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Molecular Cloning, Sequencing, and Regulation of the Rhodobacter capsulatus hemB Gene.

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MOLECULAR CLONING, SEQUENCING, AND REGULATION OF THE
Rhodobacter capsulatus hemB GENE.

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
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in partial fulfillment of the
requirements for the degree of
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in

The Department of Microbiology

by
Karl Joseph Indest
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Dedication

This work is gratefully dedicated to
my loving wife, Lisa, for inspiring me
to pursue my dream.
Acknowledgements

Words cannot express the deep appreciation and gratitude I have for my friend and wife, Lisa (the real hemB). Without her encouragement, emotional support, and love, I would have not been able to successfully complete this degree.

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Abstract

The common tetrapyrrole pathway in the photosynthetic bacterium, *Rhodobacter capsulatus*, is responsible for the synthesis of bacteriochlorophyll, heme, vitamin B-12, and siroheme. The common portion of this pathway is regulated up to 100-fold by changes in oxygen tension. This regulation is accomplished by controlling the intracellular level of porphobilinogen. One way in which porphobilinogen levels could be controlled is by regulating the synthesis of porphobilinogen. Porphobilinogen synthase, encoded by the *hemB* gene, is the second enzyme in the common tetrapyrrole pathway that catalyzes the dimerization of 5-aminolevulinic acid to form the monopyrrole porphobilinogen. In order to further investigate the mechanism by which oxygen regulates porphobilinogen levels, the *R. capsulatus* *hemB* gene was cloned and sequenced.

The *R. capsulatus* *hemB* gene was cloned by complementation of an *Escherichia coli* *hemB* mutant. Sequence analysis of the *R. capsulatus* *hemB* gene revealed that the putative porphobilinogen synthase has a metal-binding domain that more closely resembles that found in plant porphobilinogen synthases. The locations of the *hemB* and *hemA* genes on the *R. capsulatus* chromosome indicate that these gene do not form an operon.

Oxygen mediated transcriptional regulation of the *R. capsulatus* *hemB* gene was measured by dot blot analysis of mRNA from cells grown under 3%
and 23% oxygen and by hemB-cat transcriptional fusion studies. Both of these methods reveal that the synthesis of porphobilinogen synthase does not change with shifts in oxygen tension. Oxygen tension also does not appear to regulate enzyme activity since the specific activity does not change significantly with changes in oxygen tension.

Overexpression of the *R. capsulatus* hemB, in the presence of exogenous aminolevulinate, in *R. capsulatus* can overcome the normal oxygen mediated regulatory mechanisms. While the mechanism of how oxygen modulates carbon flow down the common tetrapyrrole pathway remains elusive, it is clear that the level of porphobilinogen plays a crucial part in oxygen regulation. The possibility still exist that oxygen regulates the degradation of porphobilinogen.
Introduction

The photosynthetic, purple, nonsulfur bacteria are a metabolically versatile group of organisms. One member of this group, *Rhodobacter capsulatus*, is able to obtain energy from many different sources. It can use light for photophosphorylation, or it can obtain energy from the oxidation of either organic compounds or inorganic compounds such as H₂. The presence of the Calvin cycle (47), in addition to the Entner-Doudoroff and glycolytic pathways (30), allows this organism to use CO₂ or organic carbon compounds as a carbon source. In addition, *R. capsulatus* possesses a nitrogenase enzyme which allows it to fix atmospheric nitrogen. One factor in this organism's remarkable metabolic versatility is that it is one of a small group of organisms that can synthesize all four tetrapyrrole endproducts: heme, bacteriochlorophyll, vitamin B-12 and siroheme. Bacteriochlorophyll is the primary light-harvesting pigment involved in bacterial photosynthesis and is only produced under anoxic conditions. Heme is required under all growth conditions and is present as the prosthetic group in cytochromes, catalases and peroxidases. Vitamin B-12 has been shown to be a cofactor in methionine biosynthesis (24) and siroheme serves as a prosthetic group for the enzymes sulfite reductase (98) and nitrite reductase (99).
Formation of aminolevulinate and its conversion into protoporphyrin IX

Most of the information about the tetrapyrrole pathway in the Rhodospirillaceae has come from studies using R. sphaeroides. In 1964, while working with R. sphaeroides, Lascelles proposed that bacteriochlorophyll a and heme share a common pathway that bifurcates after the formation of protoporphyrin IX (88). This common portion of the tetrapyrrole pathway, from aminolevulinate to protoporphyrin IX, is found in most organisms (Fig. 1). There are only a few examples of organisms found in nature that have an incomplete tetrapyrrole biosynthetic pathway (55). Most of these organisms have complex nutritional requirements and require exogenous heme or a tetrapyrrole precursor for growth. As far as is known, all organisms either make or require tetrapyrroles. This suggests that the tetrapyrrole molecule is evolutionarily ancient, functioning in life's most important biochemical processes.

The aminoketone, aminolevulinate, is the first common intermediate found in the tetrapyrrole pathway. Synthesis of aminolevulinate can occur by two distinct routes: the C5 pathway, which uses the carbon skeleton of glutamate (8), or the C4 pathway, which involves glycine and succinyl-CoA (80). The C5 pathway is present in plants (8), algae (141), cyanobacteria (39), archeabacteria (46) and eubacteria (41). Formation of aminolevulinate using the C5 pathway requires activation of glutamate by linking it to the tRNA_{glu}. This reaction is catalyzed by glutamyl-1-synthetase (104). Glutamyl-tRNA_{glu} is reduced to glutamate-1-semialdehyde by glutamyl-tRNA_{glu} reductase, the product of the
Figure 1. The tetrapyrrole biosynthetic pathway in *R. capsulatus*. 
hemA gene (3). Aminolevulinate is formed from glutamate-1-semialdehyde, following a transamination reaction, catalyzed by glutamate-1-semialdehyde aminotransferase, the product of the hemL gene (41).

The C4 pathway is found in the α-proteobacteria, which includes the Rhodospirillaceae, but is mainly confined to animals (4, 105). In the C4 pathway formation of aminolevulinate is catalyzed by aminolevulinate synthase, the product of the hemA gene. This enzyme forms aminolevulinate by the condensation of glycine and succinyl-CoA. This step is one of the most studied of the tetrapyrrole pathway. The R. capsulatus hemA gene has been cloned and sequenced from two independent sources. Biel et al. (143) isolated a cosmid, containing the R. capsulatus hemA gene, by complementation of a R. capsulatus hemA:Trn5 mutant. Drews et al. (63) isolated the hemA gene from R. capsulatus by probing a cosmid bank of R. capsulatus with the aminolevulinate synthase gene from R. sphaeroides. When compared with other hemA genes encoding aminolevulinate synthase, the predicted amino acid sequence is 69% to 73% similar, and 49% to 55% identical to other aminolevulinate synthases (38). The predicted molecular mass of the R. capsulatus aminolevulinate synthase is 50,491 Da, based on sequence information (38). Since there are no published reports on the purification of aminolevulinate synthase from R. capsulatus, it is not known what the actual molecular mass is of the native enzyme. The native enzyme from R. sphaeroides exist as a homodimer and has a molecular mass of 80,000 Da (103). The R. sphaeroides enzyme requires pyridoxal phosphate
for catalytic activity and is strongly inhibited by hemin, Mg-protoporphyrin or ATP (43, 151). Multiple forms of aminolevulinate synthase from *R. sphaeroides* have been isolated on DEAE-Sephadex (136). The different forms have varying degrees of activity and respond differently to environmental factors, such as light and oxygen (135). The enzyme forms have been found to be interconvertable, with the inactive form requiring cysteine trisulfide to become active (113).

Interestingly, *R. sphaeroides* has recently been found to encode two distinct aminolevulinate synthases, the products of the *hemA* and *hemT* genes (79). These two genes are located on separate chromosomes and share homology with each other, as well as all previous characterized aminolevulinate synthases. The protein products of the *hemA* and *hemT* genes, however, have not been correlated with the multiple forms of aminolevulinate synthase reported previously in *R. sphaeroides* (136).

The next step in the tetrapyrrole pathway is the formation of the monopyrrole, porphobilinogen. Porphobilinogen is formed by the condensation of two molecules of aminolevulinate with the loss of two molecules of water. This reaction is catalyzed by porphobilinogen synthase, the product of the *hemB* gene. This reaction is the second most studied of the common tetrapyrrole pathway. The *R. capsulatus hemB* gene has been cloned and sequenced and will be discussed in detail later. The *R. sphaeroides hemB* gene has also been cloned, but no sequence information is available (35). This makes the *R. capsulatus hemB* gene the only porphobilinogen synthase gene to have been sequenced
from the *Rhodospirillaceae*. Porphobilinogen synthase has been purified from *R. capsulatus* (102) and *R. sphaeroides* (101). The native conformation of the enzyme is believed to exist as a hexamer having a molecular mass of approximately 260,000 Da for the *R. capsulatus* enzyme and 240,000 Da for the *R. sphaeroides* enzyme (62). Porphobilinogen synthase from *R. sphaeroides* requires the presence of metallic cations and thiols for full activity and is strongly inhibited by hemin (101). In contrast, the *R. capsulatus* enzyme has no metal requirement, is less responsive to thiol compounds and is not inhibited by hemin (102).

The first tetrapyrrole, uroporphyrinogen III, is formed by the sequential action of two separate enzymes. Porphobilinogen deaminase, the product of the *hemC* gene, catalyzes the deamination and polymerization of four molecules of porphobilinogen to form the highly labile, linear tetrapyrrole, hydroxymethylbilane. This linear tetrapyrrole is released into solution where it serves as the substrate for uroporphyrinogen III synthase, the product of the *hemD* gene. This enzyme catalyzes the rearrangement of ring four of hydroxymethylbilane followed by its subsequent cyclization into uroporphyrinogen III. In the absence of uroporphyrinogen III synthase, hydroxymethylbilane is spontaneously converted into uroporphyrinogen I, a nonphysiological isomer. Porphobilinogen deaminase has been purified from *R. sphaeroides* (74) and *R. palustris* (82), as well as a variety of other prokaryotic and eukaryotic sources. In all cases, the deaminase exist as a monomer having a molecular mass ranging from 34,000 Da to 44,000
Da (71), with the exception of the *R. palustris* deaminase which has a molecular mass of 74,000 Da. Porphobilinogen deaminase requires a dipyrrolmethane cofactor which serves as a primer to which four substrate molecules are covalently attached (75). This cofactor is unique in that it is synthesized by the deaminase from two molecules of porphobilinogen. Now that the *E. coli* porphobilinogen deaminase has been crystallized and undergone preliminary X-ray diffraction studies greater insight into the mechanism by which this enzyme functions can be gained (76).

