Purification of Porphobilinogen Deaminase and the Sequencing and Expression of the hemC Gene of Rhodobacter Capsulatus.

Keith A. Canada
Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_disstheses/6813

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Historical Dissertations and Theses by an authorized administrator of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700  800/521-0600

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
PURIFICATION OF PORPHOBILINOGEN DEAMINASE
AND
THE SEQUENCING AND EXPRESSION OF THE \textit{hemC} GENE
OF \textit{Rhodobacter capsulatus}

A Dissertation
Submitted to the Graduate Faculty
of the Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

by
Keith A. Canada
B.S., Purdue University, 1992
December 1998
DEDICATION

To my loving wife
Darla
who's strength and encouragement
have helped me to achieve my dreams
ACKNOWLEDGMENTS

I would first like to thank my parents and family for their support (financial & emotional) throughout my life. Without their encouragement and love I would have never made it to where I am now.

To my wife, Darla, I am forever in debt. She has helped at every stage of my graduate career, from designing experiments, to running experiments, to proofreading this dissertation. I am very fortunate to have a wife that can understand me as I talk about the ups and downs of the tetrapyrrole pathway. Her faith and encouragement have helped me become a better scientist.

I would like to thank my major professor, Alan J. Biel, for taking me under his wings and teaching me his knowledge of microbiology. I deeply appreciate all the time and effort he has invested. His patience and encouragement have made my graduate career a successful one.

I would like to express my sincere appreciation to the members of my graduate committee: Dr. Donal Day, Dr. Randall Gayda, Dr. Gregg Pettis, Dr. Ding Shih and Dr. James Krahenbuhl for their support and helpful advice throughout my graduate career.

To all of my laboratory colleagues past and present, Karl Indest, Georgia Ineichen, Katya Kanazireva, David Huang, Alok Khanna and Karen Sullivan, I would like to express my deepest gratitude for being so helpful and being such good friends.

Special thanks to Cindy Henk and Ron Bouchard for their help in photography and slide preparation.
# TABLE OF CONTENTS

**DEDICATION** ................................................................. ii

**ACKNOWLEDGMENTS** .................................................. iii

**LIST OF TABLES** .......................................................... vi

**LIST OF FIGURES** ......................................................... vii

**ABSTRACT** ................................................................. viii

**INTRODUCTION** .......................................................... 1

- Tetrapyrrole Pathway of *R. capsulatus* ........................ 3
  - Aminolevulinate Synthase ........................................... 3
  - Porphobilinogen Synthase ........................................... 7
  - Porphobilinogen Deaminase ....................................... 9
  - Uroporphyrinogen III Synthase ................................. 12
  - Uroporphyrinogen III Decarboxylase ....................... 13
  - Coproporphyrinogen III Oxidase .............................. 15
  - Protoporphyrinogen IX Oxidase ............................... 17
  - Tetrapyrrole Biosynthesis Regulation ....................... 20

**MATERIALS AND METHODS** ....................................... 30

- Strains and Plasmids .................................................. 30
- Media ........................................................................... 30
- Growth Conditions ..................................................... 30
- Protein Determination ............................................... 34
- Porphobilinogen Deaminase Assay ............................... 34
- Isolation and Purification of Porphobilinogen Deaminase .................................................. 37
  - (NH₄)₂SO₄ Fractionation and Heat Treatment .......... 37
  - Anion-Exchange Chromatography ......................... 38
  - Hydroxylapatite Chromatography ......................... 38
  - Gel-Filtration Chromatography .............................. 39
- Molecular Weight Determination by Electrophoresis ........ 39
  - Denaturing Conditions ......................................... 39
  - Nondenaturing Conditions .................................... 40
- Isoelectric Focusing .................................................. 42
- Two-Dimensional Gel Electrophoresis ....................... 43
- Protein Blotting ........................................................ 43
- Plasmid DNA Isolation .............................................. 44
- Restriction Enzyme Digestion ................................. 45
- DNA Ligation .......................................................... 45
- Agarose Gel Electrophoresis .................................... 46
- DNA Recovery from Agarose Gels ......................... 46
Electroporation ................................................................. 46
Triparental Matings .............................................................. 47
DNA Sequencing ................................................................. 48
Chloramphenicol Acetyltransferase Assay ................................. 48

RESULTS ................................................................................ 50
Purification of Porphobilinogen Deaminase ................................. 50
Molecular Weight Determination of Porphobilinogen Deaminase 50
Nondenaturing Gel Electrophoresis ............................................. 50
SDS-PAGE ........................................................................ 53
Determination of Optimum pH and Isoelectric Point ....................... 57
Kinetic Analysis of Porphobilinogen Deaminase ............................ 57
Stoichiometry ...................................................................... 57
Inhibitors ........................................................................... 60
Effects of Oxygen on Porphobilinogen Deaminase Activity ............... 60
N-terminal Sequence Analysis ............................................... 62
Cloning of the R. capsulatus hemC Gene .................................. 62
Sequencing of the hemC Gene ............................................... 64
Overexpression of the hemC Gene ......................................... 68
Construction of a hemC-cat Transcriptional Fusion Plasmid 71
Effects of Oxygen on Transcription of the hemC-cat Fusion Plasmid 75

DISCUSSION ........................................................................ 78

REFERENCES ........................................................................ 87

VITA ................................................................................... 118

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
LIST OF TABLES

1. Strains used in this study ................................................................. 31
2. Plasmids used in this study ................................................................. 32
3. Purification of porphobilinogen deaminase ........................................... 51
4. Effects of additives on the *R. capsulatus* porphobilinogen deaminase .... 61
5. Overexpression of *hemC* in *E. coli* .................................................. 72
6. Overexpression of *hemC* in *R. capsulatus* ......................................... 73
7. Effects of oxygen on chloramphenicol acetyltransferase activity ............ 76
LIST OF FIGURES

1. Tetrapyrrole biosynthetic pathway of *R. capsulatus* .......................... 4

2. Analysis of the *R. capsulatus* porphobilinogen deaminase by non-denaturing polyacrylamide gel electrophoresis .......................... 52

3. Mobility of proteins in nondenaturing gels ........................................ 54

4. Molecular weight determination of the non-denatured *R. capsulatus* porphobilinogen deaminase .................................................. 55

5. Analysis of the *R. capsulatus* porphobilinogen deaminase by SDS-PAGE .......................................................... 56

6. Effects of pH on the *R. capsulatus* porphobilinogen deaminase activity .... 58

7. Kinetic analysis of the *R. capsulatus* porphobilinogen deaminase ........ 59

8. Two-dimensional gel electrophoresis of the purified *R. capsulatus* porphobilinogen deaminase .................................................. 63

9. Plasmid pCAP154 containing the *R. capsulatus* hemC and hemE genes ........................ 65

10. Restriction map of *R. capsulatus* hemC derivatives .......................... 66

11. Nucleotide sequence of the *R. capsulatus* hemC gene ........................ 67

12. Alignment of conserved cofactor binding sites ................................... 69

13. Palindromic sequences upstream of *R. capsulatus* genes ................ 70

14. The hemC-cat fusion plasmid constructed to study transcriptional regulation by oxygen .................................................. 74

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
ABSTRACT

*Rhodobacter capsulatus* uses the common tetrapyrrole pathway to synthesize heme, bacteriochlorophyll, siroheme and vitamin B-12. As part of our studies on the regulation of this pathway, the product of the *hemC* gene, porphobilinogen deaminase was purified approximately 200-fold. The molecular weight, isoelectric point, pH optimum and $K_m$ of the enzyme were all similar to other porphobilinogen deaminases. The stoichiometry of the enzyme was found not to be the expected four porphobilinogens used per one uroporphyrin formed. Rather a 9:1 ratio was typically seen. Further study of this atypical ratio uncovered evidence of possible *in vivo* regulation of porphobilinogen deaminase dependant on the cellular redox state. The protein was isolated from a two-dimensional gel and the sequence of the first twenty amino acids from the N-terminus was determined. This protein sequence matched a putative amino acid sequence 110 bp upstream of the *R. capsulatus hemE* gene. The putative *hemC* locus was subcloned from the plasmid pCAP154 which contains the *R. capsulatus hemE* gene with 1.2 Kb of upstream DNA. The sequence of the upstream DNA was determined by dideoxy sequencing of both strands. A single open reading frame of 951 bp was found which could code for a 317 amino acid protein of 34,082 Da. The putative protein has 49% identity with the *E. coli* porphobilinogen deaminase and 44% identity with the *B. subtilis* enzyme. When a plasmid carrying the *R. capsulatus hemC* was inserted into *E. coli* and *R. capsulatus*, a four-fold and ten-fold increase in porphobilinogen deaminase activity was seen respectively.
The DNA sequence revealed that the hemC and hemE genes are divergently transcribed. A palindrome was identified between the two start sites, 47 bp upstream of hemE and 42 bp upstream of hemC. This palindrome, found upstream of many *R. capsulatus* genes, has been proposed to be a transcriptional regulatory site. The effects of oxygen on the transcription of hemC were studied using a hemC-cat fusion vector. Under the conditions tested, oxygen had no effect on the transcription of the *R. capsulatus* hemC gene.
INTRODUCTION

*Rhodobacter capsulatus* is a purple nonsulfur bacteria, in the alpha-subgroup of proteobacteria, that is capable of anoxygenic photosynthesis. Unlike the photosynthesis in cyanobacteria or eukaryotic algae, *R. capsulatus* uses only one photosystem. Like all anoxygenic phototrophic bacteria, *R. capsulatus* is unable to photolyse water and no oxygen is produced. Reducing power must be provided for CO$_2$ fixation by reduced compounds from the environment that have lower redox potential than water. Most commonly, hydrogen or organic compounds such as aliphatic acids or alcohols are used as photosynthetic electron donors. To harness the energy from light, the photosynthetic pigments and the photosynthetic apparatus are located in an extended system of intracytoplasmic membranes that originate and are continuous with the cytoplasmic membrane. The major photosynthetic pigments are bacteriochlorophyll $a$ or $b$ and various carotenoids of the spheroidene series. These photopigments are responsible for the distinct coloration of *R. capsulatus* cultures.

*R. capsulatus* is an extremely metabolically versatile bacteria often described as the most versatile of the prokaryotes (186). The preferred growth mode for *R. capsulatus* is photoheterotrophically under anaerobic conditions in the light using a variety of organic compounds as electron donors and carbon sources (57). This mode of growth depends on the absence of oxygen, because photopigments are not synthesized in the presence of oxygen. Under these phototrophic conditions respiration is inhibited, yet *R. capsulatus* still
exhibits a considerable respiratory capacity. This allows *R. capsulatus* to switch immediately from phototrophic to respiratory metabolism when environmental conditions change. *R. capsulatus* is capable of photoautotrophic growth under anaerobic conditions in the light with CO₂ as the sole carbon source and H₂ as the electron donor (197). *R. capsulatus* can also grow under anaerobic conditions in the dark, as a chemoautotroph (186), using hydrogen as the energy source (312). Since *R. capsulatus* is quite tolerant of oxygen, it can grow well as a chemoheterotroph under aerobic conditions in the dark using a variety of organic compounds for carbon and energy, and O₂ as the final electron acceptor. *R. capsulatus* can also perform a respiratory metabolism anaerobically in the dark using various sugars for carbon and either nitrate, nitrous oxide, dimethyl sulfoxide (DMSO), or trimethylamine-N-oxide (TMAO) as electron acceptors (184, 185, 197). Along with these specialized metabolic processes, *R. capsulatus* can fix nitrogen in both light and dark conditions (13). This makes it possible to grow *R. capsulatus* in the simplest of medium, containing CO₂ for carbon, light for energy, and ammonium for nitrogen, or in the most complex of medium such as Peptone-Yeast Extract (PYE).

Tetrapyrroles are essential molecules in energy metabolism and are found in almost all organisms in nature. Humans are able to produce the tetrapyrrole heme, while plants, algae, and cyanobacteria are able to synthesize the tetrapyrroles heme and chlorophyll. *R. capsulatus* is uniquely versatile in that it has the ability to form four tetrapyrrole end products:
bacteriochlorophyll, heme, siroheme, and vitamin B_{12}. Bacteriochlorophyll is used as the major light harvesting pigment. Heme is used as the prosthetic group for cytochromes, catalase, and peroxidase and is required for all growth modes. Siroheme is the prosthetic group for sulfite and nitrite reductase (206, 207). Vitamin B_{12} is used as the cofactor for homocysteine methyltransferase, an enzyme involved in methionine biosynthesis (50).

**Tetrapyrrole Pathway of *R. capsulatus***

The four tetrapyrrole end products made by *R. capsulatus* are the products of a common pathway (Figure 1) that branches after the formation of protoporphyrin IX to form bacteriochlorophyll and heme (130, 167). Except for a few cases, all organisms synthesize tetrapyrroles by some form of this common pathway. Those organisms that have incomplete pathways require exogenous sources of heme or a tetrapyrrole precursor molecule (99). In humans, defects in this common pathway lead to a group of disorders called porphyrias (205). These disorders, most commonly inherited as autosomal dominant traits, are characterized by excess production of porphyrin precursors.

**Aminolevulinate Synthase**

The first intermediate molecule common to all tetrapyrroles is the five carbon aminoketone, δ-aminolevulinate (ALA). Depending on the organism, two pathways leading to the formation of δ-aminolevulinate are known. One production pathway called the C_{5}-pathway involves the conversion of glutamate into ALA via a three step process (23). This pathway is widely distributed
Figure 1. Tetrapyrrole biosynthetic pathway of *R. capsulatus*. 

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
among the phototrophic bacterial groups and is probably the more primitive and evolutionarily earlier pathway (12). First, the enzyme glutamyl-tRNA synthetase links a tRNA\textsuperscript{Glu} to the alpha-carboxyl group of glutamate through an ATP and Mg\textsuperscript{2+} dependent ligation (221). Second, a NADPH-dependent glutamyl-tRNA dehydrogenase reduces the tRNA bound glutamate to glutamate-1-semialdehyde (11). Third, glutamate-1-semialdehyde aminotransferase interchanges the positions of the nitrogen and oxygen atoms on the glutamate-1-semialdehyde through a pyridoxal phosphate dependent transamination reaction to form \(\delta\)-aminolevulinate. The \(C_5\) pathway for ALA biosynthesis is utilized by the green sulfur bacteria \textit{Chlorobium vibrioforme} (242), the green nonsulfur bacteria \textit{Chloroflexus aurantiacus} (224), the purple sulfur bacteria \textit{Chromatium vinosum} (225), the unicellular red algae \textit{Cyanidium caldarium} (303), cyanobacteria (75, 221, 241) and higher plants (24, 233). The \(C_5\) pathway is also used by the non-chlorophyll containing organisms \textit{Bacillus subtilis} (220, 228), \textit{Clostridium thermoaceticum} (226), \textit{Escherichia coli} (11, 174, 220), \textit{Salmonella typhimurium} (79), and the archaebacterium \textit{Methanobacterium thermoautotrophicum} (89).

