2000

Mutagenesis and Cloning of Photosynesis Genes of the Filamentous Bacterium, Chloroflexus aurantiacus

Kristi L. Berger '00
Illinois Wesleyan University

Mutagenesis and Cloning of Photosynthesis Genes of the Filamentous Bacterium, *Chloroflexus aurantiacus*

Kristi L. Berger
Illinois Wesleyan University

Research Honors Designated: April 14, 2000
Thesis Submitted: April 28, 2000

ABSTRACT

Chloroflexus aurantiacus is a green non-sulfur bacterium that preferentially grows photosynthetically under lighted, anaerobic conditions. The biosynthetic pathway for one of the photosynthetic pigments, bacteriochlorophyll *c,* is not well understood but may share common steps with the better understood bacteriochlorophyll *a* pathway. Cultures of *C. aurantiacus* were mutagenized in order to produce photosynthetic pigment mutants to be used later in the determination of intermediate compounds in the pathway for bacteriochlorophyll *c* synthesis. However, mutants with photosynthetic pigment deficiencies were not produced due to experimental difficulties. The photosynthesis genes of *C. aurantiacus,* such as the *bchXYZ* genes, are of evolutionary interest due to the similarities this bacterium has with the distantly related green sulfur and purple non-sulfur photosynthetic bacteria. The unknown *bchXYZ* sequences of *C. aurantiacus* could be used in comparisons with different families of bacteria to understand phylogenetic relationships between photosynthetic bacteria. Using degenerate PCR primers designed from known *bchZ* sequences, regions of the genome from *C. aurantiacus* were amplified, cloned, and sequenced. Sequence analysis indicated that the cloned regions corresponded neither to *bchZ* nor to other photosynthesis genes. Repetition of cloning experiments may yet reveal amplified regions corresponding to the *bchZ* gene.

INTRODUCTION

Chloroflexus aurantiacus is a filamentous green non-sulfur photosynthetic bacterium that was first reported by Pierson and Castenholz in 1974 (7). It is the most biochemically and genetically studied phototrophic thermophile in the *Chloroflexaceae* family. This filamentous bacterium was first isolated from Japanese hot springs and forms dense, brown or orange-colored bacterial mats often together with thermophilic cyanobacteria (12). The holotype, strain J-10-fl, was isolated from alkaline hotsprings from the Hakone district in Japan and has unbranched filaments that are 0.6 – 0.7 μm in width and 30- 300 μm in length, although length can be indefinite. The filaments are divided by septa formed by the in-growth of the cytoplasmic membrane and inner cell wall layers. *C. aurantiacus* filaments have no flagella yet can move by

gliding at a rate of 0.01 – 0.04 μm/sec (12). Other strains of *C. aurantiacus* have been found in hotsprings in areas such as the United States at Yellowstone and in Oregon, Guatemala, Iceland, and New Zealand.

C. aurantiacus has an interesting but ambiguous evolutionary position as a green non-sulfur bacterium (*Chloroflexaceae*). *Chloroflexaceae* includes a large and diverse group of anoxygenic, filamentous phototrophs and is difficult to place in a meaningful taxonomic position on the bacterial evolutionary tree. The ambiguous categorization is in part due to the fact that only two strains of *C. aurantiacus* have been studied (13). Phylogenetic relatedness of bacteria has long been based on the sequences of proteins and nucleic acids. 16S rRNA is widely used to trace bacterial phylogeny. The nucleotide sequence of rRNA is used for understanding evolutionary relationships because it is found in all prokaryotes and is phylogenetically conservative (9). Sequence comparisons of 16S rRNA between various families of bacteria suggest that *C. aurantiacus* diverged from its photosynthetic ancestors much earlier than those of any other eubacterial phototrophs (Fig. 5), (15). Evidence by Henderson et. al. that *C. aurantiacus* has a eubacterial type 30S ribosomal subunit justifies the placement of *C. aurantiacus* on the eubacterial evolutionary line (8). However, *C. aurantiacus* does not appear related to any eubacterial group, and therefore it represents a deep division within the eubacterial line of descent (18). The division is a result of the similarities that *C. aurantiacus* shares with both green sulfur bacteria (*Chlorobiaceae*) and purple non-sulfur bacteria (*Rhodospirillaceae*). Such an early divergence of an anoxygenic photosynthetic bacterium makes *C. aurantiacus* an interesting organism to study the early processes of photosynthesis and its evolution.

In its chemical composition, *C. aurantiacus* contains various photosynthetic pigments including bacteriochlorophylls and carotenoids. The C_5 pathway of tetrapyrrole formation from glutamate is used to synthesize two different bacteriochlorophylls (Fig. 1) and is found in a wide range of bacteria including green non-sulfurs, green sulfurs, and cyanobacteria (11). Purple non-sulfur bacteria use the alternate ALA synthase pathway (18). The C_5 pathway is considered the phylogenetically oldest pathway for chlorophyll and bacteriochlorophyll synthesis (18). Acting as the primary light-harvesting pigment, bacteriochlorophyll *c* (BChl *c*) absorbs light around 740 nm and is contained in cellular organelles called chlorosomes; accessory light-harvesting

structures attached to the cytoplasmic membrane. Bacteriochlorophyll *a* (BChl *a*) comprises the reaction center (abs. 860 nm) in the cytoplasmic membrane and a light-harvesting complex (abs. 800 nm) (11). There is also a specialized BChl *a*-containing complex (abs. 792 nm) that associates with the base of the chlorosome and serves as an attachment to the cytoplasmic membrane (13). The major carotenoids present in *C. aurantiacus* include β-carotene, γ-carotene, and hydroxy-γ-carotene-glucoside under anaerobic conditions and echinenone and myxobactone under aerobic conditions (13).

The steps in the BChl *a* biosynthetic pathway are well understood (Fig. 2). Various enzymes encoded by the photosynthesis *bch* genes catalyze these steps. The biosynthetic pathway for BChl *c* is unknown but may share common intermediate compounds with the BChl *a* pathway up to a certain branch-off point. This is a likely hypothesis because BChl *a* and BChl *c* are structurally similar (Fig. 3). Both share a Mg-tetrapyrrole ring organization but have different side groups. The specific structural differences are outlined in figure 3. Photosynthesis mutant analysis is necessary to determine the intermediates involved in BChl *c* synthesis.

The regulation of the photosynthetic capabilities of *C. aurantiacus,* including BChl synthesis, depends largely on light and oxygen levels of the environment since this bacterium can function either as an anaerobic photoheterotroph or an aerobic chemoheterotroph. The ability to switch between metabolic pathways allows *C. aurantiacus* to adapt to a wide range of environmental conditions, although it grows best as an anaerobic photoheterotroph (13). Regardless of the presence of oxygen, *C. aurantiacus* depends on an external source of reduced carbon and electron donors, likely acquired from the photoautotrophic cyanobacteria found proximal to the bacterial mats (13). BChl *c* is synthesized maximally under anaerobic phototrophic conditions, and BChl *a* is synthesized to a lesser extent. Specifically under these conditions, Pierson and Castenholz reported that BChl *c* concentration exceeded the amount of BChl *a* by a ratio of 10:1 (12). The production of both bacteriochlorophylls, β-carotene, γ-carotene, and hydroxy-γ-carotene-glucoside under anaerobic conditions gives cultures a dull greenish orange coloration. Bacteriochlorophyll synthesis is stimulated by a decrease in oxygen levels and an increase in light intensity, characteristic of anaerobic photoheterotrophic metabolism (19). BChl *c* synthesis was found to be more sensitive to oxygen concentrations than

BChl *a,* suggesting independent regulation of the synthesis of the two bacteriochlorophylls (11). This was determined by Oelze who analyzed the initial, rate limiting step in the C_5 biosynthetic pathway in which 5-aminolevulinic acid (ALA) is formed (Fig. 4). Gabaculine inhibits ALA formation and also BChl *c* synthesis, to a larger extent than BChl *a* synthesis. Gabaculine is a synthetic analog that can bind to GSA aminotransferase and terminate ALA formation (2). Oelze found that oxygen and light had similar effects as gabaculine in inhibiting ALA synthesis, and thus causing the BChl *c*/BChl *a* ratio to decrease (11). It has been proposed that control by oxygen overrides the effects of light (19). Therefore, bacteriochlorophylls are optimally synthesized anaerobically in the light but very minimally in the dark.