Information concerning uroporphyrinogen III synthase has lagged behind the other enzymes of the tetrapyrrole pathway due to the instability of the protein. It was first demonstrated by Bogorad and Granick that heating spinach extracts prior to incubation with porphobilinogen led to the production of uroporphyrinogen I (17). The authors later concluded that a labile protein, which they termed uroporphyrinogen isomerase (uroporphyrinogen III synthase) was responsible for the isomerization reaction (16). So far uroporphyrinogen III synthase has been purified to homogeneity from *E. coli* (1), human erythrocytes (133), rat liver (125), and *Euglena gracilis* (60). The synthases appear in monomeric form having molecular masses ranging from 28,000 Da to 31,000 Da. There are scattered reports of the copurification of uroporphorinogen III synthase with porphobilinogen deaminase to form a complex called porphobilinogenase (2, 77, 112). Jordan maintains that these enzymes do not form a complex but rather function independently and sequentially (72). The cloning and sequencing of the genes
encoding porphobilinogen deaminase and uroporphyrinogen III synthase have been reported for *E. coli* (73, 130), human erythrocytes (109, 132), *Bacillus subtilis* (59), *Pseudomonas aeruginosa* (97) and other prokaryotic and eukaryotic sources. There is greater homology exhibited among the porphobilinogen deaminases than among the uroporphyrinogen III synthases. DNA sequence information from *E. coli*, *B. subtilis*, and *P. aeruginosa* further reveal that the *hemC* and *hemD* genes form an operon (59, 97, 117). This arrangement may be advantageous when considering that these enzymes function sequentially.

Uroporphyrinogen III is at a major branch point in the tetrapyrrole pathway. Upon decarboxylation, uroporphyrinogen III gives rise to heme and bacteriochlorophyll. If uroporphyrinogen III is methylated, siroheme and vitamin B-12 will be the final endproducts. Uroporphyrinogen decarboxylase, the product of the *hemE* gene, catalyzes the stepwise decarboxylation of the acetate groups on all four rings of uroporphyrinogen III to form coproporphyrinogen III. Currently, it is believed that the decarboxylation reaction occurs in an orderly, clockwise fashion starting with ring D (92). The enzyme has been purified to homogeneity from only one bacterial source, *R. sphaeroides* (70). A 600-fold purification revealed that the native enzyme exists as a monomer having a molecular mass of 41,000 Da. These findings are consistent with other reports regarding eukaryotic uroporphyrinogen decarboxylases, which have molecular masses ranging from 39,000 Da to 46,000 Da (44, 126, 137). The enzyme is susceptible to inhibition by a variety of porphyrins; the more hydrophilic the side chain the
greater the degree of inhibition. In addition, the enzyme is stimulated by thiol compounds and inhibited by the metal ions, Hg$^{+2}$ and Cu$^{+2}$. The hemE gene has been cloned from *R. capsulatus* by complementing an *E. coli* hemE mutant (65). DNA sequencing analysis has revealed that the gene encodes a 344 amino acid protein having a predicted molecular mass of 43,611 Da (65). The putative amino acid sequence shares an overall identity of 34% with uroporphyrinogen decarboxylases from yeast (48) and *B. subtilis* (57) and is 36% identical to the rat enzyme (111).

The formation of protoporphyrinogen IX occurs when the propionyl groups on the A and B rings of coproporphyrinogen III are oxidatively decarboxylated to vinyl groups. This reaction is catalyzed by coproporphyrinogen oxidase, the product of the hemF gene. Eukaryotes and aerobically growing bacteria require molecular oxygen in order to transform coproporphyrinogen III into protoporphyrinogen IX (115), whereas anaerobically grown cells use a different enzyme to complete this transformation. Using extracts of *R. sphaeroides*, Tait (127, 128) showed that the anaerobic enzyme required NADH, ATP, L-methionine or S-adenosyl-methionine for activity. He concluded that *R. sphaeroides* had two different enzymes which possessed the ability to convert coproporphyrinogen III to protoporphyrinogen IX: an aerobic coproporphyrinogen oxidase, which required O$_2$ as an electron acceptor, and an anaerobic coproporphyrinogen oxidase, which used NADH as an electron acceptor. Neither one of these enzymes has been purified to homogeneity from a bacterial source. The cloning of a putative
anaerobic coproporphyrinogen oxidase gene from *R. sphaeroides* has recently been reported (31). Using chemical mutagenesis, Hunter et al. (31) isolated a mutant that when grown anaerobically was unable to synthesize bacteriochlorophyll and excreted large amounts of coproporphyrin. This same mutant, under aerobic conditions, accumulated only trace amounts of coproporphyrin. By using conjugative gene transfer and transposon mutagenesis, the authors were able to clone the gene responsible for this phenotype which they believed was due to the lack of an anaerobic coproporphyrinogen oxidase. Sequence analysis of this gene revealed the molecular mass of the enzyme to be 34,185 Da. A putative oxygen-independent coproporphyrinogen oxidase has also been reported in *Salmonella typhimurium* (145). Using chemical mutagenesis, the authors isolated mutant strains they believed were defective in both the aerobic and anaerobic coproporphyrinogen oxidase. By complementing these mutants, it was possible to clone both the aerobic (147) and anaerobic (146) coproporphyrinogen oxidases, known as the *hemF* and *hemN* genes, respectively. The *hemN* gene codes for a 52.8 kDa protein and has a 38% amino acid sequence identity to the putative anaerobic *R. sphaeroides* coproporphyrinogen oxidase. The aerobic coproporphyrinogen oxidase codes for a 31.6 kDa protein and is 90% identical to the *E. coli* enzyme (131) and 44% identical to the yeast enzyme (152). The yeast coproporphyrinogen oxidase has been purified to homogeneity and is a homodimer having a molecular mass of 70,000 Da (23). Some have suggested that the presence of these two enzymes
may indicate that the branch point separating heme and bacteriochlorophyll synthesis may not occur at the chelation step but higher up in the tetrapyrrole pathway, at the level of coproporphyrinogen III (31).

The final intermediate that is common to heme and bacteriochlorophyll biosynthesis is protoporphyrin IX. Protoporphyrinogen oxidase, the product of the hemG gene, is a membrane bound protein, which catalyzes the six electron oxidation of protoporphyrinogen IX to form protoporphyrin IX. The formation of protoporphyrin IX can also proceed spontaneously, however, the enzymatic reaction is significantly increased over the non-enzymatic reaction (115). Protoporphyrinogen oxidase requires molecular oxygen for the oxidation of protoporphyrinogen IX in higher eukaryotes and aerobic bacterial systems. Jacobs et al. (66, 67) were the first to report an oxygen-independent mechanism for protoporphyrinogen IX oxidation in extracts of E. coli, in which nitrate or fumarate served as the electron acceptor. The same authors reported protoporphyrinogen oxidase activity in R. sphaeroides under photosynthetic and aerobic growth conditions (68). The R. sphaeroides protoporphyrinogen oxidase activity was subject to inhibition by cyanide and azide, indicating the enzyme was coupled to the respiratory chain. Protoporphyrinogen oxidase has been purified and characterized from only one bacterial source, Desulfovibrio gigas (81). The enzyme is a membrane bound protein that has a molecular mass of 148,000 Da and consist of three nonidentical subunits having molecular masses of 12,000 Da, 18,500 Da, and 57,000 Da. These three subunits are believed to be held
together by sulfhydryl bonds. In contrast, the mammalian protoporphyrinogen oxidases that have been purified and characterized so far are monomers with a molecular mass of about 65,000 Da (34, 84, 124). The hemG gene has been cloned and sequenced from E. coli (116) and B. subtilis (56). The E. coli hemG gene is 546 nucleotides, coding for a protein with the predicted molecular mass of 21,202 Da, whereas the B. subtilis enzyme has a predicted molecular mass of 51,200 Da. The apparent disparity between the sizes of the mammalian and bacterial enzymes may indicate that these are two separate enzymes. Perhaps, there are two kinds of protoporphyrinogen oxidases, much like the two coprophyrinogen oxidases activities reported in R. sphaeroides.

The ultimate fate of protoporphyrin IX is determined by whether an Fe\(^{2+}\) ion or a Mg\(^{2+}\) ion is incorporated into the porphyrin ring. Bacteriochlorophyll will be the final endproduct if magnesium is incorporated into protoporphyrin IX, whereas, if a ferrous iron is incorporated into protoporphyrin IX, heme will be the final endproduct. Ferrochelatase, the product of the hemH gene, catalyzes the insertion of Fe\(^{2+}\) into protoporphyrin IX. The hemH gene has recently been cloned in R. capsulatus and codes for a protein with a predicted molecular mass of 46,000 Da (Kanazireva and Biel, unpublished). Ferrochelatase has been purified to homogeneity from R. sphaeroides (32). The enzyme is an intrinsic membrane protein, consisting of a single polypeptide of molecular mass 115,000 Da. This value is rather high when compared to other bacterial ferrochelatases which have molecular masses of about 35,000 Da (58, 96). The R. sphaeroides
ferrochelatase has a broad substrate specificity and is capable of using Cu\(^{++}\) and Zn\(^{++}\), in addition to iron, in the chelation reaction (33, 69). The enzyme is also sensitive to inhibition by heme, protoporphyrin, Mg-protoporphyrin, and N-methylporphyrins (32). The inactivating effects of N-ethylmalamide and iodacetamide suggest that the enzyme contains sulfhydryl residues essential for activity (33).

The enzyme(s) responsible for inserting magnesium into protoporphyrin IX are less characterized. The magnesium chelatase reaction was first reported in *R. sphaeroides* by Gorchein (51), who demonstrated that the reaction was dependent on membrane integrity, low \(O_2\) tension, ATP, and coupling of magnesium insertion to methylation. Weinstein and Walker (140), working with pea chloroplast, proposed that magnesium chelation was a two step process. In the first step, magnesium chelatase components come together in the presence of ATP and undergo activation. This activated enzyme complex then catalyzes the magnesium insertion into protoporphyrin IX. Recently, Gorchein (52), using *R. sphaeroides*, has assigned the magnesium chelatase activity to the *bchD* and *bchl* loci and the methyltransferase activity to the *bchH* locus.