The second ALA production pathway, called the \(C_4\) or the Shemin pathway (153, 267), involves a one step reaction using the product of the \textit{hemA} gene, aminolevulinate synthase (EC 2.3.1.37). Glycine is first bound as a Schiff-base to the pyridoxal phosphate cofactor of ALA synthase, to which a succinate from succinyl-CoA is transferred. Subsequently CO\textsubscript{2} is split off and ALA is released from the enzyme. The \(C_4\) pathway first found in \textit{Rhodobacter}
*sphaeroides* (96, 153) is also utilized by humans (41), yeast (23), *Rhizobium* (170), and other photosynthetic bacteria (12, 231).

The protein ALA synthase has been highly purified and characterized from *R. sphaeroides*. Initial studies found the enzyme to be a soluble protein with a molecular mass of 57 to 61 kDa (299, 323), however the native enzyme was later proven to be a homodimer with a molecular mass of 80 to 100 kDa (82, 213). The *Rhodopseudomonas palustris* ALA synthase was found to be a monomer with a native molecular mass of 61 to 65 kDa (295). The ALA synthases purified from yeast (298) and rat liver (261) were found to have similar characteristics to the *R. sphaeroides* enzyme. Inhibition studies done on the *R. sphaeroides* ALA synthase have shown the enzyme is inhibited by hemin and sulfhydryl reagents (46, 82, 299, 323). ATP was found to be a strong inhibitor of the *R. sphaeroides* enzyme with 60 to 88 percent inhibition at 1 mM concentration (81). The existence of two distinct ALA synthases was demonstrated in *R. sphaeroides* (82). It was reported that one form of ALA synthase is cytoplasmic and is functionally associated with dark metabolism. The other form of ALA synthase found in the chromatophores, could be specifically induced by light and is functionally associated with photometabolism. The two forms of the ALA synthase protein may be encoded by different genes as mentioned below.

The *hemA* gene encoding ALA synthase has been cloned and sequenced from many organisms. The *hemA* gene of *R. capsulatus* was cloned using a cosmid that complemented a *hemA*:Tn5 mutant (33). The *R.*
capsulatus hemA gene was later cloned and sequenced by probing a R. capsulatus cosmid bank with the hemA gene from R. sphaeroides (113). The R. capsulatus sequence predicts a 401 amino acid protein with a molecular mass of 44.1 kDa. There have been two ALA synthase encoding genes reported in R. sphaeroides with the second encoded by hemT (280). The two genes hemA and hemT, which respectively are located on the large and small chromosomes, express isozymes with 53% amino acid identity (216). Under photosynthetic conditions, the R. sphaeroides hemA transcript levels are threefold higher than under aerobic conditions, suggesting the hemA isozyme may encode the ALA synthase associated with photometabolism (216). Currently the hemT gene does not seem to be expressed under any physiological conditions so far tested. The R. sphaeroides hemA sequence was 76% identical to the R. capsulatus sequence, while only 50 to 76% identical to other hemA genes sequenced. Other organisms utilizing the C₄ pathway from which the hemA gene has been sequenced include human (22), chicken (187), mouse (260), yeast (291), Agrobacterium radiobacter (73), Rhizobium meliloti (170), and Bradyrhizobium japonicum (196).

Porphobilinogen Synthase

The first monopyrrole in the common tetrapyrrole pathway, porphobilinogen (PBG), is formed by the condensation of two ALA molecules with the subsequent loss of two water molecules. This reaction is catalyzed by the enzyme PBG synthase (ALA dehydratase) (EC 4.2.1.24), the product of the hemB gene. PBG synthase from all sources examined has a native molecular
mass of 250 to 320 kDa. PBG synthase was first purified from *R. sphaeroides*
(209, 211, 212) and found to be a hexamer of 240 to 250 kDa that requires
thiols and K⁺ (110). Various metallic cations stimulated enzymatic activity and
16 µM protoheme inhibited 96% of the PBG synthase activity. The PBG
synthase from *R. capsulatus* was also found to be a hexamer of 260 kDa (209),
however, it was active without the addition of metallic cations and thiols.
Protoheme was not inhibitory (only 9% inhibition at 50 µM) to the *R. capsulatus*
enzyme. The PBG synthase has been purified from many plant sources
including spinach (177), maize (190), and radish (259), all of which have a
strict requirement for either Mg²⁺ or Mn²⁺. Many of the eukaryotic PBG
synthases have also been purified, such as cow liver (15), human erythrocytes
(5), mouse liver (56), and yeast (40). The eukaryotic enzymes all have a strict
requirement for Zn²⁺. The purified *E. coli* PBG synthase was found to be an
cornered with a native molecular mass of 290 kDa, with 36.5 kDa subunits, that
was dependant on thiols and Zn²⁺ (276). Suprisingly, the *E. coli* enzyme was
also found to be stimulated by Mg²⁺ (200). PBG synthase was
also purified from *Mycobacterium phlei* (316) and *B. japonicum* (229), both of
which required Mg²⁺.

The *hemB* gene coding for PBG synthase has been cloned and
sequenced from many organisms including animals, plants, yeast, and
bacteria. All sequences code for a putative protein of 35 to 45 kDa. The *R.*
capsulatus *hemB* gene sequenced by Indest and Biel was found to have a Mg²⁺
binding site but no Zn²⁺ site (120). The nucleotide sequence of the
R. sphaeroides *hemB* gene was determined and expressed in *E. coli* making a 39 kDa product (68). The sequence has also been determined for *B. subtilis* (104), *C. vibrioforme* (240), *Clostridium josui* (94), cyanobacterium (128), *E. coli* (74, 175, 176), human (304, 305), the pea *Pisium satvium* (36), *Pseudomonas aeruginosa* (88), spinach (258), *Staphylococcus aureus* (146), rat liver (35), and yeast (208). All *hemB* sequences examined have 36 to 40% identity to each other (176).

**Porphobilinogen Deaminase**

The first tetrapyrrole, uroporphyrinogen III, is formed by the sequential action of two enzymes. Porphobilinogen deaminase (E.C. 4.1.3.8), the product of the *hemC* gene, joins four PBG molecules together to form the linear tetrapyrrole hydroxymethylbilane (preuroporphyrinogen) (16, 47, 80). The released hydroxymethylbilane is cyclized to form uroporphyrinogen III by the product of the *hemD* gene uroporphyrinogen III (co-)synthase (18). In the absence of uroporphyrinogen III synthase, hydroxymethylbilane spontaneously cyclizes into the non-physiological isomer uroporphyrinogen I.

Some groups believe the enzymes PBG deaminase and uroporphyrinogen III synthase exist as a complex called porphobilinogenase (111). The two enzymes have been copurified and the resultant mixture characterized from avian erythrocytes (179), *Euglena gracilis* (248, 249), *R. palustris* (144), soybean callus tissue (180), and yeast (6, 7). Jordan maintains that these enzymes do not form a complex, but rather function independently and sequentially (134).
The PBG deaminase, also called hydroxymethylbilane synthase or uroporphyrinogen I synthase, has been extensively studied because of the complexity of the enzyme conversion mechanism. The PBG deaminase enzyme has been purified and characterized from *E. coli* (105, 138), *R. sphaeroides* (66, 137, 247) and *R. palustris* (157, 158). The eukaryotic PBG deaminases isolated include cow liver (252), human erythrocytes (4, 90, 201), rat liver and spleen (195, 307), unicellular algae (143, 270, 308), and yeast (60). Many plant PBG deaminases have also been purified, such as from *A. thaliana* (131), *P. sativum* (275), spinach leaves (111), and wheat germ (91, 111, 250). The characterized PBG deaminases from most sources is a monomer with a molecular mass of approximately 35 kDa. The only exception is the *R. palustris* PBG deaminase, which had a native molecular mass of 74 kDa and may be a dimer (158). No characterized PBG deaminase requires metal ions or other cofactors for activity.

PBG deaminase has been found to contain a dipyrrromethane cofactor essential for activity that is not turned over (139). The dipyrrromethane cofactor was subsequently found to be attached to the protein, via the sulfur of a conserved cysteine residue in the active site (108, 109, 141, 198). This same dipyrrromethane cofactor was found to be the site of attachment for the four molecules of PBG during the catalytic cycle (262, 301). Being that the dipyrrromethane cofactor has the structure of two pyrrole units it was postulated that the cofactor was synthesized from two PBG units. However, it has recently been found that PBG deaminase can synthesize its own
dipyrromethane cofactor from hydroxymethylbilane much faster than from PBG (14, 272). PBG deaminase has been found to undergo large conformational changes during the assembly of the four PBG subunits (300). The assembly of the four PBG units occurs in a sequential order starting with ring A, followed by rings B, C, and D (17, 263). Sequentially formed intermediate enzyme-substrate complexes have been identified (26, 60, 133) and purified (4).

Many sites in the PBG deaminase enzyme have been found to be vital for activity. Pyridoxal phosphate has been found to inhibit the enzyme by modifying lysines (100, 107, 199). Conserved arginine residues have been found to be involved in binding the carboxylate side chains of the dipyrromethane cofactor and the growing oligopyrrole chain (162). Site directed mutagenesis of the arginine residues affected dipyrromethane cofactor assembly and tetrapyrrole chain initiation and elongation (142). An aspartate near the active site was found to play a key role in catalysis. When substituted with a glutamate, the $K_{cat}$ was reduced by two orders of magnitude (309).

The X-ray crystal structure has been determined for the E. coli PBG deaminase under oxidized conditions (140, 181) and again under reduced conditions (101). The enzyme has three flexible domains with a single active site in between. The dipyrromethane cofactor is covalently attached to domain 3 and it sticks into the cleft between domains 1 and 2 (181). Comparison of the structure and ligand showed that the E. coli PBG deaminase is very similar to transferrin, maltose-binding protein, and the sulfate and phosphate binding proteins (227). Comparison of the crystal structures has shown the cofactor
moves in position depending upon the redox conditions of the crystals. Under reduced conditions the cofactor occupies a position near the rear of the active site, while under oxidized conditions the cofactor is found in the active site occupying a position postulated to be a substrate binding site (161).

The hemC gene encoding PBG deaminase has been cloned and sequenced from many sources including A. thaliana (178), B. subtilis (104), C. josui (94), C. vibrioforme (189, 202), E. gracilis (265), E. coli (2, 285), human erythrocytes (239), mouse (25), P. aeruginosa (204), rat cDNA (279), S. aureus (145), and yeast (152). All hemC sequences code for a putative protein of 33 to 37 kDa.

**Uroporphyrinogen III Synthase**

The linear tetrapyrrole hydroxymethylbilane released from PBG deaminase is cyclized by the enzyme uroporphyrinogen III (co-)synthase (EC 4.2.1.75) to form the first intermediate with a tetrapyrrole macrocycle, uroporphyrinogen III. During the cyclization, hydroxymethylbilane goes through a spiro-intermediate to invert ring D (62, 169). Without uroporphyrinogen III synthase present, the hydroxymethylbilane spontaneously cyclizes into the non-physiological isomer uroporphyrinogen I. The presence of the uroporphyrin III synthase enzyme has been found to facilitate the release of tetrapyrrole product from the PBG deaminase (247).

The purification of uroporphyrinogen III synthase has proved difficult because of the extreme heat sensitivity of the enzyme. The synthase is incapable of forming uroporphyrinogen III from PBG or uroporphyrinogen I (37).
It was shown that *Chlorella* extracts heated to 55°C prior to incubation with PBG would only form the uroporphyrinogen I isomer (39). The uroporphyrinogen III synthase has been characterized from many sources including *B. subtilis* (277), cow liver (252), *E. coli* (3, 62), *E. gracilis* (107), human erythrocytes (290), mouse spleen (172, 173), and rat liver (274). All of the uroporphyrinogen III synthase enzymes have a molecular mass of 28 to 32 kDa. The enzyme has no metal or cofactor requirements, yet is sensitive to both lysine and arginine modifying agents.

The gene coding for uroporphyrinogen III synthase, *hemD*, has been sequenced from many organisms. The *E. coli* *hemD* and *hemC* exist in an operon (2, 135, 136, 256). *HemD* is expressed from the same promoter as *hemC*, with *hemC* being transcribed first. It is proposed this coordinate expression of *hemC* and *hemD* exists to avoid accumulation of porphyrins. The *hemD* gene has also been sequenced from *B. subtilis* (104), *C. josui* (94), *C. vibrioforme* (202), cyanobacterium (128), human (289), *P. aeruginosa* (204), and *S. aureus* (145). All of the genes coded for a putative protein of 27 to 29 kDa.

**Uroporphyrinogen III Decarboxylase**

The intermediate uroporphyrinogen III is located at a branch point in the common tetrapyrrole pathway. A small quantity of uroporphyrinogen III is methylated, committing it to siroheme and vitamin B$_{12}$ biosynthesis. The majority of uroporphyrinogen III is decarboxylated into the product
coproporphyrinogen III, which eventually is converted into heme or bacteriochlorophyll.

Uroporphyrinogen III decarboxylase (EC 4.1.1.37) catalyzes the removal of four molecules of CO$_2$ from the acetic acid side chains of uroporphyrinogen III to form coproporphyrinogen III (76). Intermediates containing 5, 6, and 7 carboxylic acid groups have been isolated, indicating that the decarboxylation of uroporphyrinogen III occurs in a sequential manner (112, 194). At physiological substrate concentrations the decarboxylations start on ring D of uroporphyrinogen III and continue in a clockwise fashion (182). The decarboxylation becomes random when higher substrate levels are present (182).

Uroporphyrinogen III decarboxylase has been purified from *R. sphaeroides* (132) and *R. palustris* (156). The protein has also been purified from many eukaryotes including avian erythrocytes (150), bovine liver (278), human erythrocytes (77, 293), tobacco leaves (52), and yeast (83). All uroporphyrinogen decarboxylases examined are monomers of 38 to 46 kDa, except the bovine (57 kDa) and avian (79 kDa) decarboxylases. Unlike most decarboxylases, the uroporphyrinogen decarboxylase has no metal or coenzyme requirements. The recently determined crystal structure of the human erythrocyte uroporphyrinogen decarboxylase shows a 40.8 kDa protein with a single domain that exists as a dimer in solution (306).

The *hemE* gene that encodes uroporphyrinogen III decarboxylase has been cloned and sequenced from *R. capsulatus* by Ineichen and Biel (122).
The sequence is also available from *B. subtilis* (103), *E. coli* (217), human (243, 246), mouse (311), rat (244, 245), yeast (69, 95), tobacco and barley (203). All of the *hemE* sequences code for a putative peptide of 40 to 44 kDa in size.

