separation with the RC are well characterized, and are schematized in Figure 1. Electron flow in the bacterial photosystem begins when the antenna (light harvesting) pigments absorb a photon of light energy. The energy is then passed on as an exciton to the special pair of Bchl molecules in the reaction center (denoted P870 because of its near-infrared absorption maximum), and an electron within the special pair becomes excited. This excited electron is quickly transferred to the BPh molecule in the L subunit of the reaction center. The electron transfer process leaves the special pair with a positive charge which is subsequently neutralized by an electron from

Figure 1: Schematic of the Photosynthetic Apparatus. See Section 1.1.4 for a description of the events subsequent to photo-oxidation that lead to cyclic electron flow and the production of ATP from light energy.


hv
~3H+
Periplasm
Cyt c2 / cY
Cyt c2 / cY
2 X 1 e- + 2H+
2 X 1e-
BPh
A
Cyt bc1
UQH2
UQ
RC
QB
QA
2 X 1e-
LH I
LH II
Cytoplasm
ATPase
RC (H)
2H+
2H+
ADP + Pi
ATP

a reduced cytochrome c_2 molecule on the periplasmic side of the bacterial photosynthetic membrane. The pathway continues with the reduced BPh molecule reducing the ubiquinone molecule bound at the Q_A site, resulting in a semiquinone radical. As the electron passes from Q_A to Q_B, a ubiquinone at the end of the other prosthetic group spiral, Q_B, is converted to a semiquinone radical. A second exciton is absorbed and the flow of a second electron to Q_B is repeated. Two protons from the cytosol are simultaneously added to Q_B^{2-} forming the fully reduced ubiquinol species. Finally the reduced ubiquinol is released from the bacterial reaction center into a mobile pool of ubiquinone which shuttles electrons to the cytochrome bc_1 oxidoreductase complex that transports protons from the cytoplasm to the perplasmic space. The F_oF_1 ATP synthase complex subsequently utilizes this proton gradient; thus, the electrochemical potential gradient created by the photosytem is stored as chemical energy in the form of ATP.

1.2 The Biosynthesis of Bacteriochlorophyll *a* and Carotenoids

Photosynthetic ability requires the capacity to synthesize pigment molecules in ratios that are necessary for photosynthesis. The majority of genes required for photosynthesis encode enzymes that synthesize these pigment cofactors. These genes are divided into three main classes: *hem* genes which synthesize the heme precursor protoporphyrin IX (Proto), *bch* genes, which synthesize Bchl from Proto, and the *crt* genes, which synthesize carotenoids from isopentyl pyrophosphate and dimethylallyl pyrophosphate. The organization of the *bch* and *crt* genes in the chromosome will be discussed in greater detail in Section 1.3.1.

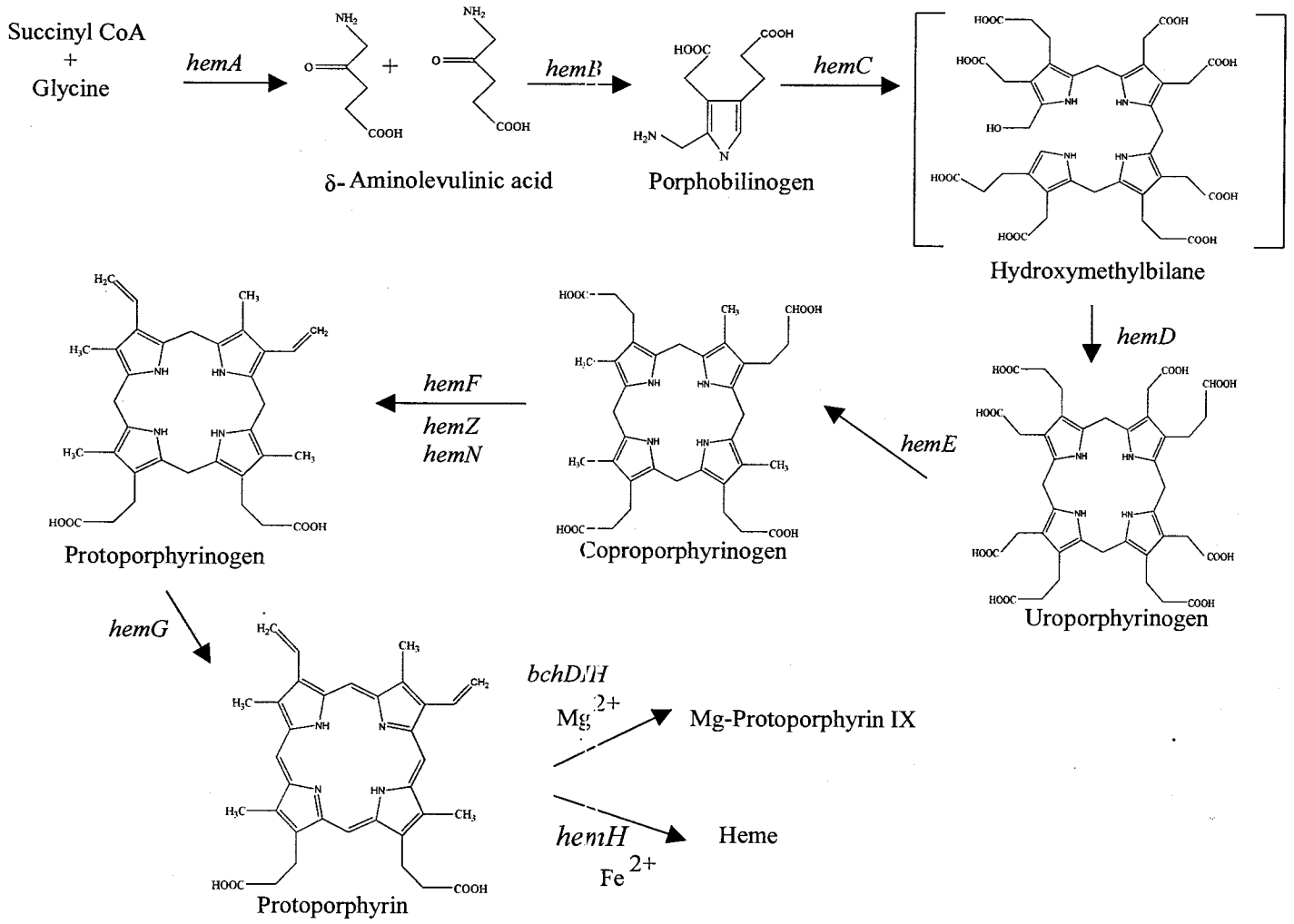
1.2.1 Enzymes and Intermediates Shared by the Heme and Bacteriochlorophyll *a* Biosynthetic Pathways

The early stages of the biosynthesis of Bchl in purple bacteria shares a pathway with heme synthesis, as outlined in Figure 2. The synthesis of either end product begins with the condensation of glycine and succinyl-CoA to form δ-aminolevulinic acid (ALA). This reaction is catalyzed by ALA synthase, encoded by *hemA*. Two molecules of ALA are then condensed by porphobilinogen (PBG) synthase, encoded by the *hemB* gene, to form one molecule of PBG. Four PBG molecules are first condensed in a linear chain by hydroxymethylbilane (HMB) synthase, encoded by *hemC*. HMB is then cyclized by uroporphyrinogen III (Urogen) synthase, encoded by *hemD*, to form the first cyclic tetrapyrrole intermediate, Urogen. The enzymatic activity of these two steps is commonly referred to as PBG deaminase. The synthesis of coproporphyrinogen III (Coprogen) from Urogen is accomplished by Urogen decarboxylase, encoded by *hemE*. Protoporphyrinogen IX (Protogen) is synthesized from Coprogen by Coprogen oxidase, which in aerobic organisms is encoded by *hemF*, and in anaerobic organisms is encoded by the *hemN* and *hemZ* genes. The final intermediate common to heme and Bchl, Proto, is synthesized from Protogen by Protogen oxidase, encoded by *hemG* or *hemY*. The pathways then diverge with heme biosynthesis accomplished with the insertion of Fe^{2+} into Proto by ferrochelatase (encoded by *hemH*).

1.2.1.1 Formation of Porphobilinogen by Porphobilinogen Synthase

PBG synthase, a metalloenzyme catalyzing the condensation of two molecules of ALA with the elimination of two water molecules to form PBG, has been extensively

Figure 2: Early Intermediates of the Biosynthetic Pathway Common to Heme and Bacteriochlorophyll Biosynthesis. The enzymes that catalyze each of these steps are encoded by the *hem* genes and are annotated above and below the arrows.


Succinyl CoA
+
Glycine
hemA
δ- Aminolevulinic acid
hemB
Porphobilinogen
hemC
Hydroxymethylbilane
hemD
Uroporphyrinogen
hemE
Coproporphyrinogen
hemF
hemZ
hemN
Protoporphyrinogen
hemG
Protoporphyrin
bchDIH
Mg 2+
Mg-Protoporphyrin IX
hemH
Fe 2+
Heme

studied by Spencer and Jordan (1994). The enzyme has been isolated from *E. coli* (Spencer and Jordan, 1993) human erythrocytes (Gibbs *et al.*, 1985), bovine liver (Jordan and Seehra, 1955), avian erythrocytes (Schmidt and Shemin, 1955) and yeast (Borralho *et al.*, 1990). The *E. coli* enzyme, like the mammalian enzyme, is octameric and binds two zinc ions per subunit at sites α and β distinguished by high affinity for Zn^{2+} at pH8 and 6, respectively (Spencer and Jordan, 1994). The enzyme from *R. sphaeroides* binds two Mg^{2+} ions per subunit as occurs with many other bacterial PBG synthases.

The enzyme has two substrate-binding sites denoted P and A that sequentially bind ALA. The P active site contains a lysine ε-amino group. This group binds the C-4 oxo group of the first ALA molecule in a Schiff's base that eventually supplies the propionate (P) side chain and pyrrole nitrogen of PBG. The nature of the A binding site is less clear but thought to contain a divalent metal ion, a zinc or magnesium ion at the "active" α site, to which the second ALA molecule is bound, possibly through a second Schiff's base with its C-4 oxo group, and thus supplies the acetate (A) and methylamino groups of PBG. Occupancy of the α site by a divalent metal ion at the β "non-catalytic" metal binding site is probably required for proper protein conformation and is only essential for activity when the α site is occupied by a magnesium ion. The PBG synthase from *R. sphaeroides* has magnesium ions at the α and β sites but only reaches full activity in the presence of potassium ions that bind two multimers to give the larger optimal multimeric form of the enzyme.

1.2.1.2 Formation of Protoporphyrinogen IX by Coproporphyrinogen III Oxidase

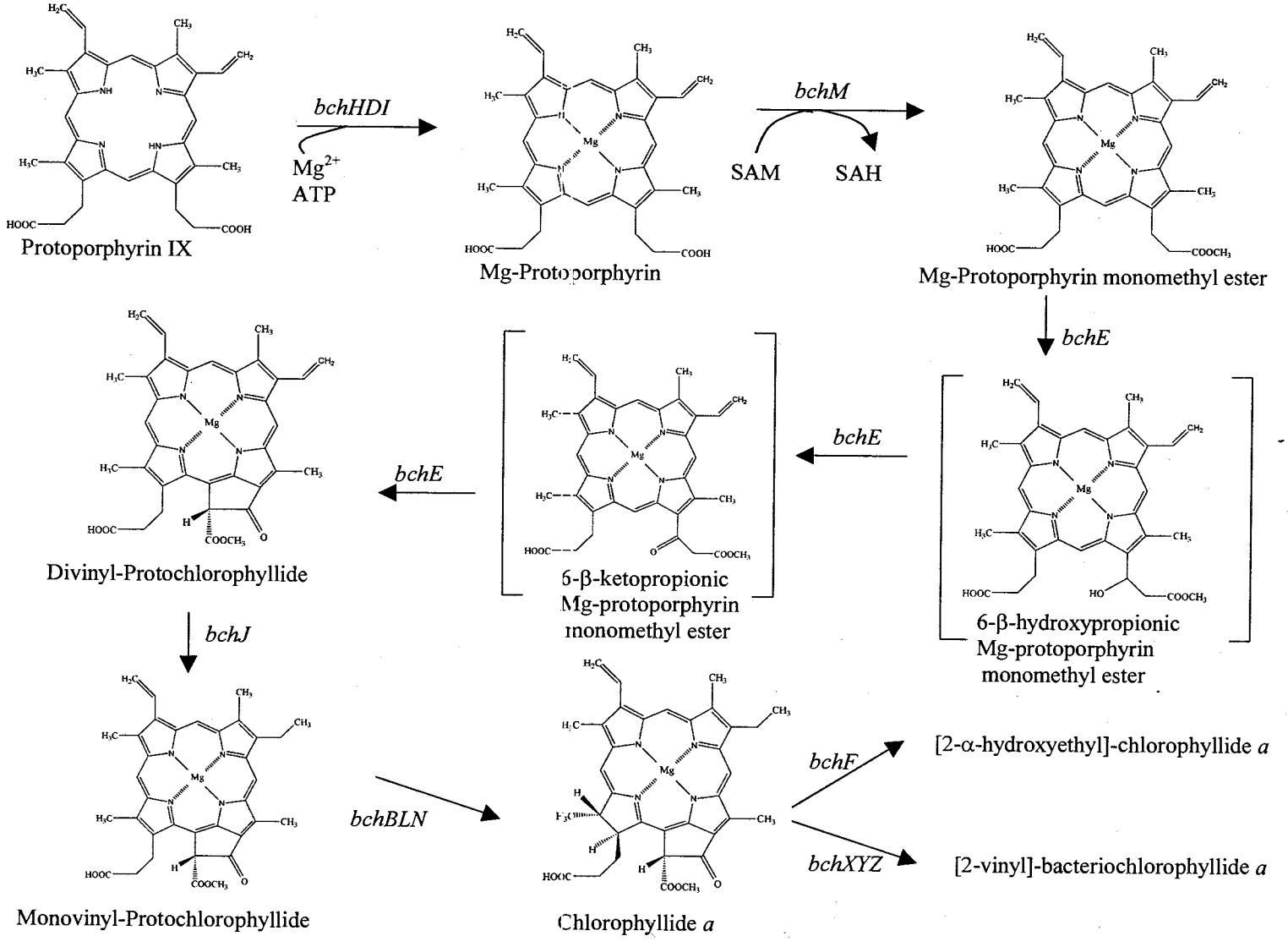
Studies with *E. gracilis* showed that Coprogen oxidase catalyzed the oxidative decarboxylation of the C-3 and C-8 propionate substituents of Coprogen, in that order, to form the C-3 and C-8 vinyl groups of Protogen (Figure 2). This reaction occurs aerobically in cells from mammalian, avian, higher plant, some algal and some bacterial sources. In extracts of *R. sphaeroides*, the anaerobic conversion requires ATP, L-methionine and $NADP^+$ (Tait, 1972), and is enhanced by the inclusion of NADH.

1.2.2 Biosynthesis of Bacteriochlorophyll *a* From Protoporphyrin IX

The latter part of the Bchl pathway, encoded by *bch* genes, involves the synthesis of Bchl from Proto. The pairing of a particular enzyme activity (and its cognate intermediate substrate) with an open reading frame has been suggested by phenotypic mapping studies (Richards and Lascelles, 1969; Yen and Marrs, 1976; Biel and Marrs, 1983; Taylor *et al.*, 1983; Yang and Bauer, 1990; Bollivar *et al.*, 1994). In such cases, a mutation that renders a particular gene product inactive causes the accumulation of an intermediate upstream of the enzymatic step in question. The distinct spectral properties of these intermediates facilitate the identification of the intermediate and consequent correlation with the mutation responsible for its accumulation.

The first committed step of Bchl synthesis employs a magnesium chelatase to insert Mg^{2+} into Proto forming magnesium protoporphyrin IX (Mg Proto) (Figure 3). The chelatase is a complex of the *bchD, H* and *I* gene products (Gibson *et al.*, 1995). The BchH protein appears to be necessary to efficiently accomplish the methyltransferase step

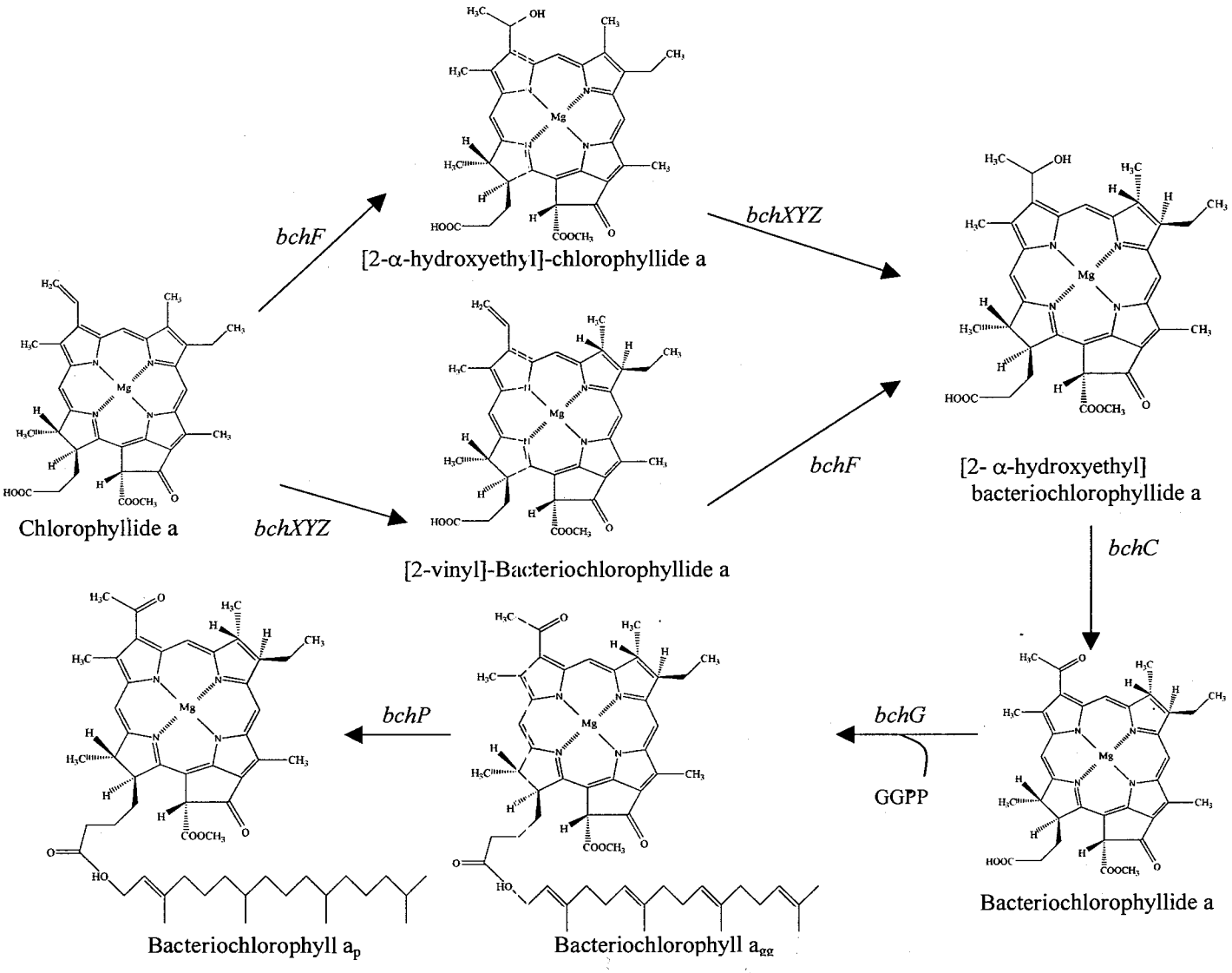
Figure 3: Biosynthesis of Chlorophyllide *a* From Protoporphyrin IX. The enzymes that catalyze each of these steps are encoded by the *bch* genes, and are annotated above the arrows. The structures of proposed intermediates are indicated by square brackets. SAM and SAH represents S-L-adenosyl-methionine and S-L-adenosyl-homocysteine, respectively.


Protoporphyrin IX
bchHDI
Mg2+
ATP
Mg-Protoporphyrin
bchM
SAM
SAH
Mg-Protoporphyrin monomethyl ester
bchE
6-β-hydroxypropionic
Mg-protoporphyrin
monomethyl ester
bchE
6-β-ketopropionic
Mg-protoporphyrin
monomethyl ester
bchE
Divinyl-Protochlorophyllide
bchJ
Monovinyl-Protochlorophyllide
bchBLN
Chlorophyllide a
bchF
[2-α-hydroxyethyl]-chlorophyllide a
bchXYZ
[2-vinyl]-bacteriochlorophyllide a

(Gorchein *et al.*, 1993; Hinchigeri *et al.*, 1997), a step that is catalyzed by the *bchM* gene product, S-adenosyl-L-methionine: Mg Proto methyltransferase (Bollivar *et al.*, 1994). These four proteins, most probably, form an active, stable complex that accomplishes the first steps of the magnesium branch of the Bchl synthesis pathway. The synthesis of Bchl continues by the formation of a fifth ring derived from the methyl propionate side chain attached to ring C, converting Mg Proto monomethyl ester to 2,4-divinylprotochlorophyllide. Studies involving the intermediate that accumulates in a *bchE* mutant has suggested that this is probably a multi-step reaction catalyzed by the *bchE* gene product (Bollivar *et al.*, 1994). Similar reasoning has been applied to the function of the *bchJ* gene product and the 2,4-divinylprotochlorophyllide 4-vinyl reductase step. A second double bond reduction step in D ring is accomplished by the *bchL, bchB,* and *bchN* gene products, to produce chlorophyllide *a* (Bollivar *et al.*, 1994; Yang and Bauer, 1990). These three enzyme subunits have recently been overexpressed in *R. capsulatus,* purified, and have also been shown to form a tight complex (Fujita and Bauer, 2000).

The next two reactions, the conversion of the 2-vinyl group attached to ring A to a 2-α-hydroxymethyl group, and the reduction of a double bond in ring B, are performed by the *bchF* and *bchXYZ* gene products, respectively (Figure 4). These assignments were concluded when the simultaneous elimination of *bchF* and one of *bchX, bchY*, or *bchZ* were deemed necessary to elevate levels of chlorophyllide *a* in the cell (Bollivar *et al.*, 1994). The oxidation of the 2-α-hydroxyethyl group on ring A to an acetyl group is catalyzed by the *bchC* gene product. The penultimate modification (transfer of a geranylgeranyl group from geranylgeranyl pyrophosphate (GGPP) (Figure 4) to the ring

Figure 4: Biosynthesis of Bacteriochlorophyll *a* From Chlorophyllide *a*. The enzymes that catalyze each of these steps are encoded by the *bch* genes, and are annotated above the arrows. GGPP represents geranylgeranyl pyrophosphate.


bchF
[2-α-hydroxyethyl]-chlorophyllide a
bchXYZ
Chlorophyllide a
bchXYZ
[2-vinyl]-Bacteriochlorophyllide a
bchF
[2- α-hydroxyethyl]
bacteriochlorophyllide a
bchC
bchG
GGPP
Bacteriochlorophyllide a
bchP
Bacteriochlorophyll a_gg
Bacteriochlorophyll a_p

15b

D propionate) is achieved by the *bchG* gene product (Oster *et al.*, 1997). The final modification is accomplished by the *bchP* gene product, which catalyzes three reductions within this side chain to ultimately produce a phytyl tail (Addlesee and Hunter, 1999).

1.2.3 Biosynthesis of Carotenoids

Carotenoids represent one of the most widely distributed and structurally diverse classes of natural pigments. There are two major roles for carotenoids in bacterial photosynthesis. Firstly, they absorb light in the visible region of the spectrum and transfer it via Bchl molecules to the photosynthetic RC, the site of primary charge separation. Carotenoids thereby increase the cross section of radiant energy ultimately transferred. Secondly, they provide photo-oxidative protection. The isolation of pigment mutants of *R. sphaeroides* more than 40 years ago allowed the photo-protective role of carotenoids to be identified. Although carotenoids are not absolutely required for viability under strictly anaerobic photosynthetic conditions, they protect against the potentially damaging combination of oxygen, light and photosensitizing Bchl molecules by quenching both the triplet excited states of the photosensitizers and the singlet excited state of oxygen. Hence, they are essential during the transition from aerobic to photosynthetic growth conditions.

The *crt* designation for genes that encode carotenoid biosynthesis enzymes has now been adopted by most researchers in the field (Armstrong, 1994; Botella *et al.*, 1995; Hirschberg, and Chamovitz, 1994). The first biochemical reaction that is specific to the production of carotenoids is the formation of C_{40} phyotene by the condensation of two

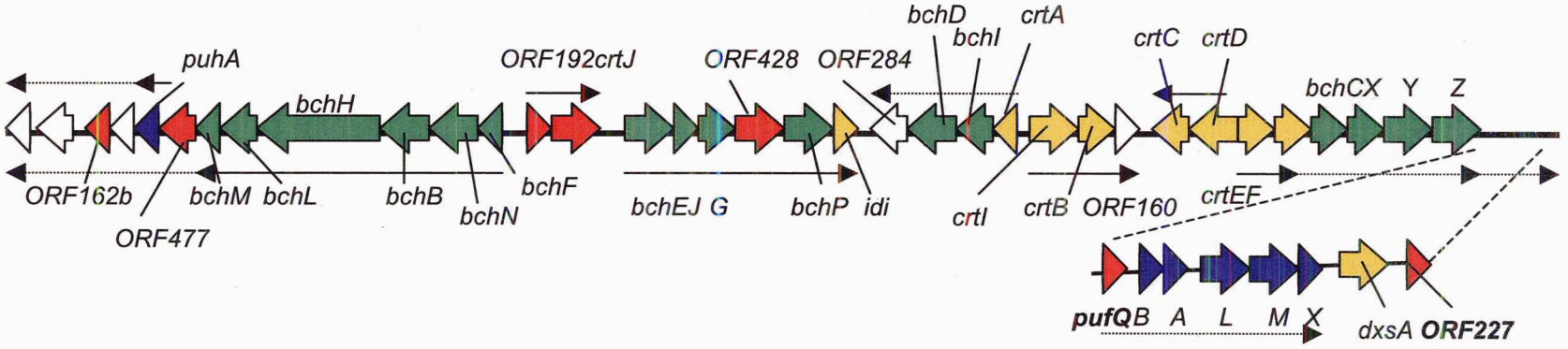
molecules of GGPP catalyzed by CrtB, the phytoene synthase. Thereafter, the products of five *crt* genes are thought to be required for a series of reactions that include desaturations mediated by the structurally related CrtI and CrtD proteins, hydration carried out by CrtC, methylation conducted by CrtF, and the addition of an oxo group catalyzed by CrtA, yielding the red-colored end products.

1.3 Genes Required for Photosynthesis

1.3.1 The Photosynthetic Gene Cluster

The first photosynthetic genes to be mapped in a photosynthetic bacterium were those of *R. capsulatus* (Yen and Marrs, 1976) (Figure 5). These essential genes, including *bch* genes for the magnesium branch of Bchl biosynthesis and *crt* genes for carotenoid biosynthesis, are contained within a relatively small portion of the chromosome. In *R. capsulatus*, these core genes are all contained within roughly 50 kilobases (kb) of the chromosome, representing slightly more than 1% of the total genome (Alberti *et al.*, 1995). The cluster extends from the *puh* operon (containing *puhA* encoding the H subunit of the RC) to the *puf* operon. The various *bch* and *crt* genes are organized into several operons grouped in between the *puf* and *puh* operons. The *puf* operon consists of six genes, *pufQBALMX*. The gene product of *pufQ* appears to be involved in the regulation of Bchl synthesis (Section 1.7). The next two genes, *pufA* and *pufB* encode the α- and β-polypeptides of the B875 LHI complex, while *pufL* and *pufM* encode the L and M polypeptides of the RC complex. The gene product of *pufX*, has recently been shown to play a critical role in facilitating the interaction between the RC-LHI supercomplex and other components required for light-driven cyclic electron transfer (Recchia *et al.*, 1998).

Figure 5: The Photosynthetic Gene Cluster of *Rhodobacter capsulatus*. Physical map of the photosynthetic gene cluster, showing the location of open reading-frames within the cluster, and the promoters required for their transcription. Arrows denoting open reading-frames are filled according to functions assigned to each reading frame. Bold type identifies two of the three genes (*pufQ* and *ORF227*) investigated in this thesis.



18b

Regulation

Structural Genes

BchI Biosynthesis

Carotenoid Biosynthesis

Unassigned Function

Promoter and limit of operonic transcription

Proposed superoperonic transcription limit

The association of PufX with the α-subunit of LHI in the membrane facilitates efficient exchange of reduced ubiquinone from the Q_B site of the RC with the quinone pool in the membrane (Barz *et al.*, 1995; Francia *et al.*, 1999). PufX may also be involved in structural organization of the LHI-RC complex (Frese *et al.*, 2000).

The molecular genetics of carotenoid biosynthesis in anoxygenic photosynthetic bacteria have been analyzed in detail in two species of the genus *Rhodobacter* (Alberti *et al.*, 1995; Armstrong, 1995). Clusters of seven carotenoid biosynthesis genes, *crtA, crtB, crtC, crtD, crtE, crtF* and *crtI* were first molecularly characterized in *R. capsulatus* (Armstrong *et al.*, 1989; Armstrong *et al.*, 1993; Bartley *et al.*, 1989; Young *et al.*, 1992), and, shortly thereafter, in *R. sphaeroides* (Toledo *et al.*, 1992; Lang *et al.*, 1994; Lang *et al.*, 1995). The genomic organization of the *crt* genes suggests the existence of a minimum of four operons: *crtA, crtIB, crtDC, crtEF* (Armstrong *et al.*, 1989; Lang *et al.*, 1995). However, the phenotypes resulting from polar mutations, in addition to transcript mapping experiments, indicate the possible existence of separate promoters for *crtI, crtB, crtD, crtC*, with two promoters for *crtE* (Armstrong *et al.*, 1990; Beatty, 1995; Giuliano *et al.*, 1988; Lang *et al.*, 1994; Lang *et al.*, 1995).

Some of the pigment biosynthetic operons are organized into superoperons that have read-through transcription from one discrete operon into another. Indeed, the operons containing *crtA, crtE*, and *crtF* are embedded within superoperons that allow their cotranscription with *bch* genes, and with the *puf* genes that encode the B870 LHI and RC polypeptides (Lang *et al.*, 1995; Young *et al.*, 1989; Young *et al.*, 1992).

Isolation of these gene superclusters by *in vivo* functional complementation of point mutations, led to the generation of transposon- and interposon-tagged pigment-deficient mutants, and to the alignment of physical and genetic maps of chromosomal regions required for Bchl and carotenoid biosynthesis (Coomber *et al.*, 1990; Giuliano *et al.*, 1986; Giuliano *et al.*, 1988; Marrs, 1981; Pemberton and Harding, 1986; Scolnik *et al.*, 1980; Taylor *et al.*, 1983; Yen and Marrs, 1976). One example of superoperon activity comes from Wellington *et al.* (1992) who showed read-through transcription from *bchFNBHLM* through ORF477 to *puhA* and including several uncharacterized ORF's downstream of *puhA*. The promoter sequence of the *bchFNBHLM* operon is not yet characterized. An *E. coli* promoter sequence upstream of the *bchF* gene has been identified but it is not yet clear if this sequence functions as a promoter in *R. capsulatus* (Beatty, 1995). The long superoperon transcript is trimmed to produce the more stable 1.1 kb *puhA* transcript (Bauer *et al.*, 1991), which also has a promoter of its own (Wong *et al.*, 1996). A further example of superoperonal organization is shown by the overlapping transcripts of the *crtEF, bchCXYZ* and *pufQBALMX* operons in *R. capsulatus*. Wellington *et al.* (1991) demonstrated that though each of the operons can be expressed from its own promoter, read-through transcription is possible starting at a promoter upstream of *crtEF* (Armstrong *et al.*, 1989; Giuliano *et al.*, 1988) and continuing through the *bchCXYZ* and *pufQBALMX* operons (Beatty, 1995).