**Regulation of tetrapyrrole biosynthesis.**

*R. sphaeroides* synthesizes tremendously different quantities of tetrapyrroles depending on its mode of growth. Cells of *R. sphaeroides* growing photosynthetically produce an estimated 25 nmoles bacteriochlorophyll per mg dry weight, 0.3 nmoles heme per mg dry weight, and 0.07 nmoles vitamin B-12
per mg dry weight (86). Upon aeration bacteriochlorophyll synthesis ceases, while heme and vitamin B-12 synthesis continue unabated (86). Bacteriochlorophyll levels in photosynthetically grown cultures reach 40 to 100 times that of bacteriochlorophyll levels in aerobically grown cultures (87), whereas hemoprotein content varies only two to four-fold with changes in oxygen tension (107). The sudden shift from anaerobic, photosynthetic growth to aerobic, dark growth does not result in the accumulation of biosynthetic intermediates. This indicates that the cell has the ability to reduce carbon flow over the common tetrapyrrole pathway 100-fold while continuing to synthesize the other tetrapyrrole endproducts. Multiple mechanisms are in place that enable the cell to adapt its biosynthetic machinery to changes in the environment. So far at least three environmental factors have been identified that modulate carbon flow over the common tetrapyrrole pathway in *R. capsulatus*: oxygen, heme, and c-type cytochromes. While each of the factors appear to act independently, the mechanisms by which they alter carbon flow are poorly understood.

Cohen-Bazire et al. (29) were the first to present evidence of an efficient mechanism for the regulation of tetrapyrrole biosynthesis. Using extracts of *R. sphaeroides* and *R. rubrum* they showed that introducing oxygen into cultures growing anaerobically in the light resulted in the abrupt cessation of bacteriochlorophyll synthesis. They proposed that the redox state of components of the respiratory chain could be influenced by oxygen and were critical in regulating bacteriochlorophyll synthesis. Marrs and Gest (94) have indicated
these components may not be directly involved in regulating bacteriochlorophyll synthesis since mutants of *R. capsulatus* with defects in the respiratory chain are still capable of preventing bacteriochlorophyll synthesis in the presence of oxygen. A genetic and physical map of the photosynthetic region of *R. capsulatus* has made it possible to study bacteriochlorophyll synthesis at the molecular level (129). Biel and Marrs (11) examined oxygen regulation of bacteriochlorophyll gene expression by isolating and studying fusions between Mud d1 (Ap",lac) and various bacteriochlorophyll biosynthetic (*bch*) genes. Transcription of the *bch* genes were two to three-fold higher in cultures grown with 2% oxygen than in those grown with 20% oxygen. Measurements of *bch* mRNA from cultures grown under high versus low oxygen tensions confirm these results (27). A two-fold change in bacteriochlorophyll gene expression may be significant, but it does not account for the 100-fold difference in bacteriochlorophyll levels between anaerobically and aerobically grown cultures. In addition, studies with cells of *R. sphaeroides* indicate that the coupling of magnesium insertion and methylation to form magnesium protoporphyrin monomethyl ester is sensitive to oxygen (51). Biel (10) has demonstrated that a *R. capsulatus bchE* strain does not accumulate magnesium protoporphyrin monomethyl ester when grown under high oxygen tension. The fact that the organism is still synthesizing heme under these growth conditions indicates that oxygen controls the formation of magnesium protoporphyrin monomethyl ester from protoporphyrin. Exactly how oxygen is regulating this step is still unknown.
Mutational analysis of the photosynthetic gene cluster has revealed that PufQ is essential for bacteriochlorophyll synthesis (149). While the role of PufQ remains obscure, strains deficient in PufQ exhibit a severe reduction in steady-state levels of bacteriochlorophyll under anaerobic conditions. PufQ expression is highly regulated by aeration, with a 30-fold increase in gene expression under anaerobic conditions (7). One model envisions PufQ acting as a carrier molecule that interacts with protoporphyrin IX (6). The resulting tetrapyrrole-protein complex serves as the substrate for magnesium chelatase leading to bacteriochlorophyll synthesis. While this model offers a plausible explanation for how oxygen regulates the magnesium chelatase reaction, it does not explain how carbon flow is diverted away from the common pathway to accommodate the overall decrease in tetrapyrrole biosynthesis. Kaplan (50), on the other hand, suggested that PufQ is specifically involved in the insertion of bacteriochlorophyll into developing spectral complexes and is not involved in the magnesium chelatase reaction.

A hint as to how carbon flow through the common pathway is reduced during aerobic growth was provided by a R. capsulatus bchH mutant. These mutants are unable to carry out the magnesium chelatase reaction and as a result cannot make bacteriochlorophyll. Interestingly, these mutants accumulate more protoporphyrin IX when grown under low oxygen than high oxygen, suggesting there is a point in the common tetrapyrrole pathway that is regulated by oxygen (11). Until recently, a likely candidate for this second control point
was thought to be aminolevulinate synthase. The formation of aminolevulinate has been reported to be rate-limiting for the entire pathway and is generally considered to be the first committed step in tetrapyrrole biosynthesis (89). Lascelles proposed that oxygen inhibits magnesium chelatase, resulting in the accumulation of protoporphyrin IX (22). Excess protoporphyrin IX would then be converted into heme by ferrochelatase. The rise in levels of heme would feedback inhibit aminolevulinate synthase reducing carbon flow over the common pathway. Evidence supporting this model has come from in vitro studies using purified aminolevulinate synthase from *R. sphaeroides*. The enzyme is inhibited by hemin, and to a lesser extent, magnesium protoporphyrin and protoporphyrin (151). While aminolevulinate synthase activity in cultures of *R. sphaeroides* vary five-fold with oxygen tension (85), the observed specific activity may not reflect the in vivo situation since the enzyme undergoes activation by endogenous trisulfides (113). However, aminolevulinate synthase specific activity in aerobically and anaerobically grown cultures of *R. capsulatus* is indistinguishable. Transcriptional studies using a *hemA-lacZ* fusion carried on a plasmid reveal only a two-fold change in *hemA* gene expression in response to changes in oxygen tension (144). These relatively small changes in enzyme activity and gene expression are not enough to account for the large increase in tetrapyrrole biosynthesis that accompanies anaerobic growth. The effects of exogenous aminolevulinate on a *R. capsulatus bchH* mutant further support the notion that aminolevulinate formation is not regulated by oxygen. Biel (10) has reported that
protoporphyrin levels are still subject to oxygen-mediated regulation in the presence of exogenous aminolevulinate. However, when a \textit{bchH} mutant is grown under high oxygen in the presence of exogenous porphobilinogen, protoporphyrin accumulates to the level normally seen in anaerobically grown cultures. This indicates that oxygen regulates the accumulation of porphobilinogen. Perhaps the formation of porphobilinogen is the first true committed step in tetrapyrrole biosynthesis. Supporting this idea is the observation that only 20% of $^{14}$C aminolevulinate goes to make tetrapyrroles in \textit{R. capsulatus} (Biel, unpublished). While working with \textit{R. rubrum}, Shigesada (123) demonstrated that only 10% of the aminolevulinate is used for tetrapyrrole biosynthesis, while the rest is metabolized by a second metabolic pathway he termed the succinate-glycine cycle.

Recent reports in \textit{R. capsulatus} and \textit{R. sphaeroides} on the isolation of regulatory mutants deficient in photosynthetic gene expression has further complicated the picture as to the role oxygen plays in bacteriochlorophyll synthesis. Bauer (120) has isolated a regulatory protein, designated RegA, that positively activates gene expression of the light harvesting and reaction center polypeptides, but not the bacteriochlorophyll biosynthetic enzymes. Mutations in RegA exhibit only a five-fold reduction in total bacteriochlorophyll content when growing anaerobically in the dark. Kaplan's group has isolated a similar protein, called PrrA, in \textit{R. sphaeroides} (42). Interestingly, wild-type cells overproducing PrrA actually induce the formation of photosynthetic clusters under aerobic
conditions, although not at the same level that occurs during anaerobic growth. Penfold and Pemberton (106) have reported isolating a repressor of carotenoid and bacteriochlorophyll synthesis they named PpsR. Mutations at this locus lead to a five-fold increase in bacteriochlorophyll and carotenoid synthesis under aerobic conditions. While these regulatory mutants are intriguing, they still exhibit oxygen-mediated regulation of bacteriochlorophyll synthesis.

Experimental results indicate that porphobilinogen levels are subject to oxygen regulation. In order to further investigate the mechanism by which oxygen regulates porphobilinogen levels, the *R. capsulatus hemB* gene was cloned and sequenced. Enzyme activity and gene expression were measured under different oxygen tensions to evaluate the contribution that synthesis of porphobilinogen may play in oxygen regulation. Expression of the *hemB* gene was measured by Dot blot analysis and by *hemB-cat* transcriptional fusion studies. The physical location of the *hemB* gene on the *R. capsulatus* chromosome was also determined.
Materials and Methods

Strains and plasmids

Bacterial strains and plasmids used in this study are listed in Tables 1 and 2.

Media

*R. capsulatus* was routinely grown in either a malate minimal salts medium (RCV) (128) or 0.3% Bacto-Peptone-0.3% yeast extract (PYE; Difco Laboratories, Detroit, Mich.). *Escherichia coli* was grown in Luria broth (9) modified by omitting glucose and decreasing the sodium chloride concentration to 0.5%. Solid media contained 1.5% agar (Difco). Antibiotics and other chemicals were added at the following concentrations (µg/ml): ampicillin 25; kanamycin 10; rifampicin 75; spectinomycin 10; streptomycin 10; chloramphenicol 25; tetracycline 10 for *E. coli* and 0.5 for *R. capsulatus*; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) 40; Isopropyl-β-D-thiogalactopyranoside 5. Stocks of bacterial strains were stored in 10% glycerol at -70°C.