**Coproporphyrinogen III Oxidase**

The enzyme coproporphyrinogen III oxidase (EC 1.3.3.3) oxidatively decarboxylates coproporphyrinogen III to form protoporphyrinogen IX. The enzyme converts the two propionic sidechains on rings A and B of coproporphyrinogen III into vinyls, releasing two molecules of CO$_2$ and two H$^+$ atoms, producing protoporphyrinogen IX. Intermediates with one vinyl group and three carboxylic acid groups have been identified (232, 254). The side chains are converted in a sequential manner with the 2-propionic acid side chain of coproporphyrinogen III being converted to the vinyl group before the 4-propionic acid group is modified (51).

Coproporphyrinogen oxidases from aerobic organisms, prokaryotic and eukaryotic, have a strict requirement for oxygen. Extracts from aerobically or anaerobically grown *R. sphaeroides* have a coproporphyrinogen oxidase activity (282). The aerobic activity was localized to the supernatant, while the anaerobic activity was found to need both the pellet and supernatant fractions. The aerobic activity, which used O$_2$ as an electron acceptor, was purified and found to have a molecular mass of 44 kDa (281). The anaerobic activity, which used NAD or NADP as the electron acceptor, required S-adenosyl-L-methionine (SAM) or L-methionine, ATP and Mg$^{2+}$, suggesting that SAM is a cofactor (281). The anaerobic extracts of yeast (236) and *B.*
japonicum (151), have similar requirements as the R. sphaeroides enzyme.
The R. sphaeroides aerobic and anaerobic coproporphyrinogen oxidase mechanisms were studied using stereospecifically-labeled porphobilinogen (264). Both the aerobic and anaerobic activities were able to oxidize coproporphyrinogen III into protoporphyrinogen IX. This led to the postulation of a branch in the common pathway separating heme and bacteriochlorophyll synthesis at the level of coproporphyrinogen III oxidase (58). Purification of coproporphyrinogen III oxidase from yeast (48) and bovine liver (155, 318) found the enzyme to be a 70 to 75 kDa homodimer.

The first gene cloned and sequenced that encoded coproporphyrinogen III oxidase was HEM13 obtained from yeast (324). In R. sphaeroides, a gene was cloned and sequenced that when overexpressed in E. coli causes increased coproporphyrinogen oxidase activity (58). The gene product was a protein of 34,185 Da with 44.7% similarity to the yeast protein. If the gene was disrupted, R. sphaeroides cells were unable to form bacteriochlorophyll under anaerobic conditions. It was proposed that the gene product is an anaerobic coproporphyrinogen oxidase dedicated to bacteriochlorophyll synthesis. In E. coli and S. typhimurium two gene sequences encoding a coproporphyrinogen III oxidase, hemF and hemN, have been identified. Anaerobic heme synthesis required hemN function, while either hemN or hemF was sufficient for aerobic heme synthesis (287, 313). The S. typhimurium hemF sequence codes for a putative protein of 34.4 kDa and is 44% identical to the yeast sequence (315). The hemN gene of S. typhimurium codes for a putative protein of 52.8 kDa,
which is 38% identical to the *R. sphaeroides* enzyme (314). The *E. coli* hemF sequence codes for a putative protein of 34.3 kDa, with 43% identity to the yeast enzyme and 90% identical to the *S. typhimurium* hemF sequence (288). The *E. coli* hemN sequence codes for a putative protein of 52.8 kDa that is 92% identical to the *S. typhimurium* hemN and 35% identical to the *R. sphaeroides* sequence (287). Using the amino acid sequence of the bovine liver coproporphyrinogen III oxidase as a probe, a mouse cDNA library was probed and the sequence of the mouse hemF determined (155). The mouse hemF sequence is 52% identical to the yeast HEM13 sequence. The human placental hemF sequence coding for a protein of 36.8 kDa is 86% identical to the mouse sequence (283). The hemF sequence has also been determined from the plant sources tobacco and barley, each coding for proteins of approximately 39 kDa (160). No hemN sequence has been found in any of the eukaryotic sources which only grow aerobically.

**Protoporphyrinogen IX Oxidase**

The ultimate reaction of the common tetrapyrrole pathway is catalyzed by the membrane bound protoporphyrinogen IX oxidase (EC 1.3.3.4), which removes six hydrogens from the protoporphyrinogen IX ring to form protoporphyrin IX. The first characterized protoporphyrinogen IX oxidase, from yeast mitochondria, required O₂ as an electron acceptor, had a molecular mass of 180 kDa and was sensitive to hemin (50% at 20 μM) (237). The *E. coli* protoporphyrinogen IX oxidase was the first that demonstrated the ability to carry out an anaerobic oxidation process using fumarate instead of oxygen as
the physiological electron acceptor (125). Nitrate was also found to work for anaerobic oxidation, but not as effectively as fumarate (126). They reported the protoporphyrinogen IX oxidase in anaerobic E. coli extracts to be 90% inhibited by 2-heptyl-4-hydroxyquinoline-N-oxide, a quinine inhibitor, suggesting the enzyme may be linked to quinone in the anaerobic electron transport chain.

The protoporphyrinogen IX oxidase of R. sphaeroides was found in the membrane fractions of aerobically and photosynthetically grown cells (127). Interestingly, this enzyme was not able to use O₂ directly as an oxidant. The protoporphyrinogen oxidation was found to be strongly inhibited by cyanide and azide, suggesting the R. sphaeroides enzyme is coupled to the electron transport system. When the quinones were extracted, 80% of the protoporphyrinogen oxidase activity was inhibited, yet only minor inhibition was seen with 2-heptyl-4-hydroxyquinoline-N-oxide. These results are much different from those seen in R. capsulatus where protoporphyrinogen IX oxidation was not inhibited by cyanide or azide, but strongly inhibited by 2-heptyl-4-hydroxyquinoline-N-oxide (R. Cardin and A. Biel, unpublished results).

The membrane bound protoporphyrinogen IX oxidase of the anaerobic bacterium Desulfovibrio gigas was purified and found to have a native molecular mass of 148 kDa (154). When solubilized with detergent, three nonidentical subunits with molecular masses of 12, 18.5, and 57 kDa were revealed, which could be linked by sulfhydryl bonds to form a hexamer. The D.
gigas enzyme was found to have no aerobic activity and could donate electrons to 2,6-dichlorophenol-indophenol but not to NAD, NADP, FAD, or FMN. The natural electron acceptor was not identified. The protoporphyrinogen IX oxidase has also been purified from the eukaryotic sources of mammalian mitochondria (235), mouse liver mitochondria (63, 84), and bovine liver (273). All of these eukaryotic proteins were found to be monomers of 57 to 65 kDa. The bovine enzyme was unique in that it was found to be associated with FAD or FMN. The protoporphyrinogen IX oxidase has been purified from plants (123) such as barley, where it is a chloroplast associated enzyme with a molecular mass of 210 kDa, composed of 36 kDa subunits (124). In contrast to other protoporphyrinogen IX oxidases, the B. subtilis enzyme was found to be a soluble enzyme of 52 kDa (64).

The hemG gene encoding the enzyme protoporphyrinogen IX oxidase has been sequenced from A. thaliana (214), B. subtilis (64, 102, 103), E. coli (255), human (218), and tobacco (171). All of the hemG sequences code for a protein with predicted molecular mass of 50 to 57 kDa except for the E. coli sequence which codes for a protein of only 21,202 Da.

The tetrapyrrole protoporphyrin IX resides at the second branch point of the common tetrapyrrole pathway. Insertion of a magnesium ion (Mg$^{2+}$) into the protoporphyrin IX ring, a reaction catalyzed by a magnesium chelatase, commits the product to bacteriochlorophyll synthesis. If a ferrous iron (Fe$^{2+}$) is incorporated into the protoporphyrin IX ring, the product is committed to heme
biosynthesis. The enzyme ferrochelatase (EC 4.99.1.1) catalyzes the insertion of Fe$^{2+}$ into protoporphyrin IX to form protoheme.

**Tetrapyrrole Biosynthesis Regulation**

*R. capsulatus*, like other phototrophic organisms, is able to regulate the content and composition of its tetrapyrroles. The four tetrapyrrole end products formed by *R. capsulatus* serve very different functions. For this reason, the common tetrapyrrole pathway can be expected to have many complex regulatory mechanisms. In the last three decades serious efforts have been made to determine how various environmental factors influence tetrapyrrole biosynthesis. To date three environmental factors have been shown to influence the levels of the tetrapyrrole end products: light, heme, and oxygen.

The environmental factors can cause many changes in the *R. capsulatus* cell structure. While aerobically grown *R. capsulatus* has a membrane structure that is morphologically and functionally like that of a Gram negative cell, when oxygen tensions are lowered below 2.5% many changes start occurring in the *R. capsulatus* cell. An extensive series of invaginations of the cytoplasmic membrane develops as well as induction of the photosynthetic apparatus, bacteriochlorophyll and carotenoids. Each photosynthetic apparatus is composed of three distinct protein complexes that convert light to chemical energy. Two light-harvesting complexes LH-I (B870) and LH-II (B800-B850) absorb visible and near-infrared radiation and transfer the energy to the reaction center complex. The reaction center is where photochemical charge separation occurs transferring electrons through the electron transport...
system, which produces a transmembrane potential that drives ATP synthesis. Each component of the photochemical apparatus has bacteriochlorophyll and carotenoids attached. Most of the R. capsulatus bacteriochlorophyll genes (bch), which encode enzymes responsible for converting Mg-protoporphyrin into bacteriochlorophyll, were identified by complementation of bch mutants with R'-plasmids carrying various bacteriochlorophyll genes (191). Eventually a physical map was created localizing the bacteriochlorophyll and carotenoid genes (284). The R. capsulatus bch and the carotenoid biosynthetic genes (crt) were found to be clustered in a 46 kb region of the genome referred to as the photosynthetic gene cluster (322, 326). Within this region two operons were identified, the puh operon encoding the polypeptides of the light-harvesting complex I and the puf operon encoding the reaction center polypeptides (319). The genes of the puc operon, encoding the light-harvesting complex II polypeptides, were not localized within the photosynthetic cluster (321). The entire R. capsulatus photosynthetic gene cluster has been sequenced (1, 320). The many complex regulatory circuits controlling photosynthetic gene expression have recently been reviewed by Bauer (19).

The influences of light on pigment synthesis were initially studied by Cohen-Bazire (55) in R. sphaeroides. Highly illuminated photosynthetically grown cultures of R. sphaeroides were found to contain low levels of bacteriochlorophyll and carotenoids, while dimly lit cultures contained cells packed with membranes and large amounts of photopigment per cell mass. No
matter what the initial pigment concentration, the same steady state of bacteriochlorophyll synthesis was eventually established at any given light intensity (55). Influences of light on enzymes of the common pathway was demonstrated on ALA synthase and PBG synthase, which were both found to be four to five fold more active in cultures grown anaerobically in the light than aerobically in the dark (164). In \textit{R. palustris} the enzymatic levels of ALA synthase were also higher in anaerobic light grown cells compared to cells grown aerobically in the dark (294). In contrast to these results, Oelze and Arnheim (223) using chemostat cultures of \textit{R. sphaeroides}, found light had no significant effect on ALA synthase, although it clearly regulated bacteriochlorophyll levels. Oelze (222) later found bacteriochlorophyll, LH-I and LH-II complex levels reduced 50% by high light intensity. The assumption was that light somehow regulates bacteriochlorophyll synthesis. Evidence that this assumption was incorrect was provided by Biel (28) who found that light regulates the degradation of bacteriochlorophyll. Light was found to have no effect on the transcription of many oxygen-regulated bacteriochlorophyll genes of \textit{R. capsulatus} (31). The \textit{R. capsulatus} genes for light-harvesting antenna I proteins LH-I and LH-II; the reaction center proteins L, M, and H; and bacteriochlorophyll and carotenoid biosynthesis were shown by dot-blot analysis to be repressed by high light (325). Recently, the trans-acting regulator gene, \textit{hvrA}, was discovered in \textit{R. capsulatus} that activates LH-I and reaction center gene expression (not LH-II or bacteriochlorophyll expression) in response to reductions in light intensity (45).
The c-type cytochromes have historically been implicated to influence carbon flow down the common tetrapyrrole pathway. This was based on the fact that c-type cytochrome mutants accumulate photopigments (65,159). The Tn5 insertion mutant AJB530, isolated by Biel and Biel (32), was found to have a disruption in the gene encoding cytochrome c synthetase which completely stopped c-type cytochrome biosynthesis. This mutant made only 19% of the normal amount of bacteriochlorophyll, but accumulated 18-fold more coproporphyrin and nine-fold more protoporphyrin. When the total porphyrin levels (bacteriochlorophyll, coproporphyrin, and protoporphyrin) were examined, they were found to be the same in wild-type PAS100 and the mutant AJB530, indicating c-type cytochromes have no influence on carbon flow over the pathway (116).

Heme has been shown to inhibit many enzymes of the common tetrapyrrole pathway. The R. sphaeroides ALA synthase was found to be inhibited in vitro by hemin (iron-protoporphyrin) and a possible negative feedback mechanism was proposed (46). This in vitro inhibition of the R. sphaeroides ALA synthase by hemin was confirmed in many other studies: 1) 57% inhibition by 5 μM hemin (299), 2) 50% by 0.4 μM hemin and 50% inhibition with 2.2 μM Mg-protoporoporphyrin and 30 μM protoporphyrinogen (323), and 3) complete inhibition by 0.1 mM hemin (82). The PBG synthase of R. sphaeroides was also found to be inhibited by hemin, with 50% inhibition occurring at 40 μM (46, 209). Interestingly, the R. capsulatus PBG synthase was found to be insensitive to hemin (210). The R. sphaeroides enzyme,
uroporphyrinogen III decarboxylase, has been shown to be slightly inhibited by hemin, with 20% inhibition occurring at 8 μM (132). Unfortunately, higher concentrations of hemin were not tested. The *R. sphaeroides* ferrochelatase was also found sensitive to hemin with 50% inhibition occurring at 10 μM (129).

To elucidate the manner in which heme regulates tetrapyrrole biosynthesis, cells were fed various levels of heme and the production of various tetrapyrroles monitored. Lascelles (166) found that the addition of ferric citrate to *R. sphaeroides* cultures reduced porphyrin formation 100-fold, while increasing the bacteriochlorophyll formation eight to ten fold. *R. capsulatus* cell suspensions grown photosynthetically in iron-deficient media accumulated large amounts of coproporphyrin and Mg-protoporphyrin monomethyl ester, while synthesizing less than half the normal amount of bacteriochlorophyll (59). Using a more specific method for producing heme deficient cells, *R. capsulatus* and *R. sphaeroides* cell suspensions were incubated in media supplemented with iron and the ferrochelatase inhibitor, N-methylprotoporphyrin. In both species the production of Mg-protoporphyrin monomethyl ester was increased, while bacteriochlorophyll levels were not changed (115, A. Biel, unpublished results). Overall the heme deficient cell seems to have a higher level of carbon flow over the tetrapyrrole pathway. This has been attributed to the lack of feedback inhibition by heme on ALA synthase. This regulation was simulated *in vivo* by overexpressing a low copy number vector containing the *hemH* gene of *R. capsulatus*. This overexpression of ferrochelatase, by the *hemH* gene, caused increased heme
formation which inhibited the ALA synthase activity subsequently reducing the synthesis of porphyrins (147). In *R. capsulatus* the regulation of tetrapyrrole biosynthesis by heme was found to be separate from that exerted by oxygen (149).