The physiological consequence of this type of superoperonal gene arrangement has been reviewed (Wellington *et al*, 1988). A variety of different factors synthesized in balanced quantities are required for photosynthetic ability and the organization of

operons into superoperons allows for this coordination of transcription. The result of constitutive low-level expression of both upstream operons is that basal levels of light-harvesting and reaction center polypeptides are synthesized during aerobic growth. This is important during transitions from aerobic to anaerobic/photosynthetic growth conditions since the presence of pigment-binding polypeptides allows aerobically grown cells to more rapidly form the photosynthetic apparatus. The superoperonal organization of the photosynthetic cluster contributes to the coordinated and stoichiometrically balanced expression of the enzymes that synthesize photosynthetic pigment molecules and their associated structural apoproteins.

1.3.2 Genes Outside the Photosynthetic Gene Cluster

In *R. capsulatus*, there are genes required for photosynthesis located outside of the photosynthetic gene cluster, such as those encoded by the *puc* operon for the auxiliary LHII peripheral antenna polypeptides (Alberti *et al.*, 1995). However, it may be possible that the photosynthetic cluster itself may represent the minimal set of additional genes required for photosynthesis when combined with genes encoding proteins also required for respiration (eg. *cycA*, *hem* genes and the *pet* operon). Regulatory genes encoded elsewhere on the chromosome (*eg. reg* and *fnr* genes) are then relied upon to integrate photosynthetic capability with other forms of cellular metabolism.

The *puc* operon is located on a portion of the chromosome that is found some distance from the photosynthetic gene cluster (Fonstein *et al.*, 1992). As such, it is presumably an example of genes that are important, though not essential for

photosynthesis. Five genes are encoded by the *puc* operon, *pucBACDE*. As with the *puf* operon, PucB and PucA are the light-harvesting II β- and α- polypeptides, respectively. The *pucC* gene product has been shown to be necessary for normal LHII levels in the cell, but there is some discrepancy as to the means by which this is achieved (Tichy *et al.*, 1989). The most convincing evidence presented so far suggests that PucC is necessary for the assembly of the LHII complex (LeBlanc *et al.*, 1999). The *pucD* and *pucE* open reading frames encode proteins of yet unassigned function, although the *pucE* gene product has been shown to be necessary for stability of a functional LHII complex (Tichy *et al.*, 1991; LeBlanc *et al.*, 1999).

The *pet* operon encodes the prosthetic group-containing subunits of the cytochrome bc_1 complex. Active cytochrome bc_1 complexes have been purified to homogeneity from four photosynthetic purple non-sulfur bacteria. The complexes isolated from *R. rubrum* (Kriauciunas *et al.*, 1989; Majewski and Trebst, 1990), *R. capsulatus* (Robertson *et al.*, 1993) and *R. viridis* (Cully *et al.*, 1989) contain only three subunits. The genes encoding the three prosthetic group-containing subunits of the cytochrome bc_1 complexes from *R. capsulatus* form operons with the 5' to 3' order of these genes being: *petA*, encoding the Rieske iron-sulfur protein; *petB*, encoding cytochrome *b*; and *petC*, encoding cytochrome c_1. The cytochrome bc_1 complex isolated from *R. sphaeroides* (Yu *et al.*, 1984; Andrews *et al.*, 1990) has, in addition to these three subunits, a fourth subunit, Subunit IV, that carries no tightly-bound prosthetic group and is located outside of the *pet* operon (Chen *et al.*, 1988, Usui and Yu, 1991; Yu and Yu, 1991). During photosynthetic growth in *R. capsulatus*, electrons are conveyed from the

cytochrome bc_1 complex to the RC either by the well-studied mobile electron carrier cyt c_2 (Daldal *et al.*, 1986) or the more recently studied membrane-anchored electron carrier cyt c_y (Jenney and Daldal, 1993), encoded by *cycA* and *cycY*, respectively.

1.4 The Physiological Regulation of Bacterial Photosynthetic Membrane Formation

The regulation of ICM formation is dependent upon environmental conditions. Expression of the *puf* operon is dependent both on the dissolved oxygen concentration (Adams *et al.*, 1989; Sganga and Bauer, 1992), and on incident light intensity (Klug, 1993). However, the extent to which these factors affect the levels of the photosynthetic apoproteins assembled in the ICM are not the same. Previous research has demonstrated that light intensity modulates the levels of pigment-protein complexes in the cell, but high oxygen tension may abolish its synthesis completely (Drews and Oelze, 1981; Arnheim and Oelze, 1983). The powerful effect of oxygen on the levels of pigment-binding polypeptides encoded by the *puf* operon (the α and β subunits of B870 LHI and L and M subunits of the RC) has been well studied (Clark *et al.*, 1984; Klug and Drew, 1984; Zhu *et al.*, 1986; Belasco *et al.*, 1985). Whereas the transcription of the *puf* operon is severely repressed by oxygen, the transcription of *bch* genes is much less sensitive to the environmental partial pressure of oxygen (Biel and Marrs, 1985; Clark *et al.*, 1984; Hunter *et al.*, 1988). Since Bchl synthesis is almost completely abolished by a high dissolved oxygen content of the growth medium, the repression of *bch* genes by light or oxygen, acting alone or in combination to down-regulate transcription, cannot fully account for the down-regulation of Bchl synthesis.

The synthesis of Bchl in *R. capsulatus* also appears to be regulated by the level of intermediates present in the cell. For example, oxygen regulation of Proto accumulation (Biel, 1992) is released upon exogenous addition of PBG, suggesting that the activity of the *hem* genes is in part regulated by the concentration of early pathway intermediates. HemA levels are similarly controlled by heme concentrations (Rebeiz and Lascelles, 1982) suggesting that in the absence of *bch* expression, heme levels in the cell are low due to autorepressive end-product inhibition. Removal of tetrapyrroles from the shared portion of the pathway after induction of *bch* genes presumably increases activity of the *hem* enzymes.

Both *R. sphaeroides* and *R. capsulatus* accumulate a mixture of two specialized acyclic xanthophylls, spheroidene and spheroidenone. Their abundance and distribution, as a function of the amount of dissolved oxygen in the growth medium, strongly influence the color of the bacterial culture (Schmidt, 1978). Carotenoid accumulation in anoxygenic photosynthetic bacteria has long been known to be regulated by two key environmental factors, oxygen and light (Armstrong, 1995). Carotenoids are usually associated with specific Bchl-containing pigment-protein complexes and thus accumulate preferentially under the low oxygen and low light conditions that favor the formation of the ICM. Indeed, there seems to be a strict coupling between carotenoid accumulation and ICM development (Bartley and Scolnik, 1989; Biel and Marrs, 1985; Coomber *et al.*, 1990; Zsebo and Hearst, 1984). The expression of most *R. capsulatus* and *R. sphaeroides* *crt* genes is several-fold higher under anaerobic photosynthetic conditions than it is in

semiaerobic or aerobic conditions (Armstrong *et al.*, 1993; Giuliano *et al.*, 1988; Lang *et al.*, 1994; Ponnampalam *et al.*, 1995; Yeliseev *et al.*, 1995; Young *et al.*, 1989).

1.5 Processing of *puf* Operon mRNA

As described above, the induction of the ICM in actively growing cells is a result of a shift from high to low oxygen partial pressures. This shift also results in increased levels of LHI and RC polypeptides, though in stoichiometrically different amounts. The B870 LHI polypeptides are found to be in a 10-30-fold molar excess over the subunits of the RC (Schumacher and Drews, 1978; Kaufmann *et al.*, 1982). The cell attenuates the levels of proteins encoded by these genes by a mechanism presented by Belasco *et al.* (1985). Although the *puf* operon is transcribed as a single polycistronic message, recent Northern blot analysis has revealed that there are, in fact, two different long-lived mRNA transcripts of lengths, 0.5 kb and 2.7 kb encoding *puf* operon sequences. The full-length *pufQBALMX* transcript (the promoter of which is located within the *bchZ* coding sequence) is rapidly (<1 minute) degraded to produce a processed transcript of 2.7 kb that lacks the *pufQ* gene. Two RNaseE consensus sequences within the *pufQ* gene contribute to its instability (Fritsch *et al.*, 1995; Klug *et al.*, 1992). The full-length transcript (containing the *pufQ* sequence) has been estimated to exist no longer than 30 seconds (Klug *et al.*, 1987). RNaseE consensus sequences within the *pufL* and *pufM* genes (Fritsch *et al.*, 1995) are most likely responsible for initiating the degradation of the 2.7 kb *pufBALMX* transcript, ultimately producing the most stable 0.5 kb *pufBA* transcript, encoding only the LHI β and α polypeptides. Also, the 0.5 kb fragment was about 9 times more abundant than the 2.7 kb fragment. The presence and relative amounts of both

transcripts was attributed to the differential stability of segments of the mRNA transcribed from the *puf* operon (Klug, 1995). These differences were shown to be a result of a large stem-loop structure located between the *pufA* and *pufL* genes which serve to block 3' to 5' exoribonuclease processing of the *puf* operon transcript (Chen *et al.*, 1988). The stability of these *puf* operon segments has been further shown to be dependent on oxygen levels; the 2.7 kb *puf* transcript displays a half-life of 8 minutes under low oxygen levels and 3 minutes under high oxygen conditions (Klug, 1991). Thus, the stability (stem loops and terminators) and instability (RNAse consensus sites) within the *puf* operon contributes to the different transcript segment concentrations within the cell. These, in turn, are responsible for the synthesis of the required concentrations of LHI and RC proteins. As described above, approximately 16 LHI complexes are required for each RC, with only smaller amounts of the PufX and PufQ proteins present.

1.6 The Transcriptional Regulation of Bacterial Photosynthetic Gene Expression

As previously mentioned, expression of the structural proteins for LHI, LHII, and RC complexes, encoded by the *puf*, *puc*, and *puh* operons, is regulated coordinately with respect to oxygen. At the onset of anaerobiosis, the transition to photosynthetic growth is accompanied by a very large induction of *puf*, *puh*, and *puc* transcription, which is derived primarily from promoters specific to these operons. In *R. capsulatus*, there are three levels of regulation involved in the expression of the photosynthetic apparatus. The first level of regulation is the activation of the reaction center and light-harvesting structural protein genes (*puf*, *puh* and *puc*) under anaerobic conditions by a two-

component signal transduction system consisting of RegA and RegB. The second level of regulation is the repression of all photosynthesis gene expression under aerobic conditions by CrtJ/PpsR. The third level of transcription regulation is manifested under low light conditions, where the level of expression of the *puf* and *puh* operons is increased in dim light by the HvrA circuit.

1.6.1 RegA and RegB

Genetic evidence that RegA and RegB constitute a cognate two-component system includes the observation that knockout mutations in *regA* and/or *regB* abolish anaerobic induction of the *puf*, *puh*, and *puc* operons (Sganga and Bauer, 1992; Mosley *et al.*, 1994). The *in vivo* evidence is supported by *in vitro* analyses that demonstrated that a truncated form of the RegB polypeptide autophosphorylates in the presence of ATP and transfers the phosphate moiety to RegA (Inoue *et al.*, 1995). Within the RegB/RegA system, the RegB protein represents a redox sensor kinase, which appears to directly monitor the respiratory activity of the cytochrome oxidase, while the RegA protein is the response regulator. RegB transmits information regarding the redox state of the environment by phosphotransfer to RegA. The autophosphorylation of RegB is a rate-limiting step, and the end result, phosphorylation of RegA, increases the binding of the regulator to DNA by 20-fold.

1.6.2 SenC and HvrA

In *R. capsulatus, regB* is divergently transcribed from a three-gene operon comprised of *senC, regA*, and *hvrA*. Mutational analysis indicated that SenC is involved

in the formation of a functional cytochrome *c* oxidase complex which is required for proper sensing of anaerobiosis by RegB (Buggy and Bauer, 1995; Buggy *et al.*, 1994). HvrA appears to be a transcription factor that facilitates RegA binding to DNA under dim-light growth conditions (Buggy *et al.*, 1994). This gene seems to activate transcription of only the *puf* and *puh* operons, which collectively encode LHI and the RC, but not the *puc* operon, which encodes LHII. Most probably, HvrA may act in a global manner to increase the number of photosynthetic units available to the cell as light intensity decreases.

1.6.3 CrtJ/PpsR and CrtK/TspO

Two *R. sphaeroides* regulatory genes, *ppsR* and *tspO* have recently been demonstrated to encode products that normally repress not only carotenoid and Bchl pigment levels, but also *crt, bch* and *puc* gene expression several-fold under aerobic growth conditions (Gomelsky and Kaplan, 1995; Yeliseev *et al.*, 1995). The homologous genes to *ppsR* and *tspO* in *R. capsulatus* are referred to as *crtJ* (or ORF 469) and *crtK* (or ORF 160), respectively, because they were initially thought to be specifically required for carotenoid biosynthesis, based on the results of mutant studies (Armstrong *et al.*, 1989; Armstrong *et al.*, 1990; Giuliano *et al.*, 1988; Zsebo and Hearst, 1984). They are, in fact, not directly involved in carotenoid biosynthesis (Bollivar *et al.*, 1994; Yeliseev and Kaplan, 1995). The mechanism by which TspO functions in *R. sphaeroides* is unknown, although it has been proposed to serve as an environmental sensor. Both this hydrophobic outer membrane protein (Yeliseev and Kaplan, 1995) and its mRNA (Armstrong *et al.*, 1993), are more abundant in anaerobic photosynthetic cells than they are in aerobic cells.

Interestingly, TspO displays about 35% deduced amino acid sequence identity with mammalian peripheral-type benzodiazepine receptors localized in the mitochondria (Armstrong,1995; Baker and Fanestil, 1991). PpsR is a DNA-binding protein that contains a helix-turn-helix motif conserved in other eubacterial transcriptional repressors (Armstrong, 1995; Penfold and Pemberton, 1994). The protein recognizes a conserved palindromic sequence found 5' to several operons containing *crt, bch*, and *puc* genes (Alberti *et al.*, 1995; Penfold and Pemberton, 1994). CrtJ is the repressor that inhibits the expression of all the photosynthetic operons. CrtJ responds to redox by forming an intramolecular disulphide bond under oxidizing, but not reducing, growth conditions (Masuda *et al.*, 2002). The presence of the disulphide bond stimulates DNA binding activity of the repressor. There is also a flavoprotein that functions as a blue-light absorbing anti-repressor of CrtJ in the related bacterial species *R. sphaeroides* called AppA. AppA exhibits a novel long-lived photocycle that is initiated by blue-light absorption by the flavin. Once excited, AppA binds to CrtJ thereby inhibiting the repressor activity of CrtJ.

1.6.4 FnrL

FnrL is the *R. sphaeroides* homolog of the *E. coli* anaerobic regulatory protein, FNR, which acts as a redox-responsive transcriptional regulator that activates genes whose products are involved in anaerobic respiration and represses other genes required for aerobic respiration in that organism. *R. sphaeroides* FnrL mutants are incapable of photosynthetic growth (Zeilstra-Ryalls and Kaplan, 1995), suggesting that FnrL is

involved, directly or indirectly, in the regulation of photosystem genes. Indeed, it has been recently shown that FnrL is essential for the activation of a variety of genes required for photosynthetic growth (Oh *et al.*, 2000), such as *hemA, hemN, hemZ, bchE*, and the *pucBAC* operon (Zeilstra-Ryalls and Kaplan, 1998). A role for FnrL in the expression of the *hemA* gene, one of the two genes (along with *hemT*) in *R. sphaeroides* coding for isozymes of ALA synthase, has been demonstrated by using transcriptional fusions involving the *hemA* gene (Zeilstra-Ryalls and Kaplan, 1995). In these studies, transcriptional fusions involving the *puc* operon suggested that FnrL appears to act both directly, at the FNR binding site, and indirectly via a mechanism that is currently under investigation, to activate *puc* transcription when oxygen tension is reduced. Thus, it appears that FnrL acts at multiple levels to regulate photosystem gene expression. However, in *R. capsulatus*, FnrL has no apparent effect on photosynthetic growth, but does appear to play a critical role in the induction of the dimethyl sulfoxide (DMSO)-inducible cytochrome *c* and DMSO-reductase, which together as required for anaerobic-dark growth using DMSO as an alternative electron acceptor (Zeilstra-Ryalls *et al.*, 1997).

1.7 Significance of the *pufQ* Gene in Photosynthesis

The *R. capsulatus pufQ* gene is the focus of this thesis. Since it is the first gene in the *puf* operon, its expression is regulated by the oxygen-sensitive *puf* promoter. The open reading frame encodes a 74 amino acid protein. The *pufQ* gene has been previously implicated as a potential candidate in the regulation of Bchl biosynthesis (Adams *et al.*, 1989; Bauer and Marrs, 1988; Bauer *et al.*, 1991; Fidai *et al.*, 1995; Fidai *et al.*, 1994a;

Forrest *et al.*, 1989). Strains of *R. capsulatus* have been created in which the entire *puf* operon had been deleted. These mutants were photosynthetically incompetent as they were lacking the LHI polypeptides and the reaction center and almost completely deficient in Bchl. However, when a plasmid-borne *pufQ* was reintroduced into this strain, wildtype levels of Bchl and LHII (800-850) complex could be detected (Bauer and Marrs, 1988); Forrest *et al.*, 1989). Thus, the presence of the *pufQ* gene is necessary at least for the synthesis of Bchl. The *pufQ* gene is present in at least three organisms: *R. capsulatus*, *R. sphaeroides*, and *Rhodovulum sulfidophilum*. The primary amino acid sequence of the PufQ protein and conservation between these species is shown in Figure 6.

A substantial amount of evidence has led to the theory that PufQ is involved in Bchl synthesis. Evidence began accumulating when Bauer and Marrs (1988) deleted the entire *puf* operon resulting in minimal pigment synthesis and it was only the *trans* addition of the *pufQ* gene that could restore pigment synthesis to wildtype levels. To test whether the *pufQ* gene is involved in regulating transcription of the *puc* operon, Northern blot analysis of the *puc* operon in the presence and absence of the *pufQ* gene was performed by Forrest *et al.* (1989). These studies involved the construction of an artificial expression system for the *pufQ* gene, in which *pufQ* and *lacZ* were fused to the nitrogen fixation promoter. The results indicated that there was no effect by PufQ on the levels of *puc*-specific transcripts. Furthermore, results demonstrated that the synthesis of Bchl was proportional to *lacZ* expression, and by extension, to *pufQ* expression. These results confirmed the notion that the PufQ protein may be involved in Bchl biosynthesis, and also suggested its possible role in the assembly of the B800-850 LHII complex. Further

Figure 6: ClustalW alignment of the PufQ Primary Sequence. The primary protein sequence from *R. capsulatus*, *R. sphaeroides*, and *R. sulfidophilum* are shown. Conserved residues are shown in yellow, and conservative substitutions are shown in green. The putative membrane span and the conserved proline at residue 35 is outlined.


R. capsulatus ---MQSQRLRAHGVQHVDRVPRPEFALYFSLILIVAVPFALVGWVMALVR 47
R. sphaeroides MSDHAVNTPVHAARAHGHRAPRAEFYVYFAVILLGGFPVAFVSWIVSTIR 50
R. sulfidophilum ----MTDQTSDVHMVRGHRPPKAEFMVYFTIIFIAALPLAFIASFLAMVR 46
R. capsulatus ERRIPECGPFARAWREAGEITPEIFRP 74
R. sphaeroides HRRLPKRGPFASAWFDAKAITPLIFRA 77
R. sulfidophilum QGDLKTKGPIARAWSQARIITPMIFSA 73

evidence of PufQ function in Bchl biosynthesis was demonstrated in *bch* mutants, which normally accumulate an intermediate of the Bchl pathway, but fail to do so when *pufQ* is deleted from the chromosome. The hypothesis that PufQ may be involved in the stimulation of the Bchl synthesis pathway, perhaps acting as a carrier protein of tetrapyrrole intermediates, was reinforced by the observation that liposome-reconstituted PufQ protein binds protochlorophyllide (Fidai *et al.*, 1994b)

A number of features of the *pufQ* gene, and its expression, suggest that PufQ plays a primarily regulatory role. Although the protein has been detected in wildtype *R. capsulatus* (Fidai *et al.*, 1994a), and the gene that encodes this protein is essential for Bchl synthesis, the mRNA encoding PufQ has yet to be detected in wildtype cells (Adams *et al.*, 1989, Klug *et al.*, 1987). Therefore, PufQ may be functional at extremely low concentration, which is more conducive to a catalytic or regulatory role. It has also been previously suggested that in *R. sphaeriodes*, PufQ may act as an assembly factor for the LHI and LHII complexes (Gong *et al.*, 1994). These genetic studies involved the construction of a number of site-directed *pufQ* mutants and the observation of varying levels of LHI and LHII in each mutant. It was concluded by Gong *et al.* (1994) that PufQ is involved in light-harvesting complex assembly. However, the idea that accumulation of either the polypeptides for the light-harvesting complexes, or their pigment cofactors results in the degradation of the unpaired components, allows this inference to be questioned. It may be reasonable to predict that the PufQ protein functions in both assembly and Bchl regulation.

Previous research by Smart (2001) resulted in the creation of a mutant of *R. capsulatus* (JS01) in which *pufQ* was disrupted by a kanamycin-resistance-encoding cassette. This mutant forms only a small amount of Bchl and is unable to grow photosythetically. Restoration of the *pufQ* gene to JS01 in *trans* restored Bchl synthesis but did not rescue photosynthetic competence, indicating that the insertion of the cassette into the *pufQ* gene was polar on the transcription of downstream *puf*-operon genes. Photosynthetically-competent colonies were isolated at a very low frequency, however, indicating that recombination of the *pufQ* gene from the vector to the chromosome had occurred in these colonies. The isolation of mutants of JS01 suppressing the *pufQ* null allele suggested that there are two main mutations necessary to affect suppression (Smart, 2001). One mutation, which may have involved a repressor-binding site upstream of *hemZ* (encoding Coprogen oxidase), was present on all suppressing elements. Also, expression assays conducted on JS01 suggested that the transcription of *hemB*, *hemH* and *hemZ* was reduced from two to twenty-fold. In addition, transcription of *hemZ* was observed to be reduced twenty-fold in *bchH*, *bchD* and *bchI* mutants, possibly indicating that Mg Proto (along with PufQ) also specifically stimulated *hemZ* transcription. Therefore, it was hypothesized that PufQ could be a component of the system that regulates one or more *hem* genes encoding enzymes catalyzing the conversion of ALA to Proto in the *hem* pathway of tetrapyrrole synthesis (Smart, 2001).

1.8 Objectives of This Thesis

This thesis explores two primary objectives in unraveling the functionality of PufQ during the early part of the Bchl biosynthetic pathway. The first involves assaying

the activity of Coprogen oxidase in wildtype and various mutant strains of *R. capsulatus* (Table 1), under both aerobic and semiaerobic growth conditions. JS01 and JS02 were strains in which the *pufQ* gene was disrupted either by insertion of a kanamycin-resistance encoding cassette (JS01) or by deletion (JS02). Mutant ZY6 (Yang and Bauer, 1990) and ZY4 (Yang and Bauer, 1990) are Bchl biogenesis (*bch*) mutants that accumulate Proto and Mg Proto, respectively. Mutant B10Δ227 and JS02Δ227 contain a chromosomal knockout of *orf227*, which is hypothesized to be an aerobic repressor of *hemZ* in wildtype and JS02, respectively. Similarly, JSΔN contains a chromosomal knockout of the subunit E of NADH dehydrogenase, which is hypothesized to be an electron acceptor in the reaction that Coprogen oxidase catalyzes. An *R. capsulatus* mutant containing a Pro35Ala substitution in *pufQ* was also created in this thesis. This strain, HUP35A, was constructed in order to examine the functional effect of a point mutation in the membrane spanning region of the of the PufQ protein (Figure 6). HUP35A, as well as the aforementioned strains, were all assayed for PBG synthase, Coprogen oxidase and Bchl assays during aerobic and semiaerobic growth. Analysis of the induction enzyme activity in aerobically and semiaerobically grown wild-type cells may provide some clues to the mechanisms by which the transcription of genes involved in heme and Mg tetrapyrrole biosynthesis are controlled.

The second objective of this thesis involves an enzymatic kinetic study of PBG synthase (HemB). The PBG produced by PBG synthase is the substrate for HMB synthase (HemC), which catalyzes the successive condensations of four PBGs to a covalently bound dipyrrolmethane, initiated in each case by elimination of the amino

Table 1: Strains of *Rhodobacter capsulatus*. Characteristics of the various strains of *R. capsulatus* used in this thesis project. See Section 2.6 for relevant characteristics and growth conditions.

***Rhodobacter capsulatus* Strain**	**Genotype**
B10	Wild-type *R. capsulatus*
JS01	B10Δ*pufQ*::Km^R
JS02	B10 Δ*pufQ*
HUP35A	B10*pufQ*(Pro35Ala)
ZY6	B10Δ*bchH*::Km^R
ZY4	B10Δ*bchM*::Km^R
B10Δ227	B10Δ*ORF227*::Gm^R
JS02Δ227	B10Δ*pufQ*Δ*ORF227*:: Tc^R
JSΔN	B10Δ*nuoE*::Km^R

group. Once four PBGs have condensed, the enzyme has a bound hexapyrrole derived from six PBGs. Hydrolysis of the link to the dipyrrolmethane then yields free HMB. Then Urogen synthase (HemD) converts the linear tetrapyrrole HMB to the macrocyclic Urogen followed by decarboxylation of all four acetic acid groups by Urogen decarboxylase (HemE) to yield Coprogen. Autoxidation of the porphyrinogens can also lead to the formation of the nonphysiological (and phototoxic) side products, Uro and Copro. Taking these reactions into account, there was the possibility of feedback inhibition on PBG synthase by porphyrins and/or porphyrinogens. Therefore, it was deemed necessary to determine the extent of the inhibition on PBG synthase activity by the addition of Copro, Coprogen, Uro and Urogen at varying concentrations to PBG synthase assay mixtures from semiaerobically grown wildtype *R. capsulatus*. The results (Section 3.6) suggested that the physiological porphyrinogens (Urogen and Coprogen) inhibited PBG synthase activity to a greater extent than the non-physiological porphyrins (Uro and Copro). Uro actually appeared to activate PBG synthase activity. These results initiated my study of the enzyme kinetics of PBG synthase in the presence of these two inhibitors. However, in order to study PBG synthase enzyme activity, a partial purification of the PBG synthase enzyme preparation was carried out in order to remove any detectable HemC activity so that endogenous inhibitory porphyrinogens were not being produced.

The third objective of this thesis involves an indirect search for the *hemD* gene and sequence in *R. capsulatus*. All the known *hem* genes in the biosynthetic pathway of *R. capsulatus* have been sequenced and characterized, except for *hemD* and *hemG/Y*. In

bacterial organisms such as *Bacillus subtilis*, *Chlorobium vibrioforme*, *Salmonella typhimurium*, and *E. coli*, *hemD* has been found to be transcribed in an operon with *hemC*. Also, HemC, Hem D and HemE are enzymes that are required in sequence upon the induction of the photosynthetic apparatus and, therefore, it could be suggested that they may be in close proximity to each other in the genome. Based on this information, it was hypothesized that *hemD* could be located next to *hemC* or *hemE*, and could be transcribed in a *hemCD* or *hemED* operon, respectively.

2.0 Materials and Methods

2.1 Materials and Instruments

Chemicals were purchased from the following suppliers: Chemicals were purchased from Sigma-Aldrich Co., Anachemia Chemicals Inc, or BRL Scientific, and were of the highest grade commercially available. Uro octamethyl ester, Copro tetramethyl ester, and Proto dimethyl ester, and δ-aminolevulinic acid hydrochloride was obtained from Sigma-Aldrich Co. The suppliers of kits and enzymes used in this thesis project are stated in the text. All centrifugations were conducted in a Sorvall RC-5B Refrigerated Superspeed Centrifuge, unless otherwise stated, and all ultracentrifugations were conducted in a Beckman 68-80 Ultracentrifuge. All cell density readings were measured on a Beckman DU-600 spectrophotometer.

2.2 Buffers and Other Solutions

20X SSC: 0.3 M Tri-sodium Citrate and 3 M NaCl, pH 7.0.

DNA Loading Buffer (11X): 11X TAE containing 20% (w/v) glycerol, and 0.25% (w/v) bromophenol blue and xylene cyanol.

Ehrlich's reagent: 0.1 M ρ-dimethylaminobenzaldehyde, 3.5 M perchloric acid in glacial acetic acid.

Lysis Buffer: 20mM Tris-Cl pH 7.4, 200mM NaCl, 1mM EDTA, 10mM β-mercaptoethanol, and 1mM NaN_3.

TAE buffer: 40mM Tris-acetate and 1mM EDTA, pH 8.0.

2.3 Bacteriological Media

2.3.1 RCV

RCV medium: 4g maleic acid, 1 g $(NH_4)_2SO_4$, 2g K_3PO_4, 0.12g $MgSO_4 \cdot 7H_2O$, 0.075g $CaCl_2 \cdot 2H_2O$, 0.02g Na_2EDTA, 1mg Vitamin B1, and 3g H_3BO_3 per litre double-distilled water (ddH_2O), adjusted to pH 6.8 (Weaver *et al.*, 1975). This medium was used for semiaerobic and phototrophic growth of all strains of *R. capsulatus* including B10, JS01, JS02, HUP35A, B10Δ227, JS02Δ227, ZY6, ZY4 and JSΔN. To prepare solid media plates, 15- 20 g/L of agar was added to the media prior to autoclaving.

2.3.2 RCV+

RCV+ medium: As above, with the addition of 5g pyruvate, 6g glucose and 3.5mL DMSO (per liter ddH_20) (McEwan *et al.* 1998; Solomon *et al*, 1999). As DMSO can provide an alternative (anaerobic) electron acceptor in the presence of DMSO reductase, this medium was occasionally used to support anaerobic growth for strains incapable of photosynthesis.

2.3.3 YPS

YPS medium: 3g casamino acids, 3g yeast extract, 1mM final concentration each of $CaCl_2$ and $MgS0_4$ (Wall *et al.*, 1974).

2.3.4 2/3 YR

For routine growth of *R. capsulatus* cultures, a mixture of 2/3 YPS and 1/3 RCV (2/3 YR) was used.

2.3.5 2XTY

2XTY medium: 16 g casamino acids, 10g yeast extract, and 5g NaCl per liter ddH_20. This medium was typically used for growth of *E. coli* cultures. To prepare solid media plates, 15- 20 g/L of agar was added to the media prior to autoclaving.

2.3.6 SOB

SOB media: 20 g casamino acids, 5 g yeast extract, 0.584 g NaCl, and 0.186 g KCl per 1 L distilled water. This media was used for the growth of *E. coli* strain cultures in preparation of electrocompetent cells.

2.3.7 SOC

SOC media: 20 g casamino acids, 5 g yeast extract, 0.584 g NaCl, 0.186 g KCl, and 20 mM sterilized glucose per 1 L distilled water. This media was used for the rescue of electrocompetent *E. coli* strain cultures after immediately after electroporation.

2.4 Antibiotics

Ampicillin: this antibiotic was only used for *E. coli*, at a final concentration of 200 μg/mL.

Gentamycin: this antibiotic was used at a final concentration of 10 μg/mL for *R. capsulatus.*

Kanamycin: this marker was used at a final concentration of 10 μg/mL for *R. capsulatus*.

Tetracycline: this antibiotic was used at a final concentration of 0.5 μg/mL for *R. capsulatus* and 10μg/mL for *E. coli.*

2.5 *Escherichia coli* Strains

2.5.1 *Escherichia coli* Strain DH5α

Genotype: *supE44 ΔlacU169 (ø80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*

Growth conditions and relevant characteristics: Strain DH5α (Sambrook *et al*, 1989) was maintained and grown on 2XTY plates or in broth at 37°C. DH5α was used for transformation of plasmid.