C. aurantiacus can also grow in conditions that are less productive including aerobic and semi-anaerobic environments. Under aerobic conditions in light or dark, the photosynthetic apparatus is disassembled as bacteriochlorophyll synthesis diminishes and the production of primary aerobic carotenoid pigments, echinenone and myxobactone, increases. The carotenoid pigments give aerobic cultures a distinct orange color. Aerobic growth in the dark is slower and has lower yields than anaerobic growth (13). In semi-anaerobic conditions, the cells in the oxygenated top layer of the growth medium will produce the orange carotenoids, whereas the green bacteriochlorophylls are produced further down in layers where oxygen concentration diminishes. The transitions between anaerobic, semi-anaerobic, and aerobic environmental conditions are complex and are important in understanding the regulation of pigment biosynthesis.

Sprague et.al. studied the semi-anaerobic induction of photosynthetic apparatus assembly and analyzed changes in growth and pigment synthesis during the transitions between aerobic and anaerobic conditions (19). When aerobic cultures were transferred from dark-adapted to light conditions, rapid and substantial cell growth occurred. When these aerobic-adapted cultures were introduced to semi-anaerobic conditions, BChl *a* and BChl *c* were synthesized immediately, but cell growth was arrested. In contrast, when the aerobic-adapted cultures were subjected to light, anaerobic conditions, BChl *a* and BChl *c* were again synthesized immediately but growth was considerably stimulated. This is expected because light, anaerobic conditions are optimal for assembly of the photosynthetic apparatus. Sprague et. al. showed that during an

eight hour semi-anaerobic transition period, cultures of *C. aurantiacus* increased their BChl *c*:BChl *a* ratio to 6:1 since more photosynthetic units are synthesized, yet the cultures did not increase cell density. Only after the eight hour semi-anaerobic incubation did a small increase in cell density occur, but still at a rate less than that observed under aerobic conditions (19). Therefore, it is important to recognize that an incubation time longer than eight hours is needed to make a sufficient transition from aerobic to semi-anaerobic conditions.

C. aurantiacus, a green non-sulfur bacteria, shares similarities with green sulfur and purple non-sulfur bacteria. Green sulfur bacteria have characteristic photoautotrophic growth and are separated from other phototrophic bacertia because they form a compact phylogenic group (9). Some strains of *C. aurantiacus,* a green non-sulfur bacterium, have demonstrated photoautotrophic growth like green sulfurs. A major characteristic green sulfurs have in common is the presence of chlorosomes in lieu of an intracytoplasmic membrane system (13). The primary light-harvesting pigment in both types of bacteria, BChl *c*, is contained in the membrane bound chlorosomes. Comparisons made by Gruber et.al. of RecA sequences from *Chlorobium tepidum*, a green sulfur, and *C. aurantiacus* further supported the phylogenic closeness of green sulfur bacteria to the green non-sulfurs (6). One difference between these groups is that *C. aurantiacus* (non-sulfur) has a light harvesting complex I (LHI) and reaction center like that of purple bacteria which is distantly similar to that of photosystem II of cyanobacteria. However, green sulfur bacteria have reaction centers similar to those of photosystem I of cyanobacteria (6). *C. aurantiacus* has similar carotenoid composition and mat forming behavior characteristic of cyanobacteria (5). A larger difference between *Chlorobiaceae* and *Chloroflexaceae* is due to contrasting cell wall compositions. Green sulfur bacteria are gram-negative but green non-sulfurs have a gram-positive cell wall type (20). The difference in cell wall type is because lipopolysaccharide (LPS), specifically lipid A, is absent in *C. aurantiacus* (20). Interestingly, *C. aurantiacus* is the only anoxygenic phototroph that has a cell wall more similar to gram-positive bacteria than gram-negative.

The purple non-sulfur family consists of a very diverse group of phototrophic bacteria. Both purple non-sulfur bacteria and *C. aurantiacus* share the metabolic flexibility in switching

between preferred anaerobic photoheterotrophic growth and aerobic chemoheterotrophic growth in order to adapt to changing environmental conditions (13). As stated earlier, purple non-sulfurs differ from green sulfurs and green non-sulfurs like *C. aurantiacus* in that they use the ALA synthase pathway for tetrapyrrole formation (see Fig. 1). Comparison of pheophytin-quinone type reaction centers places *C. aurantiacus* close to purple non-sulfur bacteria. Green non-sulfur bacteria and purple non-sulfur reaction centers have similar amino acid residues that interact with the BChl special pair and accessory BChl (4). One difference is that purple non-sulfur bacteria contain an internal membrane system whereas green sulfurs, and green non-sulfurs do not (13). Purple non-sulfurs also use BChl *a* and *b* as the primary light harvesting pigments instead of BChl *c*. Unlike the gram-positive *C. aurantiacus*, purple non-sulfur bacteria are gram-negative and incorporate LPS into the cell well (20).

Genetic analysis of the photosynthesis genes from *Rhodobacter capsulatus*, a purple non-sulfur, encoding for reaction centers, light harvesting complexes, and pigment biosynthesis has been well studied. It was noticed that the photosynthesis genes were located in a cluster, approximately 46 kilobase pairs in size (Fig. 6), (1). The sequence of this cluster in *R. capsulatus* has helped in understanding the genes that encode enzymes and proteins involved in bacterial photosynthesis.

One group of genes found in the photosynthesis gene cluster of *R. capsulatus* includes *bchX, bchY*, and *bchZ* (see Fig. 4). These genes code for components of the chlorin reductase used in the bacteriochlorophyll biosynthesis pathway, which catalyzes the reaction converting chlorophyllide *a* to 2-desacetyl-2-vinyl bacteriochlorophillide (see Fig. 6). These genes share similarities with the gene group *bchBLN,* components of a protochlorophyllide reductase that convert protochlorophyllide into chlorophyllide *a.* Both the *bchXYZ* and *bchBLN* bacteriochlorophyll biosynthesis enzyme complexes share similarities at the structural level with nitrogenases of nitrogen-fixing bacteria. The relationship between these enzymes has been studied extensively by Robert E. Blankenship. Phylogenetic analysis based on the similarities between *bchXYZ, bchBLN,* and nitrogenases from various groups of bacteria is being done by Blankenship to construct a bacterial evolutionary tree (personal communication). The genes involved in forming the *bchXYZ* complex have already been cloned from various green sulfur

bacteria, heliobacteria, and other groups. The only bacteria currently under-represented in this phylogenic analysis are the green non-sulfur bacteria.

One objective of this study was to create bacteriochlorophyll mutants of *C. aurantiacus* and use spectral analysis to determine intermediate compounds in the BChl *c* pathway. This study was based on a selection and isolation procedure for pigmentation mutants of *C. aurantiacus* described by Pierson et. al. (1984). Another objective of this study was to clone the *bchZ* gene from *C. aurantiacus*. By cloning *bchZ* and determining its sequence, we can begin to fill in informational gaps about the *bchXYZ* complex from green non-sulfurs in order to understand the phylogenetic relatedness of bacteria in the eubacterial evolutionary tree.