Oxygen shows the greatest influences over the common tetrapyrrole pathway of *R. capsulatus*. Initial studies by Cohen-Bazire showed the synthesis of carotenoids and bacteriochlorophyll was rapidly inhibited by molecular oxygen (55). They proposed the regulation by oxygen comes from the redox conditions of some carrier of the electron transport system, which could influence bacteriochlorophyll synthesis. When the influences of oxygen on the hemoprotein content of cells was examined it was found to be two to three times higher in cells grown anaerobically in the light rather than that of aerobically grown organisms (234). Examination of bacteriochlorophyll mutants revealed that tetrapyrroles only accumulated when cells were grown anaerobically (163). When the levels of the tetrapyrroles were quantified in *R. sphaeroides*, anaerobically grown cells made 25 nmoles of bacteriochlorophyll per mg of dry weight, 0.3 nmoles heme per mg dry weight, and 0.07 nmoles of vitamin B₁₂ per mg of dry weight, while aerobically grown cells produced no bacteriochlorophyll yet the same amounts of heme and vitamin B₁₂ (165). This regulation over bacteriochlorophyll levels by oxygen was found to work independently from the light regulation (10). Biel and Marrs (31) analyzed the transcription of several *R. capsulatus* bacteriochlorophyll genes using lacZ fusions. They found transcription to increase two to three fold with a drop of
oxygen tension from 23% to 2%. Analysis of *R. capsulatus* and *R. sphaeroides* mRNA transcript levels of the *bch* genes also revealed transcription increases two to three fold upon anaerobisis (53, 118). The regulation of carotenoids by oxygen is different than that influenced on bacteriochlorophyll. Biel and Marrs found oxygen doesn't directly regulate the production of certain carotenoids (30). Yet examination of the transcription of the *crt* genes, by *lacZ* fusions and by looking at mRNA transcript levels, found all synthesis to increase 2 to 12 fold upon anaerobisis (9).

A novel regulatory factor, synthesized as the product of the *pufQ* gene located at the 5' end of the *puf* operon, was found to influence bacteriochlorophyll synthesis. Using a *pufQ-lacZ* fusion, *pufQ* synthesis was found to be 30-fold regulated by oxygen and to be essential for bacteriochlorophyll synthesis (21). A linear relationship exists between *pufQ* and bacteriochlorophyll synthesis, which has led to speculation that the *PufQ* protein may function as a carrier for intermediates in bacteriochlorophyll synthesis (20). *PufQ* purified from *R. capsulatus* was found to be a integral membrane protein (86, 87). The *PufQ* protein has been found to effect the early steps in the common tetrapyrrrole pathway synthesis somewhere in the conversion of porphobilinogen to coproporphyrinogen III. It was found that PBG deaminase activity was two-fold higher when *pufQ* was present (85).

Lascelles and Hatch (168) proposed a model for the mechanism of the regulation of bacteriochlorophyll synthesis by oxygen. They proposed the magnesium and iron branches of the biosynthetic pathway compete for
protoporphyrinogen IX. They suggested oxygen inhibits magnesium chelation into the protoporphyrin IX ring. This would lead to a larger protoporphyrin IX pool, which ferrochelatase makes into heme. The heme then feedback inhibits ALA synthase thus slowing carbon flow over the pathway. Biel and Marrs (31) verified that oxygen regulates the synthesis of protoporphyrin IX using a *R. capsulatus* *bchH* mutant, which cannot insert magnesium into protoporphyrin IX. The accumulation of protoporphyrin IX by the mutant was measured under high and low oxygen tensions. In the *bchH* mutant there was a dramatic increase in protoporphyrin IX levels under low oxygen tension compared to high oxygen tensions.

Oxygen has been found to exert influences at many steps of the common pathway. In *R. sphaeroides* the ALA synthase activity increased four to five fold upon a shift from high to low oxygen (164, 223) and it was later found that oxygen regulates the transcription of *hemA* (165). Northern analysis of the *hemA* and *hemT* transcripts in *R. sphaeroides* showed both transcripts are expressed three times higher under anaerobic rather than aerobic conditions (215). In *R. capsulatus* there is no evidence of ALA synthase activity regulation by oxygen (A. Biel, unpublished results). Examination of *hemA* transcription in *R. capsulatus* by use of a *hemA-lacZ* fusion showed promoter activity increased of two to three fold after reducing oxygen tension (114, 310). These small changes are not enough to explain the dramatic increase in tetrapyrrorole synthesis during photosynthetic growth.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Evidence that the major step regulated by oxygen is after ALA synthesis, came when the addition of exogenous ALA given to a \textit{R. capsulatus bchH} mutant did not alter the accumulation of protoporphyrin IX under anaerobic conditions (29). Therefore, a \textit{R. capsulatus bchH} mutant was grown with exogenous PBG under high and low oxygen tensions. This resulted in the accumulation of protoporphyrin IX under all oxygen conditions, suggesting that PBG disrupted oxygen-mediated control of the common tetrapyrrole pathway. It was concluded that oxygen regulates PBG levels and that perhaps ALA is not the committed precursor to tetrapyrroles (29).

In \textit{R. capsulatus} there exists considerable evidence that indicates ALA is not committed to tetrapyrrole synthesis. Feeding [\textsuperscript{14}C]-aminolevulinate to \textit{R. capsulatus} cells led to 85\% of the radioactivity being distributed to other compounds than tetrapyrroles (A. Biel, unpublished results). In a similar study [\textsuperscript{14}C]-aminolevulinate was fed to \textit{Rhodospirillum rubrum} and the radioactivity was found in tetrapyrroles as well as in aminohydroxyvalerate, hydroxyglutamate, and glutamate (269). Recently in \textit{R. capsulatus} an ALA dehydrogenase activity, which converts ALA to aminohydroxyvalerate in the presence of NADPH, was detected (A. Khanna and A. Biel, unpublished results). All of these facts provide substantial evidence for ALA not being a committed precursor to tetrapyrrole biosynthesis.

Realizing ALA is not the major control point by oxygen, the influence of oxygen over porphobilinogen synthesis was examined. \textit{R. sphaeroides} cultures were shown to have a lower PBG synthase activity in anaerobic
cultures (268). Using a hemB-chloramphenicol acetyltransferase fusion vector and northern analysis of the R. capsulatus hemB gene, no transcriptional regulation was seen by oxygen (119). Overexpression of the R. capsulatus hemB gene in the bchH mutant showed similar results to those seen when exogenous PBG was added (119).

The goal of the present study was to further investigate how porphobilinogen is involved in the oxygen regulatory scheme of tetrapyrrole biosynthesis. To achieve this goal, the enzyme porphobilinogen deaminase was purified from R. capsulatus and characterized. The N-terminal sequence of the protein was used to clone the R. capsulatus hemC gene. Sequencing of the hemC gene revealed the R. capsulatus hemC and hemE are divergently transcribed. The hemC gene was overexpressed in E. coli and R. capsulatus. The transcription of the hemC gene was measured by monitoring the chloramphenicol acetyltransferase activity of a hemC-cat fusion vector.
MATERIALS AND METHODS

Strains and Plasmids

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

Media

*E. coli* was grown in L-Broth (27) modified by decreasing the sodium chloride concentration to 0.5% and omitting glucose. *R. capsulatus* was routinely grown in either RCV, a malate minimal salts medium (302), or in 0.3% Bacto Peptone-0.3% Yeast Extract (PYE) (Difco Laboratories, Detroit, MI). Solid media contained 1.5% agar (Difco). Antibiotics and other supplements, when necessary, were added to the following concentrations (μg/ml): ampicillin, 250; chloramphenicol, 25; kanamycin, 10; streptomycin, 75; tetracycline, 10 (*E. coli*) and 0.4 (*R. capsulatus*); IPTG (isopropyl-β-D-thiogalactopyranoside), 5; X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 40. Strains of both *E. coli* and *R. capsulatus* were stored in 10% glycerol at -85°C.

Growth Conditions

*E. coli* and *R. capsulatus* strains were routinely grown aerobically in the dark with shaking at 37°C. *R. capsulatus* was also grown anaerobically, in the light, in completely filled Vacutainer tubes incubated in front of 100W light bulbs at 30°C, and anaerobically, in the dark, in completely filled Vacutainer tubes in RCV-media supplemented with 0.25% glucose and 20 mM dimethyl sulfoxide. When growth of *R. capsulatus* under defined oxygen tension was
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>$\Delta hsd5(r_k^m,m^*)\sup E\thi-1\Delta(lac-proAB)F'[proAB lacOZ\Delta M15]$</td>
<td>Gough and Murray (98)</td>
</tr>
<tr>
<td>HB101</td>
<td>ara14 galK2 hsdS20(r_k^m,m^*) lacY1 leuB6 mtl-1 proA2 recA13 rpsL20 supE44 thi-1 xyl-5 $\Delta(mcrC-mrr)$ F$^-$</td>
<td>Boyer and Roulland-Dussoix (42)</td>
</tr>
<tr>
<td>MC1061</td>
<td>araD139 galK galU hsdR2(r_k^m,m^*) rpsL thi-1 $\Delta(ara-leu)7696\Delta lacX74$</td>
<td>Cassadaban and Cohen (49)</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. (284)</td>
</tr>
<tr>
<td>SB1003</td>
<td>rif-10</td>
<td>Yen and Marrs (317)</td>
</tr>
</tbody>
</table>
Table 2. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant Characteristics</th>
<th>Reference or Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322Ω</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, Tc&lt;sup&gt;R&lt;/sup&gt;, Sm&lt;sup&gt;R&lt;/sup&gt;, Spc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Omega cartridge cloned from pHP45Ω (238) into EcoRI site of pBR322.</td>
</tr>
<tr>
<td>pCM4</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, Tc&lt;sup&gt;R&lt;/sup&gt;, cat*</td>
<td>Close and Rodriguez (54)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>mob&lt;sup&gt;+&lt;/sup&gt;, tra&lt;sup&gt;+&lt;/sup&gt;, Kn&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Ditta et al. (71)</td>
</tr>
<tr>
<td>pRK404</td>
<td>mob&lt;sup&gt;+&lt;/sup&gt;, P&lt;sub&gt;lac&lt;/sub&gt;, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Ditta et al. (70)</td>
</tr>
<tr>
<td>pUC18</td>
<td>P&lt;sub&gt;lac&lt;/sub&gt;, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Vieria and Messing (296)</td>
</tr>
<tr>
<td>pUC19</td>
<td>P&lt;sub&gt;lac&lt;/sub&gt;, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Vieria and Messing (296)</td>
</tr>
<tr>
<td>pCAP145</td>
<td>Tc&lt;sup&gt;R&lt;/sup&gt;, Sm&lt;sup&gt;R&lt;/sup&gt;, Spc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Omega cartridge cloned from pBR322Ω into the HindIII site of pRK404 (This study)</td>
</tr>
<tr>
<td>pCAP153</td>
<td>Tc&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;, cat*</td>
<td>cat gene cloned into pRK404 (121)</td>
</tr>
<tr>
<td>pCAP154</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>4 kb <em>R. capsulatus</em> genomic BamHI fragment in pUC18 (This study)</td>
</tr>
<tr>
<td>pCAP155</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>2 kb <em>R. capsulatus</em> genomic EcoRI fragment in pUC18 (121)</td>
</tr>
<tr>
<td>pCAP167</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>2.1 kb EcoRI fragment removed from pCAP154 (This study)</td>
</tr>
<tr>
<td>pCAP168</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>0.5 kb HincII-EcoRI fragment of pCAP155 in pUC18 (This study)</td>
</tr>
<tr>
<td>pCAP169</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>3 kb HincII fragment removed from pCAP154 (This study)</td>
</tr>
<tr>
<td>pCAP171</td>
<td>mob&lt;sup&gt;+&lt;/sup&gt;, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>1 kb HindIII-BamHI fragment of pCAP169 cloned into pRK404 (This study)</td>
</tr>
</tbody>
</table>

(Table 2 continued)
<table>
<thead>
<tr>
<th>pCAP172</th>
<th>$mob^{+}$, $Tc^{R}$</th>
<th>1.3 kb PstI-BamHI fragment of pCAP154 cloned into pRK404 (This study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCAP175</td>
<td>$Ap^{R}$</td>
<td>1.3 kb PstI-BamHI fragment of pCAP154 cloned into pUC19 (This study)</td>
</tr>
<tr>
<td>pCAP176</td>
<td>$Ap^{R}$, $Cm^{R}$, $hemC::cat^{*}$</td>
<td>Chloramphenicol gene from pCM4 cloned into BclI site of pCAP175 (This study)</td>
</tr>
<tr>
<td>pCAP178</td>
<td>$Ap^{R}$</td>
<td>1 kb HindIII-BamHI fragment of pCAP169 cloned into pUC19 (This study)</td>
</tr>
<tr>
<td>pCAP182</td>
<td>$mob^{+}$, $Tc^{R}$, $Sm^{R}$, $hemC::cat^{*}$</td>
<td>2.3 kb PstI-BamHI fragment of pCAP176 containing $hemC::cat^{*}$ cloned into pCAP145 (This study)</td>
</tr>
</tbody>
</table>
required, a 10 ml overnight culture was subcultured into 100 ml of media in a 100 ml graduated cylinder at an initial Klett (red filter) value of approximately ten and incubated in a 37°C water bath for four generations under high or low oxygen tension. Low oxygen tension was obtained by sparging the culture with a mixture of 3% oxygen, 5% carbon dioxide, and 92% nitrogen. High oxygen tension was obtained by sparging the culture with compressed air supplemented with 3% oxygen, resulting in an initial oxygen tension of 23%.

**Protein Determination**

The protein concentrations in cell extracts used for various enzyme assays were determined using the BioRad Protein assay (BioRad Laboratories, Hercules, CA) which is based on the method of Bradford (43). Purified bovine serum albumin (BSA) was used to create a protein standard curve with concentrations between 10 and 50 μg per 100 μl total volume. The cell extract to be tested for protein concentration was also diluted in a 100 μl total reaction volume in a separate tube. Five milliliters of a one-fifth dilution of BioRad Dye Concentrate was added to each tube and the mixture was vortexed. After five minutes incubation at room temperature the absorbance at 595 nm was recorded. The protein concentration of the cell extract was determined by comparison to the BSA standard curve.