2.5.2 *Escherichia coli* Strain S17-1

Genotype: *ΔlacU169 (ø80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*

Growth conditions and relevant characteristics: Strain S17-1 (Simon *et al.*, 1983) was used for the mobilization of broad-host range plasmids into *R. capsulatus*. This strain was maintained and grown on 2XTY plates or broth at 37°C.

2.6 *Rhodobacter capsulatus* Strains

2.6.1 *Rhodobacter capsulatus* B10

Genotype: wildtype *Rhodobacter capsulatus*.

Growth conditions and relevant characteristics: Strain B10 (Weaver *et al.*, 1975) was maintained and grown in RCV (Weaver *et al.*, 1975) or 2/3YR at 28°C. B10 was used as a wildtype *R. capsulatus* control strain, and is the parent strain to some of the mutants used in this thesis.

2.6.2 *Rhodobacter capsulatus* Strain JS01

Genotype: B10Δ*pufQ*::Km^R

Growth conditions and relevant characteristics: JS01 was routinely maintained and grown in RCV or 2/3YR in the presence of kanamycin at 28°C.

2.6.3 *Rhodobacter capsulatus* Strain JS02

Genotype: B10Δ*pufQ* (bp 41120 –40882 of Genbank submission number Z11165).

Growth conditions and relevant characteristic: JS02 contains an in-frame deletion of *pufQ* from the start codon to the *Eco*RI site downstream of *pufQ*. JS02 was routinely maintained and grown in RCV or 2/3 YR at 28°C.

2.6.4 *Rhodobacter capsulatus* Strain HUP35A

Genotype: B10*pufQ*(Pro35Ala)

Growth conditions and relevant characteristics: HUP35A was routinely maintained and grown in RCV or 2/3YR at 28°C. The construction of strain HUP35A is described in Section 2.12.

2.6.5 *Rhodobacter capsulatus* Strain B10Δ227

Genotype: B10Δ*orf227*::Gm^R

Growth conditions and relevant characteristics: B10Δ227 contains an insertional mutation of the gentamycin resistance marker in *orf227*. This strain was routinely maintained and grown in RCV or 2/3YR in the presence of gentamycin at 28°C.

2.6.6 *Rhodobacter capsulatus* Strain JS02Δ227

Genotype: B10Δ*pufQ*::Δ*orf227*::Tc^R

Growth conditions and relevant characteristics: JS02Δ227 contains an insertional mutation of the tetracycline resistance marker in *orf227,* as well as an in frame deletion of *pufQ*. This strain was routinely maintained and grown in RCV or 2/3YR in the presence of tetracycline at 28˚C.

2.6.7 *Rhodobacter capsulatus* Strain ZY6

Genotype: B10Δ*bchH*::Km^R

Growth conditions and relevant characteristics: ZY6 contains an insertional mutation of the kanamycin resistance marker in *bchH*. This strain was routinely maintained and grown in RCV or 2/3YR in the presence of kanamycin at 28˚C.

2.6.8 *Rhodobacter capsulatus* Strain ZY4

Genotype: B10Δ*bchM*::Km^R

Growth conditions and relevant characteristics: ZY4 contains an insertional mutation of the kanamycin resistance marker in *bchM*. This strain was routinely maintained and grown in RCV or 2/3YR in the presence of kanamycin at 28˚C.

2.6.9 *R. capsulatus* Strain JSΔN

Genotype:B10Δ*nuoE*::Km^R

Growth conditions and relevant characteristics: JSΔN contains an insertional mutation of the kanamycin resistance marker in *nuoE*. This strain was routinely maintained and grown in RCV or 2/3YR in the presence of kanamycin at 28˚C.

2.7 Previously Constructed Plasmids

2.7.1 pXCA935

Plasmid pXCA935 (Adams *et al.*, 1989) is a low-copy incP reporter construct containing the *pufQ* gene and a transitional fusion of *pufB* and *lacZ*. This vector also bears a tetracycline resistance marker.

2.7.2 pLAB2

Plasmid pLAB2 (L.A. Behie, unpublished) is a low-copy incP reporter construct containing the *pufQ* gene with a nucleotide substitution which changed proline35alanine and a transitional fusion of *pufB* and *lacZ*. This vector also bears a tetracycline resistance marker.

2.8 Cloning Procedures

2.8.1 Preparation of Electrocompetent *Escherichia coli*

10 mL of SOB broth was incubated with a single colony of *E. coli* strain S17-1 from a plate grown overnight and incubated until cell density measure at A_{600} reached 0.6. This culture was then used to inoculate 500 mL SOB (prewarmed to 37°C) in a 1 L flask, which was grown until cell density measured at A_{600} reached 0.5. This culture was then chilled on ice for 15 minutes at 4°C, and centrifuged at 8000 rpm in a JA-14 rotor for 15 minute at 4°C. The cell pellet was then washed with 200 mL sterile water, and centrifuged as above. Cells were washed a total of three times with sterile water, followed by three washes with sterile 10% glycerol in distilled water. The cells were finally resuspended in 0.5% original volume. The cells were immediately frozen by a dry

ice/ethanol bath, and 50 µL aliquots in 0.6 mL microcentrifuge tubes at stored at -80°C until needed for transformation.

2.8.2 Transformation by Electroporation

DNA was ensured to be salt-free by ethanol precipitation. Electrocompetent cells to be transformed were thawed on ice, and added to the DNA. This mixture was then placed in a sterile electroporation cuvette (0.2 cm gap). Electroporation was performed using a Bio-Rad Gene Pulsar, set at 2.5 kV, resulting in a time constant of <4.5 ms. Cells were rescued by addition of warm SOC media. The cultures were then incubated for 30 minutes at 37°C and plated onto 2XTY plates containing the appropriate antibiotics. The plates were dried and incubated overnight at 37°C.

2.8.3 Transformation of *Rhodobacter capsulatus* by Conjugal Transfer

Competent *E. coli* strain S17-1 (Simon *et al.* 1983) cells were transformed with the broad-host range plasmid to be mobilized, and a diparental mating was performed . 100µL of S17-1 bearing the plasmid of interest was spun at 4000 rpm in a Beckman tabletop centrifuge for 2 minutes, and the supernatant removed by aspiration. The resultant cell pellet was resuspended with 1 mL mid-log *R. capsulatus* culture. This mixture was spun as above for 2 minutes, and the supernatant removed. The mixed cell pellet was resuspended in 100 µL of RCV and the resultant suspension was dropped in 10-15 µL aliquots on a dried RCV plate, and the mating spots were allowed to dry. Mating spots were incubated at 28°C for 12-24 hours, or until *R. capsulatus* growth was evident. The mating spots were then streaked on selective media (RCV plus antibiotic

appropriate to the transferred plasmid). *E. coli* hosts were typically auxotrophic, to allow selection for *R. capsulatus* at this point on minimal RCV media.

2.9 Agarose Gel Electrophoresis

Agarose gels were prepared by the addition of agarose powder to TAE running buffer. Typically, gels contained 1% agarose for routine DNA separations. Ethidium bromide was added to a final concentration of 1 μg/mL from a 10 mg/mL stock. DNA was compared to a standard 1 kb ladder (Invitrogen Inc., Burlington, Ont.). Gels were electrophoresed at 80-100V constant voltage for 1-2 hours. DNA fragments were visualized by monitoring the fluorescence of the ethidium bromide-DNA complex under ultraviolet light, and permanent records were taken using a gel documentation system.

2.10 Purification of DNA

Genomic DNA from wildtype and HUP35A *R. capsulatus* was purified according to the following protocol to prepare DNA for sequence analysis. Liquid cultures were grown semiaerobically to mid-log phase in 100 mL flasks containing RCV medium, and a 10 mL fraction of this culture was pelleted by centrifugation at 8000 rpm for 10 minutes. The supernatant was removed and the pellet was resuspended in 500 μL of 1X SSC, and then incubated with 400 μg of lysozyme (Sigma-Aldrich Canada Ltd., Oakville, Ont.), and 50 μL of 10% SDS for 30 minutes at 37°C. The sample was then put into a boiling water bath for one minute and shocked on ice for 5 minutes. The sample was extracted four times with a mixture of 24:24:1 phenol:chloroform:isoamyl alcohol, and the supernatant was collected and re-extracted after each step. The DNA was precipitated

by the addition of 1 mL of -20°C 95% ethanol, followed by centrifugation for 15 minutes at 15,000 rpm. Supernatant ethanol was removed by aspiration, and the precipitated pellet was then washed with 1 mL of -20°C 70% ethanol, and centrifuged for 5 minutes as above. Again, the supernatant ethanol was removed by aspiration, and the pellet was dried briefly under vacuum. The washed, dried DNA pellet was then resuspended in 100 µL of water. A 1/100 dilution of this sample was used to determine DNA concentration and purity by measuring the absorbance of the sample at 260 nm and 280 nm. DNA fragments were purified on agarose gels, using a QIAquick Gel Extraction Kit (Qiagen, Mississauga, Ont.) according to the manufacturer's instructions to elute a fragment of DNA from an agarose slice.

2.11 Polymerase Chain Reaction (PCR)

2.11.1 5'*bchZ* Primer

Primer Sequence: 5'dTCA TGG GTT ACA TGG GTA GC 3'

This primer is sense to base-pairs 40549 through 40568 of Genbank submission Z11165.

2.11.2 3'*pufB* Primer

Primer Sequence: 5' dAAT TCC GTA CCG GCT CAG GG 3'

This primer sequence is anti-sense to base-pairs 41101 through 41120 of Genbank submission Z1165.

2.11.4 PCR Conditions

PCR conditions were determined empirically for the primer pairs mentioned above and for template DNA. Melting temperatures for the primer pair was estimated with the use of OLIGO software. A range of annealing temperatures were chosen near and slightly above the calculated melting temperatures. A final Mg^{2+} concentration of 2 mM was used. Other components of the PCR reaction were as follows: dNTP's, 500 μM each, 2 units Taq polymerase, 1X PCR buffer (Invitrogen Inc. Burlington, Ont.), and 500 ng of genomic DNA template.

PCR reactions were cycled on a Biometra Trio-Thermoblock PCR machine, using a hot start protocol (mixing of template, enzyme, and primers was not attempted until sample temperature were greater than 80°C). Denaturization for 45 s at 95°C was followed by 60 s of annealing at 54°C, and extension was carried out at 72°C for 60 s. PCR products were visualized and purified by gel electrophoresis.

2.12 Construction of HUP35A

Plasmid pLAB2 (L.A. Behie, unpublished), which bears the *pufQ* gene with a proline to alanine mutation at residue 35, was used as the point-mutational construct to create strain HUP35A from *R. capsulatus* strain JS01. This plasmid was transformed into *E. coli* strain S17-1 and transferred to JS01 by conjugal transfer. The resulting strain, JS01pLAB2, was resistant to both kanamycin and tetracycline when grown aerobically,

but could not grow photosynthetically. However, upon incubation anaerobically at 28°C in the presence of light for 5 days in 15 mL of RCV medium containing both antibiotics, a pigmented culture eventually grew. A single-cell pigmented colony was selected and grown aerobically for several days on RCV plates in the absence of antibiotics, transferring it four times. A single-cell colony, HUP35A, from the resultant growth was isolated and found to be able to grow quickly overnight under photosynthetic conditions, but was sensitive to both tetracycline and kanamycin. To confirm the amino acid substitution, primers were designed near the 3' end of *bchZ* and near the 5' end of *pufB* (Section 2.11). Primers were synthesized by the Nucleic Acid Protein Services Unit at the University of British Columbia, Vancouver BC. The PCR reaction would produce a 500 bp fragment which was sequenced by the Centre for Molecular Medicine and Therapeutics (CMMT) DNA Sequencing Core Facility in Vancouver, BC.

2.13 Bradford Protein Assay

Protein concentrations were estimated by Bradford assay (Bradford, 1976). Samples were diluted to 800 μL with ddH_20, to which 200 μL of Bio-Rad (Mississauga, Ont.) Bradford dye reagent was added. This mixture was then incubated at 37°C for 20-40 minutes, after which the A_{595} was measured. A dilution series of bovine serum albumin was prepared in the range of 0-20 μg to generate a standard curve.

2.14 Growth of *Rhodobacter capsulatus* and Preparation of Cell Lysates

R. capsulatus was grown at 28°C aerobically in 2 L flasks filled with 250 mL of RCV shaking at 300 rpm or semiaerobically in 2L flasks filled with 1.6L of RCV shaking

at 150 rpm, until the cultures had attained mid-log phase (A_{680} = 0.4-0.6). For the adaptation experiments described below, after aerobically grown cultures had reached mid-log phase, 150 mL samples were removed and the flasks were refilled to 1.6 L with fresh RCV, and growth was continued semiaerobically by shaking at 150 rpm until the culture had reached mid-log phase. Similarly, photosynthetic cultures were grown by filling a 2 L flask to the brim with RCV and stoppered to ensure the exclusion of air. These cultures were incubated, with a stir-bar, at 28°C in the presence of a 100-watt light bulb about 25 cm away from the flask. Fully grown cultures were harvested by centrifugation at 8,000 rpm for 10 minutes at 4°C, washed twice with nitrogen-saturated 0.05M Tris-HCl, either pH 8.0 or 8.8 depending on growth conditions, and resuspended in a minimal amount of the same buffer. The suspension was either used immediately or kept frozen at -20°C until required. Concentrated suspensions of organisms, supplemented with 1mM β-mercaptoethanol, were subsequently disrupted by passing through a French pressure cell at 15,000 lb/in^2. Broken-cell suspensions were then centrifuged for 10 minutes at 25,000 x g in stoppered tubes filled with nitrogen. The supernatant was removed and subjected to ultracentrifugation for 2 hours at 105,000 x g in a Ti80 rotor. Aliquots of the supernatants (soluble fraction) obtained were either used immediately or kept frozen at -20°C. The membrane fractions obtained were resuspended in nitrogen-saturated 0.05M Tris-HCl, pH 8.0, in a volume one-half of that of the original crude extract, and were either used immediately or kept frozen at -20°C.

2.15 Pigment Analysis of *Rhodobacter capsulatus* Strains

Pigments were extracted from mid-log *R. capsulatus* cultures, grown either aerobically or semiaerobically at 28°C, as described in Section 2.14. Following disruption in the French pressure cell, the broken cell suspension was centrifuged for 2 hours at 105,000 x g in a Ti80 rotor, the supernatant solution discarded, and the pellet resuspended phosphate buffer (KH_2PO_4-NaOH), pH 6.8. This mixture was mixed with an equal volume of 7:2 acetone:methanol and centrifuged at 8,000 rpm in a JA-14 rotor for 10 minutes (Cohen-Bazire *et al.*, 1957). The concentration of Bchl was determined from this supernatant by measuring A_{769}. An extinction coefficient of 76 $mM^{-1}cm^{-1}$ was used to calculate Bchl content (Richards, 1994), and the resultant values were normalized for protein concentration as estimated by Bradford assay.

2.16 Preparation of Coproporphyrinogen III and Uroporphyrinogen III

The free acid form of Copro was prepared by dissolving Copro tetramethyl ester in 25% (w/v) HCl and allowing the solution to stand at room temperature, in the dark, for 20-24 hours for complete ester hydrolysis. Once hydrolysis is complete, the acid solution is overlayed with a relatively large volume of ether, the pH adjusted to 4 and the mixture shaken vigorously before precipitation occurs. This method results in the transference of the hydrolyzed porphyrin from aqueous acid to ether. The ether layer is removed, evaporated and the Copro is resuspended in distilled water and a drop of 1.5 M HCl. This solution is stored at 4°C in the dark until required. A similar hydrolysis and recovery protocol was followed for Uro octamethyl ester, however, the aqueous solution was allowed to stand at room temperature for 48 hours, instead of 24 hours.

Coprogen and Urogen were prepared immediately before use by treating a suspension of hydrolyzed Copro or Uro in water with sodium borohydride (Nishida *et al*, 1959; Schwartz *et al*., 1960). To 1 mg of either porphyrin suspended in 1 mL of water, one drop of 1.5 M HCl was added; nitrogen gas was bubbled through the solution and about 4 mg of sodium borohydride added. The flow of gas through the solution was maintained until the solution changed from bright pink to colourless. The solution was then neutralized by careful addition of 1.5 mL HCl until the solution could be pipetted into a 200 μL pipette-tip without the formation of gas bubbles.

2.17 Assaying Coproporphyrinogen III Oxidase Activity in *Rhodobacter capsulatus*

2.17.1 Aerobic activity

This activity was assayed essentially as described by Sano *et al*. (1961). Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as standard. The standard assay mixture contained, in a total volume of 1.0 mL, 100 mM of phosphate buffer (KH_2PO_4-NaOH), pH 7.5, approximately 30 nmol of freshly prepared Coprogen, and 10-15 mg of *R. capsulatus* soluble enzyme extract. When indicated in Table 3a and 3b, the soluble fraction and membrane fraction of the extracts were recombined to equal the total protein content of the original crude extract. Incubation was at 37°C, in the dark, for 1 hour in 125 mm X 15 mm glass test tubes and stoppered. The reaction was stopped by adding 3 mL of ethyl acetate-acetic acid (3:1, v/v). After mixing, the tubes were centrifuged and the pellet was washed with an additional 3 mL of ethyl acetate-acetic acid. To the combined extracts 5 mL of ether was

added and the resulting solution was washed with 3 mL of distilled water. The aqueous layer was discarded and the porphyrins removed from the organic layer by extracting twice with 2 mL portions of 3 M HCl. To the combined extracts, 5 mL of ether and 3 mL of saturated sodium acetate solution were added and the mixture was shaken. The ether layer, which contained all the porphyrins, was then extracted twice with 2 mL portions of 0.05 M HCl to remove Copro and once with 2 mL of 0.15M HCl to remove the Proto. The amounts of Copro and Proto were determined by measuring the spectra of the acid solutions between 390 nm and 420 nm. The amounts of each were determined by using an extinction coefficient of 489 $mM^{-1}cm^{-1}$ at 401 nm for Copro and 262 $mM^{-1}cm^{-1}$ at 407 nm for Proto.

2.17.2 Anaerobic activity

Anaerobic activity was assayed essentially as described by Tait (1972). The standard assay mixture contained, in a total of 1.0 mL, 100 mM phosphate buffer (KH_2PO_4-NaOH), pH 7.5, 10 mM of $MgSO_4$, 4 mM of ATP (added as a solution at pH 8.0), 0.3 mM NADH, 0.2 mM $NADP^+$, 1mM of L-methionine, approximately 30 nmol of freshly prepared Coprogen and 10-15 mg *R. capsulatus* soluble enzyme extract. The normal assay contained both the soluble and membrane fractions of the extracts recombined to equal the total protein content of the original crude extract. The values obtained for this activity were carried out during the adaptation described in Section 2.14. When indicated in Tables 3a and 3b, only the soluble fraction was used. Solutions of all the constituents, and the water used to make up the volume, had been previously saturated with nitrogen gas. After making all the additions to 125 mm X 15 mm glass test

tubes, nitrogen gas was blown over the surface of the solutions, the tubes were tightly stoppered, and they were incubated at 37°C for 1.5 hours in the dark. The reaction was stopped and Copro and Proto were isolated and determined as above. The only difference in procedure was that tubes were left 30 minutes in the light after addition of ethyl acetate-acetic acid mixture, to allow for the oxidation of the porphyrinogens to porphyrins. For each experiment, data were expressed as the mean of triplicate observations.

2.18 PBG Synthase Partial Purification from *Rhodobacter capsulatus* Cell Lysates

PBG synthase was isolated from crude cell extracts of *R. capsulatus* essentially as described previously (Nandi *et al.*, 1968). *R. capsulatus* strains were initially grown aerobically and then semiaerobically, as described in Section 2.14. The preparation of the extract and all subsequent purification steps were carried out at 0-4°C unless otherwise stated. The cells were harvested by centrifugation at 8000 rpm, washed twice with 0.9% NaCl solution and resuspended in a minimal (typically 1/10 culture volume) amount of 0.1 M potassium phosphate buffer, pH 6.8, containing 0.01M β-mercaptoethanol. The cell suspension was then disrupted by passing them through a French pressure cell at 15,000 lb/in^2. The broken cell suspension was centrifuged in a Ti80 rotor at 105,000 x g for 2 hours, and the supernatant solution collected, aliquoted and stored at -20°C.

Aliquots (50 mL) of crude extract were stirred and heated to 55°C in a period of about 5 minutes, and the heating and stirring were continued for another 5 minutes. At

the end of the heating period, the flask was immediately cooled in an ice bath. The aliquots were combined, and the precipitate was removed by centrifugation. Acetone at -20°C was added dropwise to the extract, which was stirred and maintained at -5°C until the acetone concentration was 40% by volume. After removal of a slight precipitate by centrifugation, the addition of the cold acetone was continued in a similar fashion until its concentration reached 60% by volume. The mixture was gently stirred for another 10 minutes, and the precipitate collected by centrifugation. The precipitate was dissolved in about 15 mL of 0.1 M potassium phosphate buffer, pH 7.6, containing 0.01 M β-mercaptoethanol. This solution was clarified by centrifugation, and solid ammonium sulphate was added to the supernatant until its concentration reached 35% of saturation. The pH was adjusted to 6.0 with 2 M acetic acid, and the solution was centrifuged after it was gently stirred for 30 minutes. The precipitate was dissolved in 5 mL of the above phosphate-β-mercaptoethanol buffer, pH 7.6, and clarified by centrifugation.

A Bradford protein assay (Section 2.13) was conducted to estimate protein quantity, and for each milligram of protein in the solution, 15 μL of a cool 2% solution of protamine sulphate, pH 7.0, was added slowly, and the mixture was gently stirred for 10 minutes after addition. The precipitate was removed by centrifugation, and solid ammonium sulphate was added to the gently stirred supernatant until the concentration of the salt reached 30% saturation. The pH of the solution was adjusted to 6.0 with 2 M acetic acid and the ammonium sulphate concentration was readjusted to 30% saturation. The mixture was stirred gently for another 30 minutes and the precipitate collected by

centrifugation was dissolved in about 5 mL of the above mentioned phosphate-β-mercaptoethanol buffer, pH 7.6 and clarified by centrifugation.

After purification, PBG synthase assays were conducted (Section 2.19) to ensure the lack of porphyrinogens being synthesized and, therefore, reconfirm the absence of HemC and/or HemD.

2.19 PBG Synthase Assay from Partially Purified *Rhodobacter capsulatus* Cell Lysates

Cell lysates partially purified to remove HemC and/or HemD activity (see Section 2.18) were prepared from cultures of wildtype and mutant strains of *R. capsulatus* adapted from aerobic to semiaerobic growth conditions (see Section 2.14). These partially purified lysates were assayed for PBG synthase activity essentially as described previously (Jordan and Shemin, 1973). PBG synthase assay mixtures consisted of, unless otherwise stated, 0.1 mg of protein, 100 mM Tris-HCl buffer, pH 8.5, 3.33 mM aminolevulinic hydrochloride pH 7.0, 5 mM β-mercaptoethanol, and 50 mM potassium chloride in a 3 mL total assay volume. The assay mixture was incubated for 2.5 hours at 37°C in the dark in a 125 mm X 15 mm culture tube filled with nitrogen gas and stoppered. After a timed incubation, the reaction is stopped by addition of 1 mL of a 20% TCA, 0.1 M mercuric chloride solution. A 1 mL aliquot is then removed, clarified by centrifugation, and added to 1 mL of freshly prepared Ehrlich's reagent. This mixture was incubated for 5 minutes to allow the colour of the PBG derivative to develop and then A_{555} was read and the amount of PBG calculated using an extinction coefficient of

61 $mM^{-1}cm^{-1}$. For each experiment, data were expressed as the mean of triplicate observations.

2.20 Inhibition Studies of Wildtype *Rhodobacter capsulatus* PBG Synthase

PBG synthase inhibition studies were initially performed on crude cell extracts of semiaerobically grown wildtype *R. capsulatus* as described in Section 2.19, except with the addition of Copro, Coprogen, Uro, and Urogen at 5μM, 16μM or 33μM as final concentrations in each assay. Once the results had been collected for these initial inhibition studies, a more complete kinetic and inhibition study was performed with a partially purified extract essentially as described in Section 2.19, except that the concentrations of Coprogen or Urogen were varied between 10 and 30 μM and the concentrations of ALA were varied between 1 and 50 mM. For each experiment, data were expressed as the mean of triplicate observations.

2.21 RNA Isolation and Northern Blotting

Wildtype *R. capsulatus* was grown aerobically at 28°C in 2L flasks until A_{680} = 0.5, after which the contents of three flasks were combined into one 2L flasks and grown under semiaerobic growth conditions for up to 1.5 hours. After the shift, 30mL samples were collected at T = 0, 15, 30, 45, 60 and 90 minutes, and centrifuged at 8000 rpm at 4°C for 10 minutes. The supernatant was then removed and the remaining pellet was immediately stored at -80°C. Within 24 hours, total RNA was isolated using the RNeasy RNA Isolation Kit (Qiagen Inc. Mississauga, Ont) according to the manufacturer's

instructions. A_{260} was determined for calculations of RNA concentration and A_{260}/A_{280} were obtained to determine RNA quality. RNA (10 μg per well) was fractionated on a 1.2% w/v formaldehyde-agarose gel and blotted onto nylon membrane (Hybond-XL, Amersham Pharmacia Biotech Inc. Buckinghamshire, UK) (Koetsier, 1993). DIG-labeled DNA probes of *hemC* and *hemE* (isolated from pCAP154) were generated using the DIG High Prime DNA labeling and Detection Starter Kit II (Roche Molecular Biochemicals. Mannheim, Germany) according to the manufacturer's instructions. After cross-linking, the membrane was prehybridized for 30 minutes and hybrized overnight at 50°C, washed, and DIG-labeled DNA-mRNA hybrids were immunologically detected according to the same manufacturer's instructions. The membranes were exposed to film (Kodak Biomax ML, USA) for 20 minutes and developed.

3.0 Results

3.1 Bacteriochlorophyll Content of Mutant Strains of *Rhodobacter capsulatus*

Under aerobic growth conditions, Bchl content values for all *R. capsulatus* mutant strains, JS01, JS02, HUP35A and JSΔN, are similar to that of wildtype (Table 2). However, under semiaerobic growth conditions, this is clearly not the case. JS01, in which *pufQ* has been disrupted in the chromosome with a kanamycin cassette, is photosythetically incompetent because the kanamycin cassette interferes with the transcription of the remainder of the downstream *puf* operon genes. On the other hand, JS02, which contains an in-frame deletion of *pufQ*, is photosynthetically competent under high light intensity. Nevertheless, both mutants form very little Bchl under semiaerobic growth conditions when compared with the wildtype strain, JS01 containing only 2.7% and JS02 only 3.6% of the amount of Bchl formed by wildtype (Table 2). Having a wildtype copy of *pufQ* present in *trans* on plasmid pXCA935 in JS01 does not rescue the photosynthetic capability of the mutant, but does increase its Bchl content to 28% of the wildtype under semiaerobic growth conditions. Finally, JSΔN, which is a mutant strain of *R. capsulatus* that lacks subunit E of the NADH dehydrogenase, is also photosynthetically incompetent, but produces 26.1% the Bchl formed by the wildtype under semiaerobic growth conditions (Table 2).

3.2 Phenotype of strain HUP35A

In order to examine the functional effect of a point mutation in the membrane-spanning region of the PufQ protein, a strain of *R. capsulatus* (HUP35A) with a mutation

Table 2: Bacteriochlorophyll Content in *pufQ*-Disrupted Strains. Pigments were extracted from mid-log phase *R. capsulatus* cultures and analyzed for Bchl content as described in Section 2.15. Values are tabulated as specific Bchl content and as a percentage of wildtype Bchl content. Values are represented as the mean and standard deviation of triplicate samples.

Rhodobacter capsulatus Strain	Bchl content - Aerobic (nmol Bchl/mg membrane protein)	% Bchl content of Wildtype	Bchl content - Semiaerobic (nmol Bchl/mg membrane protein)	% Bchl content of Wildtype
B10	10.6 ± 0.7	---	169.1 ± 9.0	---
JS01	10.4 ± 0.2	98 %	4.6 ± 0.4	2.7 %
JS02	12.5 ± 1.5	118 %	6.1 ± 0.9	3.6 %
HUP35A	8.9 ± 1.1	84 %	30.4 ± 2.0	18.0 %
JSΔN	8.3 ± 0.3	79 %	44.1 ± 2.2	26.1 %
JS01pXCA935	10.5 ± 1.6	99 %	47.4 ± 3.5	28.0 %
JS01pLAB2	10.6 ± 2.1	100 %	53.6 ± 2.7	31.6 %

in the *pufQ* gene encoding a proline35alanine substitution in PufQ was constructed by the recombination technique described in Section 2.12. It has been shown that chromosomal DNA carrying selectable markers can also be used to transform *R. capsulatus* strains. The incoming DNA recombines with homologous sequences on the resident chromosome. Transformation by such chromosomal recombination can be effected by homologous DNA presented as a linear fragment or as a cloned insert in a recombinant vector. This property was exploited to insert a heterologous piece of DNA into the chromosome of JS01. Bracketing the heterologous DNA by homologous DNA directs it to a specific chromosomal locus. Applications of this system include gene activation by recombination with a cloned altered allele (Golden *et al.*, 1986), gene duplication (Williams and Szalay, 1983; Golden *et al.*, 1987) and insertional mutagenesis (Buzby *et al.*, 1985; Labarre *et al.*, 1989).

In order to verify the accuracy of the point mutation in HUP35A, a 500 bp portion of the genome from *bchZ* to *pufB* was sequenced. Sequencing of this fragment confirmed a single base-pair substitution (C to G) at base-pair 103 of *pufQ*. Since no other mutations were identified, the rest of *pufQ*, *pufB* and *bchZ* was concluded to be wildtype and, therefore unaffected by the recombination event. The parent strain to HUP35A is JS01; however, unlike JS01, strain HUP35A is photosynthetically competent, and is similar in appearance to wildtype *R. capsulatus*. Under aerobic growth conditions, Bchl content in both JS01 and HUP35A is similar to that of wildtype (Table 2). However, under semiaerobic growth conditions, the Bchl content of HUP35A is 18% of that of the wildtype grown under similar conditions, while that of JS01 is only 2.7% of that of the

wildtype (Table 2). Therefore, in the absence of *pufQ*, Bchl synthesis is greatly affected; however, in a strain that contains *pufQ* with a proline35alanine mutation (HUP35A), Bchl synthesis is somewhat restored, but not to wildtype levels. When HUP35A is grown under anaerobic photosynthetic growth conditions, Bchl levels increase to 65.4% to that of wildtype (data not shown). Since JS01 is photosynthetically incompetent, it cannot be grown under these growth conditions.

Strain JS01pLAB2 was obtained before the chromosomal recombination event had occurred in the construction of HUP35A. In this strain, *pufQ* containing the proline35alanine point mutation is only present in *trans* as a single copy on plasmid pLAB2. This strain is also photosynthetically incompetent. However, when JS01pLAB2 is grown semiaerobically, it synthesizes 31.6% the quantity of Bchl that wildtype synthesizes under the same growth conditions (Table 2). However, since JS01 with wildtype *pufQ* in *trans* (JS01pXCA935) only produces 28.0% of the Bchl of the wildtype under the same conditions, any effect on Bchl synthesis of the point mutation in *pufQ* when it is present in *trans* is not apparent.