MATERIALS AND METHODS

Culture maintenance. Cultures of *C. aurantiacus*, J-10-fl, grow optimally at a pH between 7.6 and 8.4 at temperatures between 52°C-60°C (12). Hanada et. al. demonstrated that PE medium enhanced long term growth of *Chloroflexus* cultures (7). Using screw-cap tubes filled to the rim with media simulated anaerobic conditions, thus stimulating bacteriochlorophyll production for photosynthesis and minor carotenoid production. Stock cultures were maintained in anaerobic conditions in light (40 W-incandescent bulbs) at 55°C in screw-cap tubes filled completely with PE medium, pH 7.5. Stocks were subcultured weekly, transferring approximately 1/4 of the old culture to fresh PE media. To eliminate the necessity of weekly subculturing, the viability of cells stored long term at –80°C was tested. Aliquots of cultures where frozen in 20% glycerol. Using the –80°C aliquots, 1/500 and 1/1000 dilutions were prepared in 5 mL PE top agar (0.7% agar), plated onto PE bottom agar (1.5% agar), incubated at 55°C in the dark, and analyzed by colony counts after a weeks time.

Growth media. Different growth media were inoculated with *C. aurantiacus* stock culture to test which media promoted rapid growth in anaerobic conditions (completely filled screw cap tubes) compared to aerobic conditions (half-filled tubes) over the course of seven days. Full tubes contained 8.0 mL of media to 1 mL of wild-type culture. Half-filled tubes contained 4 mL of media to 0.44 mL of culture. In addition to PE media, PEGP and acetate

media were tested. PEGP media was formulated based on the RVC+ media used for *Rhodobacter capsulatus* and had a final concentration of 0.6% glucose, 0.5% pyruvic acid, and base of PE medium. Acetate media was formulated based on PE media but with 0.15% acetate substituted for the succinate and glutamate carbon sources.

Mutagenesis – selection for photosynthetic mutants. For mutagenesis experiments, subcultures (9.4 mL) were grown anaerobically in PE media and incubated at 55°C for 24 hours. Cells were collected within an acceptable absorbance range (A_{max} = 650 nm) of 0.2-0.7 and then fragmented with a Glas-Col® BIONEB™ Cell Disruptor. The fragmented cells were centrifuged for 7 minutes at 15,000 rpm at 20°C, followed by a wash, centrifugation, and resuspension in 0.1 M Tris, pH 8.0.

Cells were mutagenized using the chemical agent N-methyl-N'-nitro-N-nitrosoguanidine (NTG). NTG (5 mg/mL stock in sterile water) was added to actively growing semi-anaerobic subcultures so that the final NTG concentration in solution was 30 µg/mL. Cultures were then incubated at 55°C under light. After 15 minutes, cultures were centrifuged for 7 minutes at 15,000 rpm at 20°C and resuspended in PE media. Next, the mutagenized cultures were transferred to the bottom of a 150 mL Erlenmeyer flask and incubated for 17 hours at 55°C in the dark. By using an Erlenmeyer flask, cultures were spread over a larger surface area to allow for aerobic growth conditions. Because of the possibility of mutagenized cells producing photosensitive biosynthetic intermediates, the flask was covered in foil to keep out light.

Ampicillin selection was then used to eliminate wild-type cells from mutagenized cultures. To allow the cells to adjust from aerobic to anaerobic conditions, small screw cap tubes were filled to the rim with the cultures from the Erlenmeyer flask and then incubated at 55°C in darkness for 7 hours. Ampicillin was added to cultures so that the final concentration in solution was 50 µg/mL. Two methods of ampicillin selection were tested. In one method, samples were covered in foil and incubated anaerobically at 55°C for 24 hours. In the second method of ampicillin selection, samples were placed in a closed container behind an attached interference filter from Acton Research Corp. which had approximately 57% peak transmittance at 740 nm (10 nm band-pass), the wavelength specifically absorbed by BChl *c*. Light from a flood lamp

was filtered through 5 cm of water before passing through the 740 nm filter. The entire apparatus containing the mutagenized samples was incubated at 55°C for 24 hours.

After the 24 hour incubation, samples were centrifuged for 7 minutes at 15,000 rpm at 20°C and washed two times in PE media. The pellet was resuspended in 5 mL of PE top agar and plated onto PE bottom agar. The mutant culture plates were then incubated at 55°C in the dark and inspected daily for growth.

Isolation methods and spectral analysis of mutants. Two different methods of isolating and growing cells from the mutant culture plates were analyzed. In both methods, plates were visually and microscopically inspected for colonies that had different pigmentation compared to that of orange colored wild-type controls. The first isolation method tested involved restreaking colonies with a sterile toothpick onto two identical sets of PE bottom agar plates, and incubating them at 55°C. One set of plates was incubated aerobically in the dark. A duplicate set of plates was incubated in anaerobic conditions using a brewer's jar and the GasPak System in the presence of light to stimulate the photosynthetic machinery.

The second method involved removing an entire isolated colony from the plate, utilizing the backend of a sterile Pasteur pipette. Agar plugs from mutagenized cultures, which did not undergo ampicillin selection in the presence of a filter, were ejected into tubes filled with a minimal amount of PE media (approximately 1/5 of the tube) to allow for aerobic conditions. The samples were incubated at 55°C in foil. No colonies were isolated from the mutagenized cultures which were incubated behind an interference filter

Growth of cells was visually monitored until a density appropriate for spectral analysis could be performed. A Shimadzu spectrophotometer was used to determine the whole-cell absorption spectrum of potential mutants to ascertain any deficiencies in BChl *c* and BChl *a* production compared to a wild-type control. Samples were prepared in 30% BSA to decrease background and absorbance readings were taken between 400 nm – 900 nm.

DNA isolation. DNA from *C. aurantiacus* was isolated and purified by cesium chloride gradient centrifugation according to the protocol described by Robinson and Redlinger (16). Approximately 5 grams of filaments were resuspended in 12.5 mL of 50 mM Tris-HCL, 100mM EDTA, pH 8.0. 50 mg of lysozyme were added to the suspension and incubated for 2 hours at

37°C. Then 25 mL of freshly prepared lysis solution was added and incubated for 2 hours at 50°C. Lysis solution consisted of 0.7M EDTA, 0.75% Sarkosyl, 0.6 mg/mL *Staphlococcus* proteinase (25 mg/mL stock). Cesium chloride was baked at 160°C for 16 hours. To the solution now containing free DNA, 1 g of CsCl per 1 mL DNA solution was added for a final CsCl concentration of 1.5 g/mL. The DNA solution was heated to 30°C to dissolve CsCl. The solution was then centrifuged at 18,000xg for 30 minutes. Bacterial debris (i.e. not DNA-containing) from the top of the meniscus was removed and more CsCl was added to bring the solution to a final density of 1.55 g/mL. Ethidium bromide was added to a final concentration of 740 µg/mL. To check for the necessary CsCl concentration, the refractive index of the DNA solution was compared to the index expected of a 1.55 g/mL CsCl solution (index = 1.3860). The solution was then centrifuged for 5 minutes at 8000 rpm at room temperature. The solution under the top debris layer was transferred to a Beckman ultracentrifuge tubes. The samples were then centrifuged at 45,000 rpm for 16 hours (Ti65 rotor) at 20°C in a Beckman ultracentrifuge.

After CsCl density gradient centrifugation, the presence of a DNA band in the ultracentrifuge tubes was located under UV light and extracted. To remove the ethidium bromide, an equal volume of isoamyl alcohol was added to the DNA solution and vortexed. The solution was centrifuged at 1500 rpm for 3 minutes at room temperature and the aqueous phase was transferred to a new eppendorf tube. Washings with isoamyl alcohol were repeated until the pink EtBr color disappeared from the aqueous phase. Residual CsCl was removed by diluting the DNA solution with 3 volumes of sterile water. The DNA was precipitated with 2 volumes of 100% ethanol for 15 minutes at 4°C followed by centrifugation at 10,000xg for 15 minutes at 4°C. After removing the supernatant, 1 mL of TE, pH 8.0 was added to dissolve the precipitated DNA. The OD_{260} of the final DNA solution was measured and analyzed for purity. To further check purity, 10 µg of DNA was electrophoresed through a 1% agarose/TBE gel and visually analyzed.