Growth conditions

*R. capsulatus* and *E. coli* were routinely grown aerobically in the dark at 37°C with shaking. For growth of *R. capsulatus* under defined oxygen tensions, overnight cultures were subcultured into a 100 ml graduated cylinder containing
Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM522</td>
<td>supE thi Δ(lac-proAB) hsd5 F'[proAB' lacF' lacZ ΔM15]</td>
<td>Gough and Murray (53)</td>
</tr>
<tr>
<td>HB101</td>
<td>F' ara-14 proA2 lacY1 galK2 rpsL20 recA13 xyl-5 mtl-1 supE44 hsdS20(r'm)</td>
<td>Boyer and Roulland-Dussoix (18)</td>
</tr>
<tr>
<td>SHSP1</td>
<td>thr-1 leuB6 hemB1 ara-13 thi-1 lacY1 gal-3 malA1 xyl-7 myl-2 tonA2 supE44 λ'</td>
<td>Sasarman and Horodniceanu (118)</td>
</tr>
<tr>
<td><strong>R. capsulatus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAS100</td>
<td>hsd-1 str-2</td>
<td>Taylor et al. (129)</td>
</tr>
<tr>
<td>SB1003</td>
<td>rif-10</td>
<td>Yen and Marrs (148)</td>
</tr>
<tr>
<td>AJB456</td>
<td>Φ(bcH'-lacZ')700 hsd-1 str-2</td>
<td>Biel and Marrs (11)</td>
</tr>
</tbody>
</table>
Table 2. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevent Characteristics</th>
<th>Reference or description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322Ω</td>
<td>Ap(^{r}), Tc(^{r}), Sp(^{r})</td>
<td>Omega cartridge cloned from pH45Ω (108) into Eco RI site of pBR322.</td>
</tr>
<tr>
<td>pCM7</td>
<td>Ap(^{r}), Tc(^{r})</td>
<td>Close and Rodriguez (28)</td>
</tr>
<tr>
<td>pUC-4K</td>
<td>Ap(^{r}), Kn(^{r})</td>
<td>Vieira and Messing (138)</td>
</tr>
<tr>
<td>pKK223-3</td>
<td>Ap(^{r})</td>
<td>Brosius and Holy (21)</td>
</tr>
<tr>
<td>pUC18</td>
<td>Ap(^{r})</td>
<td>Vieira and Messing (138)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>mob(^{+}), tra(^{+}), Kn(^{r})</td>
<td>Ditta et al. (37)</td>
</tr>
<tr>
<td>pRK404</td>
<td>mob(^{+}), Tc(^{r})</td>
<td>Ditta et al. (36)</td>
</tr>
<tr>
<td>pRPSB105</td>
<td>Ap(^{r}), Kn(^{r})</td>
<td>Taylor et al. (129)</td>
</tr>
<tr>
<td>pCAP22</td>
<td>Ap(^{r}), Tc(^{r}), Cm(^{r})</td>
<td>0.6kb Eco RI generated DNA fragment from pCAP17 (13) cloned into pSUP202.</td>
</tr>
<tr>
<td>pCAP86</td>
<td>Ap(^{r})</td>
<td>pKK223-3 containing a 5.6 kb Eco RI generated DNA fragment from R. capsulatus able to complement SHSP1. (This study)</td>
</tr>
</tbody>
</table>

Table 2 con't.
<table>
<thead>
<tr>
<th>Vector</th>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCAP87</td>
<td>Ap'</td>
<td>pKK223-3 containing a 3.3 kb Pst I generated DNA fragment from <em>R. capsulatus</em> able to complement SHSP1. (This Study)</td>
</tr>
<tr>
<td>pCAP103</td>
<td>Tc'</td>
<td>3.3 kb Pst I generated DNA fragment from pCAP87 cloned into pRK404. (This study)</td>
</tr>
<tr>
<td>pCAP96</td>
<td>Ap', Str'</td>
<td>2.0 kb omega cartridge cloned into Eco RV site of pCAP87. (This study)</td>
</tr>
<tr>
<td>pCAP118</td>
<td>Ap'</td>
<td>3.3 kb Pst I generated DNA fragment from pCAP87 cloned into Pst I site of pUC18 (sequence generated from clone is the same direction as transcription). (This study)</td>
</tr>
<tr>
<td>pCAP119</td>
<td>Ap'</td>
<td>3.3 kb Pst I generated DNA fragment from pCAP87 cloned into Pst I site of pUC18 in the opposite orientation of pCAP118. (This study)</td>
</tr>
<tr>
<td>pCAP142</td>
<td>Tc', Sm', Sp'</td>
<td>Omega cartridge from pBR322Ω cloned into the Hind III site of pCAP103. (This study)</td>
</tr>
</tbody>
</table>

Table 2 con't.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Resistance</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCAP146</td>
<td>Ap&lt;sup&gt;′&lt;/sup&gt;, Cm&lt;sup&gt;′&lt;/sup&gt;</td>
<td>Chloramphenicol gene from pCM7 blunt-end ligated into Eco RV site of pCAP87. (This study)</td>
</tr>
<tr>
<td>pCAP147</td>
<td>Tc&lt;sup&gt;′&lt;/sup&gt;</td>
<td>Pst I generated DNA fragment from pCAP146 cloned into Pst I site of pRK404. (This study)</td>
</tr>
<tr>
<td>pCAP148</td>
<td>Tc&lt;sup&gt;′&lt;/sup&gt;, Sm&lt;sup&gt;′&lt;/sup&gt;, Sp&lt;sup&gt;′&lt;/sup&gt;</td>
<td>Omega cartridge cloned into Hind III site of pCAP147. (This study)</td>
</tr>
<tr>
<td>pCAP153</td>
<td>Tc&lt;sup&gt;′&lt;/sup&gt;</td>
<td>Chloramphenicol gene cloned into Bam HI site of pRK404. (This study)</td>
</tr>
</tbody>
</table>
fresh RCV media to an initial Klett value of approximately 20 (red filter) and grown for two generations under high versus low oxygen tension. Low oxygen conditions were obtained by sparging the culture with a mixture of 3% oxygen, 5% carbon dioxide, and 92% nitrogen, whereas high oxygen conditions were obtained by sparging the culture with compressed air supplemented with 3% oxygen. Cultures were incubated in a 37°C water bath.

**Nucleic acid isolation**

Plasmid minipreps were routinely isolated using QIAprep Spin Plasmid Kits (Qiagen Inc., Chatsworth, CA). Large plasmid isolations were performed using the Wizard Maxiprep DNA purification system (Promega Inc., Madison, WI). Both procedures are based on the modified alkaline lysis method of Birnboim and Doly (139) and the adsorption of DNA to silica in the presence of high salt (26).

Chromosomal DNA was isolated from 100 ml overnight cultures of *R. capsulatus* grown in PYE broth. Cells were harvested at 10,400 x g for 10 minutes and resuspended in 2.5 ml TES (50 mM Tris-Cl, pH 8.0, 4 mM disodium ethylenediaminetetraacetic acid (EDTA), 1% NaCl). Proteinase K was added to a final concentration of 0.5 mg per ml. Ten percent sodium dodecyl sulfate (SDS) in 1 M Tris-Cl, pH 8.0 was added to a final SDS concentration of 1% and incubated 1 hour at 42°C. The suspension was repeatedly extracted with TES-saturated phenol followed by several extractions with chloroform-isooamyl alcohol (24:1) until little or no white precipitate was seen. Pancreatic RNase (Sigma Co.,
St. Louis, MO) was added to the aqueous phase at a concentration of 25 μg/ml and incubated at 37°C for 30 minutes. Following incubation, the solution was extracted once with an equal volume of chloroform-isoamyl alcohol (24:1) and once with water-saturated diethyl ether until clear. An equal volume of isopropanol was added and the solution was incubated 20 minutes at -20°C. The solution was centrifuged at 9,750 × g for 20 minutes, and the remaining pellet was dried and resuspended in a minimal amount of water.

Total cellular RNA was isolated from 40 ml of R. capsulatus grown in RCV broth to 80 Kletts. Cells were poured over an equal volume of -80°C buffer containing 50 mM Tris-Cl, pH 7.5, 75 μg/ml rifampicin, 25 μg/ml chloramphenicol and harvested at 16,300 × g for 5 minutes. The cell pellet was resuspended in 4 ml of the same buffer containing 10 mg of lysozyme. After a 5 minute incubation at 4°C, 0.5 ml of 10% SDS was added and the solution was mixed by inversion. The suspension was extracted 2 to 3 times with an equal volume of TES-saturated phenol followed by extraction 2 to 3 times with an equal volume of chloroform/isoamyl alcohol (24:1). Nucleic acids were precipitated by adding 0.5 ml 3 M sodium acetate, pH 4.8 and an equal volume of isopropanol and incubating the solution for 30 minutes at -20°C. After centrifugation at 9,750 × g for 30 minutes, the pellet was dried and resuspended in 1.5 ml 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 10 mM magnesium chloride, 30 μg/ml DNase (Boehringer-Mannheim, grade 1) and incubated at 37°C for 30 minutes. Following incubation,
the solution was extracted separately with an equal volume of water-saturated phenol and chloroform-isoamyl alcohol (24:1). The aqueous phase was divided into 3 equal portions and the RNA was precipitated from each by adding 3 M sodium acetate, pH 4.8, to a final concentration of 1 M and ethanol. RNA pellets were dried and stored at -80°C. All nucleic acids were quantified by measuring optical density at 260 nm using a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer.

**Restriction digests, ligations, nick translations, and other recombinant DNA techniques.**

Restriction digests were routinely performed by digesting 1 µg of DNA in a 10 µl reaction consisting of 1µl (10U) of enzyme and 1 µl of the appropriate 10X buffer from New England Biolabs. Digest were incubated 1 to 2 hours at the optimal enzyme incubation temperature.

Ligation reactions were performed using T4 DNA ligase (New England Biolabs, Beverly, MA) and incubated 4 to 16 hours at 16°C. A typical reaction consisted of 1 µg DNA, 2 µl 5X ligase buffer, 1 µl DNA ligase (400U), and water up to 10 µl.

Plasmid DNA was nick translated using the BRL nick translation kit (Life Technologies, Grand Island, NY). Unincorporated nucleotides were removed by passing the DNA sample through a G-50 spin column (Worthington Biochemical Corp., Freehold, NJ). A 2 µl sample was added to 5 ml of CytoScint scintillation
fluid and counts per minute were measured on a Beckman model LS6860 Liquid Scintillation instrument.

Three prime recessed ends were made blunt using Klenow polymerase. A typical fill-in reaction consisted of 1 μg DNA, 2 μl any 10X New England Biolabs restriction enzyme buffer, 33 μM each deoxyribonucleotide, 1 unit Klenow polymerase (Promega Inc.), and water up to 20 μl. The reaction was incubated 15 minutes at 25°C and EDTA was added to a final concentration of 10 mM to stop the reaction. The polymerase was inactivated by heating the reaction to 75°C for 10 minutes.