3.3 Coproporphyrinogen III Oxidase Activity in Mutant Strains of *Rhodobacter capsulatus*

The membrane and cofactor requirements for aerobic and anaerobic Coprogen oxidase (HemZ) activities are presented in Table 3a and Table 3b. Aerobic Coprogen oxidase activity was solely found in the soluble fraction and absent in the pellet in both aerobically and semiaerobically grown wildtype *R. capsulatus*. Anaerobic activity was

Table 3a: Coproporphyrinogen III Oxidase Specific Activity in Aerobically Grown Wildtype *Rhodobacter capsulatus.* Crude extract (39.5 mg of protein/mL) of *R. capsulatus* prepared from cells as described in Section 2.14, and ultracentrifuged for 2 hours at 105, 000 x g. The clear supernatant (13 mg of protein/mL) was removed and the pellet was resuspended in 0.5 volume of that of the original crude extract (45.5 mg of protein/mL). Samples were analyzed for coprogen oxidase activity under aerobic and anaerobic incubation conditions, as described in Section 2.17. Values represent the mean of triplicate samples.

Table 3b: Coproporphyrinogen III Oxidase Specific Activity in Semiaerobically Grown Wildtype *Rhodobacter capsulatus.* Crude extract (38.4 mg of protein/mL) of *R. capsulatus* prepared from cells as described in Section 2.14, and ultracentrifuged for 2 hours at 105, 000 x g. The clear supernatant (13.1 mg of protein/mL) was removed and the pellet was resuspended in 0.5 volume of that of the original crude extract (46.9 mg of protein/mL). Samples were analyzed for Coprogen oxidase activity under aerobic and anaerobic incubation conditions, as described in section 2.17. Values below represent the mean of triplicate samples.

a)

Fraction	Wt. of Protein (mg)	Protoporphyrin Formed (nmols/hr/mg protein) Anaerobic Incubation	Protoporphyrin Formed (nmols/hr/mg protein) Aerobic Incubation
Crude Extract	15.8	0.16	0.11
Supernatant	5.2	0	0.23
Pellet	9.1	0.01	0.03
Supernatant + pellet	18.2	0.22*	0.11*

* per mg of soluble protein

b)

Fraction	Wt. of Protein (mg)	Protoporphyrin Formed (nmols/hr) Anaerobic Incubation	Protoporphyrin Formed (nmols/hr) Aerobic Incubation
Crude Extract	12.2	0.14	0.15
Supernatant	4.2	0.05	0.4
Pellet	7.5	0	0.03
Supernatant + pellet	11.3	0.23	0.17

* per mg of soluble protein

only detected when the supernatant and pellet fractions were recombined. In contrast with the aerobic activity, the anaerobic activity appears to require at least two proteins, or protein complexes, one in the supernatant and one in the pellet obtained by centrifuging the crude extract at high speed. This suggests that there is some component of the membrane fraction required for activity when oxygen is not available as the final electron acceptor. When oxygen is available, however, it can react directly with the soluble enzyme.

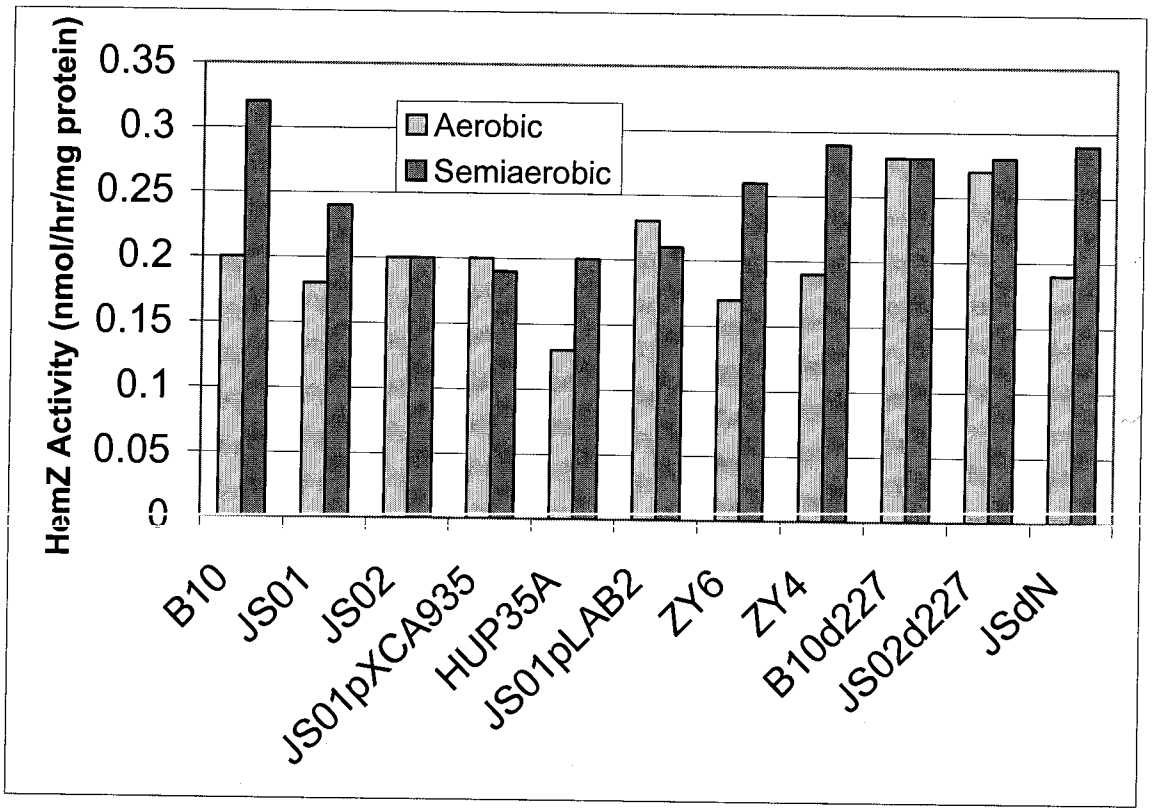
Coprogen oxidase assays involving wildtype and mutant strains of *R. capsulatus* were initially performed with and without the addition of the membrane fraction, under both aerobic and anaerobic incubation conditions. Results indicated that the presence of the membrane fraction under aerobic incubation conditions demonstrated little difference in Coprogen oxidase specific activity (on a per mg soluble protein basis) (*data not* shown), and therefore was not continued in the remainder of the experiment. Similarly, the absence of the membrane fraction under anaerobic incubation conditions demonstrated no significant Coprogen oxidase specific activity (data not shown), and, therefore, was also excluded from the remainder of the experiment. Only specific activity values for anaerobic incubations with the addition of the membrane fraction are presented below in this thesis since they were very similar to values for aerobic incubation in the absence of the membrane fraction when calculated on a per mg soluble protein basis.

According to results presented in Table 4 and summarized in Figure 7, Coprogen oxidase specific activity increases 1.6-fold in the wildtype strain during transition from

Table 4: Coproporphyrinogen III Oxidase Activity in *pufQ*-Disrupted and Other Mutant Strains of *Rhodobacter capsulatus*. Cell lysates grown under aerobic or semiaerobic growth conditions were analyzed for Coprogen oxidase specific activity (per mg soluble protein) as described in Section 2.17. Values represent the mean and standard deviation of triplicate samples.

***Rhodobacter capsulatus* Strain**	**HemZ Activity Aerobic Growth** (nmol Protogen/mgP/hr)	**HemZ Activity Semiaerobic Growth** (nmol Protogen/mgP/hr)	**Specific Activity Ratio**
B10	0.20 +/- 0.01	0.32 +/- 0.04	1.6
JS01	0.19 +/- 0.03	0.24 +/- 0.07	1.3
JS02	0.21 +/- 0.03	0.20 +/- 0.04	1.0
JS01pXCA935	0.21 +/- 0.04	0.19 +/- 0.01	0.9
HUP35A	0.13 +/- 0.04	0.21 +/- 0.01	1.5
JS01pLAB2	0.23 +/- 0.01	0.22 +/- 0.01	0.9
ZY6	0.17 +/- 0.01	0.27 +/- 0.01	1.5
ZY4	0.19 +/- 0.02	0.29 +/- 0.04	1.4
B10Δ227	0.28 +/- 0.01	0.28 +/- 0.04	1.0
JS02Δ227	0.28 +/- 0.02	0.28 +/- 0.04	1.0
JSΔN	0.19 +/- 0.01	0.30 +/- 0.03	1.5

Figure 7: Coproporphyrinogen III Oxidase Activity in *pufQ*-Disrupted and Other Mutant Strains of *Rhodobacter capsulatus*. Summary of the numerical results presented in Table 4. Cell lysates grown under aerobic and then semiaerobic growth conditions were analyzed for Coprogen oxidase specific activity (per mg soluble protein) as described in Section 2.17.


HemZ Activity (nmol/hr/mg protein)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Aerobic
Semiaerobic
B10
JS01
JS02
JS01pXCA935
HUP35A
JS01pLAB2
ZY6
ZY4
B10d227
JS02d227
JSdN

aerobic to semiaerobic growth conditions. In mutant strains containing chromosomal knockouts of the *pufQ* gene (JS01 and JS02) and in HUP35A, there appears to be an incomplete induction of Coprogen oxidase activity under semiaerobic growth conditions. In none of these cases was Coprogen oxidase induced more than 1.2-fold over the average aerobic level, attaining only 63-75% of the level of wildtype Coprogen oxidase activity. Similar results were obtained when *pufQ* was present *in trans* on plasmids, pXCA935 or pLAB2 (the latter with the site-directed mutation), where Coprogen oxidase activity under semiaerobic growth conditions remains essentially the same as those under aerobic growth conditions. Coprogen oxidase activities in JSΔN, and in the *bch* mutants ZY6 (*bchH*) and ZY4 (*bchM*), reveal that there still appears to be normal induction of Coprogen oxidase when transferred from aerobic to semiaerobic growth conditions. However, specific activity values in both B10Δ227 and JS02Δ227 indicate that Coprogen oxidase activity remains at highly induced levels under both aerobic and semiaerobic growth conditions.

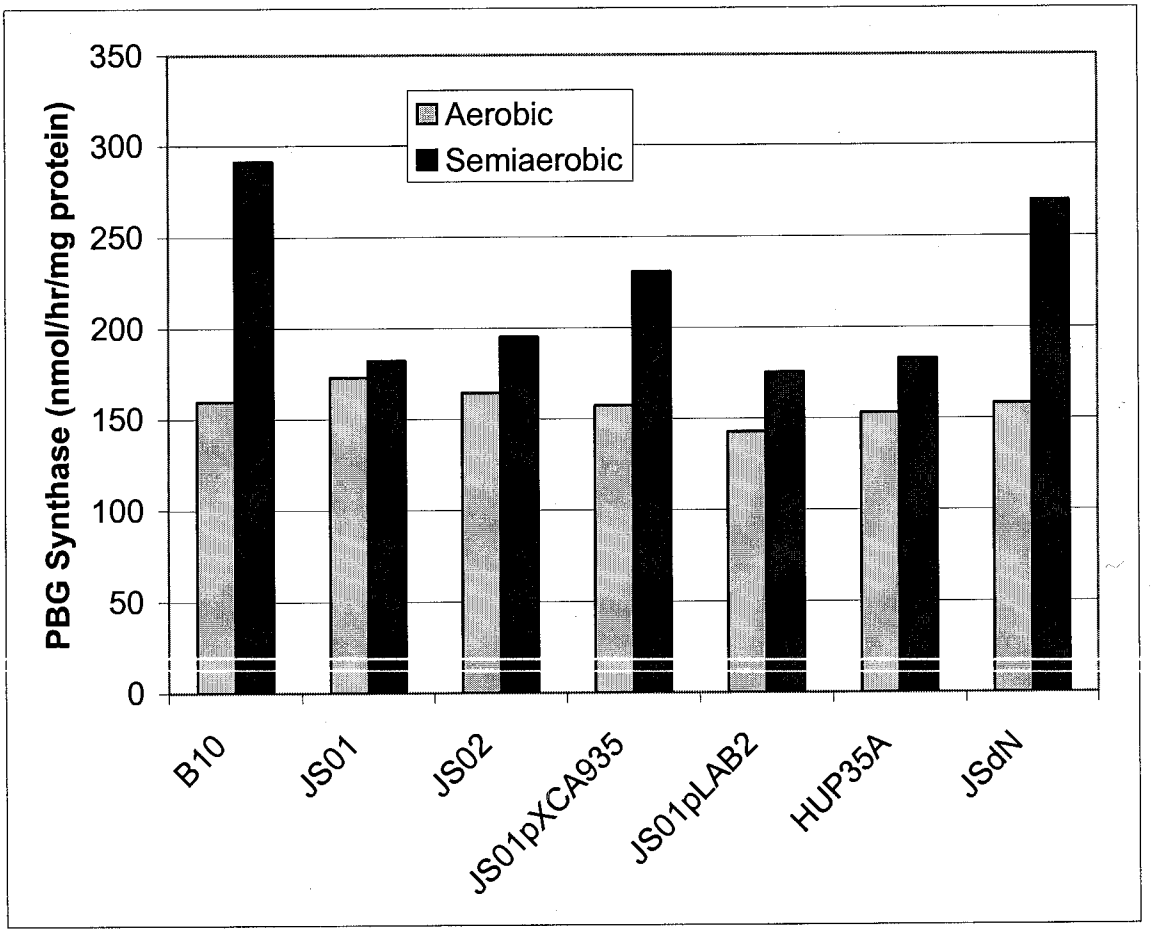
3.4 PBG Synthase Activity in Mutant Strains of *Rhodobacter capsulatus*

Crude enzyme extracts of B10, JS01, JS02, JS01pLAB2, JS01pXCA935, HUP35A and JSΔN were partially purified according to Section 2.19, to eliminate the condensation of PBG to Urogen by HemC and HemD. Specific PBG synthase activities for each strain were determined before and after transfer of aerobically grown cultures to semiaerobic growth conditions. Results are presented in Table 5 and summarized in Figure 8. PBG synthase specific activity in B10 is induced 1.8-fold during the transition. In strains containing a chromosomal knockout of the *pufQ* gene (JS01 and JS02), or a

Table 5: PBG Synthase Activity in *pufQ*-Disrupted and Other Strains of *R. capsulatus*. Cell lysates grown under aerobic or semiaerobic growth conditions were analyzed for PBG synthase specific activity as described in Section 2.19. Values below represent the mean and standard deviation of triplicate samples.

R. capsulatus Strain	PBG Synthase Activity Aerobic Growth (nmol PBG/mgP/hr)	PBG Synthase Activity Semiaerobic Growth (nmol PBG/mgP/hr)	Specific Activity Ratio
B10	159.6 +/- 8.7	291.4 +/- 7.5	1.8
JS01	172.9 +/- 12.7	182.0 +/- 7.8	1.0
JS02	164.3 +/- 8.2	195.1 +/- 15.0	1.2
JS01pXCA935	157.3 +/- 6.9	230.8 +/- 7.4	1.5
HUP35A	152.9 +/- 10.7	182.8 +/- 24.5	1.2
JS01pLAB2	142.8 +/- 21.8	175.3 +/- 7.5	1.2
JSΔN	158.1 +/- 5.2	269.5 +/- 8.5	1.7

Figure 8: PBG Synthase Activity in *pufQ*-Disrupted and Other Mutant Strains of *Rhodobacter capsulatus*. Summary of the numerical results presented in Table 5. Cell lysates grown under aerobic and semiaerobic growth conditions were analyzed for PBG synthase specific activity as described in Section 2.19. Values represent the mean of triplicate samples.


PBG Synthase (nmol/hr/mg protein)
350
300
250
200
150
100
50
0
Aerobic
Semiaerobic
B10
JS01
JS02
JS01pXCA935
JS01pLAB2
HUP35A
JSdN

mutant copy of *pufQ* (HUP35A), there appears to be very little induction (1.1-1.2 times) of PBG synthase specific activity under semiaerobic growth conditions. Similar results were obtained when *pufQ* with a proline35alanine point mutation is introduced in *trans* on plasmid, pLAB2. However, when *pufQ* is introduced to JS01 in *trans* on plasmid pXCA935, PBG synthase is induced 1.5-fold, which was still, however, less than that observed for the wildtype. Induction of PBG synthase specific activity under semiaerobic growth conditions does not appear to be affected in mutant JSΔN.

3.5 Kinetic Analyses of PBG Synthase

For kinetic studies, a preparation of PBG synthase was used which had been partially purified from semiaerobically grown *R. capsulatus* according to the technique presented in Section 2.19. The kinetics of enzymatic condensation were studied under standard assay conditions with an excess of substrate over the enzyme (Figure 9). The dependence of the initial rate on the substrate concentration was measured and the experimental data were analyzed by a Lineweaver-Burk plot (Figure 10a), Hanes plot (Figure 10b) and Eadie-Hofstee plot (Figure 10c). The K_m and V_{max} were determined from the Lineweaver-Burk plot of $1/v_o$ against $1/[S]_o$ (Figure 10a); the enzyme demonstrated classic Michaelis-Menten kinetics. The K_m for ALA was 2.66 mM and the V_{max} was 243.9 nmol PBG hour $^{-1}$ (mg of protein)$^{-1}$. According to the Hanes plot (Figure 10b), the K_m for ALA was 3.31 mM and the V_{max} was 256.4 nmol PBG hour $^{-1}$ (mg of protein)$^{-1}$. And according to the Eadie-Hofstee plot (Figure 10c), the K_m for ALA was 2.77 mM and the V_{max} was 246.75 nmol PBG hour $^{-1}$ (mg of protein)$^{-1}$.

Figure 9: Plot of PBG Synthase Activity vs. ALA Concentration in the Absence of Inhibitor. Initial rate of PBG synthase activity at constant enzyme concentration and varying concentrations of ALA (1 mM – 50 mM). Cell lysate preparation and assay conditions as described in Section 2.19. Values represent the mean of triplicate samples.

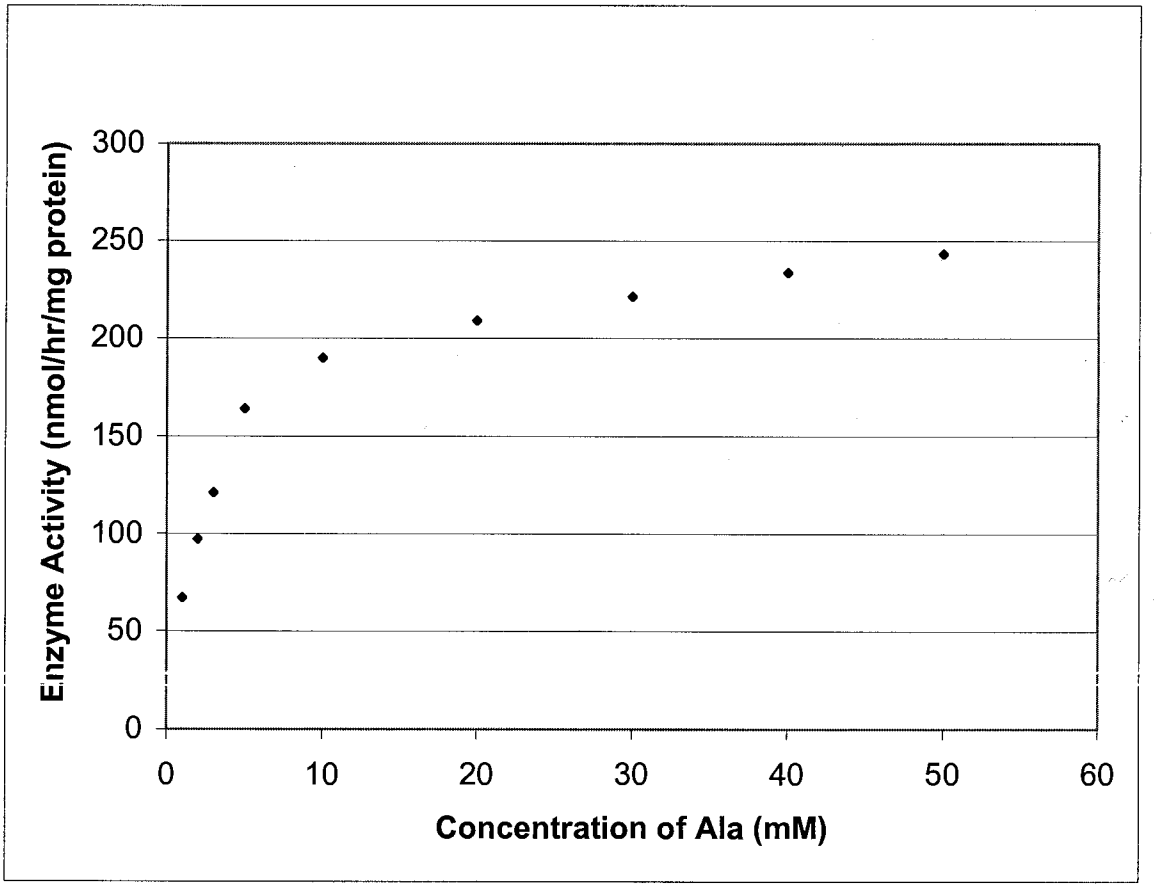

Enzyme Activity (nmol/hr/mg protein)
300
250
200
150
100
50
0
0
10
20
30
40
50
60
Concentration of Ala (mM)

Figure 10a: Lineweaver-Burk Plot for the Kinetics of PBG Synthase in the Absence of Inhibitor. $1/v_o$ is plotted against $1/[S]_o$, and a straight line is obtained where the slope is equal to K_m/V_{max} and the y-intecept is equal to $1/V_{max}$. The K_m for ALA was 2.66 mM and the V_{max} was 243.90 nmol PBG/hr/mg of protein.

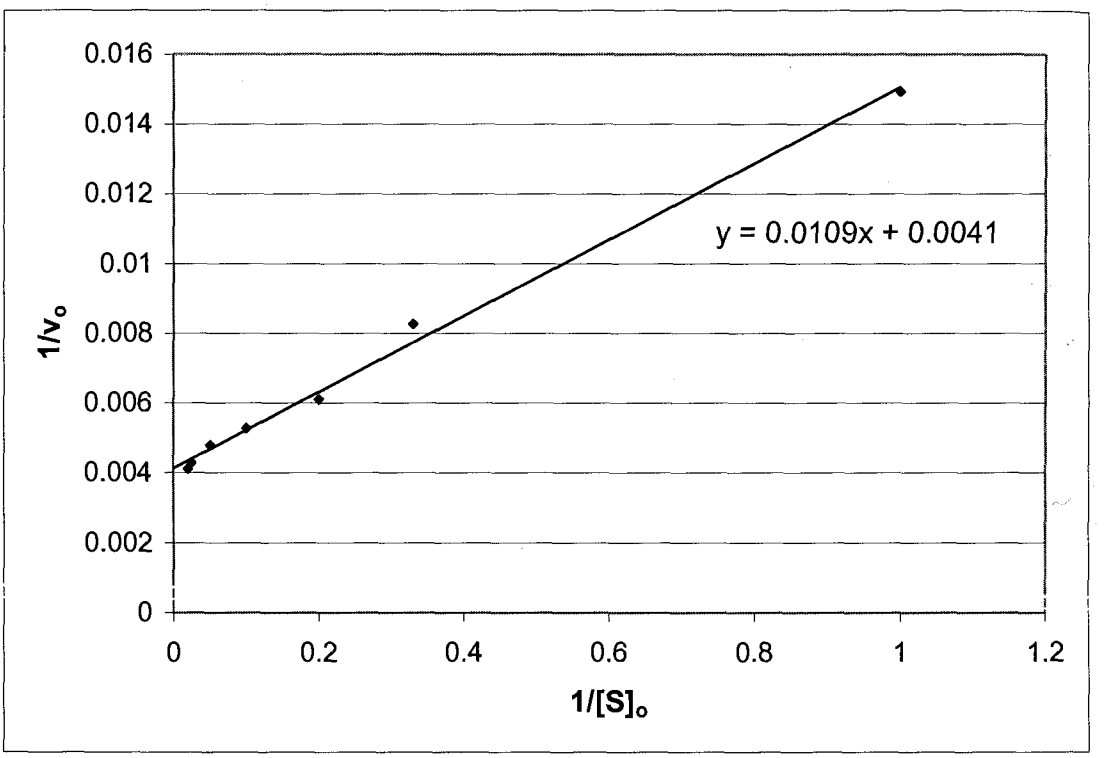

0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
1/v$_o$
y = 0.0109x + 0.0041
0
0.2
0.4
0.6
0.8
1
1.2
1/[S]$_o$

Figure 10b: Hanes Plot of PBG Synthase Kinetics in the Absence of Inhibitor. $[S]/v_o$ is plotted against $[S]_o$, and a straight line is obtained where the slope is equal to $1/V_{max}$ and the y-intercept is equal to K_m/V_{max}. The K_m for ALA was 3.31 mM and the V_{max} was 256.4 nmol PBG/hr/mg of protein.

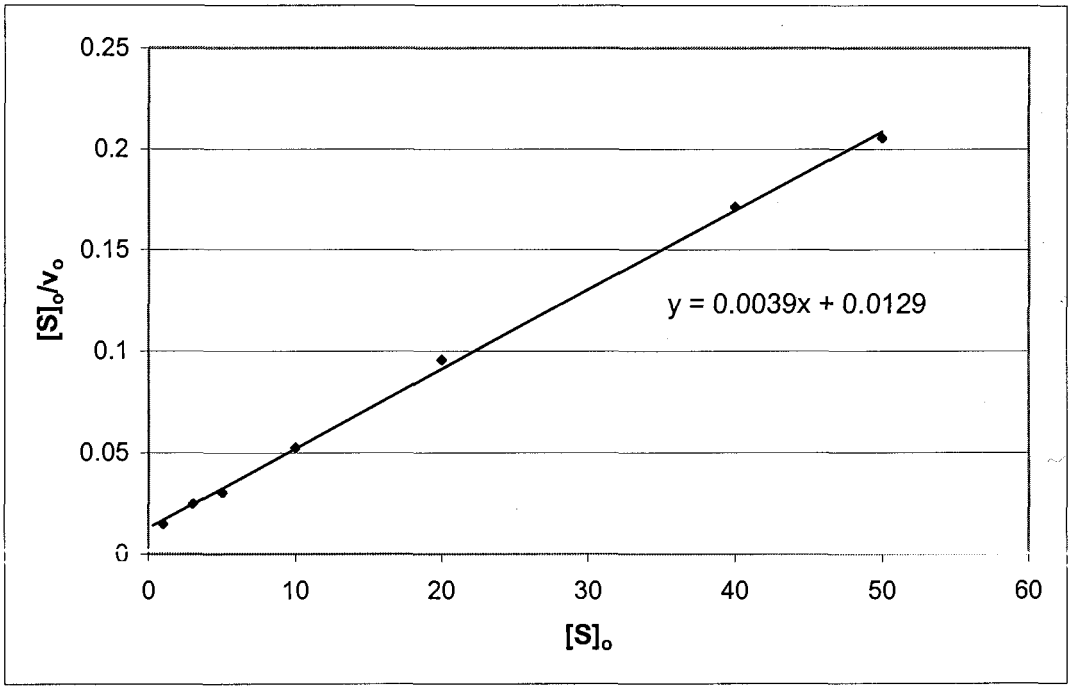
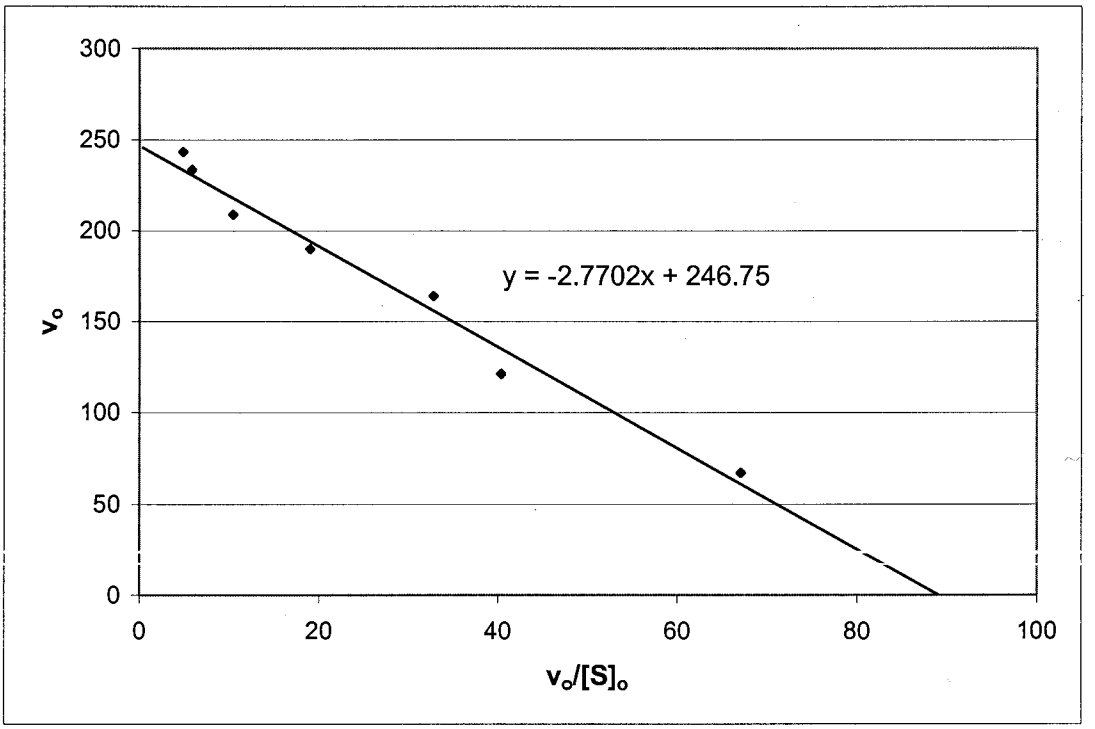

0.25
0.2
0.15
0.1
0.05
0
[S]o/vo
y = 0.0039x + 0.0129
0
10
20
30
40
50
60
[S]o

Figure 10c: Eadie-Hofstee Plot of PBG Synthase Kinetics in the Absence of Inhibitor. v_o is plotted against $V_o/[S]_o$, a straight line is obtained where the slope is equal to $-K_m$ and the y-intercept is equal to V_{max}. The K_m for ALA was 2.77 mM and the V_{max} was 246.75 nmol PBG/hr/mg of protein.


300
250
200
150
100
50
0
v_o
y = -2.7702x + 246.75
0
20
40
60
80
100
$v_o/[S]_o$

3.6 Inhibitors of PBG Synthase

In order to screen various Bchl precursors as potential inhibitors, test compounds were screened in the micromolar range rather than the millimolar range (Figure 11), with a 20 minute enzyme-inhibitor preincubation. Copro, Uro, and their physiological, reduced forms, Coprogen and Urogen, were screened in this manner. Of the four possible inhibitors, Copro did not inhibit the production of PBG at concentrations of 5 μM and 16 μM, and did not inhibit enzyme activity more than 25% at 33 μM. In addition, it appears that Uro seems to activate PBG synthase by two-fold (Figure 11). Coprogen provided substantial inhibition of PBG synthase activity. At a concentration of 16μM, PBG synthase activity had been reduced to 21% of that of control PBG synthase activity, and at a concentration of 33μM, total inactivation seems to have occurred. Subsequently, Urogen and Coprogen were then tested in more detail in order to determine their inhibitor constants. Lineweaver-Burk plots, Hanes plots and Eadie-Hofstee plots were constructed using data obtained for the condensation of six different concentrations of ALA to form PBG by PBG synthase in the presence of either 10μM, 15μM or 30μM Coprogen, or Urogen. Results for Urogen, and Coprogen are presented in Figures 12a, 12b, 12c and Figures 13a, 13b, 13c, respectively. Once the type of inhibition had been established, inhibitor constants K_i and K_I were determined for Urogen and Coprogen using secondary plots (Figures 14a and 14b, and Figures 15a and 15b, respectively). The plots of the slopes vs. [I] allows calculation of K_i values and the plots of the $1/v_o$ intercept vs. [I] allows calculation of K_I values. In the case for the secondary plots of Coprogen, the data for the 30μM concentration (slope of the Lineweaver-Burk, and $1/v_o$-intercept) was excluded from the best-fit line because the slope and $1/v_o$ data points of the uninhibited

Figure 11: PBG Synthase Specific Activity at Various Concentrations of Potential Inhibitors. The initial PBG synthase activity study that determined the effects of Copro, Coprogen, Uro, and Urogen on specific enzyme activity. Assays were preformed as described in Section 2.20, except with the addition of Copro, Coprogen, Uro, and Urogen at 5μM, 16μM or 33μM final concentration in each assay. Values represent the mean of triplicate samples.