PCR amplification of *bchZ*. Degenerate PCR primers for the unknown *bchZ* DNA sequence of C. *aurantiacus* were synthesized by Integrated DNA Technologies, Inc. and based on a conserved sequence found among known amino acid sequences of *bchZ* in purple non-sulfur bacteria. The amino acid sequences of *bchZ* from the following bacteria were obtained from the

GenBank website (10): *Rhodobacter sphaeroides* (accession no. AAF24299), *Rhodobacter capsulatus* (no. P26179), *Rhodovulum sulfidophilum* (no. BAA76943), *Rhodospirillum rubrum* (no. AAB67241), and *Phaeospirillum molischianum* (no. BAA22794). Primer 1 (Zam1, 50 μM) was a 24mer with the following sequence: 5'-CCGGTSGGYTGYGARAAYCTGCCG-3'. Primer 2 (Boni2, 50 μM) was a 27mer with the following sequence: 5'-CTGSGTSACSSNGCGCCACAGATCCCA-3'. The primers were designed to amplify approximately a 700 bp section of the *bchZ* gene. Epicentre Technologies' FailSafe™ PCR PreMix Selection Kit was used to optimize PCR reactions. Each reaction mixture contained 25 μL of FailSafe PCR 2X PreMix buffers, 1 μL of 50 μM Primer 1, 1 μL of 50 μM Primer 2, 2.67 μL of *C. aurantiacus* DNA template (0.374 μg/μl stock), 0.5 μL of FailSafe PCR enzyme mix, and 19.83 μL of sterile water. Then 20 μL of mineral oil were added to each PCR tube. The Amplitron®I thermocycler was programmed to run 40 cycles of denaturing at 95°C for 1 minute, annealing at 51°C for 1 minute, 5 seconds, and extending at 72°C for 1.5 minutes. Then 17 μL of each PCR reaction was loaded into a 1% agarose/TBE gel and electrophoresed at 95 V for 1 hour to determine the size of fragments that were amplified.

PCR product ligation into plasmid vector and transformation. Once amplified, the PCR product from the buffer H reaction mixture was ligated into a plasmid, pCR™II, that could transform *E. coli.* Using Invitrogen's TA Cloning® Kit, a ligation reaction mixture was prepared on ice by mixing 6 μL of the amplified PCR solution, 1 μL of 10X ligation buffer, 2 μL of plasmid pCR™II vector, and 1 μL of T4 DNA ligase. The ligation reaction was incubated overnight at 15°C. The pCR™II vector (Fig. 7) is a plasmid that contains an ampicillin resistance gene and a PCR product insertion site within the *lacZ* gene that allows for blue and white colony screening. The PCR product insertion site consists of 5'-poly-T sticky ends. The PCR product can insert and ligate into the poly-T sticky ends by means of a single deoxyadenosine that the PCR polymerase adds to the 3' ends of the amplified, DNA duplex PCR product. The two *Eco*RI sites surrounding the insertion site allows for the PCR product to be cleaved out in a ~700 bp fragment, approximately the initial size of the amplified PCR product.

The ligated pCR™II plasmid was then transformed into competent *E. coli* cells, strain DH5α, prepared according to the *E. coli* Transformation Kit by Zymo Research. To a 100 μL aliquot of competent cells, 5 μL of the ligated plasmid was added and then incubated on ice for 60 minutes. LB/ampicillin plates were prewarmed at 37°C and spread with 100 μL of X-Gal (20 mg/m stock) and 40 μL of isopropyl β-D-thiogalactopyranoside (IPTG, stock conc. 20 mg/mL). Then 100 μL of the transformed cells were spread onto the plates, incubated at 37°C for 12 hours, and screened for blue or white colonies; white colonies being positive for insert.

Restriction digests and sequencing to confirm *bchZ* insert. White colonies were isolated and DNA minipreps were prepared by alkaline lysis, including a phenol:chloroform purification step (17). Restriction digests were prepared as follows to determine the size of insert within the plasmid: 9 μL of the DNA miniprep, 2 μL of buffer H, 1 μL of 10X BSA, and 1 μL of *Eco*RI. The digests were incubated at 37°C for 45 minutes and electrophoresed in a 1% agarose/TBE gel.

A 6% DNA sequencing gel and electrophoresis apparatus were prepared according to the Promega Silver Sequence protocol. DNA minipreps used for sequencing were prepared from white colonies that yielded bands on a restriction digest approximately the size of the amplified portion of *bchZ*. PCR sequencing reactions were prepared according to the manufacturers instructions using M13 (-20) forward primer. The thermocycler was programmed to run 60 cycles of denaturing at 95°C for 30 seconds, annealing at 42°C for 30 seconds, and extending at 70°C for 1 minute. Additionally, purified plasmid from two, white colony-DNA minipreps, one with a small ~340 bp insert and another with a larger ~500 bp insert, were sequenced by using fluorescent-terminators on an ABI automated slab gel, courtesy of D. Rollo and C. Bauer at Indiana University in Bloomington, Indiana. The sequences of the small and large fragments were compared against GenBank's database of DNA sequences to identify what the cloned fragments encode.

RESULTS

Culture maintenance and growth media. Stocks of *C. aurantiacus* stored at -80°C in 20% glycerol were viable on PE plates after 5 weeks (Fig. 8). A large decrease in colony counts was noticed for week 4 but increased the following week. Additionally, extensive growth was noticed from one stock that was stored for 15 weeks at -80°C. Therefore, long term storage of cultures is possible at -80°C. PE, PEGP, and acetate media were tested to determine which best promoted growth of wild-type cultures. In completely filled tubes, PE media and acetate media promoted growth whereas PEGP did not under anaerobic conditions (Fig. 9). Acetate media stimulated a faster increase in growth but reached a steady state of growth sooner than cultures in PE media. As shown in figure 9, PE media allowed for a longer period of exponential growth. In half filled tubes, aerobic conditions caused a decrease of absorbance of cultures grown in all three types of media (Fig. 10).

Mutagenesis and isolation of photosynthesis mutants. For the mutagenized samples subjected to ampicillin selection in the presence of the 740 nm filter, no colonies grew on mutant culture plates. For the mutagenized samples subjected to ampicillin selection without the 740 nm filter, growth of colonies on the mutant colony plates was visible after one week. However, pigmentation of colonies did not distinctly differ from that of the orange colored wild-type. Even under a microscope, pigmentation could not be determined for wild-type or mutants. Consequently, colonies with possible different pigmentation, derived from the method of ampicillin selection without the filter, were selected to be isolated and analyzed by a spectrophotometer.

Two methods for colony isolation from mutant culture plates were tested. The first method involved streaking cells onto the surface of duplicate PE bottom agar plates and incubating one set under dark, aerobic and the other set under lighted, anaerobic conditions. A second method involved dispensing colonies into half-filled tubes of PE media and incubating them aerobically in the dark. In the first method tested, 9 of 10 samples had no growth after 6 days under anaerobic conditions. One plate exhibited extensive growth. All samples incubated aerobically showed no growth. In the second method where colonies were dispensed into a tube containing a minimal amount of PE media, either no growth or minimal growth occurred after

one week of dark incubation at 55°C in the dark. Due to minimal growth rate, the cell density was too low to perform spectral analysis. However, after two weeks of incubation at 55°C, cell density resembled that of wild-type cells. These cells were used for spectral analysis to determine whether deficiencies in BChl *a* or BChl *c* synthesis had occurred in potential mutants. The whole-cell absorption spectrum of wild-type and of the potential mutants both displayed similar absorption spectra (Fig. 11). Carotenoid pigments appeared as a broad peak with a maximum around 460 nm. A peak for BChl *c* appeared around 740 nm. The peak at 870 nm is due to BChl *a* from the reaction center, and the peak at 810 nm is due to the light harvesting complexes containing BChl *a.* BChl *a* utilized as membrane attachments for chlorosomes may also contribute to the peak noticed at 810 nm.

DNA isolation. Pure preparations of DNA have a ratio of OD_{260}/OD_{280} around 1.8 (17). For the DNA preparation from *C. aurantiacus,* the OD_{260}/OD_{280} ratio was 1.897, indicating a relatively pure sample. The concentration of DNA was determined to be 0.374 mg/mL. To further test purity, 10 μg (26.7 uL of DNA) was run on a 1% agarose/TBE gel (Fig. 12). The gel displayed a bright band indicative of an intact genome. However, smearing was evident down the entire lane, due to the possible presence of RNA and sheared DNA.