**Porphobilinogen Deaminase Assay**

*R. capsulatus* was grown in 200 ml of PYE until late log phase then harvested by centrifugation in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Sorvall, Inc., Newton, CT) at 5,900 x g in the Sorvall GSA rotor for
ten minutes. The cell pellet was resuspended in 4 ml of 0.1 M Tris-Cl, pH 8.0 and sonicated four to six times for 20 seconds with at least 30 seconds cooling between bursts using the Sonicator Ultrasonic Processor, Model W-200 fitted with the Sonicator Ultrasonic Convertor, Model C-2 (Heat Systems-Ultrasonics, Inc., Farmingdale, NY). The sonicated cells were then centrifuged in the Sorvall SS34 rotor at 27,000 x g for 20 minutes. The cleared cell extract was stored at 4°C for two hours before assaying and could be stored at -20°C for extended periods of time. Porphobilinogen deaminase was assayed either by following the disappearance of the substrate porphobilinogen (PBG) or by measuring the formation of uroporphyrinogen I (Uro-I). The assay mixture contained 50 mM Tris-Cl, pH 8.0, 0.2 mM porphobilinogen (Porphyrin Products, Logan, UT) and enzyme, in a total volume of 1 ml. The reaction was incubated at 37°C. At intervals aliquots were removed for monitoring PBG usage and Uro-I formation. To assay the disappearance of PBG, aliquots of 100 µl were transferred into 1.4 ml of 10% trichloroacetic acid. One and a half milliliters of Modified Ehrlich's reagent (193) was added and the absorbance measured at 554 nm in a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) after incubation at room temperature for ten minutes. To assay the Uro-I formed, aliquots of 200 µl were pipetted into 2 ml of 1N HCl containing 0.01% I₂. After 20 minutes incubation in the dark to oxidize the uroporphyrinogen I to uroporphyrin I, the iodine was reduced by adding 1.8 ml of 0.1% Na₂S₂O₅. The fluorescence was then measured using an excitation wavelength of 403 nm and an emission wavelength of 596 nm in a Perkin-
Elmer LS-3 Fluorescence Spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). The fluorescence value was used to determine the uroporphyrin I concentration by comparison to a standard curve prepared using uroporphyrin I standards (Porphyrin Products, Logan, UT). This assay procedure is based upon the procedure used by Jordan and Shemin (137).

When the porphobilinogen deaminase was assayed under the same conditions as the cells were grown, a different procedure was used to make the extracts. The \textit{R. capsulatus} cultures were grown aerobically under the defined high oxygen tension. The \textit{R. capsulatus} cultures were grown anaerobically by photosynthesis or anaerobic respiration as mentioned above. The cells were pelleted by centrifugation in a bench-top centrifuge for one hour. The supernatant of the aerobic cultures was decanted. The supernatant of the anaerobic cultures was sucked from the anaerobic Vacutainer tubes with a syringe, while adding argon gas. The cell pellets were resuspended in 1 ml of degassed 0.1 M Tris-Cl, pH 8.0, added using a syringe. To break open the cells, 20 \textmu l of a 10 mg per ml lysozyme solution was added using a Hamilton syringe and the suspension was swirled gently for 15 minutes at room temperature. These aerobic and anaerobic extracts were then assayed as described earlier. The anaerobic porphobilinogen deaminase assays were done in 3 ml Vacutainer tubes. The anaerobic assay mixture was degassed before the enzyme was added. The anaerobic enzyme solution was added using a Hamilton syringe.
Isolation and Purification of Porphobilinogen Deaminase

All purification procedures were carried out at 4°C and all buffers used contained 1 mM dithiotreitol (DTT). To purify the enzyme porphobilinogen deaminase from *R. capsulatus*, eight liters of PAS100 were grown in PYE medium. The cells were harvested at late-log phase by centrifuging the cell suspensions at 5,900 x g in the Sorvall GSA rotor for ten minutes. The cells were resuspended in cold 0.1 M Tris-Cl, pH 7.5 using 10 ml per liter of cell culture. The cellular suspension was sonicated 20 times (until the solution cleared) for 20 seconds with 30 second cooling periods using the Sonicator Ultrasonic Processor, Model W-200 (Heat Systems-Ultrasonics, Inc., Farmingdale, NY). The sonicated suspension was spun at 27,000 x g for 20 minutes in the Sorvall SS34 rotor and the supernatant collected. This crude *R. capsulatus* extract was frozen overnight at -20°C.

(NH₄)₂SO₄ Fractionation and Heat Treatment

The crude extract was thawed and a saturated (NH₄)₂SO₄ solution was slowly added to 45% overall saturation (818 ml of saturated (NH₄)₂SO₄ per liter of extract). The extract was stirred slowly for 30 minutes then centrifuged for 20 minutes at 27,000 x g in the Sorvall SS34 rotor. The supernatant, containing the porphobilinogen deaminase, was carefully decanted and a further quantity of the saturated (NH₄)₂SO₄ solution was added to a saturation of 60% (375 ml of saturated (NH₄)₂SO₄ per liter of extract). The extract was again stirred slowly for 30 minutes and then centrifuged, as before. The resulting pellet was resuspended in 10 ml of 20 mM Tris-Cl, pH 8.0, 1 mM DTT,
and 1 mM EDTA. The resulting suspension was then dialyzed for eight hours, against two changes of two liters of the same buffer to remove all traces of ammonium sulfate. The dialyzed suspension was then heated with gentle stirring at 60°C in a water bath for ten minutes. After heating, the extract was cooled in ice and centrifuged at 27,000 x g in the Sorvall SS34 rotor for one hour to remove all denatured proteins.

**Anion-Exchange Chromatography**

Using the BioRad Econo Chromatography Control System (Hercules, CA), the enzyme solution was loaded onto a 244 cm³ (46 cm x 2.6 cm) DEAE-Cellulose chromatography column equilibrated in the dialysis buffer at a flow rate of 1 ml per minute. The column was washed with 100 ml of the same buffer and the protein was eluted using a linear gradient up to 1 M KCl. The two milliliter fractions, collected during the entire column run, were assayed for porphobilinogen deaminase activity. The enzyme was eluted at 0.15 M KCl and the active fractions were pooled. The resulting solution was dialyzed overnight in two changes of two liters of 10 mM KPO₄, pH 7.2 buffer to remove all of the salt.

**Hydroxylapatite Chromatography**

The dialyzed DEAE-cellulose activity was applied onto a small 21 cm³ (12 cm x 1.5 cm) hydroxylapatite column equilibrated in 10 mM KPO₄, pH 6.8. At a flow rate of 0.5 ml per minute, the column was washed with 50 ml of the same buffer and eluted with a linear gradient up to 0.5 M KPO₄, pH 6.8. One milliliter fractions were collected and assayed for activity. The fractions

---

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
collected during the column wash contained all the deaminase activity. These
fractions were pooled and dialyzed against two liters of 20 mM Tris-Cl, pH 8.0
buffer overnight. The dialyzed hydroxylapatite solution was concentrated to 10
ml by covering the dialysis sack with Aquacide II (Calbiochem, LaJolla, CA) for
four hours.

Gel-Filtration Chromatography

The concentrated enzyme was loaded onto a 493 cm$^3$ (93 cm x 1.3 cm)
column of Sephadex G-75, previously equilibrated with 20 mM Tris-Cl, pH 8.0,
1 mM DTT, and 1 mM EDTA, at a flow rate of 0.6 ml per minute. Fractions (3
ml) were collected and assayed for porphobilinogen deaminase activity. The
active fractions were pooled together and concentrated by placing the resulting
solution into a dialysis sack and covering it with Aquacide II for five hours. The
resulting enzyme solution was stabilized by the addition of glycerol to a final
concentration of 20% (v/v).

Molecular Weight Determination by Electrophoresis

Denaturing Conditions

Proteins were routinely analyzed and their molecular weights
determined using polyacrylamide gel electrophoresis with sodium dodecyl
sulfate (SDS-PAGE). The procedure used was a discontinuous SDS-PAGE
system containing tricine that optimally separated proteins in the range from 1
to 100 kDa as described by Schägger (257). The composition of the gels is
defined by the total concentration (%) of both monomers (acrylamide and
bisacrylamide), denoted T, and the percent concentration of the crosslinker
relative to the total concentration, denoted C. A 10% T, 3% C gel was used for the separating gel, while overlaid by a 2 cm thick 4% T, 3% C stacking gel. The SDS molecular weight markers (Sigma, St. Louis, MO) of α-lactalbumin (14.2 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), and phosphorylase B (97.4 kDa) were loaded on every gel. All protein samples, ~2 µg per protein band, were incubated for one minute at 100°C in 4% SDS, 12% glycerol (w/v), 50 mM Tris-Cl, pH 6.8, 2% β-mercaptoethanol (v/v), and 0.01% bromophenol blue before loading onto the gel. Gels were run at 110 V (constant voltage) until the marker dye bromophenol blue reached the bottom of the gel. Gels were stained in a solution containing 50% methanol, 10% acetic acid and 0.25% Coomassie Brilliant Blue R for 30 to 60 minutes. A complete background destaining of the gels was achieved by shaking the gels in several changes of 50% methanol and 10% acetic acid.

**Nondenaturing Conditions**

To examine the proteins under conditions where characteristics of the native protein were retained, proteins were examined by electrophoresis in nondenaturing systems. The procedure which involved modification of the methods of Bryan (44) and Davis (67) allowed determination of several characteristics, most notably molecular weight determination of homogenous proteins. For non-homogenous proteins, charge-isomers or molecular weight isomers could be examined. To examine the molecular weight of a given
protein it was first separated by electrophoresis on a series of continuous gels equilibrated to pH 8.0 with Tris-Citrate containing from five to nine percent polyacrylamide concentrations. In each gel a mixture of non-denaturing molecular weight markers was also loaded. The markers used were α-lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa—characterized by three closely spaced bands (charge isomers)), chicken egg albumin (45 kDa—characterized by two closely spaced bands (charge isomers)), and bovine serum albumin that exhibits a monomer (66 kDa) and a dimer (132 kDa) band. Before the gel was loaded, all protein samples (~0.02 mg per ml) were mixed with an equal volume of 2X Tris-Citrate Sample buffer (40 mM Tris-Citrate, pH 8.0, 20% Glycerol, 0.004% Bromophenol Blue). Each gel was run at 110 V (constant voltage) at room temperature until the marker dye (Bromophenol Blue) reached 1 cm from the anodic end of the gel. To visualize the protein bands, the gel was stained by shaking it in a solution of 40% methanol, 7% acetic acid, and 0.25% Coomassie Brilliant Blue R for 30 minutes. This gel was subsequently decolorized using several changes of a 40% methanol and 7% acetic acid solution. To localize porphobilinogen deaminase activity within the gel, the lane containing PBG deaminase was incubated with 10 ml of 0.1 mg/ml PBG for 30 minutes at 37°C with shaking. The hydroxymethylbilane formed was cyclized into Uro-I by adding 10 ml of 1N HCl with 0.01% Iodine. After 20 minutes incubation in the dark, to oxidize the uroporphyrinogen to uroporphyrin, 0.1% Na₂S₂O₅ was added until the yellow iodine color
disappeared. The resulting fluorescent bands were viewed using a short wave UV light box (Fotodyne, Inc., New Berlin, WI).

**Isoelectric Focusing**

Proteins were separated by isoelectric focusing, on narrow range pH gradients, in tube gels. The tube gels contained an acrylamide solution of 7.5% T and 0.8% C with a mixture of 3/10 and 3/5 ampholytes yielding optimal separation between pH 3 and pH 5. Using riboflavin as the catalyst, polymerization was stimulated by placing the tubes in front of a light bank. The gels were run in a tube gel electrophoresis unit with 20 mM NaOH as the cathode solution and 10 mM phosphoric acid as the anode solution. Protein samples and isoelectric focusing standards were loaded under a layer of 5% glycerol in separate tube gels. The isoelectric focusing standard mixture (Sigma Chemical Co., St. Louis, MO) contained amylglucosidase (3.6), glucose oxidase (4.2), trypsin inhibitor (4.6), β-lactoglobulin (5.1), carbonic anhydrase (5.4, 5.9 and 6.6), and the anionic dye methyl red. The tube gel electrophoresis unit was run at 4°C at 200 V (constant voltage) for 18 to 20 hours, until the marker dye methyl red quit moving. Gels were removed from the tubes by rimming the top and bottom of each gel with a stream of water. Those gels containing porphobilinogen deaminase could be assayed for uroporphyrin I formation as done on nondenaturing gels. To visualize the protein bands the gels were fixed in 20 ml of 10% trichloracetic acid for four hours. The gels were then stained for protein with isoelectric focusing stain containing 30% isopropanol, 10% acetic acid, 0.04% Coomassie Blue R-250.
and 0.5% CuSO₄. The gels were then destained with a solution of 30% isopropanol, 10% acetic acid, and 0.5% CuSO₄.

**Two-Dimensional Gel Electrophoresis**

Proteins were separated using the two-dimensional gel electrophoresis system developed by O'Farrell (219). The first dimension was run in a tube gel as described in the isoelectric focusing section. After electrophoresis the isoelectric focusing tube gel was incubated in two changes of SDS transfer buffer (0.0625 M Tris-Cl, pH 6.8, 2% SDS, 10% Glycerol, and 5% β-mercaptoethanol) for 30 minutes. A SDS-PAGE gel prepared as described earlier was prerun at 90 mA (constant amperage) with the upper buffer containing 0.1 mM thioglycolic acid. The tube gel was then adhered to the top of the SDS-PAGE gel using 1% agarose containing 0.1% SDS. A sample of the SDS molecular weight markers described earlier were loaded into a well made in the agarose. The proteins were then separated in the second dimension at 90 mA (constant amperage) until the marker dye bromophenol blue migrated to the end of the gel.

**Protein Blotting**

Proteins separated by gel electrophoresis were electroblotted to Immobilon-P® (Millipore, Bedford, MA), a microporous polyvinylidene fluoride (PVDF) membrane enhanced for protein sequencing, using the procedure described by Matsudaira (192). The Immobilon-P® membrane was first wet in 100% methanol for a few seconds then transferred into electrotransfer buffer (25 mM Tris, 192 mM glycine, pH 8.3) for 10 to 15 minutes. After
electrophoresis, the separating gel was soaked in electrotransfer buffer for ten minutes. The Immobilon-P \( ^{50} \) membrane was placed next to the gel taking care to remove all air bubbles between the gel and membrane. The gel with membrane was then placed between blotter paper and sponges soaked in electrotransfer buffer in the blotting cassette. The loaded blotting cassette was placed into the TE50X Transfor Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA) filled with six liters of electrotransfer buffer. Transfer was done at 0.88 mA (constant amperage) for four hours. When finished the membrane was stained by shaking in 1% acid Coomassie stain (50% methanol, 1% acetic acid, 0.125% Coomassie Blue R-250) for exactly one minute. The membrane was destained in several changes of 12% isopropanol. To remove all salts and other contaminants that may inhibit sequencing, the membrane was rinsed in several changes of deionized water.