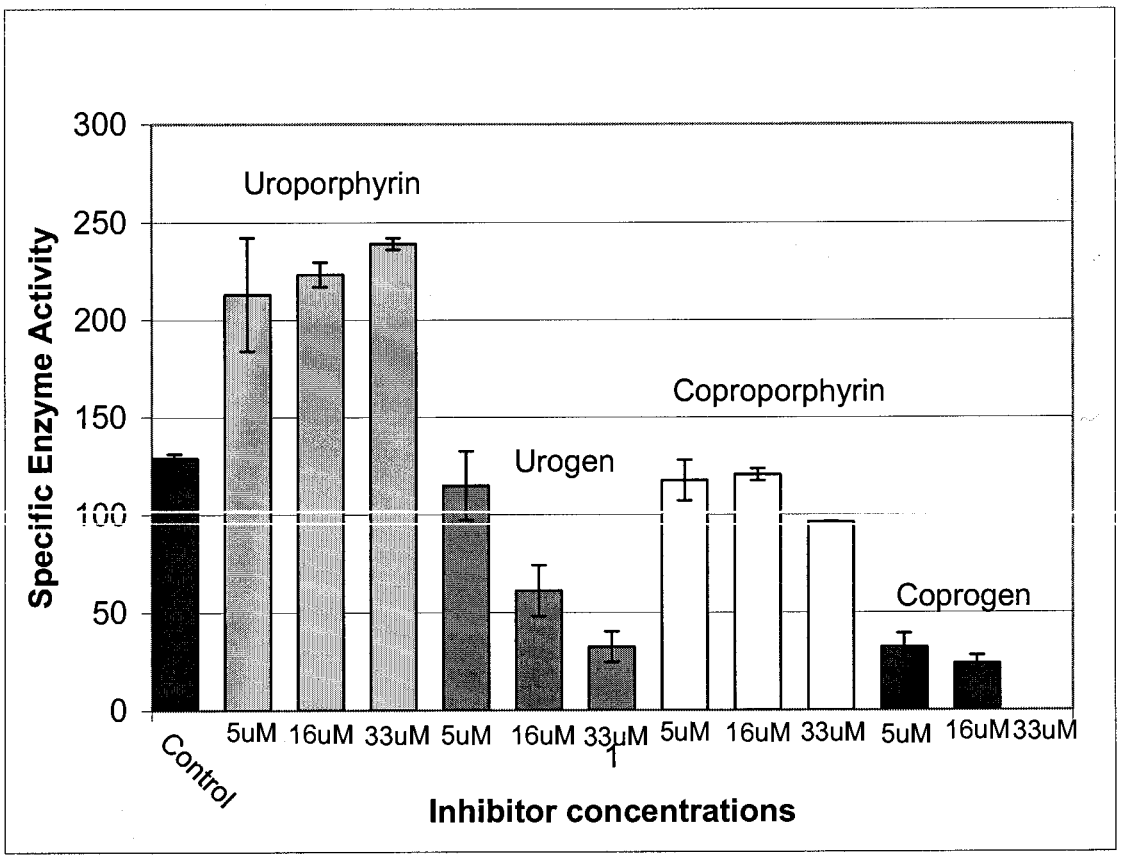

300
250
200
150
100
50
0
Specific Enzyme Activity
Uroporphyrin
Urogen
Coproporphyrin
Coprogen
Control
5uM
16uM
33uM
5uM
16uM
33uM
5uM
16uM
33uM
5uM
16uM
33uM
Inhibitor concentrations

Figure 12a: Lineweaver-Burk Plots in the Presence of Uroporphyrinogen III.

Double reciprocal Lineweaver-Burk plots for the kinetics of PBG synthase activity in the presence of different fixed concentrations of Urogen: 0 μM, 10 μM, 15 μM, and 30 μM. The intersection of the plots in the lower left quadrant is indicative of mixed-type inhibition.

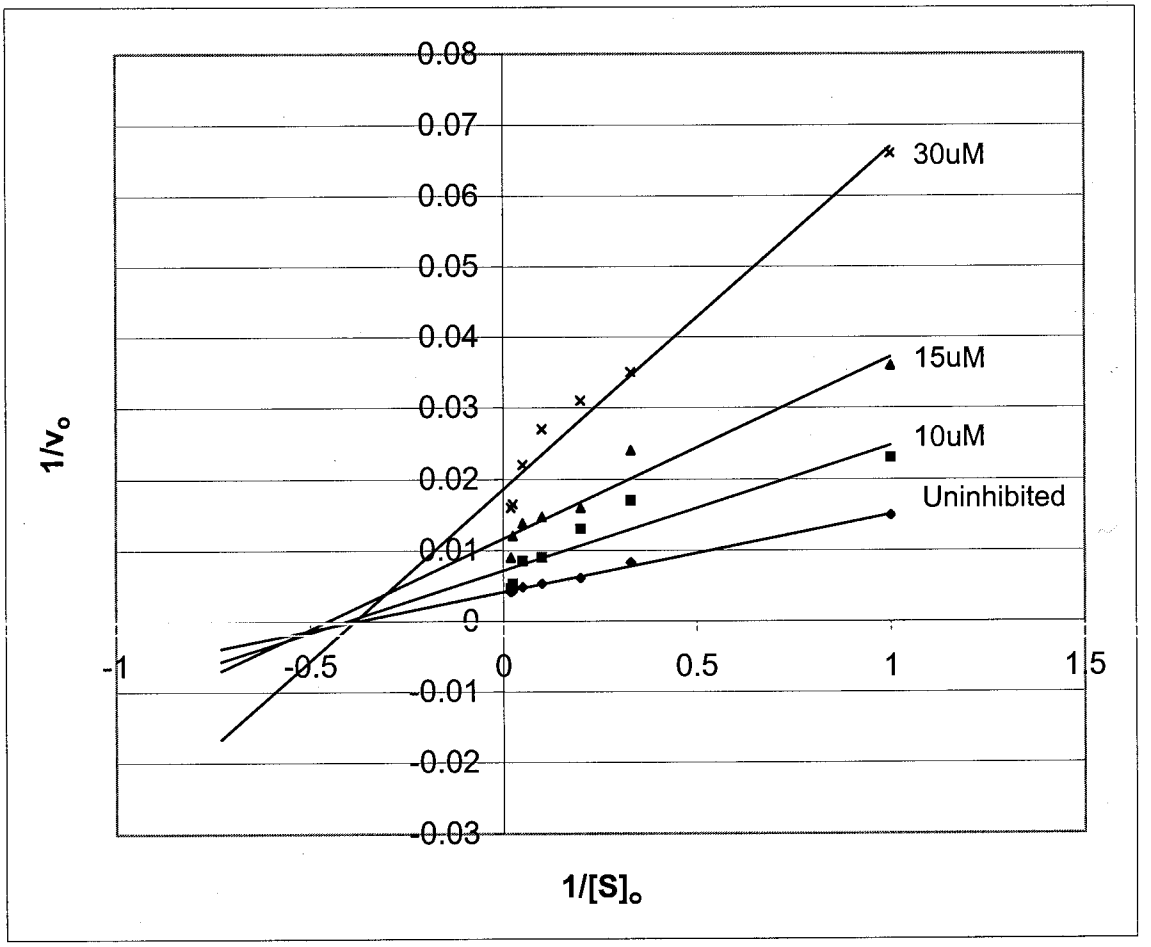

0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
-0.01
-0.02
-0.03
-1
-0.5
0
0.5
1
1.5
1/v₀
1/[S]₀
30uM
15uM
10uM
Uninhibited

Figure 12b: Hanes Plots in the Presence of Uroporphyrinogen III. Hanes plots ($[S]/v_o$ is plotted against $[S]_o$) for the kinetics of PBG synthase activity in the presence of different fixed concentrations of Urogen: 0 μM, 10 μM, 15 μM, and 30 μM.

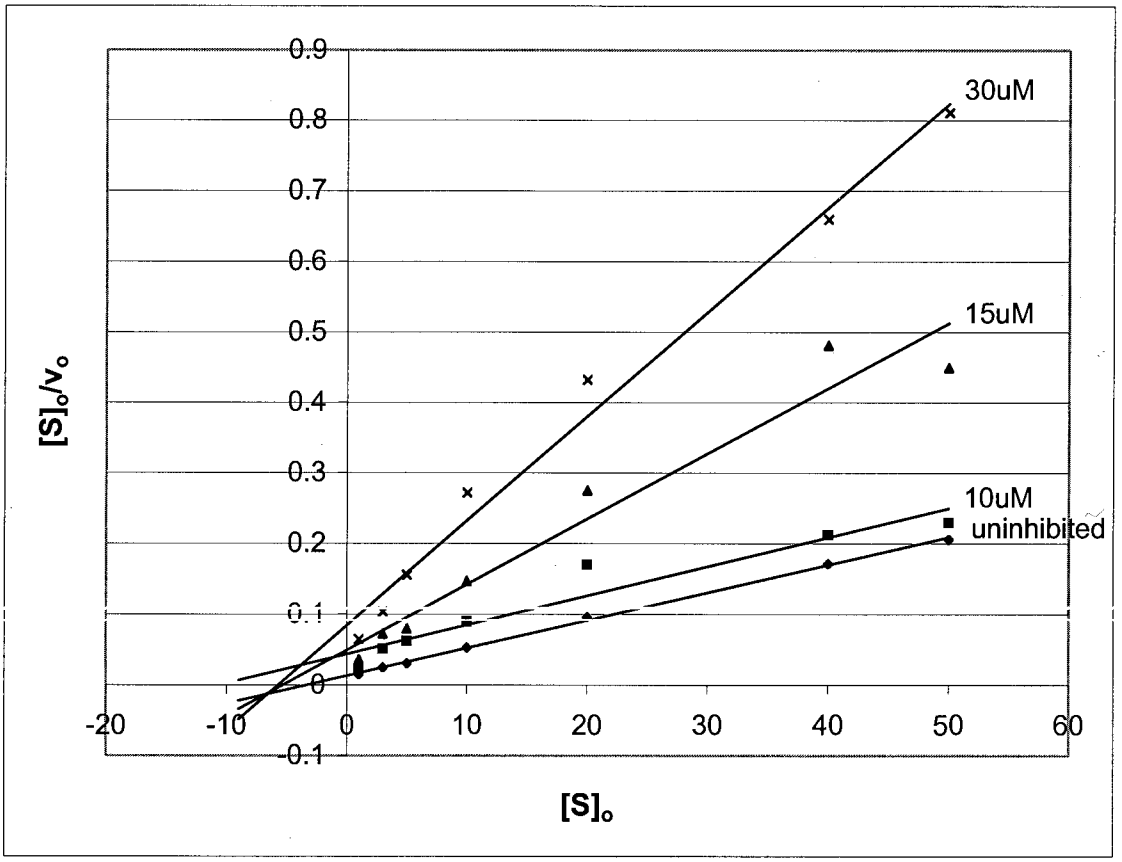

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-20
-10
0
10
20
30
40
50
60
30uM
15uM
10uM
uninhibited
[S]o/vo
[S]o

Figure 12c: Eadie-Hofstee Plots in the Presence of Uroporphyrinogen III. Eadie-Hofstee plots (v_o is plotted against $v_o/[S]_o$) of PBG synthase activity in the presence of various concentrations of Urogen: 0 μM, 10 μM, 15 μM, and 30 μM.

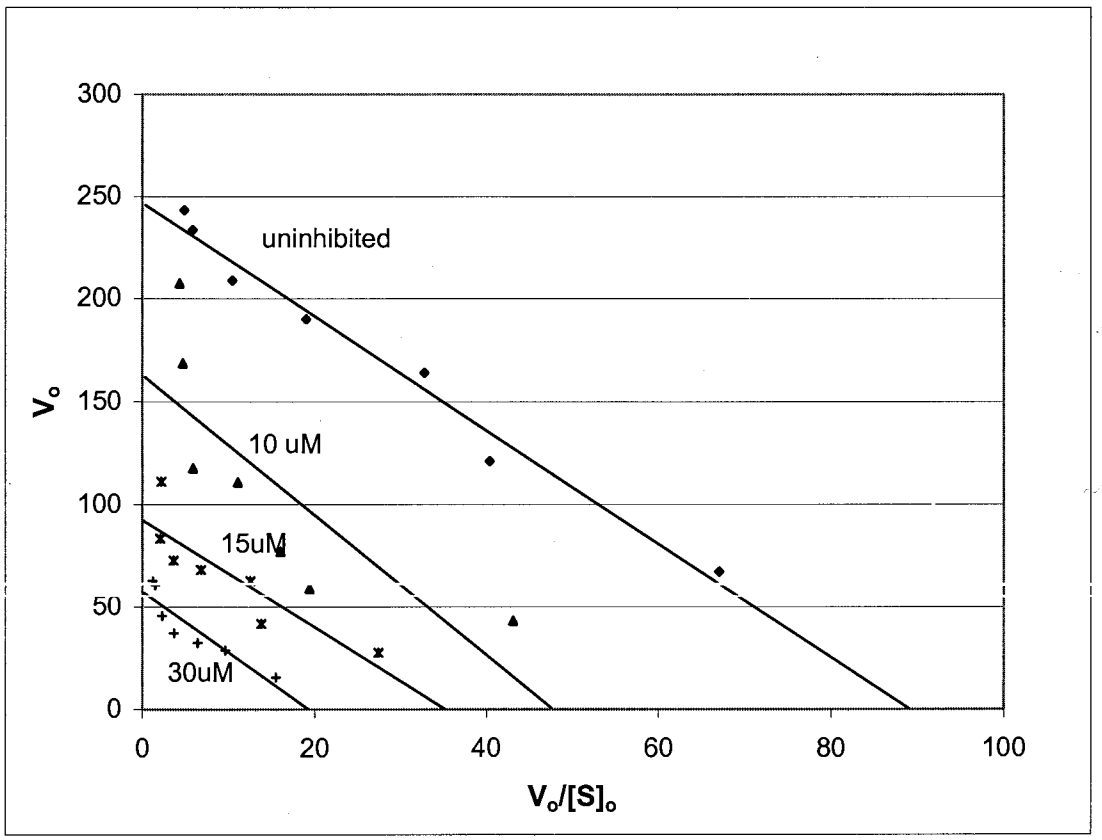
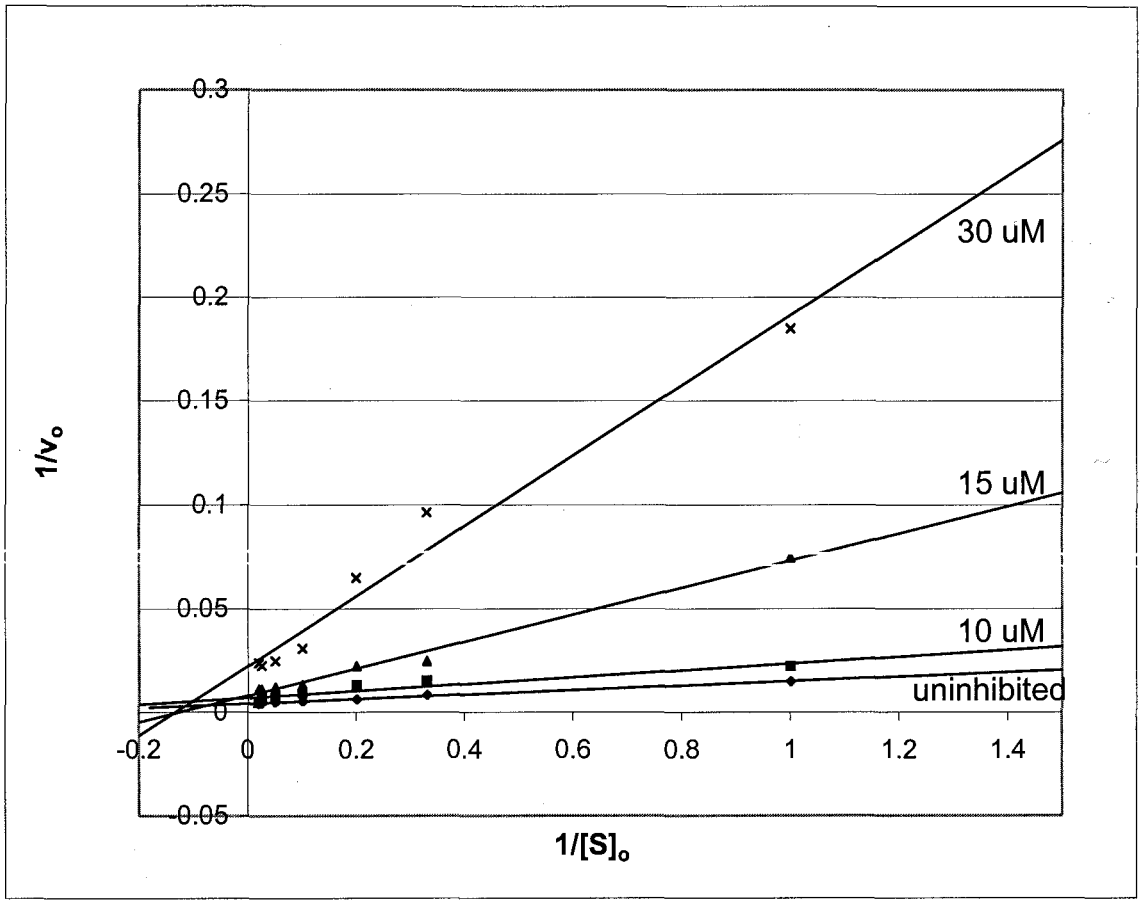

300
250
200
150
100
50
0
V_o
uninhibited
10 uM
15uM
30uM
0
20
40
60
80
100
$V_o/[S]_o$

Figure 13a: Lineweaver-Burk Plots in the Presence of Coproporphyrinogen III. Double reciprocal Lineweaver-Burk plots for the kinetics of PBG synthase activity in the presence of different fixed concentrations of Coprogen: 0 μM, 10 μM, 15 μM, and 30 μM. The intersection of the plots in the upper left quadrant is indicative of mixed-type inhibition.


0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1/v₀
1/[S]₀
30 uM
15 uM
10 uM
uninhibited

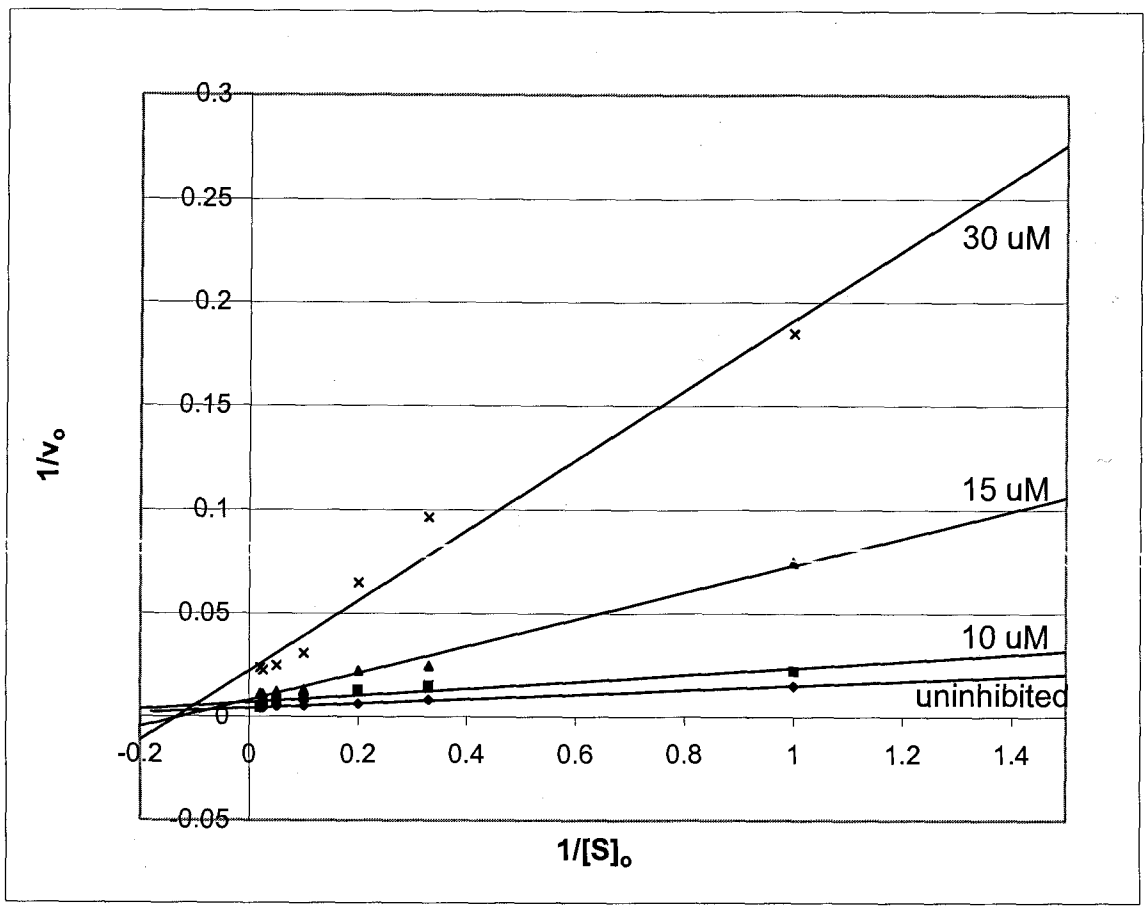

0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1/v$_o$
1/[S]$_o$
30 uM
15 uM
10 uM
uninhibited

Figure 13b: Hanes Plots in the Presence of Coproporphyrinogen III. Hanes plots ($[S]/v_o$ is plotted against $[S]_o$) for the kinetics of PBG synthase activity in the presence of different fixed concentrations of Coprogen: 0 μM, 10 μM, 15 μM, and 30 μM.

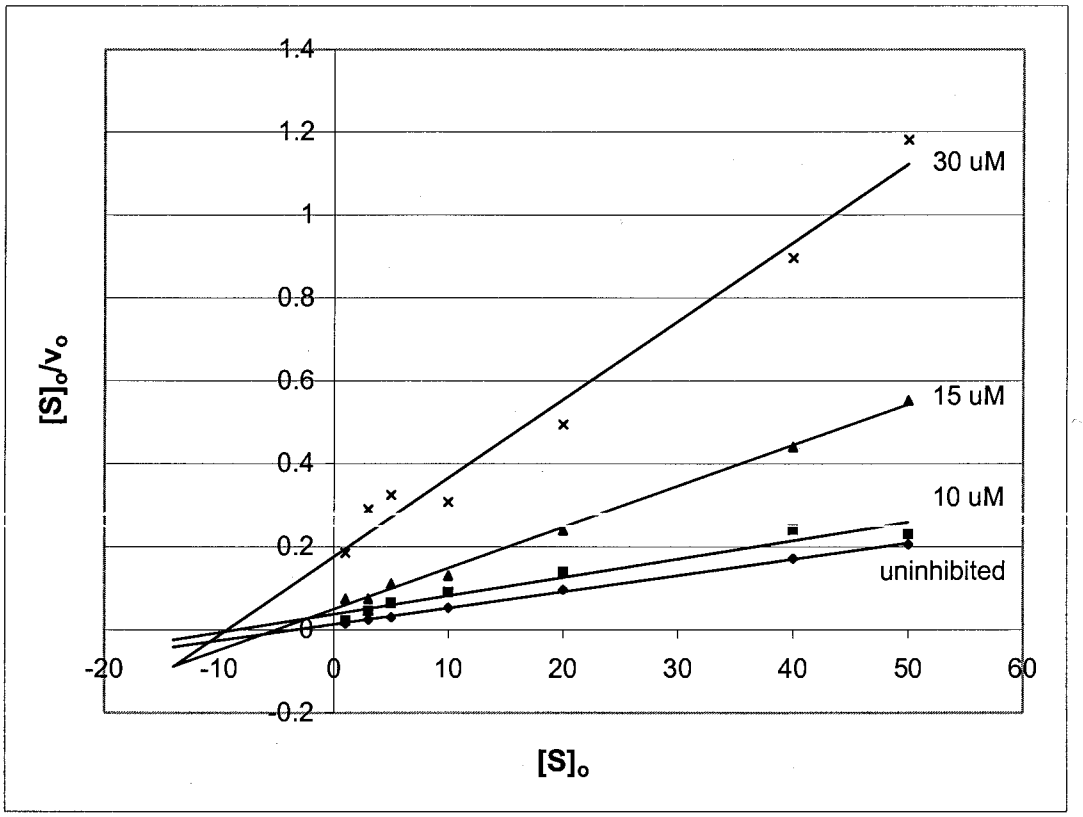

1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-20
-10
0
10
20
30
40
50
60
[S]o/vo
[S]o
30 uM
15 uM
10 uM
uninhibited

Figure 13c: Eadie-Hofstee Plots in the Presence of Coproporphyrinogen III. Eadie-Hofstee plots (v_o is plotted against $v_o/[S]_o$) of PBG synthase activity in the presence of various concentrations of Coprogen: 0 μM, 10 μM, 15 μM, and 30 μM.

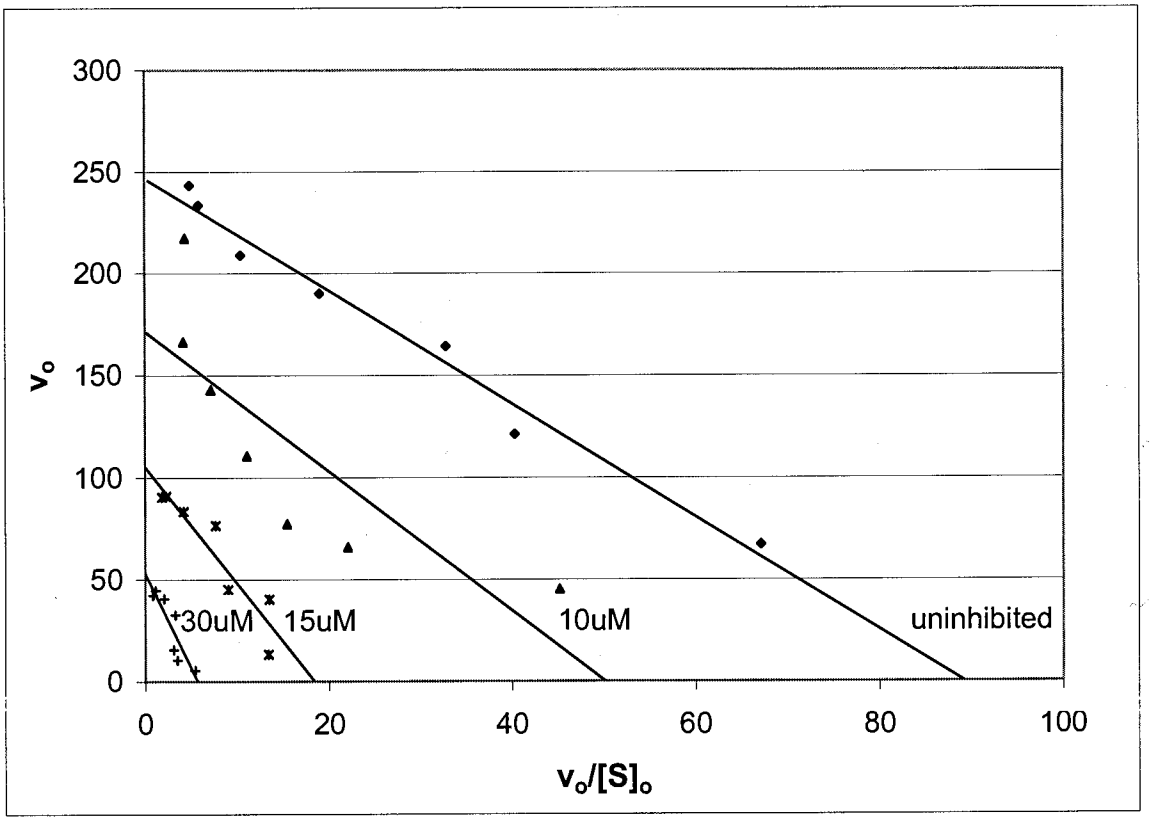
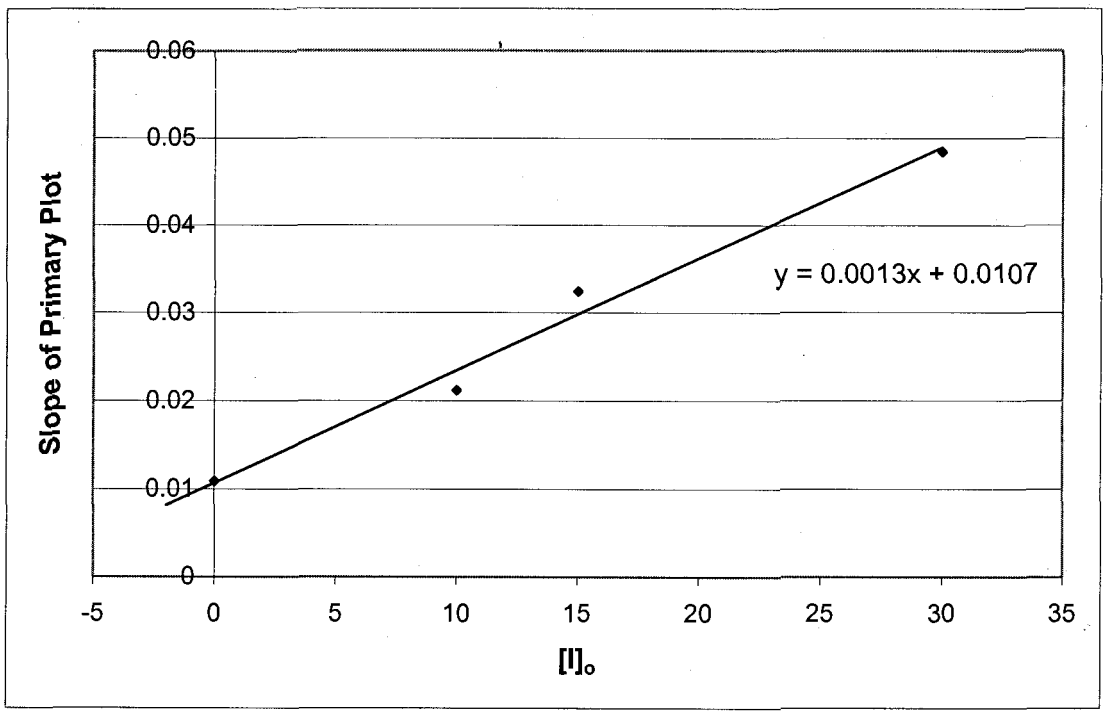

300
250
200
150
100
50
0
v_o
0
20
40
60
80
100
$v_o/[S]_o$
+30uM
15uM
10uM
uninhibited

Figure 14a: Secondary Plot (Slope vs. $[I]_o$) for Uroporphyrinogen III. Utilizing data from the Lineweaver-Burk plot, a secondary plot of the primary slope vs. $[I]_o$ allows for the calculation of a K_i value for urogen. The value of K_i was calculated to be 8.2 μM.