PCR amplification of *bchZ*. PCR primers 1 and 2 were designed to amplify approximately a 700 bp region of the unknown sequence of the *bchZ* gene in *C. aurantiacus*. PCR reactions were run with buffers A-K from the PCR PreMix Selection Kit. Buffers A, B, C, and H yielded about a 700 bp band on an agarose gel (Fig. 13). Other amplified regions include a 370 bp band and two bands less than 220 bp. The sections of amplified DNA from the PCR reaction mixture with buffer H were selected to be ligated into the pCR™II plasmid vector.

Restriction digests and sequencing to confirm *bchZ* insert. After the amplified PCR products were ligated into the pCR™II plasmid vector and transformed into competent *E. coli*, restriction digests using *Eco*RI were prepared using white colony DNA minipreps. The cloned insert was verified by agarose gel electrophoresis (Fig. 14). Plasmid minipreps from samples 4 and 13 contained an insert of approximately 500 bp, which was thought to correspond to the site of the amplified portion of *bchZ*. Samples 3, 10, and 12 contained a smaller ~340 bp insert.

Only the plasmids from samples 4 and 13 with the 500 bp insert were selected to be sequenced on a 6% sequencing gel.

The 6% sequencing gel did not exhibit readable bands and hence, it could not be used to determine the sequence of the 500 bp insert. Plasmid DNA from samples 12 and 13, containing the ~340 bp (small fragment) and ~500 bp insert (large fragment), respectively, were sequenced at Indiana University to verify what portions of the *C. aurantiacus* genome were cloned. The small fragment was approximately 225 bp and the large fragment was approximately 370 bp. Comparisons against the GenBank database indicated that both fragments did not correspond to any known consensus *bchZ* sequences or to any sequence stored in the database (10). One primer was shown to anneal to a sequence found in *bchZ.*

DISCUSSION

Growth Media – anaerobic vs. aerobic conditions. *C. aurantiacus* grows best as an anaerobic photoheterotroph. Under lighted, anaerobic conditions, the photosynthetic apparatus of *C. aurantiacus* is expected to assemble, and BChl *a* and BChl *c* synthesis is expected to increase, with BChl *c* as the predominant photosynthetic pigment. The initiation of photosynthesis is expected to stimulate cell growth. In the laboratory, a phototrophic, anaerobic environment was created by filling screw-cap tubes to the rim with media and exposing them to light. PE, PEGP, and acetate media had different effects on the anaerobic growth of cultures. Because acetate media stimulated a faster increase in growth under anaerobic conditions than PE media (see Fig. 9), acetate media could be used in spurring growth of cultures started with a small inoculum. However, since PE media allowed for longer exponential growth under anaerobic conditions, it is ideal for maintaining wild-type stock cultures for longer periods of time.

In a lighted aerobic environment, *C. aurantiacus* is expected to grow as a chemoheterotroph, mainly producing carotenoids and very minimally, the bacteriochlorophylls. Filling culture tubes half way with media simulated aerobic conditions in the laboratory. Under these conditions, it is expected that growth in aerobic tubes should have a rate of increase only

slightly slower than anaerobic tubes. However, this was not noticed. Growth of cultures unexpectedly decreased in tubes filled halfway with PE, PEGP, or acetate media. In particular, PEGP media used in aerobic conditions, as well as anaerobic conditions, did not promote growth (Fig. 9, Fig. 10). The glucose and Na pyruvate components of PEGP media were possibly too concentrated, creating a hypertonic environment and causing cells to plasmolyze. The cultures that were analyzed in this experiment were allowed to grow for five days under aerobic conditions, yet no increase in growth was noticed. In fact, cell density decreased when literature has indicated that cell density should increase under aerobic conditions.

It is still unclear exactly why wild-type stocks of C. *aurantiacus* did not grow at all under aerobic conditions. The original isolation media for *C. aurantiacus* was called Medium D, but Hanada et. al. developed PE media for enhanced primary growth (7). Hanada used PE agarose media plates to test growth of *C. aurantiacus* in anaerobic and aerobic conditions. In both conditions, cultures were able to grow on the PE agar plates. Hanada also indicated that liquid PE media, like that used in the experiments presented here, is useful for culture maintenance under anaerobic light conditions (7). From the observations made by Hanada et. al. it was assumed that PE media, in either agarose or liquid form, could be used for a wide range of incubation conditions. However, there is no mention of the ability of *C. aurantiacus* to grow aerobically in liquid PE media. It is possible that liquid PE media does not promote aerobic growth. Therefore, this could account for the decrease in cell density of cultures grown under these conditions.

NTG mutagenesis. Because of the filamentous nature of *C. aurantiacus*, cultures were first fragmented using the BIONEB™ Cell Disruptor before the cells could be mutagenized. This apparatus breaks up the filaments and frees single cells necessary in the isolation of mutant colonies. While the Cell Disruptor is not 100% effective in fragmenting every filament, it does increase the possibility of growing up colonies from single cells from mutant cultures. Once fragmented, incubation of anaerobic-adapted cultures with a chemical mutagenic agent like NTG causes random point mutations in the genome. Because of the anaerobic phototrophic environment, cells are actively transcribing photosynthesis genes. NTG could cause mutations in many of these photosynthesis genes, including those involved in bacteriochlorophyll

biosynthesis, potentially creating BChl *a* and BChl *c* mutants. After the mutagenic event, cultures were incubated aerobically in the dark to stimulate growth of mutants that may be deficient in bacteriochlorophyll synthesis, which is otherwise required for anaerobic growth. Aerobic conditions also decrease the selection pressure for mutants reverting back to the photosynthetically competent genotype. Incubation occurs in the dark because mutant pigment intermediates may be photosensitive and degrade in the presence of light.

After growth of mutants under aerobic conditions, cultures were grown in an anaerobic environment with ampicillin to select against wild-type cells as described by Pierson et. al. (14). Under anaerobic conditions, only the photosynthetically competent wild-type cells would be able to grow. Ampicillin is then able to insert into the membranes of actively growing, wild-type cells and cause them to lyse, leaving mutant cells intact. Theoretically after the ampicillin selection step, a few cells left in the culture should contain some deficiency in the genes involved in BChl *c* and/or BChl *a* synthesis. Bacteriochlorophyll mutants survive the ampicillin selection because their growth is suspended under anaerobic conditions due to defective photosynthesis genes.

In another experiment to create photosynthesis mutants, Pierson et. al. used an interference filter that only allowed transmittance of 740 nm wavelengths of light in conjunction with the ampicillin selection step. Therefore, only the cells with functional, wild-type BChl *c* would absorb the 740 nm light and transfer the energy to the reaction centers, thus stimulating active growth of photosynthetically competent wild-type cells. The wild-type cells would then be eliminated by ampicillin. After elimination of wild-type cells, the remaining cells are likely to have deficiencies in BChl *c* and reaction center synthesis. After the selection step under the filter, mutants were grown aerobically because they are expected to be photosynthetically incompetent under anaerobic conditions.

The mutant cells with possible deficiencies in BChl *a* and BChl *c* synthesis were plated onto PE bottom agar and grown under semi-aerobic conditions. To minimize gliding motility on isolation plates, Pierson et.al. used 0.7 % overlay agar to slow movement (14). The overlay agar creates a semi-aerobic environment which allows photosynthetically incompetent mutants to grow. Of the few isolated colonies on the mutant culture plates, it was difficult to determine

differences in pigmentation. Photosynthesis mutants are expected to accumulate biosynthetic intermediates with various pigment colors, allowing for easy location of mutants on the mutant culture plates. However, colonies had a dull orange coloration, characteristic of wild-type, or a slight variation of the orange color. No distinct differences in colony colors were noticed. This is likely due to the fact that the 0.7% PE top agar allowed for enough oxygen exchange to occur creating an aerobic environment. In this case, all colonies would be orange since none of the green-colored bacteriochlorophylls would be produced under aerobic conditions.