**Plasmid DNA Isolation**

Plasmid DNA was isolated from 1 to 5 ml of an overnight culture using the QIAprep Spin Plasmid Kit (Quiagen Inc., Chatsworth, CA). The plasmid extraction procedure is based on the modified alkaline lysis method of Birnboim and Doly (34) and on the adsorption of DNA onto silica in the presence of high salt (297). The procedure consisted of: 1) Preparing a clear lysate, 2) Adsorbing the DNA to a silica-gel membrane in the presence of high salt, 3) Washing away the salt , and 4) Elution of plasmid DNA with 50 to 100 μl of sterile H\(_2\)O. The concentration of DNA in each sample was ascertained by measuring the absorbance at 260 nm using a Lambda 3B UV/VIS
spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). At the wavelength of 260 nm, one optical density (O.D.) unit equals 50 µg DNA per ml. All plasmid DNA solutions were stored at -20°C.

**Restriction Enzyme Digestion**

Restriction digestion of DNA was routinely performed by mixing 1 µg of DNA in a 10 µl reaction containing 1 µl (5 - 10 units) of enzyme and 1 µl of the appropriate 10X reaction buffer from New England Biolabs (Beverly, MA). The mixture was incubated one to two hours in a dry-bath incubator at the optimal enzyme incubation temperature.

**DNA Ligation**

The DNA was cleaned by precipitation with one-tenth volume of 3 M sodium acetate, pH 4.8 and three volumes of cold 95% ethanol. After incubation at -80°C for twenty minutes the sample was centrifuged at maximum speed for four minutes in a microcentrifuge (Eppendorf, Madison, WI). The supernatant was removed and the pellet was washed with cold 70% ethanol. After incubation at -80°C for twenty minutes, the sample was centrifuged for four minutes. The pellet was dried in a vacuum desiccator and suspended in sterile deionized H₂O. Routine DNA ligations consisted of 1 to 5 µg of DNA fragments containing compatible ends in a 10 µl reaction along with 1 µl of 10X T4 DNA ligase buffer and 1 µl (400U) of T4 DNA ligase (New England Biolabs, Beverly, MA). The reactions were incubated overnight at 16°C.
**Agarose Gel Electrophoresis**

DNA was routinely separated through 0.7% 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, pH 8.0, 2 mM EDTA, pH 8.0) was diluted to 1X to serve as a solvent for gel preparation and as electrophoresis buffer. DNA samples were prepared by adding a one-tenth volume of 10X loading buffer (0.25% bromothymol blue, 40% sucrose). Electrophoresis of the gels was carried out at 80 volts for up to two hours. Upon completion of electrophoresis, the DNA was visualized using a short wave UV light box (Fotodyne, Inc., New Berlin, WI). When necessary gels were photographed with a Polaroid camera. The size of the DNA fragments was estimated by comparison to lambda DNA digested with HindIII, which served as a molecular length marker.

**DNA Recovery from Agarose Gels**

DNA was routinely recovered from agarose gels using the GENECLEAN II Kit (BIO 101, Inc., La Jolla, CA). Many of the principles of the GENECLEAN procedure are based on the data of Vogelstein and Gillespie (296).

**Electroporation**

_E. coli_ cells were grown in L-broth until mid-log phase (O.D.₆₆₀ = 0.5-1.0) then centrifuged at 5,900 x g in a Sorvall SS34 rotor for eight minutes. The cell pellet was washed twice with 10 ml of cold sterile water. In a microcentrifuge tube the pellet was washed five consecutive times with 1 ml cold sterile 10% glycerol. The cell pellet was resuspended in a final volume of 200 μl of cold...
sterile 10% glycerol. Forty microliters of the freshly prepared cells and up to 2 
µg of DNA were transferred to a sterile 0.2 cm electroporation cuvette and 
mixed. The suspension was pulsed using the BioRad Gene Pulser (BioRad 
Laboratories, Hercules, CA) set at 2.5 kV, 25 µF, and 200 ohms. Two milliliters 
of L-broth was immediately added to the cuvette and the suspension was 
transferred to a sterile test tube. The cell suspension was incubated for one 
hour at 37°C in a roller drum and 0.1 ml was plated onto the appropriate 
selective medium.

**Triparental matings**

*E. coli* strain MC1061, containing the donor plasmid and the *E. coli* strain 
HB101, containing the helper plasmid pRK2013, were grown overnight in 10 ml 
of L-broth supplemented with the appropriate antibiotics at 37°C with slow 
shaking. *R. capsulatus* recipient strains were grown overnight in 10 ml of PYE 
at 37°C without shaking. One milliliter of the *R. capsulatus* recipient culture 
and 0.2 ml of each *E. coli* culture were added to a microcentrifuge tube and 
centrifuged. After removal of the supernatant, the cells were gently 
resuspended in the remaining liquid and transferred to a nitrocellulose disc 
placed on the surface of a PYE plate. The plate was incubated at room 
temperature for four hours to overnight after which the nitrocellulose disc was 
transferred to a PYE plate supplemented with the appropriate antibiotics. The 
cells were spread with 0.1 ml of PYE and the plate was incubated at 37°C for 
two to four days.
**DNA Sequencing**

The DNA sequencing was performed using the New England Biolabs Circumvent™ Thermal Cycle Dideoxy DNA Sequencing Kit (New England Biolabs, Beverly, MA) which is based on the dideoxynucleotide chain termination method of Sanger et al. (253). The dideoxy terminated chains were labeled by the incorporation of \( [\alpha^{35}\text{S}] \text{dATP} \). Sequencing reactions were subjected to 25 cycles of incubation in a Hybaid Thermal Reactor (National Labnet Laboratories, Woodbridge, NJ). Following the initial denaturation step at 95°C for five minutes, each cycle was composed of denaturation at 95°C for one minute, annealing at 55°C for one minute, and chain extension at 72°C for one minute. The double stranded plasmid templates were extended using the thermostable VentR® (exo) DNA polymerase from *Thermococcus litoralis*. The DNA fragments generated were denatured at 80°C for five minutes and separated by electrophoresis through a 6.0% polyacrylamide-urea gel at 59°C using the Polar Bear Isothermal Electrophoresis system (Owl Scientific, Woburn, NJ). Following electrophoresis, the gel was fixed in 20% ethanol-10% acetic acid then adhered to 3M Whatman paper and dried for three to four hours at 80°C in a BioRad Gel Drier, Model 483 (BioRad Laboratories, Hercules, CA). The dried gel was exposed to Kodak BioMax X-ray film (Eastman Kodak Co., Rochester, NY) for two to three days.

**Chloramphenicol Acetyltransferase Assay**

The levels of chloramphenicol acetyltransferase were determined using the procedure described by Shaw (266). *R. capsulatus* was grown in 100 ml...
PYE containing the appropriate antibiotics until the density reached 75 to 100 Kletts. The cells were harvested then resuspended in 2 ml of 50 mM Tris-Cl, pH 7.8. The cell suspension was then sonicated five times for 20 seconds with 30 second cooling periods using the Sonicator Ultrasonic Processor, Model W-200 (Heat Systems-Ultrasoundics, Inc., Farmingdale, NY). After 20 minutes of centrifugation at 27,000 x g in the Sorvall SS-34 rotor all the cell debris was removed. Two hundred and fifty microliters of crude extract were mixed with 250 μl of 4 mg/ml 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 1 M Tris-Cl, pH 7.8, 50 μl 5mM acetyl-CoA, and 1.95 ml water. One milliliter of the reaction mixture was added to two water-jacketed cuvettes. The cuvettes were placed in the Lambda 3B UV/VIS spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) and blanked. After the cuvettes containing the enzyme and the reaction mixture had been allowed to equilibrate at 37°C, the reaction was started by adding 20 μl of 5 mM chloramphenicol to the sample cuvette. The change in absorbance at 412 nm was monitored using a chart recorder.
RESULTS

Purification of Porphobilinogen Deaminase

Table 3 summarizes the results of a typical purification of porphobilinogen deaminase from eight liters of *R. capsulatus* cell culture. At each purification step there was only one peak that could consume porphobilinogen. PBG usage co-purified with Uro-I forming activity, verifying that we were purifying porphobilinogen deaminase. The PBG usage activity was purified approximately 140-fold with 25% overall yield, while the Uro-I forming activity was purified approximately 200-fold with a 36% overall yield.

Molecular Weight Determination of Porphobilinogen Deaminase

Nondenaturing Gel Electrophoresis

The porphobilinogen deaminase and nondenaturing molecular weight standards were separated by electrophoresis on a series of nondenaturing gels containing from five to nine percent polyacrylamide. All protein bands containing PBG deaminase activity were localized in each gel (Figure 2) using the assay protocol described in the Materials and Methods. To determine the molecular weight of the *R. capsulatus* PBG deaminase, the relative mobility (R,) of the nondenaturing molecular weight standards and the PBG deaminase was determined for each gel. The R, was determined by dividing the protein migration distance by the migration distance of the marker dye. For each polyacrylamide concentration, the 100 [log (R, x 100)] value was determined for the nondenaturing molecular weight standards and the PBG deaminase. The resulting series of 100 [log (R, x 100)] values were plotted against the percent

50
Table 3. Purification of porphobilinogen deaminase.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ml</th>
<th>Total Protein (mg)</th>
<th>PBG Used Yield (%)</th>
<th>PBG Specific Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PBG Purification Factor</th>
<th>Uro-I Formed Yield (%)</th>
<th>Uro-I Specific Activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Uro-I Purification Factor</th>
<th>Specific Activity Ratio&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>102</td>
<td>1050</td>
<td>100</td>
<td>193</td>
<td>1.00</td>
<td>100</td>
<td>16.5</td>
<td>1.00</td>
<td>11.7</td>
</tr>
<tr>
<td>45-60% (NH₄)₂SO₄</td>
<td>21.5</td>
<td>323</td>
<td>73.5</td>
<td>465</td>
<td>2.41</td>
<td>98.7</td>
<td>53.0</td>
<td>3.20</td>
<td>9.11</td>
</tr>
<tr>
<td>45-60% Heated</td>
<td>20.0</td>
<td>276</td>
<td>55.3</td>
<td>428</td>
<td>2.22</td>
<td>83.2</td>
<td>52.2</td>
<td>3.16</td>
<td>9.54</td>
</tr>
<tr>
<td>DEAE-Cellulose Peak</td>
<td>25.0</td>
<td>60.0</td>
<td>43.3</td>
<td>1470</td>
<td>7.63</td>
<td>69.7</td>
<td>201</td>
<td>12.2</td>
<td>7.27</td>
</tr>
<tr>
<td>Hydroxylapatite Peak</td>
<td>12.5</td>
<td>13.8</td>
<td>32.0</td>
<td>4730</td>
<td>24.5</td>
<td>53.6</td>
<td>675</td>
<td>40.9</td>
<td>7.63</td>
</tr>
<tr>
<td>Sephadex G-75 Peak</td>
<td>5.00</td>
<td>1.93</td>
<td>25.2</td>
<td>26700</td>
<td>138</td>
<td>36.4</td>
<td>3280</td>
<td>199</td>
<td>8.63</td>
</tr>
</tbody>
</table>

<sup>a</sup> PBG used specific activity is expressed as nanomoles of porphobilinogen used per hour per mg of protein.

<sup>b</sup> Uro-I formed specific activity is expressed as nanomoles of uroporphyrin I formed per hour per mg of protein.

<sup>c</sup> The specific activity ratio is the ratio of the PBG used specific activity to the Uro-I formed specific activity.
Figure 2. Analysis of the *R. capsulatus* porphobilinogen deaminase by nondenaturing polyacrylamide gel electrophoresis. Lane 1 was stained for protein with Coomassie Brilliant Blue. Lane 2 was incubated with porphobilinogen. The resulting uroporphyrinogen band was oxidized by the addition of 0.01% I₂ in 1N HCl. The uroporphyrin I band formed was viewed using a short wave UV light box.
gel concentration (Figure 3). The absolute value of the slopes obtained from this graph were plotted against the known molecular weights of the non-denaturing molecular weight standards on two cycle log-log paper (Figure 4). The molecular weight of the non-denatured *R. capsulatus* porphobilinogen deaminase was determined from this graph to be 36,000 ± 2,000.

**SDS-PAGE**

With each purification step the fractions containing porphobilinogen deaminase activity were identified and pooled. The pooled fractions were analyzed by SDS-PAGE. After staining with Coomassie Brilliant Blue, the G-75 purified sample revealed a major protein band (Figure 5) with a mobility similar to that of glyceraldehyde-3-phosphate dehydrogenase (*M*$_r$, 36 kDa). A logarithmic plot of the molecular weights of the marker proteins against mobility yielded a straight line (results not shown). From this plot the molecular weight of the major protein band of the G-75 sample was calculated to be 35,000 ± 1,000. This result is very similar to the value obtained for the non-denatured *R. capsulatus* PBG deaminase. This indicates that the major protein band on SDS-PAGE is PBG deaminase. The data obtained from the SDS-PAGE and the non-denaturing gel analysis indicates that the *R. capsulatus* PBG deaminase is a monomeric protein. This is consistent with observations on other purified PBG deaminases, which are also reported to be monomers with molecular weights ranging from 34,000 to 44,000 (271).
Figure 3. Mobility of proteins in nondenaturing gels. The nondenaturing molecular weight standards were Bovine Serum Albumin (dimer, ▲, and monomer, ▼), Chicken Egg Albumin (■), Carbonic Anhydrase (♦), and α-lactalbumin (●). The PBG deaminase is represented by the symbol ○.
Figure 4. Molecular weight determination of the nondenatured *R. capsulatus* porphobilinogen deaminase. The solid black line is the regression line and the dashed lines display the 95% confidence interval. The square represents the porphobilinogen deaminase.
Figure 5. Analysis of the *R. capsulatus* porphobilinogen deaminase by SDS-PAGE. Lane 1: Monomeric molecular weight standards. Lane 2: Crude Extract. Lane 3: DEAE-Cellulose column peak. Lane 4: Hydroxylapatite column peak. Lane 5: Sephadex G-75 column peak. The arrow indicates porphobilinogen deaminase.
Determination of Optimum pH and Isoelectric Point

The *R. capsulatus* porphobilinogen deaminase exhibited a pH optimum at pH 8.0 (Figure 6) in 50 mM Tris buffer. A similar curve was obtained with phosphate buffer except the optimal activity was at pH 7.6. All purified porphobilinogen deaminases have optimal activities between pH 7.4 and pH 8.2 (4, 61, 131, 270). Isoelectric focusing of the partially purified deaminase from the Sephadex G-75 step revealed the presence of five bands. The band determined to contain porphobilinogen deaminase activity was found at pl 4.5. This value is similar to those reported for the *R. sphaeroides* enzyme (pl 4.46) (66) and the *E. coli* enzyme (pl 4.5) (138).