Figure 14b: Secondary Plot ($1/v_o$ intercept vs. $[I]_o$) for Uroporphyrinogen III. Utilizing data from the Lineweaver-Burk plot, a secondary plot of the $1/v_o$ intercept vs. $[I]_o$ allows for the calculation of a K_I value for urogen. The value of K_I was calculated to be 7.0 μM.

a)


Slope of Primary Plot
0.06
0.05
0.04
0.03
0.02
0.01
0
y = 0.0013x + 0.0107
-5
0
5
10
15
20
25
30
35
[I]o

b)

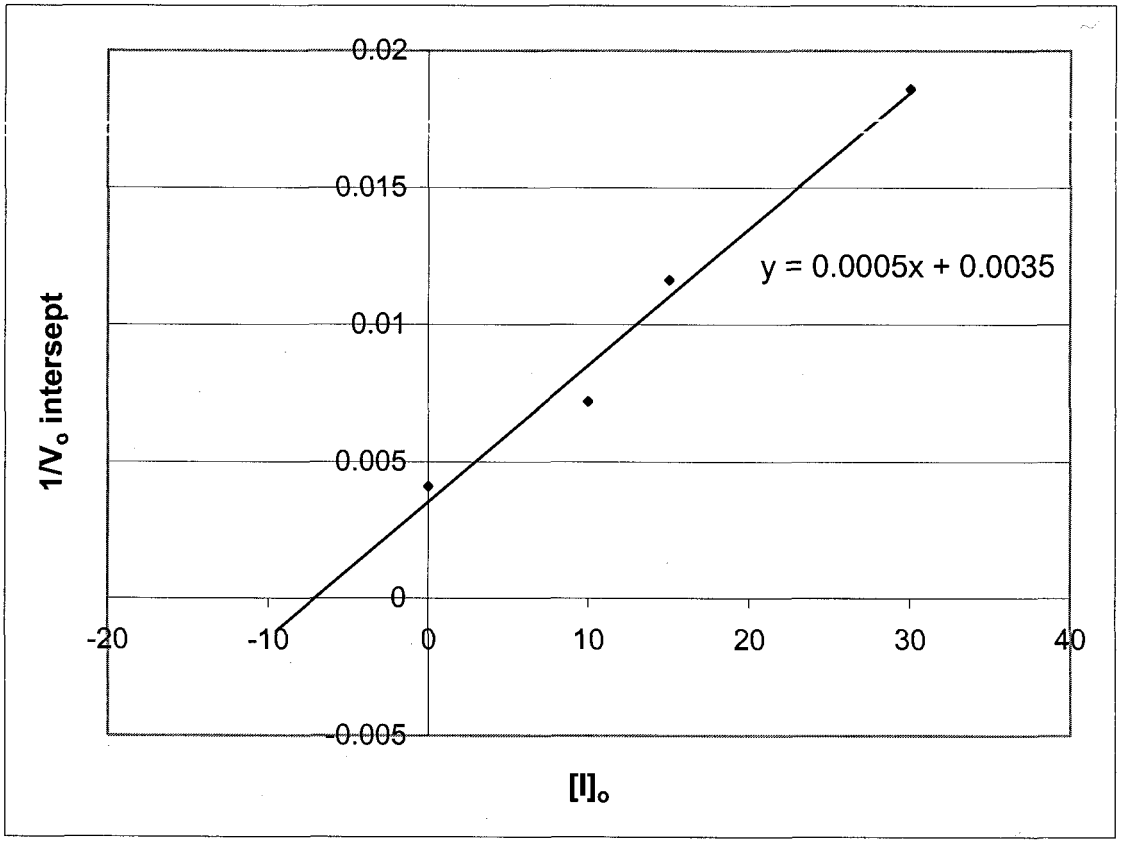
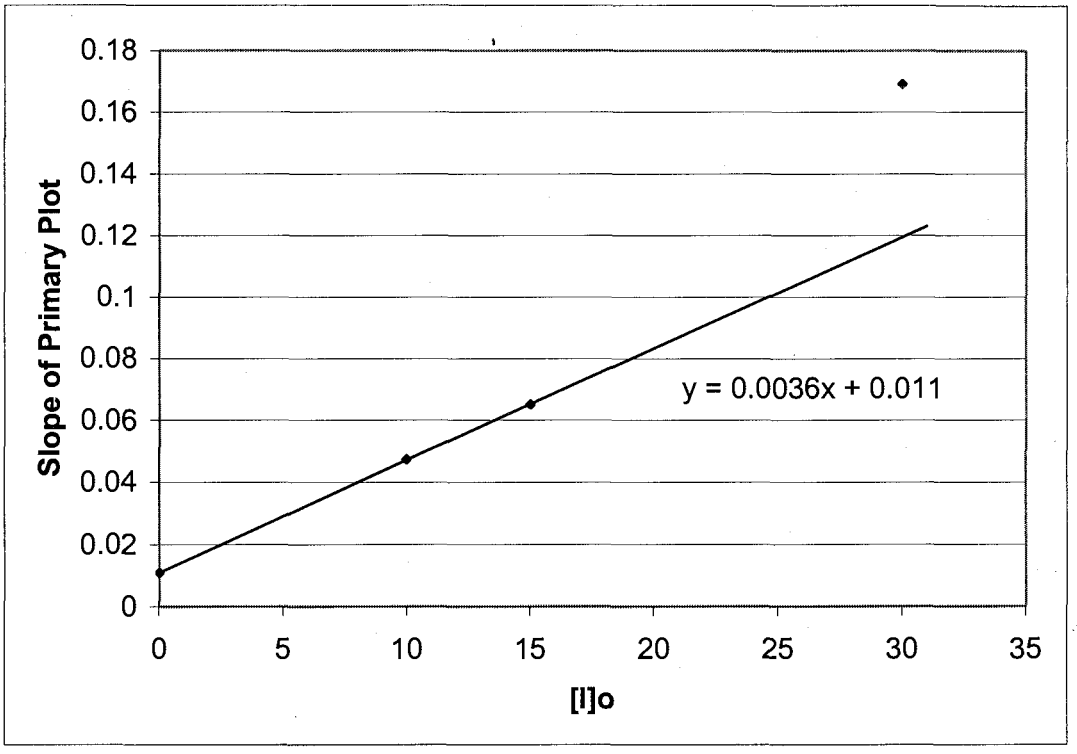

1/Vo intersept
0.02
0.015
0.01
0.005
0
-0.005
y = 0.0005x + 0.0035
-20
-10
0
10
20
30
40
[I]o

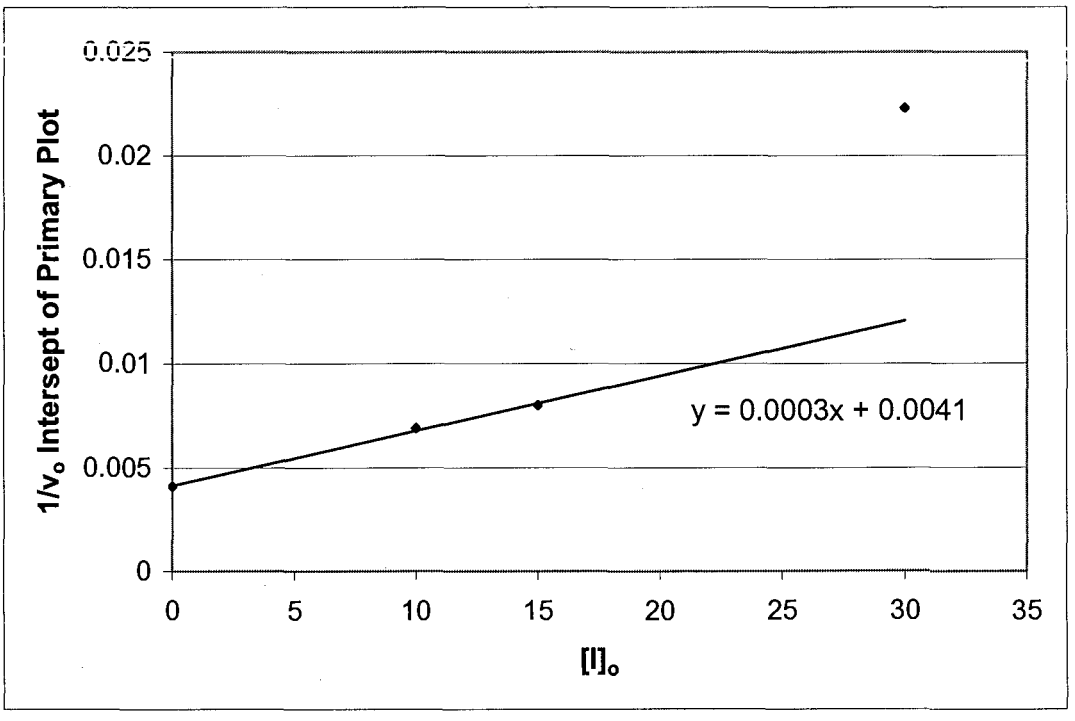
Figure 15a: Secondary Plot (Slope vs. $[I]_o$) for Coproporphyrinogen III. Utilizing data from the Lineweaver-Burk plot, a secondary plot of the primary slope vs. [I] allows for the calculation of a K_i value for Coprogen. The value of K_i was calculated to be 3.05 μM.

Figure 15b: Secondary Re-plot ($1/v_o$ intercept vs. $[I]_o$) for Coproporphyrinogen III. Utilizing data from the Lineweaver-Burk plot, a secondary plot of the $1/v_o$ intercept vs. $[I]_o$ allows for the calculation of a K_I value for Coprogen. The value of K_I was calculated to be 13.0 μM.

a)


Slope of Primary Plot
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
5
10
15
20
25
30
35
[I]o
y = 0.0036x + 0.011

b)


1/v$_o$ Intersept of Primary Plot
0.025
0.02
0.015
0.01
0.005
0
0
5
10
15
20
25
30
35
[I]$_o$
y = 0.0003x + 0.0041

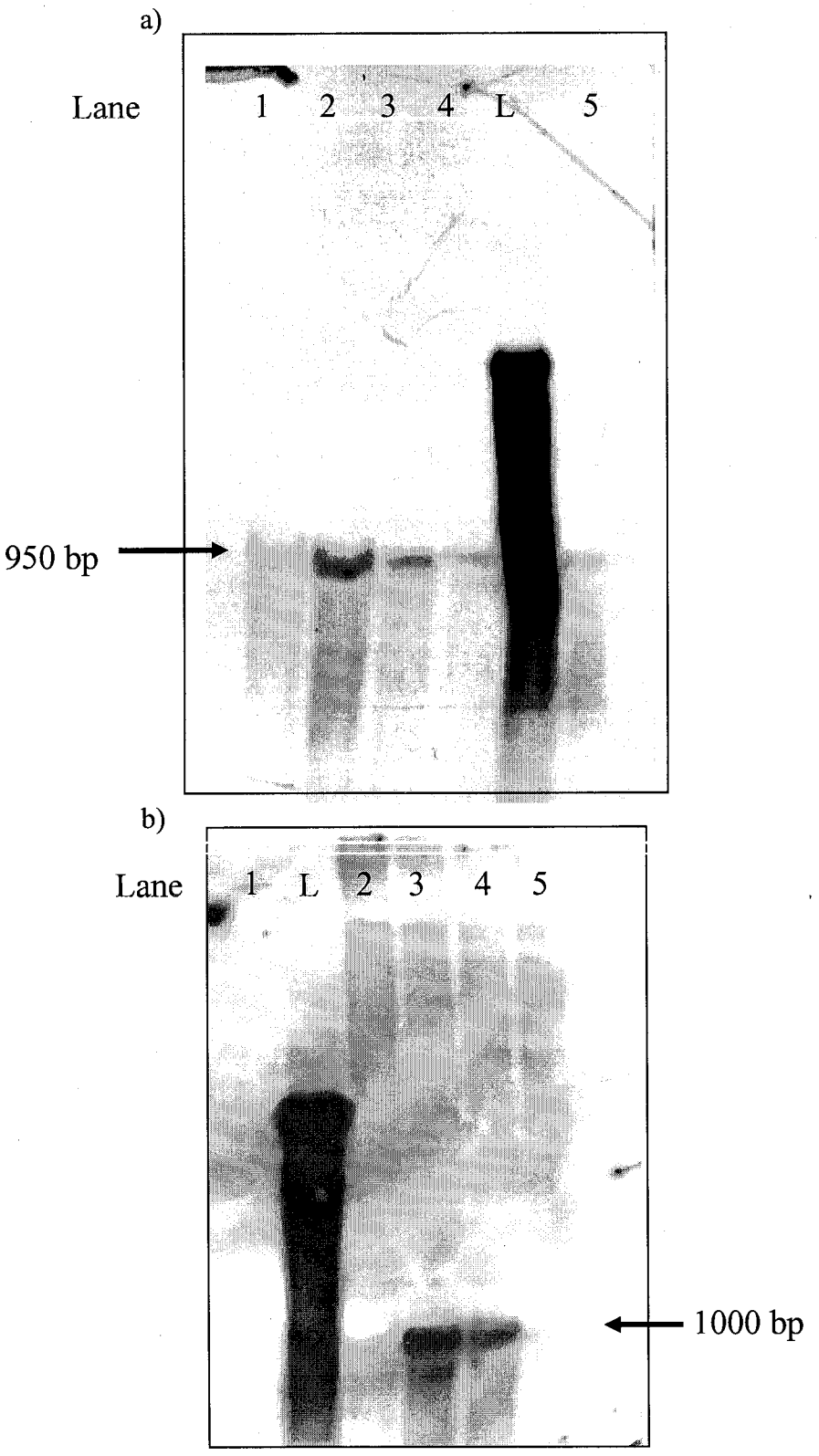
reaction are more accurate and reliable. Therefore, we concluded that the best-fit line should pass through this data point. Urogen was found to have a calculated K_i and K_I of 8.2 μM and 7.0 μM, respectively. Coprogen was found to have a calculated K_i and K_I of 3.05 μM and 13.0 μM, respectively.

3.7 The Search for *hemD*: Transcription of *hemC* and *hemE*

Northern blots of total wildtype *R. capsulatus* RNA revealed that both *hemC*, as well as *hemE*, are transcribed as single gene transcripts. The *hemC* gene is transcribed as a ~ 950 bp transcript (Figure 16a) and *hemE* (Figure 16b) is transcribed as a ~1000 bp transcript. The actual gene sequence for *hemC* and *hemE*, as obtained from Genbank submission number U16796, is 953 bp and 1034 bp, respectively.

Figure 16a: Northern Blot for *hemC*. Total RNA isolation and Northern blots were prepared as described in Section 2.21. Lane 1, 15 minutes after induction. Lane 2, 30 minutes after induction. Lane 3, 60 minutes after induction. Lane 4, 90 minutes after induction. L, RNA Ladder. Lane 5, 90 minutes after induction. *hemC* mRNA transcript shown as a 950 bp band.

Figure 16b: Northern Blot for *hemE*. Total RNA isolation and Northern blots were prepared as described in Section 2.21. Lane 1, 15 minutes after induction. Lane 2, RNA Ladder. Lane 2, 30 minutes after induction. Lane 3, 45 minutes after induction. Lane 4, 60 minutes after induction. Lane 5, 90 minutes after induction. *hemE* mRNA transcript shown as a 1000 bp band.


a)
Lane
1
2
3
4
L
5
950 bp
b)
Lane
1
L
2
3
4
5
1000 bp

4.0 Discussion

4.1 Regulation of the *hem* Pathway of Tetrapyrrole Biosynthesis

The regulation of Hem enzymes in the early part of the Bchl biosynthesis pathway is very important to *R. capsulatus*. From a cellular standpoint, it takes a considerable amount of energy and resources to grow, reproduce and maintain life. Therefore it is critical to balance the need for nutrients, metabolites, and energy. An over-abundance of one intermediate, such as Protogen, is too wasteful when considering the time, energy, and resources that are needed to synthesize it. However, the under-abundance of Protogen will prevent specific processes from occurring when needed. Therefore, the enzymes of the *hem* pathway of Bchl biosynthesis must be regulated to maintain the proper balance of resources and energy needed for life functions at the time that they are needed. There are many ways to regulate the activity of metabolic pathways. These methods include the regulation of gene transcription, the regulation of enzyme synthesis and degradation, and the regulation of enzyme activity by small molecular weight effectors (both inhibitors and activators). The regulation of enzyme activity is a direct, rapid and reversible method of the regulation of existing activity to avoid waste. This method is also faster than regulation at the gene level.

At least three factors, oxygen, heme, and *c*-type cytochromes (Biel and Biel, 1990), have been shown to have major influences on the regulation of the *hem* pathway of tetrapyrrole biosynthesis in *R. capsulatus*. It has been known for some time now that

ALA synthase activity is negatively regulated by negative feedback inhibition by heme (Warnick and Burnham, 1971). Biel and Biel (1990) reported that an *R. capsulatus* mutant lacking cytochrome oxidase activity completely lacked all *c*-type cytochromes but synthesized normal amounts of *b*-type cytochromes and nonheme iron. This mutant also excreted large amounts of Copro and Proto and synthesized reduced amounts of Bchl, suggesting a link between the synthesis of *c*-type cytochromes and the *hem* pathway.

The results of this thesis have facilitated our understanding on the role of PufQ in the oxygen-mediated regulation of tetrapyrrole biosynthesis. Results presented here indicate that oxygen tension and PufQ control the *hem* pathway leading to Bchl at a minimum of two points: (1) the formation of PBG from ALA by PBG synthase (HemB) and (2) the oxidative decarboxylation of Coprogen to Protogen by Coprogen oxidase (HemZ). The activity of both of these enzymes seems to be running at a basal level in high-oxygen-grown cells. However, during the transfer to lower-oxygen tension levels, PBG synthase and Coprogen oxidase specific activities are induced 1.8- and 1.6-fold, respectively, in the presence of a chromosomal wildtype copy of *pufQ*. On the other hand, we have also demonstrated that chromosomal *pufQ*-disrupted mutants (JS01 and JS02) fail to maximally induce the activity of both PBG synthase and Coprogen oxidase in response to a reduction in oxygen tension. The results further suggest that somehow extra copies of *pufQ* presented in *trans*, are not sufficient to rescue Coprogen oxidase activity, or completely rescue PBG synthase activity, in a *pufQ*-null mutant.

4.2 Bacteriochlorophyll Content in Mutant Strains

Previous research has reported possibilities for the involvement of PufQ in photosynthesis. Gong *et al.* (1994) suggested its role in assembly of functional light-harvesting antenna complexes in a closely related organism, *R. sphaeroides*. However, with this possibility in mind, it is difficult to distinguish between the absence of assembly of functional light-harvesting complexes and the absence of the Bchl itself, since Klug *et al.* (1986) have demonstrated that the LHI and LHII apoproteins are degraded in the absence of Bchl. And it is also possible that the function of *pufQ* may vary in *R. capsulatus* and *R. sphaeriodes*. Therefore, a more satisfying explanation for the function of *pufQ* may be that it is necessary for the synthesis of functionally high levels of Bchl in the cell.

As previously mentioned, a *pufQ* knockout of *R. capsulatus*, JS01, was created by the insertion of a kanamycin resistance gene to disrupt the chromosomal copy of *pufQ*. This mutant was shown to synthesize small amounts of Bchl and was unable to grow photosynthetically. When a wildtype copy of *pufQ* was introduced to this mutant on a plasmid in *trans*, it resulted in a higher production of Bchl, but failed to allow it to grow photosynthetically. This mutation was, therefore, concluded to be polar on the transcription of downstream *puf* genes. In other words, the insertion of the kanamycin cassette into *pufQ* also affected the transcription or post-transcriptional expression of the *puf* operon downstream of the insertion site. Another previously constructed mutant, JS02, contains an in-frame deletion of the *pufQ* gene. This mutation appears to have no effect on the transcription of downstream *puf* genes. Even though JS02 synthesizes only

slightly more Bchl than JS01, it is able to grow photosynthetically, but requires a very intense source of light to continue growth.' Using the characteristics of JS01, such as antibiotic resistance and photosynthetic incompetence, we were able to construct a strain (HUP35A) of *R. capsulatus* that contains a single amino acid point mutation (proline to alanine) at residue 35 in the transmembrane segment of the PufQ protein (Figure 6). Proline residues within transmembrane segments have unusual properties. In the middle of a helix, proline distorts ideal α-helix geometry because of steric conflicts with the preceding residue and the loss of a backbone hydrogen-bond. These proline-induced distortions in membrane proteins may be important to the function of PufQ. With this in mind, alanine was chosen for the substitution because it decreases backbone flexibility, increases α-helical tendency and may eliminate the kink in the α-helix.

HUP35A is photosynthetically competent, but is still functionally impaired with respect to maximal Bchl production relative to wildtype. In the wildtype strain, the level of Bchl was 15-fold higher in semiaerobically over aerobically grown cultures compared to only 3.5-fold in HUP35A. The Bchl content of HUP35A grown under semiaerobic conditions was higher than that found in the *pufQ* null-mutants (JS01 and JS02). Therefore, it appears that the P35A mutation has a lesser effect on Bchl synthesis than a complete null-mutation of *pufQ*. However, the data presented for Coprogen oxidase and PBG synthase activities for this mutant suggests that the point-mutated PufQ protein negatively affects the induction of both of these enzymes as much as does the *pufQ* null mutation upon transfer to lower oxygen tension growth conditions. The loss of wildtype induction of enzyme activity indicates that proline35 may be essential for proper activity

or targeting of this protein. However, other factors may also play a role in how PufQ functions to regulate Bchl synthesis which are not as negatively affected by the point mutation in PufQ. This may suggest that carbon flow as a whole through the Bchl synthesis pathway is lowered due to the absence of this protein.

4.3 Coproporphyrinogen III Oxidase Activity in *Rhodobacter capsulatus*

4.3.1 Coproporphyrinogen III Oxidase Activity in Mutant Strains

Coprogen oxidase catalyses the oxidative decarboxylation of two of the four propionate side groups of Coprogen to form Protogen. Eukaryotic Coprogen oxidases are structurally conserved and require O_2 for the oxidative reaction. In the case of the prokaryotic photosynthetic bacterium, *R. sphaeroides,* Tait (1972) previously demonstrated that Coprogen oxidase has activity under both aerobic and anaerobic conditions. Under aerobic conditions, the enzyme is found in the soluble phase and is a true oxidase as it can use oxygen directly as an electron acceptor. In the anaerobic reaction, however, there must be some acceptor other than oxygen for the electrons removed during the oxidative decarboxylation of Coprogen to Protogen. Tait (1972) found that the anaerobic activity of Coprogen oxidase in *R. sphaeroides* required the addition of the membrane fraction and additional cofactors such as $NADP^+$, NADH, L-methionine, and ATP, where S-adenosyl-L-methionine can substitute for the latter two. Other work in *R. sphaeroides* and *Bradyrhizobium japonicum*, has indicated that $NADP^+$ is the oxidant used by the O_2-independent enzyme (Seehra *et al.*, 1983; Keithly & Nadler, 1983;), but Xu & Elliott (1994) could not demonstrate that activity from the *Salmonella*

typhimurium hemN gene product; thus, identity of the physiological oxidizing substrate remains uncertain.

In *R. sphaeroides*, it has been demonstrated that there are two isozymes for this step in the pathway: HemZ (Zeilstra-Ryalls and Kaplan, 1995), induced by the FnrL system; and HemN (Coomber *et al.*, 1992; Oh *et al.*, 2000), so named for homology to the *S. typhimurium* and *E. coli* genes. There is no reported evidence for a *hemN* homolog in the *R. capsulatus* genome sequence. Indeed, it was also shown that an insertional disruption of *hemZ* is lethal unless grown in the presence of exogenously added heme (J. Smart, unpublished observations). Thus, *R. capsulatus* appears to possess only one enzyme (HemZ) for the oxidation of Coprogen to Protogen. Results presented here demonstrate that *R. capsulatus* Coprogen oxidase is a soluble enzyme that cannot function without some component of the membrane. However, it remains unclear as to which component of the membrane is required for Coprogen oxidase activity.

The proton-pumping NADH:ubiquinone oxidoreductase, also called Complex I, is the first energy-transducing complex of many respiratory chains. It catalyzes the transfer of electrons from NADH to coenzyme Q:

$$NADH + CoQ + H^+ \rightarrow NAD^+ + CoQH_2$$

Complex I of *E. coli* contains 14 gene products of the *nuoA-N* genes and can be dissociated into three fragments. One of these fragments contains a flavoprotein (NuoF)

and an iron-sulfur protein (NuoE) with NADH dehydrogenase activity. It is possible that in *R. capsulatus*, NADH dehydrogenase is the membrane-bound component that is acting as the electron acceptor in the reaction that Coprogen oxidase catalyzes. If NADPH is the final acceptor, it may be that the membrane-bound transdehydrogenase is required as well. James Smart (unpublished observations) had some evidence that subunit E (homologous to NuoE of *E. coli*) was required for Bchl synthesis. Therefore, he prepared strain JSΔN, which contains a chromosomal knockout of the gene for subunit E of NADH dehydrogenase. This mutant was assayed for Coprogen oxidase activity under anaerobic assay conditions. Coprogen oxidase was found to be active, and was also induced similarly to wildtype when transferred from aerobic to semiaerobic growth conditions. Thus, this component of NADH dehydrogenase is not involved in the reaction that oxidatively decarboxylates Coprogen. There are, however, multiple copies of NADH dehydrogenase in *R. capsulatus*. Hence, it still remains unclear as to what component of the membrane is required for Coprogen oxidase activity.

R. capsulatus Bchl biogenesis (*bch* gene) mutants, ZY6 (*bchH*) and ZY4 (*bchM*), are defective in magnesium chelatase and S-adenosyl-L-methionine: Mg Proto methyltransferase and, hence, accumulate Proto and Mg Proto, respectively. It was previously shown by James Smart (unpublished observations) that transcription of *hemZ* was observed to be reduced twenty-fold in *bchH, bchD* and *bchI* (the three subunits of magnesium chelatase) mutants but not in *bchM, bchG, or bchE* mutants, possibly indicating that magnesium Proto is required along with PufQ for the anaerobic induction

of *hemZ* transcription. However, according to Coprogen oxidase assays conducted on these mutants (Table 4), a wildtype level of activity was observed upon transfer to semiaerobic growth conditions. This shows that the absence of Mg Proto did not have any influence on the stimulation of Coprogen oxidase activity under anaerobic conditions.

PufQ has been previously demonstrated (see Section 1.7) to be involved in the synthesis of functionally high levels of Bchl in the cell. This work (Figure 7 & Table 4) has demonstrated that the *pufQ*-disrupted strains exhibited dramatically reduced Coprogen oxidase activity under semiaerobic growth conditions, when compared to the wildtype strain. It was also demonstrated (Section 3.3) that semiaerobic induction of Coprogen oxidase was not rescued when the *pufQ* gene (presented as a wildtype copy on plasmid pXCA935 or as a Pro35Ala point-mutated copy on plasmid pLAB2) was added in *trans* to JS01. This may show that Coprogen oxidase requires a chromosomal copy of PufQ to function normally, or possibly that extra copies of PufQ somehow inhibit the induction of Coprogen oxidase. It also supports the hypothesis that this step of the pathway is a major control point of the PufQ regulatory circuit.

4.3.2 ORF227 is a Possible Repressor of *hemZ*

Unpublished results by James Smart have indicated that ORF227 is an aerobic repressor of *hemZ*. The inferred protein sequence of ORF227, which contains a helix-

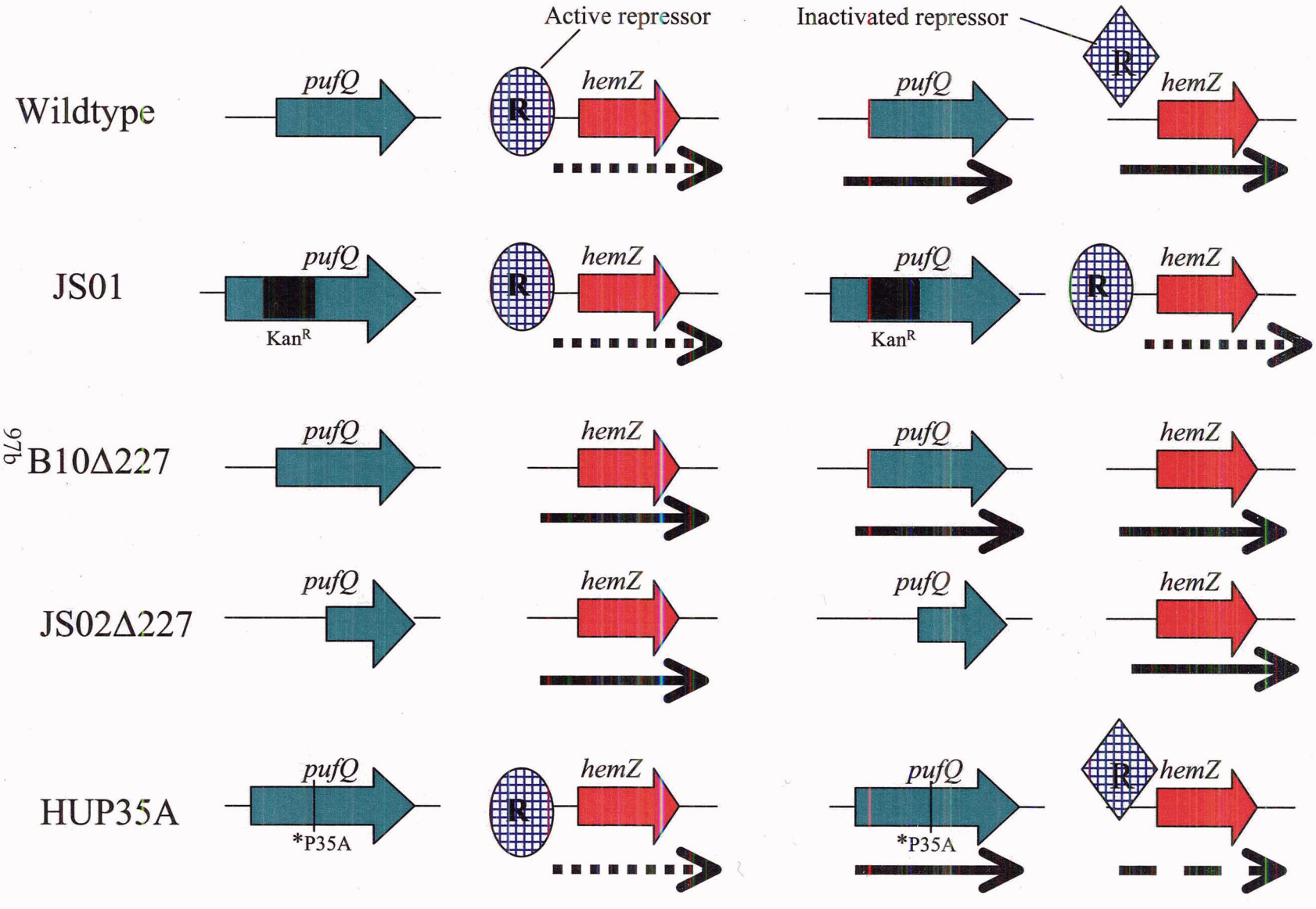
turn-helix motif, supports the hypothesis that ORF227 binds DNA and regulates transcription. James Smart (2001) observed constitutive *puf* operon expression under both aerobic and photosynthetic growth conditions in wildtype and B10Δ227 and suggested that ORF227 might be a component of the repression of the *puf* operon. However, we now have evidence that instead of being an aerobic repressor of the *puf* operon, ORF227 is an aerobic repressor of *hemZ*.

Coprogen oxidase activity was assayed for semiaerobic induction in the wildtype strain of *R. capsulatus*, containing a chromosomal knockout of *orf227* (B10Δ227), as well as a strain containing a double-chromosomal knockout of *pufQ* and *orf227* (JS02Δ227). Surprisingly, Coprogen oxidase activity was at a high (induced) level under both aerobic and semiaerobic growth conditions in both B10Δ227 and JS02Δ227. In other words, both strains showed a decreased repression of Coprogen oxidase activity under low oxygen tension conditions compared to that of wildtype. This suggested that the product of *orf227* may be an aerobic repressor of the *hemZ* gene or an aerobic inhibitor of its product, Coprogen oxidase. In addition, it may be possible that PufQ is a necessary component in the inactivation of the *hemZ* repressor to enhance transcription of *hemZ* in the transfer to photosynthetic growth conditions.