Mutant isolation and absorbance spectra. By visually screening mutant culture plates, colonies with possible different pigmentation than wild-type were selected to be isolated and grown up in mass quantity. The first isolation method, which involved streaking and incubating duplicate sets of PE plates under anaerobic and aerobic conditions, was unsuccessful. Growth was expected on the aerobic plates but not on the anaerobic plates because the mutants should not be able to grow in photosynthetic conditions. This expectation was observed except for one plate in the anaerobic jar that had extensive growth. This indicated that cells capable of photosynthesis, likely wild-type, were isolated under the anaerobic experimental conditions. The other anaerobic plates indicated that cells had been successfully mutagenized since they were unable to grow photosynthetically. On the other hand, the fact that there was no growth on the aerobic plates may just indicate a more likely explanation, that cells are not successfully transferred using the first isolation method.

There was also difficulty in growing up enough cells for spectral analysis according to the second isolation method which relied on colonies already isolated on the mutant culture plates. This method involved removing isolated colonies by stabbing the agar with a Pasteur pipette and dispensing the agar plug into tubes filled with a very minimal amount of media to simulate aerobic conditions. The mutagenized cultures that were subjected to ampicillin selection without the interference filter were then incubated in the dark since they are expected to be photosynthetically incompetent, and hence would best grow under aerobic conditions. It was observed that after one week there was only minimal or no growth of mutant cultures. This can be explained by the observation stated earlier and diagrammed in figure 3. It was experimentally shown that wild-type cultures of *C. aurantiacus* were unable to grow under aerobic conditions.

This accounts for the lack of growth after one week of the mutant cultures grown in a minimal amount of PE media.

After an incubation of two weeks at 55°C in the dark in a small amount of PE media, some mutant cultures began to undergo extensive growth with coloration similar to that of wild-type. Spectral analysis of whole-cell samples revealed that the potentially mutant cultures were not mutant at all. Instead they produced the same absorption spectrum as wild-type (see Fig. 11). This may indicate that after a two week incubation period, back mutations occurred such that the once mutant cells began to resume the wild-type genotype. Hence, the revertant cells soon took over the culture, crowding out any mutants that may have remained in the culture.

Cells that were mutagenized and subjected to ampicillin treatment in the presence of a 740 nm filter did not grow once plated onto PE plates. This may be because the mutagenized samples were not covered in foil during the aerobic incubation step (bottom of Erlenmeyer flask), thus exposing mutagenized cells to light. Pigmentation mutants may be photosensitive to light and the exposure to the light may have killed off the cells. This may explain why no colonies grew because the ampicillin selection killed off all the wild-types and the exposure to light may have killed off all the pigmentation mutants. The mutagenesis experiment involving ampicillin selection with an interference filter should be repeated and the harmful effects of photosensitivity should be avoided. In the future, if mutant cultures are able to be grown up for spectral analysis and there is an indication of defective BChl *c* synthesis, the pigments can then be extracted. Once the pigments are identified, they can used to identify the intermediate compounds accumulating in the BChl *c* pathway.

Cloning and sequencing the *bchZ* gene. The primers for PCR amplification of the unknown *bchZ* gene in *C. aurantiacus* were based on a conserved *bchZ* sequence from purple non-sulfur bacteria. While the primers were designed to amplify about a 700 bp region, there is no guarantee that the *bchZ* sequence in *C. aurantiacus* contains a region of the same size. A region slightly smaller or larger than the expected 700 bp may be amplified. Therefore, the ~700 bp band noticed using buffers A, B, C, and H (Fig. 13) was presumed to likely be the amplified region of the *bchZ* gene. The bands, including the prominent 370 bp band and two bands less than 220 bp, may be other portions of the genome that were amplified.

Restriction digests of plasmid with PCR insert derived from white *E. coli* colonies revealed that a portion of the *bchZ* gene may have been cloned (Fig. 14). The *Eco*RI site cleaves out the PCR insert as a fragment, expected to be relatively the same size as the initial amplified PCR fragment. Therefore, the bands at about ~500 bp in samples 4 and 13 are questionable as to whether they are cloned portions of the *bchZ* gene from *C. aurantiacus.* The other bands at about 340 bp from samples 3, 10, and 12 are likely due to insert of the amplified ~370 bp PCR product, taking into account that sizes of bands are approximate.

The sequence of the 500 bp insert from samples 4 and 13 could not be determined from the 6% sequencing gel. The gel had deteriorated in some sections and bands were too light to read. The light bands are due to a low concentration of DNA used for the sequencing reactions. Purified plasmid from samples 13 and 12 were sent to Indiana University to determine the sequence of the 500 bp insert (sample 13) and the 340 bp insert (sample 12). The actual size of the fragments was different than the size estimated from the agarose gel with the large fragment actually 370 bp and the small fragment 225 bp. These differences may be due to difficulty in determining the sizes of smaller sized fragments based on the agarose gel system used. Another possibility for the size difference is that the bands on the gel include extra base pairs between the PCR insert and the *Eco*RI sites (refer to Fig. 7). This would cause the PCR insert to be read at a larger size than the actual PCR insert sequence.

It is questionable what region of the *C. aurantiacus* genome was cloned. This is because comparisons of the small fragment and large fragment to known *bchZ* sequences on the GenBank database showed that both fragments neither corresponded to known *bchZ* sequences nor to other photosynthesis genes. However, sequence comparisons showed that one PCR primer did anneal to a sequence found in *bchZ.* This just indicates that the primer was annealing as designed. Since the cloned ~340 bp and ~500 bp fragments are not similar to known sequences, they are likely unknown amplified regions of the genome. One reason that the fragment sequences did not correspond to any known sequences on GenBank may be that the sequence size was too small for comparison purposes. Also, some bases of the sequence were undetermined. This problem was more pronounced for the larger fragment. Comparing sequence with unknown bases may also contribute to the lack of similarity to known sequences on GenBank. Even though these

fragment sizes lack similarity to *bchZ*, other untested fragment sizes may yet correspond to *bchZ*. Other DNA minipreps should be tested for other inserts with sizes different than the ~500 bp and ~340 bp fragments.

If *bchZ* in *C. aurantiacus* is successfully cloned in future experimentation, the next step would be to clone the other genes, *bchX* and *bchY*, involved in forming the enzyme complex with *bchZ*. In other bacteria, *bchXYZ* is clustered and hence, may also be clustered in *C. aurantiacus*. If these genes are clustered, PCR can easily be used to amplify the regions surrounding the already determined and cloned sequence of *bchZ*, and therefore amplify the *bchX* and *bchY* genes. By cloning *bchXYZ* in its entirety, the sequence can then be submitted to R.E. Blankenship for phylogenic analysis on different families of bacteria. This will help further clarify the position of *C. aurantiacus* in the eubacterial evolutionary tree and its significance in the evolution of photosynthesis.

ACKNOWLEDGEMENTS

1. Dr. David Bollivar. Biology Department, Illinois Wesleyan University. Research Honors Advisor.
2. Dr. Loni Walker. Biology Department, Illinois Wesleyan University. Research Honors Committee and for use of pCR™II vector.
3. Dr. R. Given Harper. Biology Department, Illinois Wesleyan University. Research Honors Committee.
4. Dr. Jeff Frick. Chemistry Department, Illinois Wesleyan University. Research Honors Committee.
5. Dr. Donald Bryant. Biochemistry and Molecular Biology Department, Penn State University. For stock culture of *C. aurantiacus*, wild-type strain J-10-*fl*.