**Agarose gel electrophoresis**

DNA was routinely fractionated through 0.8% agarose gels containing 0.5 μg/ml ethidium bromide using either the BRL Horizontal System Model H5 or the Hoefer Gel Unit Model HE-33. A 50X stock solution of TAE (1X TAE = 0.04 M Tris-acetate, 0.002 M EDTA) was diluted to either 1X or 4X to serve as electrophoresis buffer. Prior to electrophoresis, 1X loading buffer (0.25% bromothymol blue, 40% sucrose) was added to the DNA sample. 1X TAE agarose gels were run for 2 hours at 80 volts, whereas 4X TAE agarose gels were run overnight at 30 volts. Upon completion of electrophoresis DNA was visualized using a Fotodyne short wave UV light box and photographed with a Polaroid camera.
**Transformation**

Transformations were performed based on the procedure by Kushner (83). The *E. coli* strain was grown with vigorous shaking until a cell density of $5 \times 10^7$ cells/ml ($\text{OD}_{550} = 0.2$) was achieved and 1.5 ml of culture was briefly spun in a microfuge tube. The cell pellet was gently resuspended in 1 ml of 100 mM morpholinopropane sulfonic acid, pH 6.5, 50 mM CaCl$_2$, 10 mM RbCl and incubated on ice for 15 minutes. The solution was centrifuged for 30 seconds and the cell pellet was resuspended in 0.2 ml fresh buffer. Three microliters of dimethyl sulfoxide (DMSO) and up to 200 ng of DNA were added to the suspension. After 30 minutes incubation on ice, the cells were subjected to heat shock in a 43°C water bath for 1 minute. The suspension was immediately transferred to 1 ml of L-broth and incubated at 37°C for 1 hour without shaking. A tenth of a milliliter of transformation mix was plated on the appropriate selective medium.

**Electroporation**

*E. coli* cells were grown in L-broth until early to mid log phase ($\text{OD}_{660} = 0.5$-1.0) and transferred to a sterile 25 ml centrifuge tube where they were chilled on ice for 10 minutes. Following centrifugation for 10 minutes at 5,900 x $g$, the cell pellet was washed 2 times with 10 ml of sterile cold water and once with 10 ml sterile cold 10% glycerol. The cells were resuspended in a final volume of 200 µl of 10% glycerol. Forty microliters of freshly prepared cells and 2 µg DNA...
were added to a sterile 0.2 cm electroporation cuvette and mixed. The cell suspension was pulsed using the BioRad Gene Pulser set at 2.5 kV, 25 μF, and 200 ohms. Two milliliters of L-broth were immediately added to the cuvette and transferred to a test tube to incubate for 1 hour at 37°C in a roller drum. Cells were plated out on the appropriate selective medium.

Matings

Triparental matings were performed as described below. *E. coli* donor strains and *E. coli* strain HB101, containing the helper plasmid pRK2013, were grown overnight in L-broth with slow shaking. Recipient *R. capsulatus* strains were grown overnight in PYE broth at 37°C with no shaking. One milliliter of the *R. capsulatus* recipient strain and 200 μl of each of the *E. coli* strains were mixed together in a microfuge tube and centrifuged. The supernatant was decanted and the cell pellet was resuspended in 50 μl of medium which was spotted on a filter disc atop a PYE plate. The plate was incubated for 3-16 hours after which the plate was spread with the appropriate antibiotics and incubated at 37°C.

Southern analysis

DNA fragments were separated by electrophoresis through a 0.8% agarose gel after which the gel was trimmed to remove excess agarose. The gel was placed in a glass baking dish and covered with 1 M NaCl-0.5 M NaOH. After soaking twice for 15 minutes with agitation, the solution was discarded and
the gel was rinsed with distilled water. The gel was then submerged in 1.5 M NaCl-0.5 M Tris-Cl, pH 7.0 and soaked twice for 15 minutes with agitation. After being washed with distilled water, the gel was covered with 20X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) for 30 minutes. For transfer of DNA fragments greater than 10 kb, the gel was first treated with 0.25 N HCl for 10 minutes prior to adding 1 M NaCl-0.5 M NaOH. DNA was transferred to Nytran membranes using the S&S Turbo Blotter and Blotting Stack Assembly (Schleicher & Schuell Inc., Keene, NH). Before transfer, Nytran membranes were immersed in distilled water followed by a 5 minute soak in 20X SSC. Transfer was complete within 3 to 7 hours. Following transfer, the blot was gently washed in 2X SSC and baked for 1 hour at 80°C. Dried blots were stored in sealed plastic bags. Prehybridizations and hybridizations were performed using the Hybaid Micro-4 Hybridization Oven (National Labnet Company, Woodbridge, NJ). Blots were first rolled in mesh and placed in glass bottles where they were briefly rinsed with 2X SSC. Prehybridizations were carried out at 65°C for 4 hours to overnight in 5X SSC, 1% SDS, 10X Denhardts (1 g ficoll, 1 g polyvinylpyrrolidone, 1 g BSA, Pentex fraction V), and 0.5 mg/ml denatured salmon sperm DNA. Hybridizations were carried out for 18 to 24 hours at 65°C in 5X SSC, 1% SDS, 2X Denhardts, and 0.1 mg/ml denatured salmon sperm DNA. Both prehybridizations and hybridizations required 0.1 ml solution per cm² blot. Washes were done for 30 minutes in 2X SSC-1% SDS at room temperature, 30 minutes in 0.5X SSC-1% SDS at room temperature, and 30 minutes in 0.5X
SSC-0.1% SDS at 65°C. The blots were allowed to air dry and exposed to BioMax MR X-ray film overnight.

**Dot blot analysis**

RNA pellets were resuspended in 7.4 µl water and denatured in 5.4 µl 6 M glyoxal, 16 µl DMSO, 3.2 µl 0.1 M sodium phosphate for 1 hour at 50°C. After denaturation the solution was chilled on ice and 800 µl of cold 10 mM sodium phosphate, pH 7.0 was added to the samples. Serial dilutions of RNA samples were blotted onto Zeta-Probe (BioRad Laboratories, Hercules, CA) membranes using the BioRad Dot Blot apparatus. The membranes were baked at 80°C for 1 hour and stored in a sealed plastic bag. Prior to prehybridization, blots were washed in 0.1X SSC-0.5% SDS for 1 hour at 65°C. Prehybridizations were carried out at 65°C for 4 hours to overnight in 5X SSC, 50 mM sodium phosphate, pH 6.5, 10X Denhardts, and 1 mg/ml denatured salmon sperm DNA. Hybridizations were carried out for 18 to 24 hours at 65°C in 5X SSC, 25 mM sodium phosphate, pH 6.5, 2X Denhardts, and 0.1 mg/ml denatured salmon sperm DNA. Both prehybridizations and hybridizations were done in 0.1 ml of solution per cm² of membrane. The blots were washed twice with 500 ml of 2X SSC-0.1% SDS at room temperature, and once with 500 ml of 2X SSC-0.1% SDS at 55°C for 1 hour. Blots were air dried and exposed to BioMax MR X-ray film overnight.
DNA sequencing

Nucleotide sequencing was performed using the New England Biolabs Circumvent Kit based on the dideoxynucleotide chain termination method of Sanger (114). Double stranded plasmid templates were extended using a highly thermostable Vent<sup>r</sup> (exo<sup>-</sup>) DNA polymerase. The dideoxy terminated chains were labeled by the incorporation of [α-<sup>35</sup>S]ATP into the nascent chains. Sequencing reactions were incubated in a Hybaid Thermocycler (National Labnet Laboratories, Woodbridge, NJ) for 25 cycles (1 cycle = 95°C for 1 min, 55°C for 1 min, 72°C for 1 min). The DNA products were separated by electrophoresis through a denaturing 6% polyacrylamide gel at 60°C using the Polar Bear Isothermal Electrophoresis system (Owl Scientific, Woburn, NJ). Upon completion of electrophoresis, the gel was fixed in 20% ethanol-10% acetic acid and dried to 3M Whatman paper for 2.5 hours using BioRad gel drying system. Sequencing gels were exposed 1 day using BioMax X-ray film. Unidirectional nested deletions used in sequencing were constructed with the Erase-a-Base system by Promega. This system is based on the procedure developed by Henikoff (61) in which exonuclease III is used to digest DNA from a blunt or 5' protruding end while leaving the 3' protruding end intact.

Pulsed Field Gel Electrophoresis (PFGE)

Five milliliters of overnight culture of *R. capsulatus* SB1003 (O.D.<sub>660</sub> = 1.0) was pelleted at 3,020 x g for 6 minutes and resuspended in 5 ml 50 mM EDTA,
pH 8.0. The cells were pelleted and resuspended in 0.5 ml 50 mM EDTA, pH 8.0 and 0.5 ml 1.6% low melting point agarose cooled to 55°C. A pasteur pipette was used to transfer the solution into a section of tubing (Nalgene; Rochester, NY) approximately 24 inches long having an internal diameter of 1/16 of an inch. After the agarose solidified, the embedded cells were extruded from the tubing into 5 ml of 50 mM EDTA, pH 8.0 and allowed to incubate for 30 minutes at room temperature. The liquid was decanted and 5 ml of 3% lauryl sarcosine-0.2 M EDTA, pH 8.5, and 5 mg proteinase K were added and the suspension was incubated overnight at 55°C. The agarose 'plugs' were washed 2 times with 5 ml of 50 mM EDTA, pH 8.0 for 30 minutes. Two hundred microliters of 50 mM EDTA, pH 8.0 and 2 μl 100 mM phenylmethylsulfonyl fluoride (PMSF) were added and the solution was incubated for 1 hour. The plugs were rinsed twice with 5 ml of 50 mM EDTA, pH 8.0 for 30 minutes at room temperature to remove all traces of PMSF. One centimeter pieces of DNA plugs were placed in a microfuge tube containing 50 μl TE (10 mM Tris-Cl, pH 8.0, 1mM EDTA, pH 8.0) and incubated on ice for 20 minutes. The TE solution was removed and the DNA was rinsed twice with 50 μl of the appropriate restriction enzyme buffer for 30 minutes on ice. The buffer was removed and 50 μl of buffer solution containing the appropriate enzyme was added to the plug and incubated on ice for 30 minutes. Prior to PFGE, the 1 cm DNA plug was sealed in the well of a 1% agarose gel with 1% low melting point agarose. The BioRad CHEF system was used for electrophoresis set at the following conditions: switching intervals 8 to
16 seconds, voltage 200, temperature 13°C, run time 24 hours in 0.5X TBE (1X TBE = 0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) buffer.

Protein Determination

The protein concentration of cell extracts used for various enzyme assays was determined using the Bradford Dye-binding assay (BioRad) (19). A standard curve was constructed using purified bovine serum albumin (Pentex).