Kinetic Analysis of Porphobilinogen Deaminase

Using substrate concentrations up to 250 μM, the $K_m$ value for the substrate porphobilinogen was 94 μM as measured by its consumption and the $V_{max}$ was approximately 7.6 nmoles porphobilinogen consumed per minute. The $K_m$ value when measuring uroporphyrin formation was 81 μM and the $V_{max}$ was 1.0 nmole of uroporphyrin I formed per minute (Figure 7). Our $K_m$ value for uroporphyrin formation is similar to those reported for the deaminases from *C. regularis* (85 μM) (270), *E. gracilis* (70 μM) (308), *Scenedesmus obliquus* (79 μM) (143), and wheat germ (70 μM) (91).

Stoichiometry

The stoichiometry of the reaction was estimated by measuring the ratio of porphobilinogen consumption and the uroporphyrin I formation. Crude extracts of *R. capsulatus* not containing added reducing agents displayed
Figure 6. Effects of pH on the *R. capsulatus* porphobilinogen deaminase activity.
Figure 7. Kinetic analysis of the *R. capsulatus* porphobilinogen deaminase. The solid black line is the regression line and the dashed lines display the 95% confidence interval.
ratios of approximately 20 porphobilinogen molecules used to form one uroporphyrin. During the purification of porphobilinogen deaminase, the mild reducing agent DTT was added, which had the effect of lowering the stoichiometric ratio to approximately nine porphobilinogens used to form one uroporphyrin (Table 3). The theoretical stoichiometry, one mole of uroporphyrin from four moles porphobilinogen, was only achieved when the strong reducing agent sodium borohydride (5 mM) was added to enzymatic assays (Table 4). Reducing agents stimulated both of the activities of the purified enzyme, but the most dramatic changes were seen in uroporphyrin I formation.

Inhibitors

The effects of various compounds on enzymatic activity are shown in Table 4. Metal-chelating agents had no effect on the enzymatic activity. Sulfhydryl reactive reagents such as mercuric chloride and N-ethylmaleimide showed strong inhibition. N-ethylmaleimide inhibited only Uro-I formation, while mercuric chloride showed strong inhibition of both PBG used and Uro-I formation. Addition of ammonia or a derivative such as hydroxylamine, reduced the Uro-I production but had little effect on PBG usage.

Effects of Oxygen on Porphobilinogen Deaminase Activity

Two *R. capsulatus* wild-type strains, PAS100 and SB1003, were grown under aerobic conditions, photosynthetic conditions, and by anaerobic respiration. In both strains grown and assayed aerobically, the ratio of porphobilinogen used to uroporphyrin I formed was $23.5 \pm 5.4:1$. The
Table 4. Effects of additives on the *R. capsulatus* porphobilinogen deaminase.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc. (mM)</th>
<th>PBG Used (% of control)</th>
<th>Uro-I Formed (% of Control)</th>
<th>Ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>---</td>
<td>100</td>
<td>100</td>
<td>9:1</td>
</tr>
<tr>
<td>Sodium Borohydride</td>
<td>5</td>
<td>120±12</td>
<td>660±47</td>
<td>4:1</td>
</tr>
<tr>
<td>EDTA</td>
<td>25</td>
<td>107±4</td>
<td>107±4</td>
<td>9:1</td>
</tr>
<tr>
<td>Mercuric Chloride</td>
<td>0.05</td>
<td>22±4</td>
<td>20±4</td>
<td>9:1</td>
</tr>
<tr>
<td><em>N</em>-Ethylmaleimide</td>
<td>10</td>
<td>133±5</td>
<td>30±3</td>
<td>23:1</td>
</tr>
<tr>
<td>Ammonium Acetate</td>
<td>100</td>
<td>113±3</td>
<td>84±5</td>
<td>11:1</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>100</td>
<td>115±16</td>
<td>11±2</td>
<td>80:1</td>
</tr>
</tbody>
</table>

* The ratio of PBG used specific activity to the Uro-I formed specific activity.
photosynthetically-grown and the anaerobic respiration-grown cultures of both wild-types were assayed under strict anaerobic conditions. These anaerobic porphobilinogen deaminases displayed a ratio of $13.2 \pm 2.5$ porphobilinogen used to one uroporphyrin I formed. This is a 2-fold difference between aerobic and anaerobic cultures.

N-Terminal Sequence Analysis

The *R. capsulatus* porphobilinogen deaminase was separated using two-dimensional electrophoresis (Figure 8). The resulting gel was blotted to an Immobilon-$P^S$ membrane. The protein spot of the deaminase was cut out of the membrane and the first twenty amino acids were determined by the Baylor Core Sequencing Facility. The first twenty amino acids of the *R. capsulatus* porphobilinogen deaminase are MEQMPSPNAPLKGTRGSPL.

Cloning of the *R. capsulatus hemC* Gene

The porphobilinogen deaminase N-terminal sequence was used to clone the *hemC* gene of *R. capsulatus*. The N-terminal sequence was analyzed using the BLAST WWW Server available from the National Center for Biotechnology Information that locally aligns the query protein sequence with over 200,000 known protein sequences in the database. The *R. capsulatus* porphobilinogen deaminase N-terminal sequence was found to have 75% identity and 91% similarity to regions in the *P. sativum* and *A. thaliana* porphobilinogen deaminase sequences. Another analysis was done using the TFASTA search algorithm that back translates the protein sequence into all six possible nucleotide sequences then compares these to all nucleotide
Figure 8. Two-dimensional gel electrophoresis of the purified *R. capsulatus* porphobilinogen deaminase. The protein spot of porphobilinogen deaminase is circled.
sequences in Genbank. The *R. capsulatus* porphobilinogen deaminase N-terminal sequence was found to have 100% homology with a gene sequence located upstream of the *R. capsulatus hemE* gene, a sequence previously submitted by our lab (122). By localization of the N-terminal sequence onto the *R. capsulatus hemE* sequence, it appears the *hemC* gene is transcribed in the opposite direction of *hemE*. Clones obtained in the studies on the *R. capsulatus hemE* were available for study. The largest clone, containing 4 kb of *R. capsulatus* DNA, was pCAP154 (Figure 9) which contains the *hemE* gene with 1.2 kb of upstream DNA. The clone pCAP154 was mapped by restriction enzymes and various fragments from pCAP154 were subcloned into pUC18 (Figure 10).

**Sequencing of the hemC Gene**

The subclones pCAP155, pCAP167, pCAP168 and pCAP169 each containing small fragments of the region upstream of the *R. capsulatus hemE* gene (Figure 10) were used for sequencing the entire 1.2 kb of DNA upstream of *hemE* gene in both directions. Analysis of this nucleotide region (Figure 11) indicates there is a single open reading frame 951 bp long. The ATG codon beginning at position 137 is the most likely candidate for the start codon, based on the homology to the *R. capsulatus* porphobilinogen deaminase N-terminal sequence. The open reading frame encodes a 317 amino acid protein with a theoretical molecular mass of 34,082 Da and pl of 5.2. These values match very well to values reported in this study for the *R. capsulatus* porphobilinogen deaminase. The derived amino acid sequence of the *R. capsulatus* enzyme
Figure 9. Plasmid pCAP154 containing the *R. capsulatus* hemC and hemE genes.
Figure 10. Restriction map of *R. capsulatus* hemC derivatives. Restriction enzyme cleavage sites are BamHI (B), EcoRI (E), HincII (C), HindIII (H), Mscl (M), Ncol (N), PstI (P), PvuII (V), SphI (S), and XmnI (X). Distances are expressed in kilobases.
Figure 11. Nucleotide sequence of the *R. capsulatus* hemC gene. The upstream palindrome region is underlined with a double line and the conserved cofactor binding site sequence is underlined with a single line.
shows 49% identity with that of *E. coli*, 44% identity with that of *B. subtilis*, and 42% identity with that of *E. gracilis*. The consensus porphobilinogen deaminase cofactor binding site sequence E-R-x-[LIVMF]-x(3)-[LIVMF]-x-G-[GSA]-C-x-[IVT]-P-[LIVMF]-[GSA] (198), with C being the cofactor attachment site, was confirmed in the *R. capsulatus hemC* sequence (Figure 12). The *hemC* and *hemE* genes are divergently transcribed. Between the two start sites, 45 bp upstream of *hemC* and 47 bp upstream of *hemE*, a palindrome was discovered. This palindrome (Figure 13) is structurally similar to palindromes found in the control regions of many *R. capsulatus* genes that are reported to be regulated by oxygen tension (8, 183).

**Overexpression of the hemC Gene**

To verify the sequence could express a functional enzyme, the *hemC* gene was overexpressed in *E. coli* and *R. capsulatus*. The 1.3 kb PstI-BamHI DNA fragment containing the *hemC* gene was subcloned into pUC19 to generate pCAP175. The shorter 1.1 kb HindIII-BamHI DNA fragment containing the *hemC* gene without the upstream palindrome was subcloned into pUC19 to generate pCAP178. The orientation of the *hemC* gene in both constructs is in the same direction as transcription extending from the P₅₄ on the plasmid. These clones were electroporated into the *E. coli* host NM522 in order to determine if the *hemC* gene could express active enzyme.

NM522(pCAP175), NM522(pCAP178), and the control NM522(pUC19) were all grown with IPTG induction to stimulate P₅₄ and the porphobilinogen deaminase activities were determined as described in the Materials and Methods.
<table>
<thead>
<tr>
<th></th>
<th>R. capsulatus</th>
<th>A. thaliana</th>
<th>P. satvium</th>
<th>P. aeruginosa</th>
<th>E. coli</th>
<th>B. subtilis</th>
<th>S. cerevisae</th>
<th>Human</th>
<th>Mouse</th>
<th>Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>I S V I S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V V A T V A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>M M M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F F F</td>
</tr>
</tbody>
</table>

Figure 12. Alignment of conserved cofactor binding sites.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
<table>
<thead>
<tr>
<th>Sequence</th>
<th>N8 Position</th>
<th>Length (bp)</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGTAA</td>
<td>N8</td>
<td>63</td>
<td>bchB</td>
</tr>
<tr>
<td>TGTCA</td>
<td>N8</td>
<td>22</td>
<td>bchE</td>
</tr>
<tr>
<td>TGTAA</td>
<td>N8</td>
<td>20</td>
<td>crtA</td>
</tr>
<tr>
<td>TGTAA</td>
<td>N8</td>
<td>69</td>
<td>crtD</td>
</tr>
<tr>
<td>TGTAA</td>
<td>N8</td>
<td>51</td>
<td>crtE</td>
</tr>
<tr>
<td>TGTAA</td>
<td>N8</td>
<td>51</td>
<td>bchCXYZ</td>
</tr>
<tr>
<td>TGTAA</td>
<td>N8</td>
<td>143</td>
<td>puc</td>
</tr>
<tr>
<td>TGTCA</td>
<td>N8</td>
<td>47</td>
<td>hemE</td>
</tr>
<tr>
<td>TGTCA</td>
<td>N8</td>
<td>42</td>
<td>hemC</td>
</tr>
<tr>
<td>TGTAA</td>
<td>N8</td>
<td></td>
<td>R. capsulatus Consensus</td>
</tr>
</tbody>
</table>

Figure 13. Palindromic sequences upstream of *R. capsulatus* genes.
results summarized in Table 5 show the \textit{hemC} gene sequence was able to express porphobilinogen deaminase. NM522(pCAP175) has a two-fold higher porphobilinogen deaminase activity than the wild-type strain, while NM522(pCAP178) has nearly a five-fold higher activity.

The expression was then verified in \textit{R. capsulatus}. The 1.3 kb PstI-BamHI DNA fragment containing the \textit{hemC} gene was subcloned into mobilizable plasmid pRK404, generating pCAP171. The shorter 1.1 kb HindIII-BamHI DNA fragment containing the \textit{hemC} gene without the upstream palindrome was subcloned into pRK404 to generate pCAP172. The orientation of the \textit{hemC} gene in both constructs in the same direction as transcription extending from the P_{lac} on the plasmid. Both constructs were mated into the \textit{R. capsulatus} wild-type strain PAS100. PAS100(pCAP171), PAS100(pCAP172), and the control PAS100(pRK404) were all grown aerobically in the dark, then assayed for porphobilinogen deaminase activity. As seen in Table 6, the expression from the \textit{hemC} gene was greater than that seen in \textit{E. coli}. PAS100(pCAP171) has six-fold higher PBG usage and nine-fold higher uroporphyrin formed than the control. PAS100(pCAP172) has nine-fold higher PBG usage and 16-fold higher Uro-I formation. These results show the Uro-I forming activity was stimulated by adding extra copies of the \textit{hemC} gene more than the PBG using activity.

\textbf{Construction of a \textit{hemC-cat} Transcriptional Fusion Plasmid}

In order to test transcriptional regulation of the \textit{R. capsulatus} \textit{hemC} by oxygen, a \textit{hemC-cat} fusion vector, pCAP182 (Figure 14), was constructed as
Table 5. Overexpression of hemC in *E. coli*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PBG Used Specific Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Uro-I Formed Specific Activity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM522(pUC19)</td>
<td>10</td>
<td>0.50</td>
</tr>
<tr>
<td>NM522(pCAP175)</td>
<td>19</td>
<td>0.90</td>
</tr>
<tr>
<td>NM522(pCAP178)</td>
<td>49</td>
<td>2.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> PBG used specific activity is expressed as nanomoles of porphobilinogen used per hour per mg of protein. Results are representative of at least three trials.

<sup>b</sup> Uro-I formed specific activity is expressed as nanomoles of uroporphyrin I formed per hour per mg protein. Results are representative of at least three trials.
Table 6. Overexpression of *hemC* in *R. capsulatus*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PBG Used Specific Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Uro-1 Formed Specific Activity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS100(pRK404)</td>
<td>220</td>
<td>8.5</td>
</tr>
<tr>
<td>PAS100(pCAP171)</td>
<td>1200</td>
<td>75</td>
</tr>
<tr>
<td>PAS100(pCAP172)</td>
<td>2000</td>
<td>140</td>
</tr>
</tbody>
</table>

<sup>a</sup> PBG used specific activity is expressed as nanomoles of porphobilinogen used per hour per mg of protein. Results are representative of at least two trials.

<sup>b</sup> Uro-I formed specific activity is expressed as nanomoles of uroporphyrin I formed per hour per mg protein. Results are representative of at least two trials.
Figure 14. The *hemC-cat* fusion plasmid constructed to study transcriptional regulation by oxygen.
follows. The promoterless chloramphenicol acetyltransferase (cat) gene from pCM4 was cloned into the unique BclI site, contained within the coding region of the R. capsulatus hemC gene, of pCAP175, forming pCAP176. The cat gene was inserted so that its transcription would be in the same direction as that of the hemC gene. The Ω cartridge, which contains many strong transcriptional and translational terminator sequences, was cloned from pBR322Ω into the HindIII site of the mobilizable vector pRK404, making pCAP145. The PstI-BamHI fragment from pCAP176, containing the hemC-cat fusion, was cloned into pCAP145 downstream of the Ω cartridge, forming pCAP182. The Ω cartridge prevents readthrough transcription of cat by the P_{lac} promoter of pCAP145. The hemC-cat fusion vector, pCAP182 was mated from its E. coli host strain into R. capsulatus PAS100 as described in the Materials and Methods.