4.3.3 A Hypothetical Model for *hemZ* Transcription

In Figure 17, we propose a hypothetical model that summarizes one possible role of PufQ in *R. capsulatus*. In Figure 17, "R" represents ORF227 as a repressor that is

Figure 17: Hypothetical model for the Transcription of *hemZ*. This model summarizes one possible role of PufQ in *R. capsulatus*. Block arrows indicate individual genes. R represents ORF227 in either an active (oval) or inactive (diamond) configuration. The black box in the *pufQ* gene in JS01 represents the kanamycin-resistance cassette. The * in the *pufQ* gene in HUP35A represents the proline35alanine point mutation. A basal level of transcription is indicated by a dashed arrow. A highly induced level of transcription is indicated by a black solid arrow and a somewhat induced level by a broken arrow. See section 4.3.2 for an explanation of this model.


Aerobic
Photosynthetic
Active repressor
Inactivated repressor
Wildtype
JS01
B10Δ227
JS02Δ227
HUP35A
pufQ
hemZ
R
Kan^R
*P35A

bound to the promoter region of *hemZ* under aerobic growth conditions allowing *hemZ* to be transcribed at the basal level (indicated by a grey dashed arrow) required to maintain this type of growth. Under aerobic growth in wildtype, the *puf* gene products, which include PufQ, are not required and therefore not transcribed. But in the transfer to lower oxygen tension and photosynthetic growth conditions, the *puf* operon is induced, PufQ is synthesized (indicated by a solid black arrow), and is therefore somehow able to inactivate ORF227 and increase transcription of *hemZ* above the basal level. However, in mutant JS01, which contains a chromosomal knockout of *pufQ* and a polar disruption of the rest of the *puf* operon, PufQ is not synthesized upon transfer to photosynthetic growth conditions, and is not available to inactivate ORF227. Therefore, in this mutant strain, increased *hemZ* transcription cannot be initiated. In a mutant (B10Δ227) that harbors a chromosomal knockout of *orf227* but a wildtype copy of *pufQ*, ORF227 is not available to act as a repressor of *hemZ* under aerobic growth conditions, and *hemZ* is transcribed at fully induced levels under both growth conditions. The presence of a functional copy of PufQ has no effect of the transcription of *hemZ* in this mutant. Similar observations can be concluded in mutant JS02Δ227, which does not contain functional copies of PufQ or ORF227, and cannot regulate *hemZ* transcription as wildtype *R. capsulatus* does. In mutant HUP35A, where *pufQ* contains a point mutation that appears to partially inhibit its role in Bchl synthesis indicated by reduced Bchl content in semiaerobically grown cells. In this case, OFR227 is bound to *hemZ* and is transcribed at basal levels under aerobic growth condition just as in wildtype. However, upon transfer to lower oxygen tension conditions, since PufQ appears to be somewhat functionally impaired, it cannot function to inactivate ORF227 as efficiently as a wildtype copy of PufQ. And so, *hemZ*

transcription is increased in the transfer to photosynthetic growth conditions, but not to wildtype levels (as indicated by a broken arrow).

4.4 PBG Synthase Activity in *Rhodobacter capsulatus*

4.4.1 PBG Synthase Purification

The improved purification of PBG synthase from crude cell extracts of *R. capsulatus* has allowed the rapid production of partially purified enzyme for inhibition studies so as to eliminate HemC and/or HemD activity. The six step purification is a significant improvement over the previously published procedure which involved nine steps. This protocol can be performed in less than three hours thereby reducing the overall time involved in keeping the enzyme at 4°C. Other major advantages are the elimination of all the calcium phosphate gel treatments and elimination of the time-consuming gel filtration chromatography step, both of which tend to result in significant losses of enzyme.

4.4.2 PBG Synthase Activity in Mutant Strains

The formation of ALA, being the first step in the common tetrapyrrole pathway, is the most logical place for the pathway to be regulated. However, previous results demonstrated that the major regulatory point for oxygen-mediated control of the common tetrapyrrole pathway in *R. capsulatus* was after ALA formation (Biel, 1992). Evidence for this conclusion was found in the observation that exogenous addition of PBG, but not ALA, eliminates oxygen-mediated accumulation of Bchl intermediates (Biel, 1992). The

observation that exogenous ALA does not eliminate oxygen-mediated regulation of Proto accumulation argues that oxygen regulates some step in the common tetrapyrrole pathway after ALA formation. It would seem unusual for the second step in a pathway to be regulated, as it would result in a buildup of the first intermediate, in this case, ALA. Several studies have investigated the metabolism of ALA in different organisms. Shemin's group demonstrated that in ducks and rats the δ-carbon of ALA is incorporated into purines and suggested that ALA was actually part of a succinate-glycine cycle (Nemeth *et al.*, 1957; Shemin and Russell, 1953). Shigesada investigated the metabolism of [4-^{14}C] ALA in washed cell suspensions of photosynthetically grown *Rhodospirillium rubrum* (Shigesada, 1972). Only 10% of the incorporated radioactivity was associated with tetrapyrroles, while the rest was found in 5-amino-4-hydroxyvalerate, 2-hydroxyglutarate, 2-ketoglutarate, and glutamate, compounds closely related to ALA. Shigesada demonstrated that ALA was metabolized via a second pathway and postulated the existence of a succinate-glycine cycle in *R. rubrum* (Shigesada, 1972). In addition, Biel *et al.* (2002) reported an aerobically-induced NADPH-linked ALA dehydrogenase activity that may serve to divert ALA from the common tetrapyrrole pathway. This raises the possibility that PBG, and not ALA, is the first committed precursor in the pathway. From this, it would seem most likely that oxygen would control the conversion of ALA to PBG, and the induction of PBG synthase under anaerobic conditions appears to be an important step in providing enough intermediates in the *hem* pathway to allow Bchl synthesis.

In some organisms, the conversion of ALA to PBG has been determined to be the regulated step of the common tetrapyrrole pathway. For example, in *B. japonicum*, oxygen controls the common tetrapyrrole pathway by regulating transcription of the *hemB* gene (Chauhan and O'Brian, 1997). In other organisms, however, the pathway is regulated by controlling the synthesis of ALA. In *E. coli*, the pathway is regulated by feedback inhibition of ALA formation by heme (Beale, 1996). *E. coli*, however, utilizes a two-step tRNA-dependent pathway involving the enzymes glutamyl-tRNA reductase (encoded by *hemA*) and glutamate-1-semialdehyde-2,1-aminomutase (encoded by *hemL*) to synthesize ALA. Therefore the inhibition by heme is much different from the inhibition of ALA synthase (also encoded *hemA*) by heme in purple photosynthetic bacteria. Nevertheless, the transcription of *hemA* in *R. sphaeriodes* and *R. capsulatus* is still increased by lowering oxygen tensions, but may not involve PufQ.

With this in mind, we have demonstrated (Figure 8) that PBG synthase activity levels are also regulated by oxygen tension in *R. capsulatus*. PBG synthase specific activity is consistently higher during anaerobic than during aerobic cell culture in the wildtype strain. In addition, there was an observed significant decrease in PBG synthase activity under semiaerobic growth conditions in *pufQ*-disrupted strains of *R. capsulatus*. This, in turn, should result in a profound reduction in the synthesis of Bchl in these cells. A wildtype copy of *pufQ* present in *trans* on plasmid pXCA935 proved capable of rescuing PBG synthase activity in JS01, albeit not to wildtype levels. This observation may be due to a copy-number effect that demonstrates that extra copies of *pufQ* can compensate for the chromosomally-disrupted *pufQ* gene. On the other hand, the *pufQ*

gene containing a Pro35Ala point mutation present in *trans* on plasmid pLAB2 cannot rescue PBG synthase activity, showing that the point mutation must drastically affect PufQ functionality to the point whereby even extra copies of the gene have no rescuing effects.

4.4.3 PBG Synthase Kinetic Analysis with Inhibitors

The study of potential inhibitors is of practical importance because inhibition and activation of enzymes by key metabolites provides the normal means of metabolic 'fine' control superimposed in the 'course' control achieved by regulation of the synthesis and breakdown of active enzymes. It has been recognized for some time that heme synthesis is directly regulated by the concentration of heme (Lascelles, 1960). For example, ALA synthase activity is known to be negatively regulated by negative feedback inhibition by heme (Warnick and Burnham, 1971). Transcriptional repression of *hemB* expression has also been observed in the non-photosynthetic organism *B. japonicum* (Hamza *et al.*, 1998).

In addition, it has been known for some time now that porphyrins can be phototoxic to cells (Moan and Berg, (1992). In the presence of light and O_2, they form singlet oxygen, which will then react indiscriminately with almost any organic molecule. Therefore, it is important that porphyrin concentrations be kept to minimum levels in cells that are exposed to light and O_2. *R. capsulatus* cells that are undergoing the transition from anaerobic, photosynthetic growth to aerobic growth rapidly arrest the biosynthesis of photosynthetic components, including Bchl-binding antenna and reaction

center proteins. However, this response by itself would be insufficient to prevent the continued synthesis of Bchl and its precursors by enzymes that are active in the cells at the time of the transfer to air. Therefore, we hypothesized that there might be an additional mode of regulation to control the synthesis of porphyrins in the cell to help prevent the unnecessary accumulation of potentially lethal concentrations of Bchl and its precursors.

Our results indicate that PBG synthase, which catalyzes the second step of the early part of the tetrapyrrole biosynthetic pathway, is selectively inhibited by intermediates synthesized later in the pathway. Preliminary inhibition studies were conducted to screen two Bchl precursors as potential inhibitors of PBG synthase in wildtype *R. capsulatus* grown under semiaerobic growth conditions. These two physiological porphyrinogens, Coprogen and Urogen, appeared to drastically inhibit enzyme activity at increasing concentrations, while the corresponding non-physiological oxidized porphyrins did not. A kinetic analysis to identify the inhibition type and to estimate the kinetic parameters was applied to study inhibition among the two porphyrinogens. Results presented here show that both Urogen, and Coprogen were found to be mixed-type inhibitors of ALA condensation by PBG synthase.

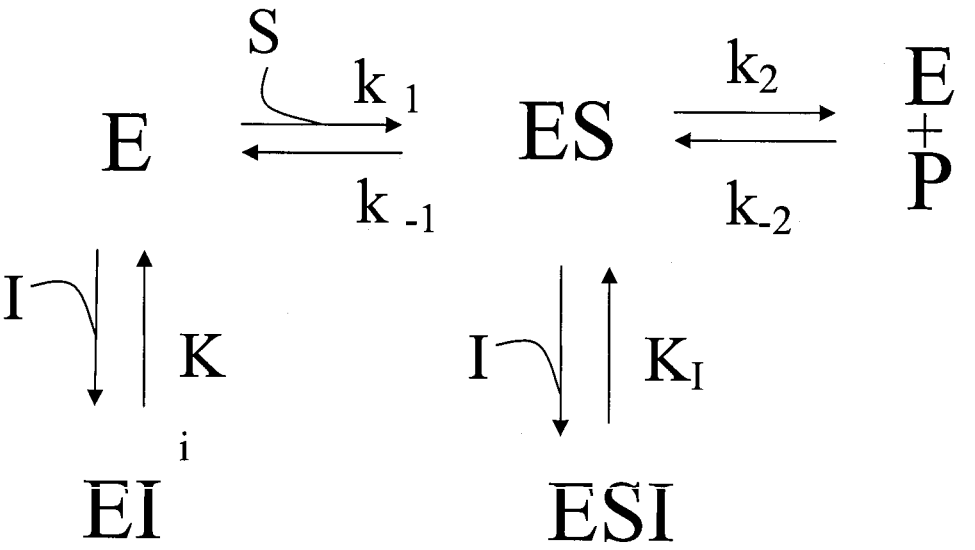
4.4.4 Coproporphyrinogen III and Uroporphyrinogen III as Mixed-Type Inhibitors

According to the data presented here, it is clear that Coprogen and Urogen both show potent inhibition of PBG synthase. The kinetic data shown in Figures 12 and 13

show clearly that the inhibition is neither competitive nor uncompetitive. The fact that the lines of the Lineweaver-Burk plots of $1/v_o$ against 1/[S] (Figure 12a and Figure 13a) intersected at a single point close to but not exactly on the abscissa, indicate that the type of inhibition is close to noncompetitive. In simple noncompetitive inhibition, the inhibitor binds both the free enzyme and the enzyme-substrate complex (ES) with the same affinity. Hence, the values of K_i and K_I are equal (Figure 18) and the K_m value is unaltered by each inhibitor so that, in the Lineweaver-Burk plot, different inhibitor concentrations lead to a family of lines fanning out from a common point of intersection on the abscissa. Otherwise, if K_i and K_I are not equal, as is the case presented here, the lines will intersect either above or below it. Since the Lineweaver-Burk plot indicated that the point of intersection for both inhibitors was not exactly on the abscissa, a case for mixed inhibition was concluded. K_i and K_I values for both Coprogen and Urogen should, therefore, not be equal; these values were obtained graphically by constructing two secondary plots for each inhibitor utilizing data from the Lineweaver-Burk plots. The secondary plots of the slopes vs. [I] (Figures 14a and 15a) allows calculation of K_i values and the plots of the $1/v_o$ intercept vs. [I] (Figures 14b and 15b) allows calculation of K_I values. The K_i and K_I values for Coprogen were determined to be 3.05 μM and 13.6 μM, respectively, while the K_i and K_I values for Urogen were determined to be 8.2 μM and 7.0 μM, respectively.

K_i values provide a useful yardstick of the relative potency of different inhibitors. In the mechanism for noncompetitive inhibition it is assumed that the presence of

Figure 18: A Model for Enzyme Inhibition. E represents the free enzyme, ES represents an enzyme-substrate complex, S represents the substrate, P represents the product, EI represents the enzyme-inhibitor complex, and ESI represents the enzyme-substrate-inhibitor complex. k represent rate constants. K_i and K_I are dissociation constants.


S
k 1
k -1
k2
k-2
E
ES
E
+
P
I
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i
EI
I
KI
ESI

inhibitor or substrate does not affect the binding of the other species. A mixed-type inhibitor can bind with different affinities to both the free enzyme and enzyme-substrate complex to form an enzyme-inhibitor complex (EI) and an enzyme-inhibitor-substrate complex (EIS). This inhibitor binding, however, does not necessarily affect the enzyme's ability to bind its substrate, but it does affect its ability to form product. In both cases, this binding causes a decrease in the catalytic activity of the enzyme. Both Coprogen and Urogen must be binding at an inhibitor binding site other than the active site (*eg.* an allosteric site). Comparing the calculated K_i and K_I values, it can be seen that Coprogen has a higher affinity for this inhibitor site on the empty enzyme than Urogen does. However the binding of substrate (either the first or second ALA molecule) changes the conformation of the inhibitor site such that Urogen now has a higher affinity for this site on the occupied enzyme-substrate complex (ES) than Coprogen does.

4.4.5 Two-fold Feedback Inhibition in the Early Pathway of Bacteriochlorophyll Biosynthesis

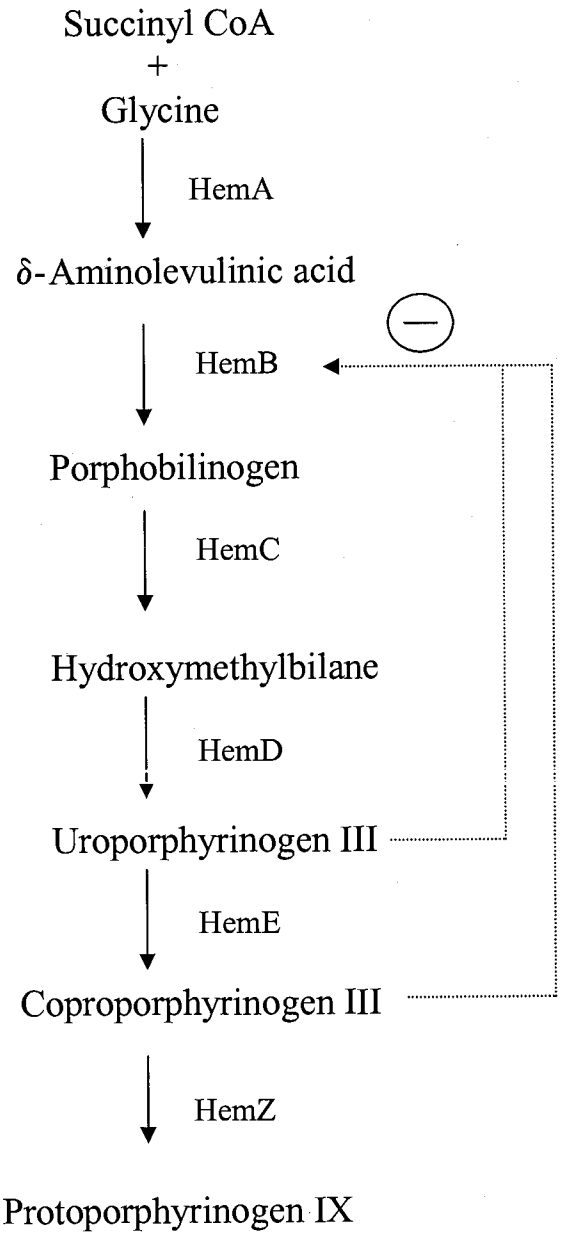
Under photosynthetic growth conditions, the concentration of the intermediates in the pathway will reach a steady state depending on the level of oxygen and incident light intensity in the bacterial culture. Upon transfer to high-oxygen conditions, the requirement for Bchl is lessened and the continued synthesis of porphyrinogens at the same rate is unnecessary. The concentration of porphyrins derived from the autoxidation of porphyrinogens could in fact rise to a level which might be toxic to the organism. In contrast, if the porphyrinogen intermediates in the *hem* pathway of Bchl biosynthesis could act as inhibitors of one or more of the first enzymes in the sequence, then as their

concentrations increased the degree of inhibition will also increase and the rate of synthesis of the porphyrinogens will fall. This thesis has shown that Coprogen and Urogen (which are produced subsequently to the reaction that PBG synthase catalyzes) demonstrate a two-fold form of feedback inhibition on the activity of PBG synthase (Figure 19). In other words, Coprogen and Urogen reduce the net rate of the reaction by inactivating a certain percentage of active PBG synthase and there would be a decrease in the apparent V_{max}. Since the remaining portion of the enzyme behaves normally, the Km value, which does not depend on active enzyme concentration, would remain approximately the same since the K_i and K_I values are very close in value. Obviously, reducing the rate of conversion of ALA to PBG would also lead to an increase in the concentration of ALA. However, ALA is a compound that can be diverted from the common tetrapyrrole pathway and reduced to 5-amino-4-hydroxyvalerate by a novel enzyme called ALA dehydrogenase (Biel *et al.*, 2002).

4.5 Search for HemD

As previously mentioned, Urogen is the universal precursor of all metabolic tetrapyrroles (Neve and Labbe, 1956). It is the branching point where the biosynthesis of vitamin B_{12} diverges from that of heme and Bchl. Its biosynthesis from PBG requires the cooperation of two enzymes, HMB synthase (HemC) and Urogen synthase (HemD), also known as Urogen cosynthetase. There are numerous reports of *hem* gene clusters, and *hemCD* operons are especially common (Alefounder *et al.*, 1988; Fujino *et al.*, 1995; Hansson *et al.*, 1991; Kafala and Sasarman, 1997). In *E. coli*, for example, h*emC* and

Figure 19: A Two-fold Form of Feedback Inhibition in the Early Part of the Bacteriochlorophyll Biosynthetic Pathway. Coprogen and Urogen (which are produced subsequently to the reaction that PBG synthase catalyzes) demonstrate a two-fold form of feedback inhibition on the activity of PBG synthase.


Succinyl CoA
+
Glycine
HemA
δ-Aminolevulinic acid
HemB
Porphobilinogen
HemC
Hydroxymethylbilane
HemD
Uroporphyrinogen III
HemE
Coproporphyrinogen III
HemZ
Protoporphyrinogen IX

hemD were shown to occur in tandem and overlap by one base pair. In *R. capsulatus*, the *hemC* gene is located next to *hemE*, which encodes Urogen decarboxylase; however, *hemC* is divergently transcribed from the *hemE* gene. Also, the sequence and location of *hemD* has not been identified in *R. capsulatus*. Since various *hem* genes have been shown to be clustered in other photosynthetic organisms, there was a chance that either *hemC* or *hemE* might have been transcribed as an operon with *hemD*. Results from Northern blotting experiments revealed that *hemC* is transcribed as a 953 bp transcript and *hemE* is transcribed as a 1034 bp transcript. These results indicate that both genes are transcribed as single-gene transcripts and, thus, not as operons.

5.0 Conclusions

This thesis project was undertaken to help understand the function of *pufQ* in *R. capsulatus* during the early part of the tetrapyrrole pathway that is common to both heme and Bchl biosynthesis. There appear to be multiple regulatory systems that govern *hem* gene expression, as well as Hem enzyme activity. The findings presented in this thesis have demonstrated that the flow of carbon through the tetrapyrrole pathway is in part controlled by regulating the activity of at least two enzymes that participate in the increased demand for Bchl upon transfer of *R. capsulatus* from aerobic to photosynthetic growth conditions. One of these enzymes, PBG synthase, catalyses the condensation of PBG from ALA, and is the first committed step in the tetrapyrrole pathway. The other enzyme is Coprogen oxidase (HemZ), which subsequently catalyzes the oxidative decarboxylation of Coprogen to Protogen, in the same pathway.

The first objective of this thesis involved assaying the extent of the induction of PBG synthase and Coprogen oxidase activity upon transfer from aerobic to semiaerobic growth conditions, and the effect that the absence of PufQ had on this induction. The lack of induction in PBG synthase and Coprogen oxidase specific activities observed in the absence of PufQ supports the previous conclusion that PufQ is necessary for the increased synthesis of Bchl in the transition to photosynthetic growth conditions (Forrest *et al.*, 1989). We also determined that an amino acid point mutation located in the transmembrane segment of PufQ affects its role in regulating Bchl synthesis. Previous research by James Smart (unpublished observations) demonstrated that *hemZ* and *hemB*

transcription under semiaerobic growth conditions had decreased dramatically in the *pufQ*-disrupted mutant JS01. Activity assays for Coprogen oxidase and PBG synthase in these same *pufQ*-disrupted mutants, JS01 and JS02 (Figures 7 & 8) demonstrated that PufQ is required for the semiaerobic induction of high-level activity of these two enzymes, and suggested that repression (characteristic of aerobic growth) of the corresponding genes occurs in its absence.

It has been previously suggested by James Smart (2001) that suppression of the *pufQ* null allele requires the mutation of at least two additional genes. One of these mutations was hypothesized to be in a repressor-binding site on *hemZ* such that the repressor was no longer able to repress *hemZ* transcription to basal levels under aerobic growth conditions. This thesis has shown that deletion of the gene for *orf227* results in high levels of enzyme activity of Coprogen oxidase under both aerobic and semiaerobic growth conditions. In addition, the high-level of Coprogen activity does not appear affected by the presence or absence of PufQ. These results support the hypothesis that ORF227 is the aerobic repressor of *hemZ.* It is not know whether ORF227 may act as a repressor of *hemB* transcription, however. Therefore, in the absence of ORF227, *hemZ* is always being transcribed at semiaerobic (induced) levels which is reflected by a higher level of Coprogen oxidase activity. Thus, a new regulatory role is suggested for PufQ in *R. capsulatus.* It may be concluded that PufQ is acting as either an anti-repressor of ORF227 or a component in a system involved in its inactivation.

It seems improbable that transcriptional regulation of either, both, or several additional steps encoded by *hem* genes could alone account for the large difference in Proto levels seen under high and low oxygen tensions. Low oxygen tensions result in a higher specific activity of PBG synthase, Coprogen oxidase, and perhaps additional Hem enzymes to meet the increased demand for Bchl. Transfer back to high oxygen levels would result in repression of the transcription of *hemB, hemZ,* and perhaps additional *hem* genes. Eventually, a return to normal basal level of enzyme activity and a decrease in Protogen synthesis would result, but this response would be fairly slow as already synthesized enzymes would remain active. However, oxygen also has an inhibitory effect on the magnesium branch of Bchl synthesis, perhaps at the magnesium chelatase step (Willows *et al.*, 2003). Hence, the concentration of Proto would immediately build up. This would cause an increased conversion of Proto to heme which would feedback-inhibit ALA synthase, resulting in a decrease in carbon flow over the common tetrapyrrole pathway. An increase in the concentration of Proto would also be reflected in an increase in the concentrations of previous intermediates in the tetrapyrrole pathway, including Coprogen and Urogen. In the execution of the second objective of this thesis, it was demonstrated that both Coprogen and Urogen act as feedback inhibitors on PBG synthase activity. This type of "two-fold feedback inhibition" is present in addition to the other forms of regulation already demonstrated for this organism. Both of these inhibitors are acting on PBG synthase at micromolar concentrations, whereas ALA has a K_m value of 2.66 mM and may be present at millimolar concentrations. Hence, Coprogen and Urogen bind with very high affinity to PBG synthase at concentrations that are 1000-fold less than that of substrate concentrations.

In summary, we have presented how PufQ appears to be regulating the synthesis of Bchl in the *hem* pathway in *R. capsulatus*. A decrease in oxygen tension in the external environment leads to the synthesis of PufQ. In turn, PufQ somehow inactivates ORF227 so that it can no longer bind to its repressor-binding site on *hemZ*. This results in the induction of *hemZ* transcription, and therefore also increasing Coprogen oxidase activity and the synthesis of Protogen. However, upon transfer back to higher oxygen levels, as described above, a build-up in the concentration of Proto and heme, and subsequently, in both Coprogen and Urogen, would result. The higher concentrations of Coprogen and Urogen would then feedback-inhibit PBG synthase (HemB). This would prevent the synthesis of excess levels of PBG and, therefore, porphyrinogens, that can be autoxidized to porphyrins which are toxic to the organism. In a slower response, the repression of *pufQ* transcription, and the absence of PufQ synthesis under aerobic conditions would result in the reactivation of ORF227 and repression of *hemZ* transcription back to basal levels. As well, the transcription of *hemB* (and other *hem* genes) would also be reduced back to aerobic levels by as yet uncharacterized means also requiring the absence of PufQ.

The conclusions presented here give further insight into the regulatory role that PufQ plays in *R. capsulatus*. We have presented evidence that supports the hypothesis that the PufQ protein fulfills a regulatory role, rather than a catalytic or structural role in the synthesis of the photosynthetic apparatus. However, there are still many questions that remain to be resolved considering the role of PufQ in the cell.

References

Adams, C.W., Forrest, M.E., Cohen, S.N., and Beatty, J.T. (1989). Structural and functional analysis of transcriptional control of the *Rhodobacter capsulatus puf* operon. J Bacteriol *171*, 473-482.

Addlesee, H., and Hunter, C.N. (1999). Physical mapping and functional assignment of the geranylgeranyl-bacteriochlorophyll reductase gene, *bchP*, of *Rhodobacter sphaeroides*. J. Bacteriol *181*, 7248-7255.

Alberti, M., Burke, D, D.H., and Hearst, J.E. (1995). Structure and sequence of the photosynthetic gene cluster. In Anoxygenic Photosynthetic Bacteria, R.E. Blankenship, M.T. Madigan and C.E. Bauer, eds. (The Netherlands: Kluwar Academic Publishers), pp.1083-1106.

Alefounder, P. R., C. Abell, and A. R. Battersby. (1988). The sequence of *hemC*, *hemD* and two additional *E. coli* genes. Nucleic Acids Res. *16*, 9871-9880.

Allan, J., Feher, G., Yeates, T., Rees, D., Deisenhofer, J., Michel, H., and Huber, R. (1986). Structural homology of reaction centers from *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas viridis* as determined by X-ray diffraction. PNAS *83*, 8589-8593.

Andrews, K.M, Crofts, A.R., and Gennis, R.B. (1990). Large-scale purification and characterization of a highly active four-subunit cytochrome bc_1 complex from *Rhodobacter sphaeroides*. Biochemistry *29*, 2645-51.

Armstrong, G.A., Alberti, M., Leach, F., and Hearst, J.E. (1989). Nucleotide sequence, organization, and nature of the protein products of the carotenoid biosynthesis gene cluster of *Rhodobacter capsulatus*. Mol Gen Genet *216*, 254-268.

Armstrong, G.A., Alberti, M., and Hearst, J.E. (1990). Conserved enzymes mediate the early reactions of carotenoid biosynthesis in non-photosynthetic and photosynthetic prokaryotes. Proc Natl Acad Sci USA *87*, 9975-9.

Armstrong, G.A., Cood, D.N., Ma, D., Alberti, M., Burke, D.H., and Hearst, J.E. (1993). Regulation of carotenoid and bacteriochlorophyll biosynthesis genes and identification of an evolutionarily conserved gene required for bacteriochlorophyll accumulation. J Gen Micobiol *139*, 897-906.

Armstrong, G.A. (1994). Eubacteria show their true colors: genetics of carotenoid pigment biosynthesis from microbes to plants. J Bacteriol *176*, 4795-4802.

Armstrong, G.A. (1995) Carotenoid biosynthesis gene and gene products in photosynthetic bacteria. In Anoxygenic Photosynthetic Bacteria, R.E. Blankenship, C.E. Bauer and M.T. Madigan, eds. (The Netherlands: Kluwer Academic Publishers), pp. 1135-1157.

Arnheim and Oelze, J. (1983). Control of the formation of bacteriochlorophyll, and B875- and B850- bacteriochlorophyll complexes in *Rhodopseudomonas sphaeroides* mutant strain H5. Arch Microbiol *136*, 312-6.

Babst, M., Albrecht, H., Wegmann, I., Brunisholz, R., and Zuber, H. (1991). Single amino acid substitution in the B870 alpha and beta light-harvesting polypeptides of *Rhodobacter capsulatus*. Structural and spectral effects. Eur J Biochem *202*, 277-84.

Baker, M.E., and Fanestil, D.D. (1991). Mammalian peripheral-type benzodiazepine receptor is homologous to CrtK protein of *Rhodobacter capsulatus*, a photosynthetic bacterium. Cell *65*, 721-2.

Bartley, G.E., and Scolnik, P.A. (1989). Carotenoid biosythesis in photosynthetic bacteria. Genetic characterization of the *Rhodobacter capsulatus* CrtI protein. J Biol Chem *264*, 13109-13.

Barz, W.P., Francesco, F., Venturoli, G., Melandri, B.A., Vermeglio, A. and Oesterhelt, D. (1995). Role of PufX protein in photosynthetic growth of *Rhodobacter sphaeroides*. 1. PufX is required for efficient light-driven electron transfer and photophosphorylation under anaerobic conditions. Biochemistry. *34*, 15235-15247.

Bauer, C.E., and Marrs, B.L. (1988). Rhodobacter capsulatus *puf* operon encodes a regulatory protein (PufQ) for bacteriochlorophyll biosynthesis. Proc Natl Acad Sci *85*, 7074-8.

Bauer, C.E., Buggy, J.J., Yang, Z.M., and Marrs, B.L. (1991). The superoperonal organization of genes for pigment biosynthesis and reaction center proteins is a conserved feature in *Rhodobacter capsulatus*: analysis of overlapping *bchH* and *puhA* transcripts. Mol Gen Genet *228*, 433-444.

Beale, S. I. (1996). Biosynthesis of hemes, p. 731-748. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.

Beatty, J.T. (1995). Organization of photosynthesis gene transcripts. In Anoxygenic Photosynthetic Bacteria, R.E. Blankenship, C.E. Bauer and M.T. Madigan, eds. (The Netherlands: Kluwer Academic Publishers), pp. 1209-1219.

Belasco, J.G., Beatty, J.T., Adams, C.W., von Gabain, A., and Cohen, S.N. (1985). Differential expression of photosynthesis genes in *Rhodobacter capsulata* results form segmental differences in stability within the polycistronic *rxcA* transcript. Cell *40*, 171-81.