Porphobilinogen synthase assay

Porphobilinogen synthase activity was assayed based on the method developed by Shemin (122). One hundred milliliters of *R. capsulatus* cells grown to 75 to 100 Klett's in RCV media were harvested and resuspended in 1 ml of 0.1 M potassium phosphate buffer, pH 7.6 with 0.1 M β-mercaptoethanol. The cells were sonicated, under aerobic conditions, 2 to 4 times for 20 seconds with 30 second cooling periods using a Heat-Systems Ultrasonics sonifier (Model w-220). The extract was centrifuged for 20 minutes at 27,000 x g. One hundred microliters of crude extract was added to 150 µl 1 M Tris-Cl, pH 8.5, 75 µl 1 M KCl, 50 µl 0.1 M aminolevulinate, pH 7.0, 0.6 µl β-mercaptoethanol, 1.125 ml water and incubated for 30 minutes at 37°C. Following incubation, 2 ml of 0.5 M 20% TCA-0.1 M HgCl₂ was added and the solution was spun for 2 minutes. Two milliliters of modified Ehrlich's reagent (0.5 g p-dimethylaminobenzaldehyde, 17 ml glacial acetic acid and 8 ml 70% perchloric acid) was added to the
supernatant and incubated at room temperature for 5 minutes. The optical
density was determined at 553 nm. The specific activity was calculated using a
molar extinction coefficient of $2.29 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

**Chloramphenicol acetyltransferase assay**

Chloramphenicol acetyltransferase assays were performed as described
by Shaw (121). One hundred milliliters of *R. capsulatus* cells grown to 75 to 100
Klett's in RCV were harvested and resuspended in 1 ml of 50 mM Tris-Cl, pH 7.8.
The cells were sonicated 3 to 4 times for 20 seconds with 30 second cooling
periods. Cell debris was removed by spinning the extract at 27,000 x $g$ for 20
minutes. Two hundred and fifty microliters of crude extract was added to 250 µl
of 4 mg/ml 5,5'-dithiobis-(2-nitrobenzoic acid) in 1 M Tris-Cl, pH 7.8, 50 µl 5 mM
acetyl CoA, 1.950 ml water and the mixture was divided into two 1 ml samples.
The samples were blanked in the spectrophotometer and 10 µl of 5 mM
chloramphenicol was added to one of the cuvettes. The change in optical density
at 412 nm was determined. The specific activity was calculated using a molar
extinction coefficient of $1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

**Porphyrin determination**

Protoporphyrin and coproporphyrin were extracted from 1 to 5 ml of culture
with 3 ml ethyl acetate-acetic acid (3:1). The bottom layer was removed and the
remaining ethyl acetate layer was extracted twice with 3N HCl. Fluorescence of
the acid layer was determined using a Perkin-Elmer LS-3 fluorescence spectrophotometer. Porphyrin concentrations were calculated by comparing fluorescence values to curves constructed using protoporphyrin and coproporphyrin standards (Porphyrin Products, Logan, Utah).

Thin layer chromatography

Total porphyrins were extracted into ethyl acetate-acetic acid (3:1) and spotted onto a Silica Gel G TCL plate (Fisher). Extracted porphyrins, coproporphyrin, and protoporphyrin standards (Porphyrin Products, Logan, Utah) were characterized by thin layer chromatography. The plates were developed in 2,6 lutidine-0.05N ammonium hydroxide (7:2) (54) and viewed under long-wave UV light. Porphyrins were identified by their comigration with the appropriate standard.
Results

Cloning of the *R. capsulatus* hemB gene

Chromosomal DNA from *R. capsulatus* strain PAS100 was isolated as described in Materials and Methods and digested to completion with either Eco RI or Pst I. The DNA was ligated into the expression vector pKK223-3, which contains the *tac* promoter and confers ampicillin resistance. The ligation mixture was transformed into *E. coli* strain SHSP1 (*hemB*) by electroporation. Because SHSP1 is *Hem−* it requires a fermentable carbon source, such as glucose, for growth. SHSP1 was routinely cultured in L-broth containing 0.2% glucose and 0.5% NaCl. It was, therefore, possible to select for *HemB*, ampicillin-resistant colonies by plating the transformed cells on L-agar without glucose in the presence of 25 μg ampicillin per ml. *HemB*, ampicillin-resistant transformants were obtained from both the Pst I and Eco RI plasmid libraries. *Hem−* colonies, originating from either the Pst I or Eco RI plasmid libraries, were picked and grown in broth for plasmid isolation. Those plasmids isolated from *Hem−* colonies, arising from the Pst I plasmid library, had identical restriction enzyme patterns when digested with Pst I, Eco RI, or Bam HI and electrophoresed through a 0.8% agarose gel. One clone was chosen to work with and the plasmid from that clone was designated pCAP87. Those plasmids isolated from *Hem−* colonies, arising from the Eco RI plasmid library, had identical restriction enzyme patterns when digested with Eco RI, Pst I, or Bam HI and electrophoresed through a 0.8%
agarose gel. One clone was selected to work with and the plasmid from this clone was named pCAP86. Plasmid DNA isolated from SHSP1(pCAP86) or SHSP1(pCAP87) was used to transform strain SHSP1 to ampicillin resistance. All of the resulting colonies from either transformations were HemB⁺, demonstrating that both plasmids carried the *R. capsulatus* hemB gene. In addition, SHSP1(pCAP86) and SHSP1(pCAP87) both exhibited porphobilinogen synthase activity, whereas SHSP1 showed minor levels of porphobilinogen synthase activity (Table 3).

**Restriction analysis of the hemB region**

Restriction digest of pCAP86 with Eco RI revealed that pCAP86 contained a 5.6 kb DNA fragment cloned into pKK223-3. Restriction digest of pCAP87 with Pst I revealed that pCAP87 contained a 3.3 kb DNA fragment cloned into pKK223-3 (Fig. 2). The restriction maps of the two plasmids indicate that they have a 3.0 kb Eco RI-Pst I generated DNA fragment in common, suggesting they are from the same region of the chromosome. A restriction enzyme map of the DNA inserts in either pCAP86 or pCAP87 indicated that both inserts had a different restriction enzyme map than the *E. coli* hemB DNA region.

The approximate location of the hemB gene was determined by subcloning various fragments from pCAP87 into pUC18. Plasmids containing either the Eco RI-Cla I generated DNA fragment (pCAP108) or the Nsp V-Bam HI generated DNA fragment (pCAP111) were able to complement SHSP1, indicating that the
Table 3. Porphobilinogen synthase activity in strains containing *hemB* plasmids.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Porphobilinogen synthase Specific activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
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<tr>
<td>SHSP1</td>
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<tr>
<td>SHSP1(pCAP86)</td>
<td>100</td>
</tr>
<tr>
<td>SHSP1(pCAP87)</td>
<td>49</td>
</tr>
</tbody>
</table>

<sup>a</sup>Specific activity expressed as nmoles of porphobilinogen formed per hr per mg protein at 25°C. Results are representative of at least two trials.
Figure 2. Restriction map of plasmids containing the *R.capsulatus hemB* gene. Restriction enzyme cleavage sites are Bam HI (B), Cla I (C), Eco RI (E), Eco RV (V), Nsp V (N), and Pst I (P). The omega cartridge (Ω) is not drawn to scale.
hemB gene is located in the 1.4 kb region between the Nsp V site and the Cla I site. This was confirmed by digesting pCAP87 with Eco RV and replacing the Eco RV DNA fragment with the omega cartridge. The resulting plasmid, pCAP96, does not complement SHSP1 (Fig. 2).

The orientation of the DNA inserts in pCAP87 and pCAP86, with respect to the tac promoter, is also an important factor in determining whether or not the plasmid can complement SHSP1. The orientation of the DNA inserts in pCAP86 and pCAP87 put the Nsp V restriction enzyme site proximal to the tac promoter. To further investigate this, the Pst I generated DNA fragment from pCAP87, was cloned into pUC18 in both orientations and named pCAP118 and pCAP119. Only pCAP118, which contains the Pst I generated DNA fragment oriented such that the Nsp V site is proximal to the lac promoter, was able to complement SHSP1. These results suggest that the R. capsulatus hemB gene is only expressed in E. coli with the help of an exogenous promoter, and that hemB transcripton proceeds from left to right as diagrammed in Fig. 2. The possibility exists that the Pst I generated DNA fragment from pCAP87 does not contain the promoter region of the hemB gene. Alternatively, the R. capsulatus hemB gene promoter may not be recognized by E. coli's DNA polymerase.

**Expression of pCAP142(hemB") in SB1003**

The 3.3 kb Pst I generated DNA fragment, from pCAP87, was subcloned into the broad host vector pRK404. The omega cartridge was placed upstream
from the Pst I generated DNA fragment to prevent readthrough transcription from the plasmid into the Pst I DNA fragment. The orientation of the omega cartridge, with respect to the Pst I generated DNA fragment, was such that the direction of transcription was extending away from the omega cartridge. This construct, pCAP142, was mated into SB1003 in order to determine whether the Pst I fragment carries the hemB control region. SB1003 and SB1003(pCAP142) were both grown under high and low oxygen tensions and porphobilinogen synthase specific activities determined as described in Materials and Methods. The results are summarized in Table 4. SB1003(pCAP142) has nearly a five-fold higher porphobilinogen synthase activity than the wild-type strain under both high and low oxygen tensions. These results indicate that the Pst I DNA fragment can express porphobilinogen synthase without the help of an exogenous promoter. In addition, the results show that porphobilinogen synthase specific activity is 1.4-fold higher in cultures grown under low oxygen. The small change in enzyme activity is not enough to account for the large increase in porphyrin synthesis that accompanies anaerobic growth.

Southern analysis

To confirm that no rearrangements occurred during the cloning process and that there was only one copy of the hemB gene in the *R. capsulatus* chromosome, Southern analysis was performed. Chromosomal DNA from *R. capsulatus* stain PAS100 was digested with either Eco RI or Pst I, separated on
Table 4. Overexpression of porphobilinogen synthase in SB1003.

<table>
<thead>
<tr>
<th></th>
<th>Specific activity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td>23% Oxygen</td>
</tr>
<tr>
<td>SB1003</td>
<td>280</td>
</tr>
<tr>
<td>SB1003 (pCAP142)</td>
<td>1300</td>
</tr>
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</table>

$^a$Specific activity expressed as nmoles of porphobilinogen formed per min per mg protein at 25°C. Results are representative of at least two trials.
a 4X TAE 0.8% agarose gel, and blotted to nitrocellulose. The blots were probed with either pCAP86 or pCAP87 that had been nick-translated. The blot of chromosomal DNA digested with Eco RI, when probed with pCAP86, yielded a single band of 5.6 kb (Fig. 3 A). This size is consistent with the size of the insert in pCAP86. To confirm that the inserts in pCAP86 and pCAP87 overlap, pCAP86 DNA was digested with Eco RI, blotted to nitrocellulose, and probed with pCAP87. The pCAP87 hybridized to the 5.6 kb band from pCAP86, indicating that the two plasmids carry DNA from the same region of the \textit{R. capsulatus} chromosome (Fig. 3 B). In addition, pCAP87 hybridized to the 4.6 kb vector band and a large partially digested band corresponding to the whole plasmid.