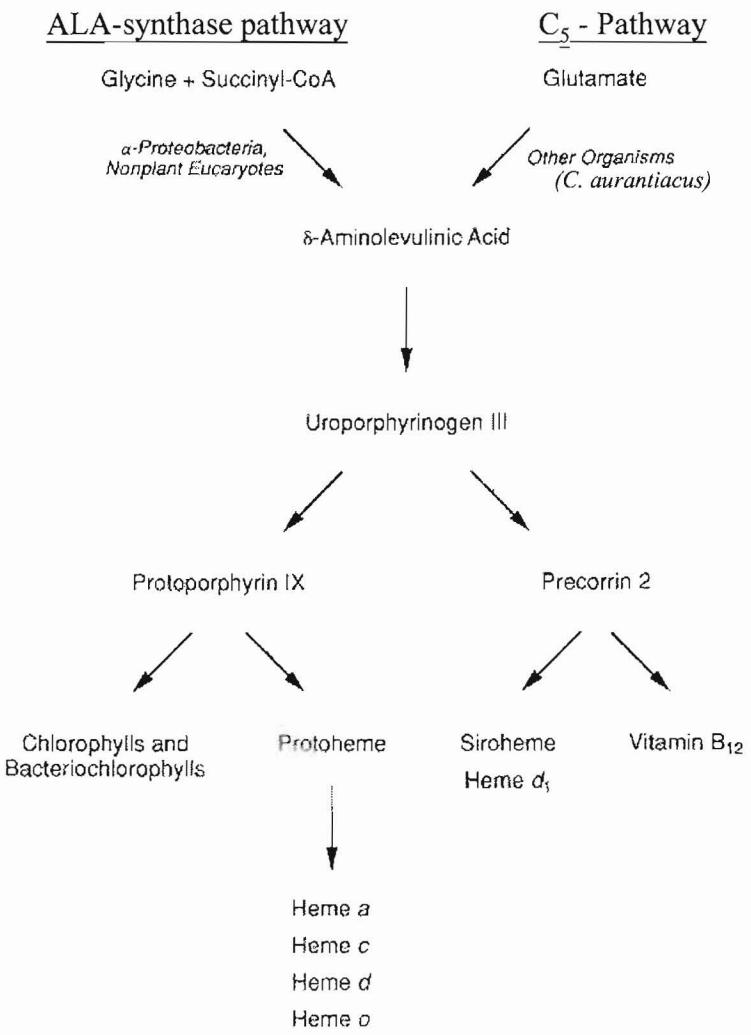

Figure 1. *C. aurantiacus* utilizes glutamate in the C_5 pathway of tetrapyrrole formation to synthesize bacteriochlorophylls. However, other bacteria, like purple non-sulfurs, utilize glycine and succinyl-CoA. ALA is the first intermediate common to all tetrapyrrole synthesis pathways. (2)

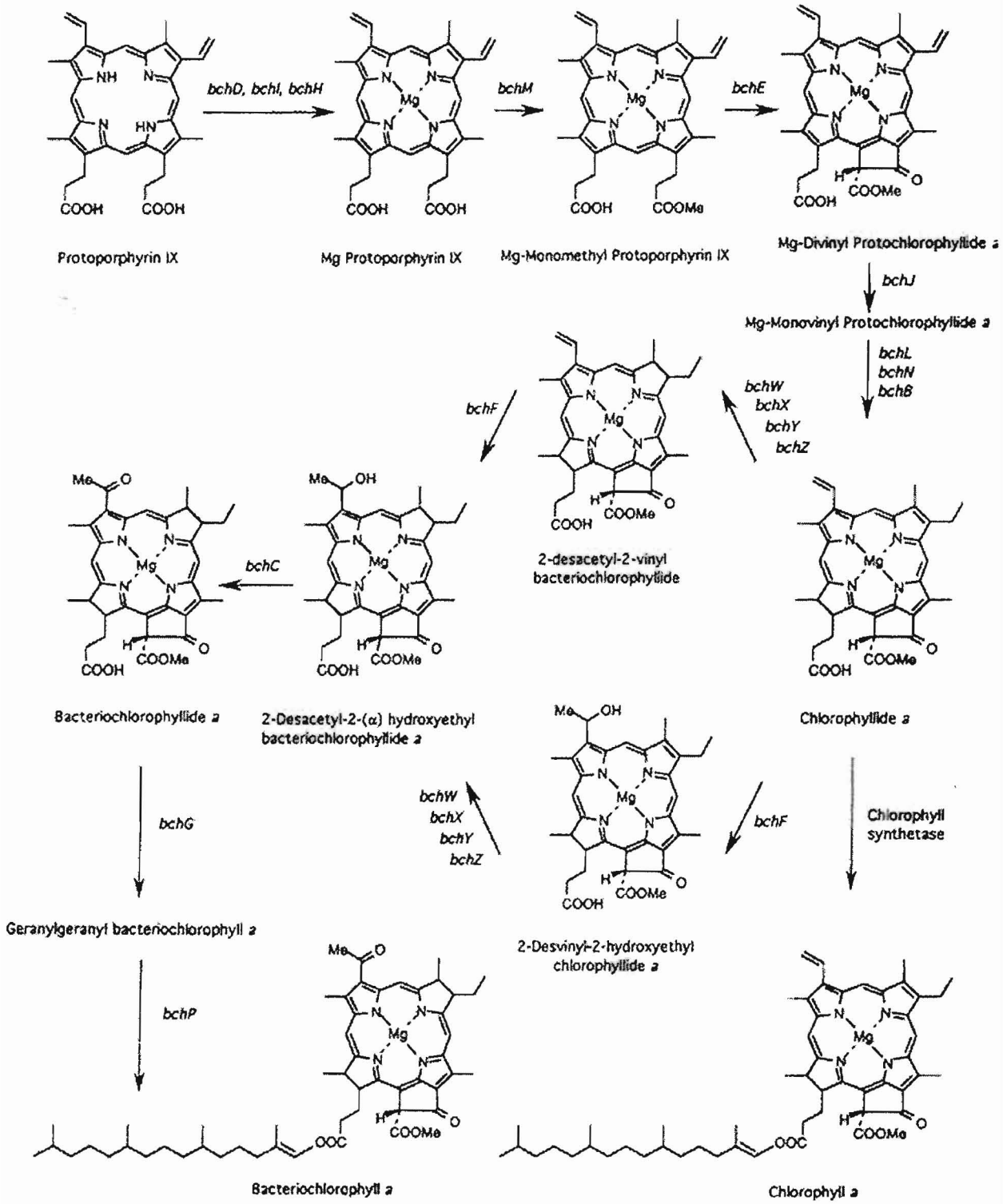

Figure 2. Biosynthetic pathway for BChl *a*. Because BChl *a* and *c* are structurally similar, they may share common biosynthetic pathway intermediates. (1)