**Effects of Oxygen on Transcription of the hemC-cat Fusion Plasmid**

The control strain PAS100(pCAP153) was created to account for differences in cat expression due to changes in plasmid structure or copy number, that may accompany changes in oxygen tension. The control strain was grown in RCV media under conditions of high and low oxygen, harvested, then assayed for chloramphenicol acetyltransferase activity. The control cultures grown under low oxygen tension exhibited 1.4-fold more chloramphenicol acetyltransferase activity than those cultures grown under high oxygen tension (Table 7). PAS100(pCAP182) was also grown in RCV media under conditions of high and low oxygen then assayed for
Table 7. Effects of oxygen on chloramphenicol acetyltransferase activity.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Specific Activity* 23% Oxygen</th>
<th>Specific Activity* 3% Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS100(pCAP153)</td>
<td>33</td>
<td>46</td>
</tr>
<tr>
<td>PAS100(pCAP182)</td>
<td>48</td>
<td>66</td>
</tr>
</tbody>
</table>

a Specific activity expressed as nanomoles of chloramphenicol acetylated per min per mg protein at 37°C. Results are representative of at least two trials.
chloramphenicol acetyltransferase activity. PAS100(pCAP182) cultures grown under low oxygen tension exhibited 1.4-fold more chloramphenicol acetyltransferase activity than those cultures grown under high oxygen tension. Since this difference is not greater than that seen in the control, the results show that chloramphenicol acetyltransferase activity from pCAP182 is not influenced by oxygen.
DISCUSSION

The goal in our laboratory has been the study of regulation of carbon flow over the common tetrapyrrole pathway of *R. capsulatus* through cloning, sequencing and characterization of the hem genes. Several of the *R. capsulatus* hem genes have been cloned and sequenced including hemA (33), hemB (120), hemE (122), and hemH (148). The *R. capsulatus* porphobilinogen deaminase was purified allowing us to clone the *R. capsulatus* hemC. The entire hemC gene was sequenced (Figure 11) revealing a 951 bp open reading frame that codes for a putative protein of 34,082 Da with a pl of 5.2. These values are very similar to those obtained for the purified deaminase, which had a molecular mass of 35,000 Da and pl of 4.5. The putative amino acid sequence of the *R. capsulatus* hemC shares from 40 to 50% identity with other porphobilinogen deaminases. The sequence revealed that hemC and hemE are divergently transcribed. The conserved cofactor binding site (Figure 12) was found in the putative amino acid sequence, indicating the cysteine residue, which binds the dipyrrromethane cofactor through a sulfhydryl linkage, is involved in the *R. capsulatus* enzyme.

The palindrome (Figure 13) identified between the hemE and hemC genes was first identified upstream of three genes in the 11 kb *R. capsulatus* crt gene cluster by Armstrong (8). A palindrome with the same motif was also identified upstream of the puc operon. These four palindromes were found to overlap putative *E. coli*-like promoters (8). The palindromes are centered from 20 bp upstream of the gene as in crtA up to 143 bp upstream as seen in the
puc operon. The palindrome consensus was found to be similar to the consensus sequence from the recognition sites of many positive and negative prokaryotic regulators including NifA, AraC, CAP, LacI, GalR, LexA, TrpR, LysR, and λcll (97). Because of this consensus similarity, Armstrong proposed the *R. capsulatus* palindrome was the binding site for a transcriptional regulatory factor. Since several of the *R. capsulatus* genes that have this palindrome located upstream are known to be regulated by oxygen, it was suggested that the palindrome may be involved in oxygen-mediated transcriptional regulation. Site-directed mutagenesis of either part or all of the palindromes upstream of some *bch* genes led to only two-fold reduction in anaerobic induction (183).

A *R. capsulatus hemC-cat* fusion plasmid was constructed to determine if the *R. capsulatus hemC* gene is transcriptionally regulated by oxygen. The constructed fusion plasmid was mated into *R. capsulatus* PAS100 and the cells were grown under high and low oxygen conditions. Extracts from the cultures grown under both high and low oxygen tensions had the same level of activity, when assayed for chloramphenicol acetyltransferase. This indicates the *R. capsulatus hemC* is not transcriptionally regulated by oxygen. Using a similar fusion plasmid constructed with the *R. capsulatus hemE* gene (121), oxygen was found not to transcriptionally regulate the *R. capsulatus hemE* gene. Since these genes are not transcriptionally regulated by oxygen, these results demonstrate that the palindrome located upstream of the *R. capsulatus hemC*
and hemE genes is not involved in oxygen-mediated transcriptional regulation. The palindrome, may however, play a role in transcription of these genes.

Interestingly, during initial experiments with crude extracts of R. capsulatus, more porphobilinogen was consumed than stoichiometrically expected. In R. capsulatus only one enzyme, porphobilinogen deaminase, is known to utilize PBG. Under stoichiometric conditions PBG deaminase utilizes four molecules of PBG to form one molecule of Uro-I. Yet, the R. capsulatus crude extracts used twenty molecules of PBG to form one molecule of Uro-I. It was postulated that there may be another enzyme utilizing PBG in R. capsulatus. A porphobilinogen oxygenase has been identified in plants and animals that oxidizes PBG into 2-hydroxy-5-oxoporphobilinogen as the major product and 5-oxoporphobilinogen as the minor product (92, 93, 286). Since porphobilinogen is one of the key intermediates in porphyrin metabolism, its oxidation to an oxypyrrolenine, which is not converted into a porphyrin, may be considered a regulatory step in porphyrin biosynthesis.

This led us to determine whether R. capsulatus had other PBG utilizing enzymes. Throughout our purification process, only one PBG utilizing activity was found, corresponding to PBG deaminase. The purification of PBG deaminase from R. capsulatus (Table 3) proved relatively straightforward. While the heating of the enzyme solution did not lead to significant increases in purity, it did ensure the inactivation of the heat labile uroporphyrinogen III synthase. Each step of the purification was equally effective, with approximately three to five fold purification occurring at each step. It is evident
from the SDS-PAGE gel (Figure 5) that the eluted fraction from the Sephadex G-75 column still contained a mixture of proteins. However, only one of the bands, as seen under nondenaturing gel electrophoresis (Figure 2), was shown to have deaminase activity. By comparing the size of the band containing PBG deaminase activity in the nondenaturing gels to the size of the major band seen on SDS-PAGE, the porphobilinogen deaminase was found to be a monomer with a molecular mass of approximately 35,000 Daltons. The PBG deaminase optimum pH of 8.0 and pi of 4.5, indicates the protein is acidic and that it works best at slightly alkaline pH levels. The low $K_m$ value obtained for PBG deaminase suggests the physiological concentration of PBG in *R. capsulatus* may be very small.

The effects of ammonium ions, the product of the deamination, or derivatives of ammonia, such as hydroxylamine, on the PBG deaminase was first described by Plusce and Bogorad (230), who found they interfered markedly and preferentially with the formation of porphyrin. Both ammonium ions and hydroxylamine at millimolar concentrations inhibited the *R. capsulatus* PBG deaminase Uro-I formation exclusively (Table 4). This is in agreement with the results of Jordan and Warren (139) who found that hydroxylamine releases bound substrate molecules, but not the dipyrrromethane cofactor.

The involvement of sulfhydryl groups in the active site of PBG deaminase has been historically demonstrated (251). It has also been demonstrated that in *E. coli* a dipyrrromethane cofactor, which is covalently linked through cysteine-242 to the enzyme, is responsible for binding the first
PBG molecule (301). The thiol alkylating reagent N-ethylmaleimide was only inhibitory to Uro-I formation (Table 4). In contrast, mercuric chloride inhibited both Uro-I formation and PBG usage, probably due to the binding of the metal to essential thiol groups. These results suggest the involvement of sulfhydryl groups in the active site of the \textit{R. capsulatus} enzyme.

The influences of various additives on the purified PBG deaminase, provided evidence that the two activities of the enzyme may be uncoupled. N-ethylmaleimide was only inhibitory to Uro-I formation, indicating the presence of more sensitive sulfhydryl groups in the part of the enzyme responsible for Uro-I formation. The addition of reducing agents like DTT or sodium borohydride increased Uro-I formation, again supporting the idea of the presence of sulfhydryl groups in the porphyrin forming part of the deaminase. The results of ammonia or hydroxylamine addition point to the same conclusion.

The \textit{R. capsulatus} enzyme is interesting in that it has a stoichiometry that is influenced by redox conditions. Abnormal stoichiometric ratios for the purified deaminases have been seen only in a few cases (38, 66, 91). The abnormal stoichiometry was probably rarely noticed, since most groups assayed the deaminase under reducing conditions. During studies on the \textit{E. coli} PBG deaminase 3D-crystal structure, it was found that the dipyrromethane cofactor shifts positions in the active site upon varied redox conditions (161). Since the stoichiometric ratio and the cofactor positioning can be altered by redox conditions, we postulated that there may be an \textit{in vivo} regulatory
mechanism controlling the activity of porphobilinogen deaminase in response to redox conditions. We found cultures grown and assayed under strict aerobic conditions formed less than half the amount of Uro-I as those grown and assayed under strict anaerobic conditions. While this observed difference is not enough to explain the change in carbon flow over the common tetrapyrrole pathway, we hypothesize that this is the major control point of the common tetrapyrrole pathway. While we cannot mimic the true intracellular conditions, the fact that the conversion of PBG into Uro-I is regulated by redox conditions is our most promising lead to date.

Our laboratory has shown that regulation of carbon flow through the common tetrapyrrole pathway is not the result of transcriptional regulation of hem genes (119, 121, 310). We have also not found any oxygen-mediated changes in the activities in either aminolevulinate synthase (A. Biel, unpublished results) or porphobilinogen synthase (119). This suggests that oxygen regulates carbon flow over this pathway by shuttling some intermediate out of the pathway. Biel (29) showed that exogenous porphobilinogen disrupted normal oxygen-mediated regulation. In the presence of exogenous porphobilinogen, carbon flow over the pathway remained high even when the organism was grown under high oxygen tension. These results were extended by Indest (119), who showed that carbon flow over the common tetrapyrrole pathway in a strain containing multiple copies of hemB, which would have increased intracellular levels of porphobilinogen, was also constitutive. Biel (29) also showed that exogenous aminolevulinate did not alter regulation of...
carbon flow over the common tetrapyrrole pathway. Taken together, these results suggest that some step in the conversion of aminolevulinate to uroporphyrinogen III is regulated by oxygen.

Recently, an aminolevulinate dehydrogenase activity has been found in _R. capsulatus_ (A. Khanna and A. Biel, unpublished results). This means that ALA is not the first committed precursor in this pathway, and suggests the pathway could be regulated by converting ALA to aminohydroxyvalerate preferentially under aerobic conditions. However, further experiments have shown that the ALA dehydrogenase activity was higher in extracts from photosynthetically grown cultures than in extracts of aerobically grown cultures. Therefore, it is unlikely that ALA dehydrogenase plays any role in regulating the common tetrapyrrole pathway.

The results presented in this study suggest a mechanism by which oxygen might regulate the common tetrapyrrole pathway. Ideally, it takes four molecules of PBG to produce one Uro-I. We obtained this ratio when sodium borohydride was added to our PBG deaminase assay. The ratio was only slightly higher (10:1) when anaerobically grown cells were kept anaerobic during the assay. When cultures were grown aerobically and assayed aerobically, the ratio increased to between 20:1 and 30:1. In other words, when the culture was grown in the presence of oxygen, it took as many as 30 molecules of PBG to form one molecule of Uro-I. This suggests that as the oxygen tension increases, more and more PBG is shunted out of the common tetrapyrrole pathway. The most likely mechanism is that the PBG is made into
dipyrrole and tripyrrole intermediates, as seen during ammonia and hydroxylamine inhibition (139). Instead of being converted into Uro-I, the intermediates are released from the enzyme. This could be due to alterations of the PBG deaminase conformation brought about by changes in redox conditions in the cell. Studies on the *E. coli* PBG deaminase 3D-Crystal structure have found the reduced conformation of the enzyme is the active form of the molecule (161). Under reducing conditions the dipyrromethane cofactor occupies a position in the rear of the active site. The internal volume of the enzyme under reduced conditions corresponds to approximately 3.5 pyrrole rings (116). This volume limitation explains why the polymerization does not proceed beyond a tetrapyrrole product. Upon oxidation the dipyrromethane cofactor shifts into a position in the front of the active site that is likely a substrate-binding site (116). The internal volume of the enzyme under oxidized conditions has not been reported, yet the oxidized position of the dipyrromethane cofactor could very likely reduce the internal volume needed for the polymerization of four PBG molecules. Instead of creating a tetrapyrrole, intermediates would be released.

To summarize, the *hemC* gene of *R. capsulatus* was cloned by alignment of the purified PBG deaminase N-terminal sequence with a sequence upstream of the *R. capsulatus hemE* gene. The *hemC* gene was overexpressed in *E. coli* and *R. capsulatus* making a functional PBG deaminase, proving the entire *hemC* sequence was cloned. Sequencing of the *hemC* gene revealed it is transcribed in the opposite direction as *hemE*.
Between the two start sites a palindrome was identified that has been implicated in oxygen-mediated transcriptional regulation of other *R. capsulatus* genes. Monitoring the chloramphenicol acetyltransferase activity of a *hemC-cat* fusion plasmid indicated that *hemC* is not transcriptionally regulated by oxygen. The purified porphobilinogen deaminase has an atypical stoichiometric ratio that is sensitive to redox conditions. We believe that PBG deaminase is the regulated point of the common tetrapyrrole pathway.
REFERENCES


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


---

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


deaminase-tetrapyrrole complex that is an intermediate in the biosynthesis of uroporphyrinogen III. Biochemistry 27:4871-4879.


110

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


309. Woodcock, S. C., and P. M. Jordan. 1994. Evidence for participation of aspartate-84 as a catalytic group at the active site of porphobilinogen
deaminase obtained by site-directed mutagenesis of the hemC gene from Escherichia coli. Biochemistry 33:2688-2695.


116


VITA

Keith Allen Canada was born on November 7, 1970, in Minot, North Dakota. He graduated from Chippewa Valley High School, Mt Clemens, Michigan, in June 1988. In May 1992, he was awarded the bachelor of science degree in biology from Purdue University, West Lafayette, Indiana. Keith was employed as a research associate from May 1992 until August 1993 at the Purdue University School of Veterinary Medicine. In August 1993, Keith entered the doctoral degree program in the Department of Microbiology at Louisiana State University, Baton Rouge, under the direction of Alan J. Biel, his graduate advisor. Keith is currently a doctoral candidate in the Department of Biological Sciences at Louisiana State University, Baton Rouge.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Keith A. Canada

Major Field: Microbiology

Title of Dissertation: Purification of Porphobilinogen Deaminase and the Sequencing and Expression of the hemC Gene of Rhodobacter capsulatus

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

October 16, 1998