**Chromosomal location of the \textit{R. capsulatus} hemB gene**

A physical map of the genome of \textit{R. capsulatus} SB1003 constructed by Fonstein et al. (45) made it feasible to map the location of the \textit{hemB} gene. Chromosomal DNA from strain SB1003 was digested en bloc with Ase I and Xba I as described in Materials and Methods. The DNA fragments were separated using the BioRad CHEF Mapper Pulsed-Field Electrophoresis System (Fig. 4 A). The DNA fragments were blotted to Nytran membranes using the Schleicher and Schuell Turbo Blotter and probed with nick-translated pCAP87. The \textit{hemB} gene was localized to Ase I fragment 2 and Xba I fragment 3' (Fig. 4 B). The chromosomal location of the \textit{R. capsulatus} \textit{hemA} gene was also determined using pCAP22 as a probe. The \textit{hemA} gene was localized to Ase I fragment 5
Figure 3. Southern analysis. A. PAS100 chromosomal DNA digested with EcoRI and probed with pCAP86. B. pCAP86 DNA digested with Eco RI and probed with pCAP87.
Figure 4. A. PFGE of SB1003 chromosomal DNA digested with Ase I (lane1) and Xba I (lane2). B. pCAP113(hemB) hybridized to a 370kb Ase I fragment (lan3) and a 350 kb Xba I fragment (lane4).
and Xba I fragment 3 (Fig. 5). The results of these mapping experiments, summarized in Figure 6, indicate that the hemA and hemB genes do not comprise an operon.

**Sequencing of the R. capsulatus hemB gene**

Nested unidirectional deletions were made from pCAP118 and pCAP119 using the Promega Erase-A-Base system. The nucleotide sequence of the Nsp V-Cla I region was determined in both directions using the New England Biolabs CircumVent kit (Fig. 7) (64). Analysis of this nucleotide region indicates that there is a single open reading frame 995 nucleotides long. Codon preference analysis, using the *R. capsulatus* codon preference table (150), shows that this reading frame contains few rare codons (Fig. 8). The ATG codon beginning at position 211 is the most likely candidate for a start codon, based on the codon preference profile and the presence of a putative ribosome binding site upstream. The ATG codons at positions 152 and 256 lack good ribosome binding sites, and the codon at position 256 is within a region conserved in the coding regions of several hemB genes. The open reading frame starting at position 211 encodes a protein with a molecular mass of 43,068 daltons, in agreement with a report of 40,000 daltons for the *R. capsulatus* enzyme (102).
Figure 5. A. PFGE of SB1003 chromosomal DNA digested with Ase I (lane 1) and Xba I (lane 2). B. pCAP22(hemA) hybridized to a 220kb Ase I fragment (lane 3) and a 350 kb Xba I fragment (lane 4).
Figure 6. Location of the *hemB* and *hemA* genes on a physical map of SB1003. *Ase I* (a) and *Xba I* (x) restriction sites are labeled. The restriction map along with the location of the *bch* region was constructed by Fonstein et al. (45). The *hemE* gene was located by Ineichen and Biel (63).
Figure 7. Nucleotide sequence of the *R. capsulatus* hemB gene.
Figure 8. Codon Preference Plot of the region containing the *hemB* gene.

The codon preference profile for *hemB* is reflected in the top panel.

Open reading frames are designated as uninterrupted boxes beneath the plot in the panel. Rare codons are shown as vertical hatch marks in the box below each panel. The dashed line in each panel represents the rare codon threshold. A plot above this line signifies a region of DNA with few rare codons. The numbering system along the top and bottom of the figure coincide with the DNA sequence presented in Figure 7.
Regulation of the *R. capsulatus hemB* gene

**Dot blot analysis**

Transcriptional regulation of the *hemB* gene by oxygen was examined by Northern analysis. RNA was isolated from cultures of *R. capsulatus* strain PAS100 grown under high and low oxygen tensions. Serial dilutions of the RNA samples were blotted onto Zeta-Probe membranes (BioRad), using a dot-blot apparatus, and hybridized to pCAP113. This construct contains the 0.6 kb Nsp V-Eco RV generated DNA fragment from pCAP87, cloned into pUC18. This DNA fragment is entirely within the coding region of the *hemB* gene. As a control for the amount of mRNA in the sample, the same serial dilutions were hybridized to pRPSB105. This plasmid carries the *crtB* gene which is not transcriptionally regulated by oxygen (27). Figure 9 is a representative example of the autoradiograms generated from the dot blot experiments. To quantitate hybridization, autoradiograms were scanned with a BioRad Video densitometer Model 620. The differences in mRNA from high to low oxygen were normalized for the *crtB* gene and a correction factor was applied to *hemB* mRNA. For example, *crtB* mRNA was determined to be four-fold higher under high oxygen when compared to low oxygen. *HemB* mRNA was also determined to be four-fold higher under high oxygen. After applying a correction factor of four to *hemB* mRNA under low oxygen, the *hemB* mRNA was the same under high and low oxygen. The results of the dot-blot experiments indicate that *hemB* mRNA do not change with oxygen tension.
Figure 9. Dot blot analysis. pRPSB105 (crtB) and pCAP113 (hemB) were hybridized to serial dilutions of total cellular RNA extracted from cells grown under high (H) oxygen and low (L) oxygen.
Construction of a hemB-cat transcriptional fusion plasmid

A hemB-cat transcriptional fusion plasmid was constructed in order to further study transcriptional regulation (Fig. 10). The Eco RV generated DNA fragment in pCAP87 was replaced by the promoterless chloramphenicol acetyltransferase gene from pCM7 via a blunt-end ligation. The Eco RV sites are both within the hemB coding region, therefore this placed the coding region of the cat gene under control of the hemB promoter. The Pst I fragment, containing the hemB-cat fusion, was subcloned into the mobilizable plasmid pRK404. To prevent upstream transcriptional interference from the lac promoter, the omega cartridge was cloned into the Hind III site upstream of the hemB-cat fusion. This plasmid was designated pCAP148.

A second plasmid was constructed as a control for differences in plasmid copy number and supercoiling. The plasmid, designated pCAP153, is pRK404 with the chloramphenicol acetyltransferase gene cloned into the Hind III site. Each plasmid was transformed into NM522 and moved into SB1003 via a triparental mating.

Effects of oxygen on transcription of the hemB-cat fusion

Strain SB1003(pCAP148) was grown in RCV media for two generations with initial oxygen tensions of 3% and 23%. Cultures grown with an oxygen tension of 3% exhibited a 1.4-fold higher chloramphenicol acetyltransferase
Figure 10. *HemB-cat* fusion plasmid constructed to study transcriptional regulation by oxygen.
activity than that found in the culture grown under 23% oxygen (Table 5). As a control for differences in the plasmid, e.g., copy number and supercoiling, that may accompany a shift in oxygen tension, chloramphenicol acetyltransferase activity was also determined in SB1003(pCAP153). Under low oxygen tension the chloramphenicol acetyltransferase specific activity was 1.4-fold higher than under high oxygen. After correcting for differences in the plasmid these results show that chloramphenicol acetyltransferase specific activity from pCAP148 is the same under high and low oxygen tensions.

**Overexpression of the hemB gene in AJB456**

It has been previously demonstrated that the addition of exogenous porphobilinogen to cultures of AJB456, growing under high and low oxygen, can eliminate oxygen-mediated regulation of protoporphyrin accumulation (10). These results suggest that the level of porphobilinogen is regulated by oxygen and that high levels of exogenous porphobilinogen can overcome this regulation. pCAP142 was moved into AJB456 in an attempt to mimic the above experiment in vivo by increasing endogenous levels of porphobilinogen. Strain AJB456(pCAP142) was grown with initial oxygen tensions of 23% and 3% as described in Materials and Methods. At 150 Kletts Units, 5 ml samples were removed and porphyrins were extracted and quantified as described in Materials and Methods. As a control, AJB456 was also grown under high and low oxygen and porphyrins were extracted and quantified. These results (Fig. 11) illustrate
Table 5. Effects of oxygen on chloramphenicol acetyltransferase activity.

<table>
<thead>
<tr>
<th>Strains</th>
<th>23% Oxygen</th>
<th>3% Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB1003(pCAP148)</td>
<td>16</td>
<td>23</td>
</tr>
<tr>
<td>SB1003(pCAP153)</td>
<td>33</td>
<td>46</td>
</tr>
</tbody>
</table>

<sup>a</sup>Specific activity expressed as nmoles of chloramphenicol acetylated per min per mg protein at 25°C. Results are representative of at least two trials.
Figure 11. Protoporphyrin accumulation in AJB456. Shaded bars refer to cultures grown with an initial oxygen tension of 3%, whereas, open bars refer to cultures grown with an initial oxygen tension of 23%. Results are representative of at least two trials, with the exception of AJB456(pCAP142) + ALA which is from a single determination.
that pCAP142 has no effect of protoporphyrin accumulation in AJB456. To ensure that pCAP142 was being expressed in AJB456, porphobilinogen synthase assays were performed. Porphobilinogen synthase specific activity was approximately five-fold higher under both high and low oxygen in the strain containing pCAP142 (Table 6). Together these results suggest that the enzyme is being expressed but there is no substrate present for it to act on. To test this idea, AJB456(pCAP142) was grown in the presence of exogenous aminolevulinate under high and low oxygen and porphyrins were extracted and quantified. As a control, AJB456 was grown in the prepense of exogenous aminolevulinate. In the prepense of exogenous aminolevulinate, AJB456 accumulates more protoporphyrin under low oxygen than high oxygen confirming earlier results (Fig. 11) (10). Interestingly, exogenous aminolevulinate stimulates porphyrin accumulation under both high and low oxygen in AJB456. AJB456(pCAP142) in the presence of aminolevulinate accumulates even larger amounts of porphyrins under high and low oxygen. In addition, oxygen-mediated accumulation of porphyrins has changed, with even more porphyrins being accumulated under high oxygen than under low oxygen. Thin layer chromatography revealed that large amounts of coproporphyrin and protoporphyrin were accumulating. These results support the notion that the level of porphobilinogen is affected by oxygen and that increasing the endogenous levels of porphobilinogen overrides this effect.
Table 6. Overexpression of porphobilinogen synthase in AJB456.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Specific activity$^a$</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23% Oxygen</td>
<td>3% Oxygen</td>
<td></td>
</tr>
<tr>
<td>AJB456</td>
<td>174</td>
<td>241</td>
<td></td>
</tr>
<tr>
<td>AJB456(pCAP142)</td>
<td>934</td>
<td>1078</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Specific activity expressed as nmoles of porphobilinogen formed per min per mg protein at 25°C. Results are representative of at least two trials.
Discussion

In 1957, Cohen-Bazire et al. (29) were the first to present evidence of an efficient mechanism for the regulation of tetrapyrrole biosynthesis. Since that time, a considerable effort has been dedicated to the identification of the major points of oxygen regulation in tetrapyrrole biosynthesis. Oxygen is believed to control the pathway leading to bacteriochlorophyll at two points: the formation of magnesium protoporphyrin monomethyl ester from protoporphyrin IX, and the formation of porphobilinogen from aminolevulinate. So far, the mechanisms by which oxygen regulates tetrapyrrole biosynthesis at these points are unknown. To investigate the mechanism by which oxygen regulates the formation of porphobilinogen, the \textit{R. capsulatus} hemB gene was cloned and sequenced.