Biel, A.J., and Marrs, B.L. (1983). Transcriptional regulation of several genes for bacteriochlorophyll biosynthesis in *Rhodopseudomonas capsulata* in response to oxygen. J Bacteriol *156*, 686-694.

Biel, A.J., and Marrs, B.L. (1985). Oxygen does not directly regulate carotenoid biosynthesis *in Rhodopseudomonas capsulata*. J Bacteriol *162*, 1320-1.

Beil SW, and AJ Beil. (1990). Isolation of *Rhobacter capsulatus* mutant that lacks *c*-type cytochromes and excretes porphyrins. J. Bacterio. *172*, 1321-1326.

Biel, A.J. (1992). Oxygen-regulated steps in the *Rhodobacter capsulatus* tetrapyrrole biosynthetic pathway. J Bacteriol *174*, 5272-4.

Biel, A.J. Canada, K., Huang, D., Indest, K., and Sullivan, K. (2002). Oxygen-mediated regulation of porphobilinogen formation in *Rhodobacter capsulatus*. J Bacteriol *184*, 1685-92.

Bollivar, D.W., Jiang, Z.Y., Bauer, C.E., and Beale, S.I. (1994) Heterologous expression of the *bchM* gene product from *Rhodobacter capsulatus* and demonstration that it encodes S-adenosyl-L-methionine:Mg-protoporphyrin IX methyl transferase. J Bacteriol *176*, 5290-5296.

Borralho, L.M., Ortiz, C.H., Panek, A.D., and Mattoon, J.R. (1990). Purification of delta-aminolevulinic dehydrase from genetically engineered yeast. Yeast *6*, 319-330.

Botella, J.A, Murillo, F.J., and Ruiz-Vazquez, R. (1995). A cluster of structural and regulatory genes for light-induced carotenogenesis in *Myxococcus xanthus*. Eur J Biochem *233*, 238-48.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*, 248-54.

Buggy, J. and Bauer, C.E. (1995). Cloning and chacterization of *senC*, a gene involved in both aerobic respiration and photosynthesis gene expression in *Rhodobacter capsulatus*. J Bacteriol *177*, 945-8.

Buggy, J.J., Sganga, M.W., and Bauer, C.E. (1994). Nucleotide sequence and characterization of *Rhodobacter capsulatus hvrB* gene: HvrB is an activator of S-

adenosyl-L-homocysteine hydrolase expression and is a member of the LysR family. J Bacteriol *176*, 61-69.

Buzby, J.S., Porter R.D., and Stevens S.E. (1985). Expression of the *Escherichia coli lacZ* gene on a plasmid vector in a cyanobacterium. Science *230*, 805-7.

Chauhan, S., and M. R. O'Brian. (1997). Transcriptional regulation of delta-aminolevulinic acid dehydratase synthesis by oxygen in *Bradyrhizobium japonicum* and evidence for developmental control of the *hemB* gene. J. Bacteriol. *179*, 3706-3710).

Chen, Y.R., Shenoy, S.K., Yu, C.A., and Yu, L. (1995). Identification of amino acid residues involved in structural and ubiquinone-binding functions of subunit IV of the cytochrome bc_1 complex from *Rhodobacter sphaeroides*. J Biol Chem *270*, 1149-501.

Chory, J., Donohue, T.J., Varga, A.R., Staehelin, L.A., and Kaplan, S. (1984). Induction of the photosynthetic membranes of *Rhodopseudomonas sphaeroides*: biochemical and morphological studies. J Bacteriol *159*, 540-554.

Chen, C.Y., Beatty, J.T., Cohen, S.N., and Belasco, J.G. (1988). An intercistronic stem-loop structure functions as an mRNA decay terminator necessary but insufficient for *puf* mRNA stability. Cell *52*, 609-619.

Clark, W.G., Davidson, E., and Marrs, B.L. (1984). Variation of levels of mRNA coding for antenna and reaction center polypeptides in *Rhodopseudomonas capsulata* in response to changes in oxygen concentration. J Bacteriol *157*, 945-8.

Coomber, S.A., Chaudhri, M., Connor, A., Britton, G., and Hunter, C.N. (1990). Localization transposon Tn5 mutagenesis of the photosynthetic gene cluster of *Rhodobacter sphaeroides*. Mol Microbiol *4*, 977-89.

Cohen-Bazire, G., Sistrom, W.R., and Stanier, R.Y. (1957). Kinetic studies of pigment synthesis by non-sulfur purple bacteria. J Cell Physiol *49*, 25-68.

Daldal F., Tokito, M.K., Davidson, E., and Faham, M. (1986). Mutations conferring resistance to quinol oxidation (Qz) inhibitors of the cyt bc_1 complex of *Rhodobacter capsulatus*. EMBO J *8*, 3951-61.

Debus, R.J., Lubitz, W., Abresch, E.C., Isaacson, R.A., Okamura, M.Y., and Feher, G. (1985). Electron nuclear double resonance of semiquinones in reaction centers of *Rhodopseudomonas spaeroides*. Biochem Biophys Acta *808*, 464-9.

Debus, R.J., Feher G., and Okamura, M.Y. (1986). Iron-depleted reaction centers from *Rhodopseudomonas sphaeroides* R-26.1: characterization and reconstitution with Fe^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}. Biochemistry *25*, 2276-87.

Deisnhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1984). Structure of the protein subunits on the photosynthetic reaction center of *Rhodopseudomonas viridis* at 3A resolution. Nature *318*, 618-624.

Drews, G., and Oelze J. (1981). Organization and differentiation of membranes of phototrophic bacteria. Adv Microb Physiol *22*, 1-92.

Drews, G. (1996). Formation of the light-harvesting complex I (B870) of anoxygenic phototrophic purple bacteria. Arch Microbiol *166*, 151-159

Ermler, U., Fritzsch, G., Buchanan, S.K., and Michel, H. (1994). Structure of the photosynthetic reaction center from *Rhodobacter sphaeroides* at 2.65A resolution: cofactors and protein-cofactor interactions. Structure *2*, 925-36.

Feher, G., and Okamura, M.Y. (1978). Reaction centers from *Rhodopseudomonas sphaeroides*. Brookhaven Symp Biol *247*, 489-92.

Fidai, S., Hinchigeri, S.B., Borgford, T.J., and Richards, W.R. (1994a). Identification of the PufQ protein of *Rhodobacter capsulatus*. J Bacteriol *176*, 7244-51.

Fidai, S., Dahl, J.A. and Richards, W.R. (1994b). Effect of the PufQ protein on early steps in the pathway of bacteriochlorophyll biosynthesis in *Rhodobacter capsulatus*. Biochem Biophys Res Commun *200*, 1679-84.

Fonstein, M., Zheng, S., and Haselkorn, R. (1992). Physical map of the genome of *Rhodobacter capsulatus* SB 1003. J Bacteriol *174*, 4070-4077.

Forrest, M.E., Zucconi, A.P., and Beatty, J.T. (1989). The pufQ gene product of *Rhodobacter capsulatus* is essential for formation of B800-850 light-harvesting complexes. Current Microbiol *19*, 123-127.

Francis, G.A., and Richards, W.R. (1980). Localization of photosynthetic membrane components in *Rhospseudomonas sphaeroides* by a radioactive labeling procedure. Biochemistry *19*, 5104-5111.

Francia, F., Wang, J., Venturoil, G., Melandri, B.A., Barz, W.P., and Oesterhelt, D. (1999). The reaction center-LHI antenna complex of *Rhodobacter sphaeroides* contains one PufX molecule which is involved in dimerization of this complex. Biochemistry *38*, 6834-45.

Frese, R.N., Olsen, J.D. Branvall, R., Westerhuis, W.H., Hunter, C.N., and van Grondelle, R. (2000). The long-range supraorganization of the bacterial photosynthetic unit: A key role for PufX. Proc Natl Acad Sci *97*, 5197-202.

Fritsch, J., Rothfuchs, R., Rauhut, R. Klug, G. (1995). Identification of an mRNA element promoting rate-limiting cleavage of the polycistronic puf mRNA in *Rhodobacter capsulatus* by an enzyme similar to RNase E. Mol Microbiol 15, 1017-29.

Fujita, Y., and Bauer, C. (2000). Reconstitution of light-independent protochlorophyllide reductase from purified BchL and BchN-BchB subunits. In vitro confirmation of nitrogenase-like features of a bacteriochlorophyll biosynthesis enzyme. J Biol Chem *275*, 23583-23588.

Fujino, E., T. Fujino, S. Karita, K. Sakka, and K. Ohmiya. (1995). Cloning and sequencing of some genes responsible for porphyrin biosynthesis from the anaerobic bacterium *Clostridium josui*. J. Bacteriol. *177*, 5169-5175.

Gibbs, P.N., Chaudhry, A.G., and Jordan, P.M. (1985). Purification and properties of 5-aminolevulinic dehydrase from human erythrocytes. Biochem J *230*, 25-34.

Gibson, L.C., Willows, R.D., Kannangara, C.G., von Wettstein, D., and Hunter, C.N. (1995). Magnesium-protoporphyrin chelatase of *Rhodobacter sphaeroides*: reconstitution of activity by combining the products of the *bchH*, *-I*, and *–D* genes expressed in *Escherichia coli*. Proc Natl Acad Sci U S A *92*, 1941-1944.

Giuliano, G., Pollock, D., and Scholnik, P.A. (1986). The gene *crtI* mediates the conversion of phytoene into coloured carotenoids in *Rhodopseudomonas capsulata*. J Biol Chem *261*, 12925-9.

Giuliano, G., Pollock, D., Stapp, H., and Scolnik, P.A. (1988). A genetic-physical map of the *Rhodobacter capsulatus* carotenoid biosynthesis gene cluster. Mol Gen Genet *213*, 78-83.

Golden, S.S., Brusslan, J., and Haselkorn, R. (1986). Expression of a family of *psbA* genes encoding a photsystem II polypeptide in the cyanobacterium *Anacystis nidulans* R2. EMBO J *5*, 2789-98.

Golden, S.S., Brusslan, J., and Haselkorn, R. (1987). Genetic engineering of the cyanobacterial chromosome. Methods Enzymol *153*, 215-31.

Gomelsky, M., and Kaplan, S. (1995). Genetic evidence that PpsR from *Rhodobacter sphaeroides* 2.4.1 functions as a respressor of *puc* and *bchF* expression. J. Bacteriol *177*, 1634-7.

Gong, L., Lee, J.K., and Kaplan, S. (1994) The Q gene of *Rhodobacter sphaeroides*: its role in *puf* operon expression and spectral complex assembly. J Bacteriol *176*, 2946-2961.

Gorchein, A., Gibson, L.C., and Hunter, C.E. (1993). Gene expression and control of enzymes for synthesis of magnesium protoporphyrin monomethyl ester in *Rhodobacter sphaeroides*. Biochem Soc Trans *21*, 2018.

Hansson, M., L. Rutberg, I. Schroeder, and L. Hederstedt. (1991). The *Bacillus subtilis hemAXCDBL* gene cluster, which encodes enzymes of the biosynthetic pathway from glutamate to uroporphyrinogen III. J. Bacteriol *173*, 2590-2599.

Hinchigeri, S.B., Hundle, B., and Richards, W.R. (1997). Demonstration that the BchH protein of *Rhodobacter capsulatus* activates S-adenosyl-L-methionine:magnesium protoporphyrin IX methyltransferase. FEBS Lett *407*, 337-342.

Hundle, B.S., and Richards, W.R. (1990). Use of the membrane-impermeable guanidinating reagent 2-S-[14^C]thiuroniumethanesulfonate to demonstrate the orientation of light-harvesting proteins in *Rhodobacter sphaeroides*. Biochemistry *29*, 6172-79.

Hunter, C.N., Ashby, M.K., and Coomber, S.A. (1988). Effect of oxygen on levels of mRNA coding for reaction-center and light-harvesting polypeptides of *Rhodobacter sphaeroides*. Biochem J *247*, 489-92.

Hunter, C.N., Fowler, G.J., Grief, G.G., Olsen, J.D., and Jones, M.R. (1993). Protein engineering of bacterial light-harvesting complexes. Biochem Soc Trans *21*, 41-43.

Ikeda-Yamasaki, I., Odahara, T., Mitsuoka, K., Fujiyoshi, Y., and Murata, K. (1998). Projection map of the reaction center-light harvesting I complex from *Rhodopseudomonas viridis* at 10A resolution. FEBS Lett *425*, 505-508.

Inoue, K., Kouadio, J.L., Moseley, C.S., and Bauer, C.E. (1995). Isolation and in *vitro* phosphorylation of sensory transduction components controlling anaerobic induction of light harvesting and reaction center gene expression in *Rhodobacter capsulatus*. Biochemistry *34*, 391-396.

Jenney, F.E., and Daldal, F. (1993). A novel membrane-associated c-type cytochrome, cyt c_y, can mediate the photosynthetic growth of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. EMBO J *12*, 1283-92.

Jones, M.R., Visschers, R.W., van Grondelle, R., and Hunter, C.N. (1992). Construction and characterization of a mutant of *Rhodobacter sphaeroides* with the reaction center as the sole pigment protein complex. Biochemistry *31*, 4458-65.

Jordan, P.M., and Shemin, D. (1973). Purification and properties of uroporphyrinogen I synthetase from *Rhodopseudomonas spheroides*. J Biol Chem *248*, 1019-24.

Kafala, B., and A. Sasarman. (1997). Isolation of the *Staphylococcus aureus hemCDBL* gene cluster coding for early steps in heme biosynthesis. Gene *199*, 231-239.

Karrasch, S., Bullough, P.A. and Ghosh, R. (1995). The 8.5 Å projection map of the light-harvesting complex I from *Rhodospirillum rubrum* reveals a ring composed of 16 subunits. EMBO J. *14*, 631–638.

Keithly, J.H., and Nadler, K.D. (1983). Protoporphyrin formation in *Rhizobium japonicum*. J Bacteriol *154*, 838-45.

Klug, G, and Drew, G. (1984). Construction of a gene bank of *Rhodopseudomonas capsulata* using a broad host range DNA cloning system. Arch Microbiol *139*, 319-25.

Klug, G., Adams, C.W., Belasco, J., Doerge, B., and Cohen, S.N. (1987). Biological consequences of segmental alterations in mRNA stability: effects of deletion of the intercistronic hairpin loop of the *Rhodobacter capsulatus puf* operon. EMBO J *6*, 3515-3520.

Klug, G. (1991). Endonucleolytic degradation of *puf* mRNA is *Rhodobacter capsulatus* is influenced by oxygen. Proc Natl Acad Sci USA *88*, 1765-9.

Klug, G., Jock, S., and Rothfuchs, R. (1992). The rate of decay of *Rhodobacter capsulatus*-specific *puf* mRNA segments is differentially affected by RNase E activity in *Escherichia coli*. Gene *121*, 95-102.

Klug, G. (1993). The role of mRNA degradation in the regulated expression of bacterial photosynthesis genes. Mol Microbiol *9*, 1-7.

Klug, G. (1995). Post-transcriptional control of photosynthesis gene expression. In Anoxygenic Photosynthetic Bacteria, R.E. Blankenship, C.E. Bauer and M.T. Madigan, eds. (The Netherlands: Kluwer Academic Publishers), pp. 1235-1244.

Koetsier, P.A., Schorr, J., and Doerfler, W. (1993). A rapid optimized protocol for downward alkaline Southern blotting of DNA. Biotechniques *15*, 260-2.

Kriauciunas, A., Yu, L., Yu, C.A., Wynn, R.M., and Knaff, D.B. (1989). The *Rhodospirillum rubrum* cytochrome bc_1 complex: peptide composition, prosthetic group content and quinone binding. Biochim Biophys Acta *976*, 70-6.

Labarre, J., Chauvat, F., and Thuriaux, P. (1989). Insertional mutatgenesis by random cloning of antibiotic resistance genes into the genome of the cyanobacterium *Synechocystis* strain PCC 6803. J Baceriol *171*, 3449-57.

Lancaster, C.R., and Michel, H. (1999). Refined crystal structure of reaction centers from *Rhodopseudomonas viridis* in complexes with the herbicide atrazine and two

chiral atrazine derivatives also lead to a new model of the bound carotenoid. J Mol Biol *286*, 883-98.

Lang, H.P., Cogdell, R.J., Gardiner, A.T., Hunter, C.N. (1994). Early steps in carotenoid biosynthesis: sequences and transcriptional analysis of the *crtI* and *crtB* genes of *Rhodobacter sphaeroides* and overexpression and reactivation of *crtI* in *Escherichia coli* and *Rhodobacter sphaeroides*. J Bacteriol *176*, 3859-69.

Lang, H.P., Cogdell. R.J., Takaichi, S., and Hunter, C.N. (1995). Complete DNA sequence, specific Tn5 insertion map, and gene assignment of the carotenoid biosynthesis pathway of *Rhodobacter sphaeroides*. J Bacteriol *177*, 2064-73.

Lascelles, J. (1960). The synthesis of enzymes concerned in bacteriochlorophyll formation in growing cultures of *Rhodopseudomonas spheroides*. J Gen Microbiol *23*, 487-98.

LeBlanc, H., Lang, A.S., and Beatty, J.T. (1999). Transcript cleavage, attenuation, and an internal promoter in the *Rhodobacter capsulatus puc* operon. J Bacteriol *181*, 4955-60.

Majewski, C., and Trebst, A. (1990). The *pet* genes of *Rhodospirillum rubrum*: cloning and sequencing of the genes from the cytochrome bc_1 complex. Mol Gen Genet *224*, 373-82.

Marrs, B. (1981). Mobilization of the genes for photosynthesis from *Rhodopseudomonas capsulata* by a promiscuous plasmid. J Bacteriol *146*, 1003-12.

Masuda, S., Dong, C., Swem, D., Setterdahl, A.D., Knaff, D.B., and Bauer, C.E. (2002). Repression of photosynthesis gene expression by formation of a disulfide bond in CrtJ. Proc Natl Acad Sci 99, 7078-83.

McEwan, A.G., Hanson, G.R., and Bailey, S. (1998). Dimethylsulphoxide reductase from purple phototrophic bacteria: structures and mechanism(s).Biochem Soc Trans *26*, 390-6.

Moan, J., and K. Berg. (1992). Photochemotherapy of cancer—experimental research. Photochem. Photobiol. *55*, 931-948.

Moseley, C.S., Suzuki, J.Y. and Bauer, C.E. (1994). Identification and molecular genetic characterization of a sensor kinase responsible for coordinately regulating light-harvesting and reaction center gene expression in response to anaerobiosis. J Bacteriol *176*, 7566-7573.

Nandi, D.L. and Shemin, D. (1968). Delta-aminolevulinic acid dehydratase of *Rhodopseudomonas spheroides*. 3. Mechanism of porphobilinogen synthesis. J Biol Chem *243*, 1236-42.

Nemeth, A.M., Russell, C.S., and Shemin, D. (1957). The succinate-glycine cycle. Metabolism of δ-aminolevulinic acid. J Biol Chem *229*, 415-422.

Neve, R. A., and Labbe, R. F. (1956). Reduced Uroporphyrinogen III in the biosynthesis of heme. *J. Am. Chem. Soc. 78*, 691-692.

Nishida, G. and Labre, R.F. (1959). Heme biosynthesis; on the incorporation of iron into protoporphyrin. Biochim Biophys Acta *31*, 519-24.

Oh, J.I. Eraso, J.M., and Kaplan, S. (2000). Interacting regulatory circuits involved in orderly control of photosynthetic gene expression in *Rhodobacter sphaeroides* 2.4.1. J Bacteriol *182*, 3081-7.

Okamura, M.Y., Eisenberger, P., and Feher, G. (1982). The electronic structure of Fe^{2+} in reaction centers from *Rhodopseudomonas sphaeroides* II. Extended X-ray fine structure studies. Biophys J *37*, 523-38.

Oster, U., Bauer, C.E., and Rudiger, W. (1997). Characterization of chlorophyll a and bacteriochlorophyll a synthases by heterologous expression in *Escherichia coli*. J Biol Chem *272*, 9671-9676.

Penfold, R.J., and Pemberton, J.M. (1994). Sequencing, chromosomal inactivation, and functional expression in *Escherichia coli* of *ppsR*, a gene which represses carotenoid and bacteriochlorophyll synthesis in *Rhodobacter sphaeroides*. J Bacteriol *176*, 2869-76.

Ponnampalam, S.N., Buggy, J.J., and Bauer, C.E. (1995). Characterization of an aerobic repressor that coordinately regulates bacteriochlorophyll, carotenoid, and light harvesting II expression in *Rhodobacter capsulatus*. J Bacteriol *177*, 2990-7.

Rebeiz, C.A., and Lascelles, J. (1982). Biosynthesis of pigments in plants and bacteria. In Photosynthesis: Energy Conversion by Plants and Bacteria (New York: Academic Press, Inc), pp. 699-780.

Recchia, P.A., Davis, C.M., Lilburn, T.G., Beatty, J.T., Parkes-Loach, P.S., Hunter, C.N., and Loach, P.A. (1998) Isolation of the PufX protein from *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*: evidence for its interaction with the alpha- polypeptide of the core light-harvesting complex. Biochemistry *37*, 11055-63.

Richards, W.R., and Lascelles, J. (1969). The biosynthesis of bacteriochlorophyll. The characterization of latter stage intermediates from mutants of *Rhodopseudomonas spheroides*. Biochemistry *8*, 3473-82.

Robertson, D.E., Ding, H., Chelminski, P.R., Slaughter, C., Hsu, J., Moomaw, C., Tokito, M., Daldal, F., and Dutton, P.L. (1993). Hydroubiquinone-cytochrome c_2 oxidoreductase from *Rhodobacter capsulatus*: definition of a minimal functional isolated preparation. Biochemistry *32*, 1310-7.

Sano, S. and Granik, S. (1961). Mitochondrial coproporphyrinogen oxidase and protoporphyrin formation. J Biol Chem *236*, 1173-80.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Habour Laboratory Press. New York.

Schmidt, D.L., and Shemin D. (1955). The succinate-glycine cycle. I. The mechanism of pyrrole synthesis. J Biol Chem *215*, 613-26.

Schumacher, A., and Drews, G. (1978). The formation of bacteriochlorophyll-protein complexes of the photosynthetic apparatus of *Rhodopseudomonas capsulata* during early stages of development. Biochim Biophys Acta *501*, 183-94.

Scolnik, P.A., Zannoni, D., and Marrs, B.L. (1980). Spectral and functional comparsions between the carotenoids of the two antenna complexes of *Rhodopseudomonas capsulata*. Biochim Biophy Acta *593*, 230-40.

Sganga, M.W., and Bauer, C.E. (1992). Regulatory factors controlling photosynthetic reaction center and light-harvesting gene expression in *Rhodobacter capsulatus*. Cell *68*, 945-954.

Shemin D., and Russell, C.S. (1953). δ-Aminolevulinic acid, its role in the biosynthesis of porphyrins and purine. J Am. Chem. Soc. *75*, 4873-4874.

Shigesada, K. (1972). Possible occurence of a succinate-glycine cycle in *Rhodospirillum rubrum*. J Biochem *71*, 961-972.

Smart, J. (2001). A Partial Characterization of Three Photosynthetic Regulatory Genes in *Rhodobacter capsulatus*. Ph.D Thesis. Simon Fraser University. BC, Canada.

Spencer, P., and Jordan, P.M. (1993). Purification and characterization of 5-aminolevelunic acid dehydrase from *Escherichia coli* and a study of the reactive thiols at the metal-binding domain. Biochem J *290*, 279-287.

Spencer, P., and Jordan, P.M. (1994). Investigation of the nature of the two metal-binding sites in 5-aminolevulinic acid dehydrase from *Escherichia coli*. Biochem J *300*, 373-81.

Sturgis, J.N., and Neidermann, R.A., (1996). The effect of different levels of the B800-850 light-harvesting complex on intracytoplasmic membrane development in *Rhodobacter sphaeroides*. Arch Microbiol *165*, 235-42.

Tadros, M.H., Suter, F., Drews, G, and Zuber, H. (1983). The complete amino-acid sequence of the large bacterichlorophyll-binding polypeptide from light-harvesting complex II (B800-850) of *Rhodopseudomonas capsulata*. Eur J Biochem *129*, 533-6.

Tadros, M.H., Suter, F., Seydewitz, H.H., Witt, I., Zuber, H., and Drews, G. (1984). Isolation and complete amino-acid sequence of the small polypeptide from light-harvesting pigment-protein complex I (B870) of *Rhodopseudomonas capsulata*. Eur J Biochem *138*, 209-12.

Tait, G.H. (1972). Coproporphyrinogenase activities in extracts of *Rhodopseudomonas sphaeroides* and *Chromatium* strain D. Biochem J *128*, 1159-69.

Takemoto, J.Y., Peterson, R.L., Tadros, M.H. and Drews, G. (1987). Transverse membrane topography of the B875 light-harvesting polypeptides of wild type *Rhodobacter sphaeroides*. J Bacteriol *169*, 4731-4736.

Taylor, D.P., Cohen, S.N., Clark, W.G., and Marrs, B.L. (1983). Alignment of genetic and restriction maps of the photosynthetic region of the *Rhodopseudomonas capsulata* chromosome by a conjugation-mediated marker rescue technique. J Bacteriol *154*, 580-590.

Tichy, H.V., Oberle, B., Stiehle, H., Schiltz, E., and Drews, G. (1989). Genes downstream from *pucB* and *pucA* are essential for formation of the B800-850 complex of *Rhodobacter capsulatus*. J Bacteriol *171*, 4914-22.

Tichy, H.V., Albien, K.U., Gad'on, N., and Drews, G. (1991) Analysis of the *Rhodobacter capsulatus puc* operon: the *pucC* gene plays a central role in the regulation of LHII (B800-850 complex) expression. EMBO J *10*, 2949-55.

Toledo, J.C., Gari, E., Gilbert, I., and Barbe, J. (1992). Nucleotide sequence of the methoxyneurosporene dehydrogenase gene from *Rhodobacter sphaeroides*: comparison with other bacterial carotenoid dehydrogenases. FEMS Microbiol Lett *72*, 103-108.

Usui, S., and Yu, L. (1991). Subunit IV (Mr = 14,384) of the cytochrome *bc*-1 complex from *Rhodobacter sphaeroides*. Cloning, DNA sequencing, and ubiquinone binding domain. J Biol Chem *266*, 15644-9.

Walz, T., Jamieson, S.J., Bowers, C.M., Bullough, P.A., and Hunter, C.N. (1998) Projection structures of three photosynthetic complexes from *Rhodobacter sphaeroides*: LH2 at 6Å, LHI and RC-LHI at 25Å. J Mol Biol *282*, 833-45.

Warnick, G.R., and Burnham, B.F. (1971). Regulation of porphyrin biosynthesis. Purification and characterization of δ-aminolevulinic acid synthase. J Biol Chem *246*, 6880-5.

Weaver, P.F., Wall, J.D., and Gest, H. (1975) Characterization of *Rhodopseudomonas capsulatus*. Arch Microbiol *105*, 207-216.

Wellington, C.L., Taggart, A.K., and Beatty, J.T. (1991). Functional significance of overlapping transcripts of *crtEF*, *bchCA*, and *puf* photosynthesis gene operons in *Rhodobacter capsulatus*. J Bacteriol *173*, 2954-61.

Williams, J.G., and Szalay, A.A. (1983). Stable integration of foreign DNA into the chromosome of the cyanobacterium *Synechococcus* R2. Gene *24*, 37-51.

Williams, J.C., Steiner, L.A., Feher, G., and Simon, M.I. (1984). Primary structure of the L subunit of the reaction center from *Rhodopseudomonas sphaeroides*. Proc Natl Acad Sci USA *81*, 7303-7.

Williams, J.C., Steiner, L.A., and Feher, G. (1986). Primary structure of the reaction center from *Rhodopseudomonas sphaeroides*. Proteins *1*, 12-25.

Willows, R.D., Lake, V., Roberts, T.H., and Beale, S.I. (2003). Inactivation of Mg Chelatase during Transition from Anaerobic to Aerobic Growth in *Rhodobacter capsulatus*. J Bacteriol *185*, 3249-3258.

Wong, D.K., Collins, W.J., Harmer, A., Lilburn, T.G., and Beatty, J.T. (1996) Directed mutagenesis of the *Rhodobacter capsulatus puhA* gene and *orf 214*: pleitrophic effects on photosynthetic reaction and light-harvesting I complexes. J Bacteriol *178*, 2334-2342.

Xu, K., and Elliott, T. (1994). Cloning, DNA sequence, and complementation analysis of the *Salmonella typhimurium hemN* gene encoding a putative oxygen-independent coproporphyrinogen III oxidase. J Bacteriol *176*, 3196-203.

Yeliseev, A.A., and Kaplan, S. (1995). A sensory transducer homologous to the mammalian peripheral-type benzodiazepine receptor regulates photosynthetic membrane complex formation in *Rhodobacter sphaeroides* 2.4.1. J Biol Chem *270*, 21167-75.

Yeliseev, A.A., Eraso, J.M., and Kaplan, S. (1996). Differential carotenoid composition of the B875 and B800-850 photosynthetic antenna complexes in *Rhodobacter sphaeroides* 2.4.1: involvement of spheroidene and spheroidenone in adaptation to changes in light intensity and oxygen availability. J Bacteriol *178*, 5877-83.

Yen, H.C., and Marrs, B. (1976) Map of genes for carotenoid and bacteriochlorophyll biosynthesis in *Rhodopseudomonas capsulata*. J Bacteriol *126*, 619-629.

Yang, Z.M., and Bauer, C.E. (1990). *Rhodobacter capsulatus* genes involved in early steps of the bacteriochorophyll biosynthetic pathway. J Bacteriol *172*, 5001-5010.

Young, D.A., Bauer, C.E., Williams, J.C. and Marrs, B.L. (1989). Genetic evidence for superoperonal organization of genes for photosynthetic pigments and pigment-binding proteins in *Rhodobacter capsulatus*. Mol Gen Genet *218*, 1-12.

Young, D.A., Rudzik, M.B., and Marrs, B.L. (1992). An overlap between operons involved in carotenoid and bacteriochlorophyll biosynthesis in *Rhodobacter capsulatus*. FEMS Microbiol Lett *74*, 213-8.

Yu, L. and Yu, L. (1984). Purification and properties of cytochrome b from photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26. Biochem Biophys Res Commun *123*, 1234-9.

Yu, L., and Yu, C.A. (1991). Essentiality of the molecular weight 15,000 protein (subunit IV) in the cytochrome bc_1 complex of *Rhodobacter sphaeroides*. Biochemistry *30*, 4934-9.

Zhu , Y.S., Cook, D.N., Leach, F., Armstrong, G.A., Alberti, M. and Hearst, J.E. (1986). Oxygen-regulated mRNA for light-harvesting and reaction center complexes and for bacteriochlorophyll and carotenoid biosynthesis in *Rhodobacter capsulatus* during the shift from anaerobic to aerobic growth. J Bacteriol *168*, 1180-8.

Zsebo, K.M. and Hearst, J.E. (1984). Genetic-physical mapping of a photosynthetic gene cluster from *Rhodospeudomonas capsulata*. Cell *37*, 937-47.

Zeilstra-Ryalls, J.H., and Kaplan S. (1995) Aerobic and anaerobic regulation in *Rhodobacter sphaeroides* 2.4.1: the role of the *fnrL* gene. J Bacteriol *177*, 6422-31.

Zeilstra-Ryalls, J.H., Gabbert, K., Mouncey, N.J., Kaplan, S., and Kranz, R.G. (1997). Analysis of the *fnrL* gene and its function in *Rhodobacter capsulatus*. J Bacteriol *179*, 7264-73.

Zeilstra-Ryalls, J.H., and Kaplan S. (1998). Role of the *fnrL* gene in photosystem gene expression and photosynthetic growth of *Rhodobacter sphaeroides* 2.4.1. J Bacteriol *180*, 1496-503.