The codon preference profile of the 1281 nucleotide region sequenced suggests that there is only one possible open reading frame (Fig. 8, panel 1). The putative amino acid sequence of porphobilinogen synthase from \textit{R. capsulatus} shares an overall identity of 43\% to 54\% with porphobilinogen synthases from several different sources (15, 20, 25, 59, 90, 119). Within the coding region of the \textit{R. capsulatus} protein, there are several regions that are highly conserved in relation to other known porphobilinogen synthases. In particular, the invariant lysine residue at position 256, proposed to be involved in Schiff-base formation within the active site (49), and the arginine residue at position 306, thought to be important in catalysis (91), are present in all
porphobilinogen synthases genes sequenced. The presence of a metal-binding domain is another highly conserved feature among porphobilinogen synthases. Most porphobilinogen synthases can be placed into two alternative groups depending on whether Zn$^{2+}$ or Mg$^{2+}$ is required for activity. The role of the metal ion is still unknown, but it is thought to have a structural and/or mechanistic function in protecting the sulfhydryl groups from oxidation or in catalysis (71). The mammalian enzymes are zinc metalloenzymes (71). They possess a metal binding domain which consist of four cysteine residues, thought to be oxygen sensitive, and two histidine residues. (Fig. 12) (134). Bacteria also contain this motif except that one of the conserved cysteines is missing. In the case of E. coli it has been shown that Zn$^{2+}$ is required for catalytic activity (95).

The corresponding metal binding domain in the plant enzymes resembles that of the nonplant enzymes except that the cysteines have been replaced with a threonine, alanine, and three aspartic acid residues thought to bind magnesium (Fig. 12) (15). The lack of cysteine in the ion binding center correlates well with the fact that the enzyme is not blocked by sulfhydryl reagents (91).

The R. capsulatus enzyme metal binding domain closely resembles the magnesium binding domain found in plants, except an asparagine residue has replaced the conserved aspartate at position 134 (Fig. 12). Interestingly, it has been reported that the R. capsulatus enzyme does not have a metal requirement (102). Perhaps by removing the negatively charged aspartate the metal ion is no longer needed.
Figure 12. Comparison of the metal-binding domain of *R. capsulatus* porphobilinogen synthase to those of plants and nonplants.

The *R. capsulatus* sequence shown corresponds to amino acids 120 to 141. Sequences from soybean (25), pea (15), spinach (119), *Selaginella martensii* (119), mouse (119), rat (14), human (142), *S. cervisias* (100), *Methanothermus sociabilis* (20), *Bacillus subtilis* (20), and *E.coli* (90) porphobilinogen synthase metal-binding domains are shown. Relevant amino acid residues are boxed.
### Non-plants

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
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<tr>
<td>Rat</td>
<td>L L V A C D V C L C P Y T S H G H C G L L S</td>
</tr>
<tr>
<td>Human</td>
<td>L L V A C D V C L C P Y T S H G H C G L L S</td>
</tr>
<tr>
<td>Yeast</td>
<td>L Y I I C D V C L C E Y T S H G H C G V L Y</td>
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<td><em>M. sociabilis</em></td>
<td>L V V I T D V C L C Q Y T E H G H C G I V K</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
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<tr>
<td><em>E. coli</em></td>
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<td><em>R. capsulatus</em></td>
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</tbody>
</table>

### Plants

<table>
<thead>
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<th>Sequence</th>
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<td>Pea</td>
<td>L I I Y T D V A L D P Y S S D G H D G I V R</td>
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<td>Spinach</td>
<td>L I I Y T D V A L D P Y Y Y D G H D G I V T</td>
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<tr>
<td><em>S. martensii</em></td>
<td>L V I Y T D V A L D P Y S S D G H D G I V R</td>
</tr>
</tbody>
</table>
The effects of multiple copies of the *R. capsulatus hemB* gene on protoporphyrin accumulation in AJB456 was examined by moving pCAP142 into AJB456. It was rationalized that AJB456(pCAP142) would generate an endogenous pool of porphobilinogen, capable of eliminating oxygen mediated accumulation of protoporphyrin. Previous results have demonstrated that exogenous porphobilinogen can eliminate oxygen mediated accumulation of protoporphyrin in AJB456 (10). However, AJB456(pCAP142), when grown under both high and low oxygen, accumulates protoporphyrin to the same extent as AJB456 (Fig. 11), even though porphobilinogen synthase specific activity is five-fold higher in the strain with the plasmid (Table 6). This suggests that the levels of aminolevulinate, and not porphobilinogen, are limiting. Interestingly, protoporphyrin accumulation in AJB456(pCAP142) is still regulated by oxygen. When AJB456(pCAP142) is grown in the presence of aminolevulinate, the accumulation of porphyrins is dramatically increased (Fig. 11). Porphyrin accumulation was so large under both conditions that porphyrins were excreted into the medium, reminiscent of AJB530 (12). Thin layer chromatography indicated that both coproporphyrin and protoporphyrin were being excreted. Most importantly, oxygen mediated accumulation of porphyrins has been altered from what is normally seen in AJB456. These results suggest that the overproduction of porphobilinogen can overcome the normal oxygen mediated regulatory mechanisms present in the cell. The excretion of excess porphyrins may be a safety measure to prevent photooxidative damage (110).
Expression of the *hemB* gene and porphobilinogen synthase specific activity were measured under different oxygen tensions to evaluate the contribution that synthesis of porphobilinogen may play in oxygen regulation. Enzyme synthesis was measured by dot blot analysis and *hemB-cat* transcriptional fusions. Dot blot analysis (Fig. 8) shows that *hemB* steady-state mRNA levels are the same under high and low oxygen. The results from the *hemB-cat* fusion studies, presented in Table 5, show that the initiation of transcription of the *hemB* gene does not change with shifts in oxygen tension. Oxygen tension also does not appear to regulate enzyme activity. Our measurements indicate that the specific activity of porphobilinogen synthase does not change significantly with changes in oxygen tension (Table 4). It has been reported that porphobilinogen synthase from bovine sources is oxygen labile and requires thiols for full activity (134). It is therefore possible, that the assay conditions employed may not reflect the in vivo situation. While the cultures used in this assay were grown under defined oxygen tensions, crude extracts and enzyme assay conditions were performed under aerobic conditions. However, the *R. capsulatus* enzyme may not be oxygen labile, like it's bovine counterpart, because it lacks the cysteine rich metal-binding domain believed to be responsible for oxygen sensitivity of the bovine enzyme.

While the mechanism of how oxygen modulates carbon flow down the common tetrapyrrrole pathway remains elusive, it is clear that the level of porphobilinogen plays a crucial part in oxygen regulation. The addition of
exogenous porphobilinogen (10), as well as overexpression of porphobilinogen synthase can eliminate or alter oxygen regulation of the common tetrapyrrole pathway. The results of the gene expression studies and the enzyme assay studies overwhelmingly indicate that the synthesis of porphobilinogen synthase is not regulated by oxygen and suggest that oxygen does not regulate porphobilinogen synthase activity. A more likely possibility is that oxygen regulates the degradation of porphobilinogen. Porphobilinogen serves as the substrate for the enzyme porphobilinogen deaminase. This enzyme converts four molecules of porphobilinogen into the linear tetrapyrrole, hydroxymethylbilane. This linear tetrapyrrole is released into solution where it serves as the substrate for uroporphyrinogen III synthase. Four molecules of porphobilinogen are, therefore, converted into one molecule of uroporphyrinogen. Preliminary results regarding the purification of the \textit{R. capsulatus} porphobilinogen deaminase, reveal that the ratio of porphobilinogen used to uroporphyrinogen III formed is about 12 to 1, instead of the expected four to one (Canada and Biel, unpublished). This high ratio suggest that about 2/3 of the total carbon flow going down the common tetrapyrrole pathway is lost at this step. Perhaps oxygen regulates the pathway by influencing the ratio of porphobilinogen used to uroporphyrinogen III formed. To determine if this is a real possibility, the \textit{R. capsulatus} porphobilinogen deaminase needs to be assayed under strictly aerobic and anaerobic conditions and the ratios of porphobilinogen used to uroporphyrinogen III formed compared.
If the ratio of porphobilinogen used to uroporphyringen III formed decreases under anaerobic conditions, this may explain how oxygen regulates the pathway.

Genetic mapping of the hem genes of bacteria have led to the discovery of different gene arrangements in Gram-negative and Gram-positive organisms. In the Gram-positive organism Bacillus subtilis, hem genes are clustered together to form an operon (59). The hem genes in Staphlococcus aureus are also clustered and thought to be in an operon (78). However, the hem genes in Gram-negative organisms, including R. capsulatus, appear to be scattered along the chromosome with the exception the hemC and hemD genes (5, 40, 78). The isolation of an R' factor carrying the entire R. capsulatus photosynthesis region, including the bacteriochlorophyll and carotenoid biosynthetic genes, prompted the idea that the hem genes of the common tetrapyrrole pathway may also be clustered in an operon (93). The results presented from the mapping experiments indicate these genes are not arranged in an operon.

In conclusion, the hemB gene has been cloned and sequenced in an effort to resolve how porphobilinogen levels are regulated by oxygen. Dot blot analysis and hemB-cat fusion studies indicate that transcription of the hemB gene is not regulated by oxygen. These results suggest that the formation of porphobilinogen is not regulated by oxygen. The mechanism by which porphobilinogen levels are regulated by oxygen remains open.


Karl Joseph Indest was born on October 20, 1968, in Fort Campbell, Kentucky. He graduated from Brother Martin High School in May, 1986. In May, 1990 Karl was awarded the degree of Bachelor of Arts in Biology from the University of New Orleans. In January of 1991, Karl entered the doctoral program in the Department of Microbiology at Louisiana State University, Baton Rouge under the direction of Alan Jay Biel as his graduate advisor. Karl is currently a Ph.D. candidate in the Department of Microbiology at L.S.U.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Microbiology

Title of Dissertation: Molecular Cloning, Sequencing, and Regulation of the Rhodobacter capsulatus hemB Gene

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