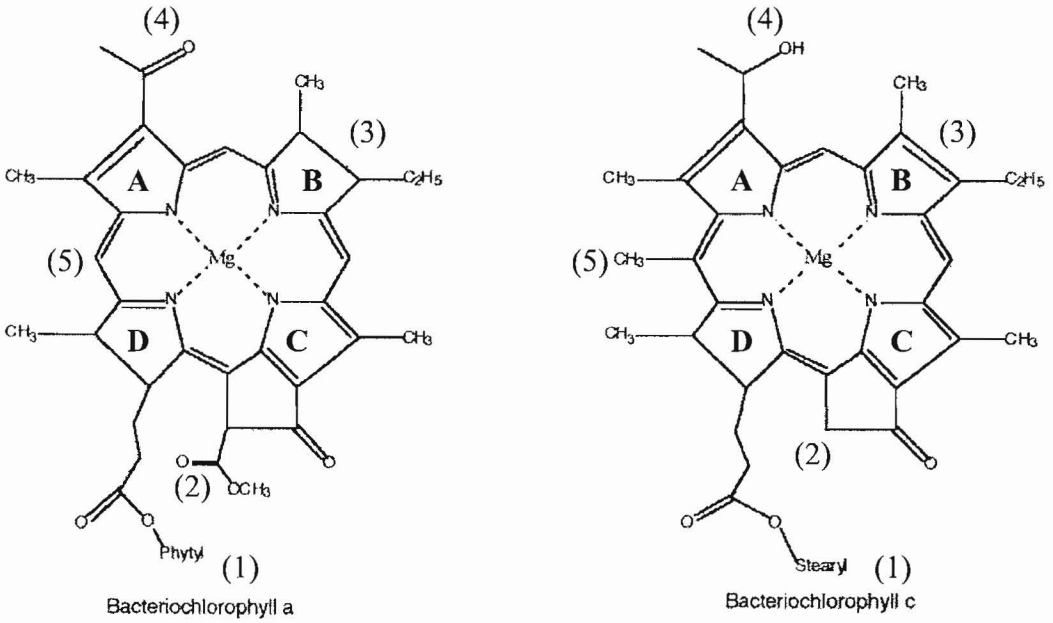

Figure 3. Mg-tetrapyrrole structures of BChl *a* and BChl *c*. In *C. aurantiacus*, BChl *a* is localized to reaction centers and a light harvesting complex. BChl *c* is found in special accessory light harvesting structures called chlorosomes. The differences between both BChls include (1) a phytyl vs. stearyl group, (2) presence of carboxymethyl group on the cyclopentone ring in BChl *a*, (3) a single vs. double bond on Ring B, (4) an alcohol or ketone on the side chain from Ring A, and (5) the presence of a methyl group between Rings A and D in BChl *c*.

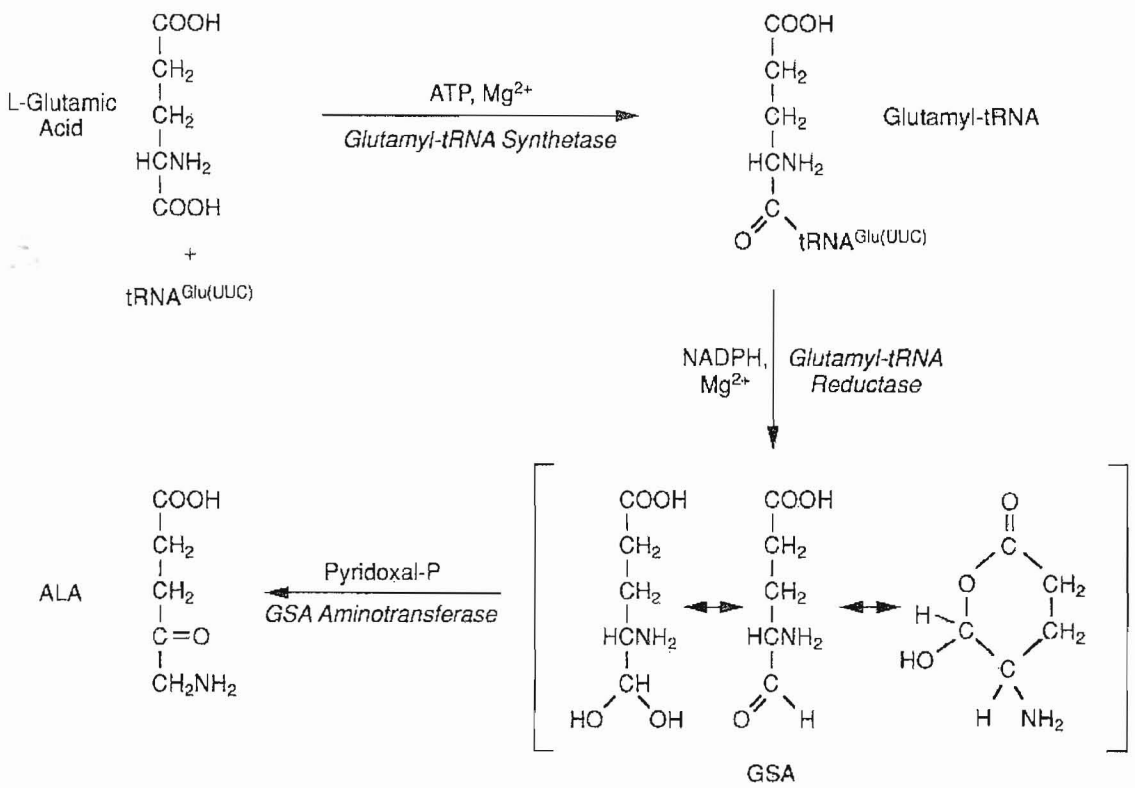

Figure 4. In the C_5 pathway of tetrapyrrole synthesis, glutamate is converted to ALA in three enzymatic steps. Oxygen and light have inhibitory effects on ALA formation in *C. aurantiacus.* (2)

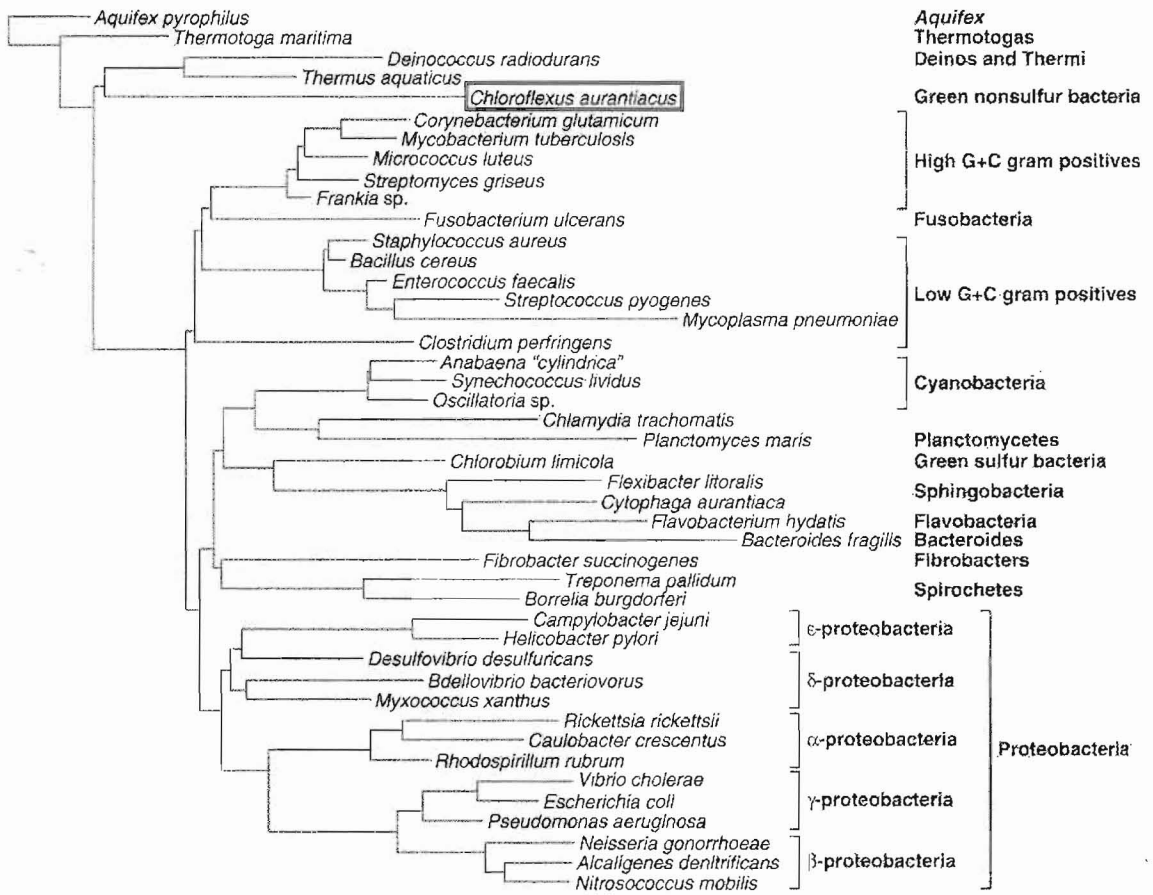

Figure 5. This evolutionary tree of *Eubacteria* is based on 16S rRNA comparisons. *C. aurantiacus*, a green non-sulfur bacterium, represents a deep division within the eubacterial line. 16S rRNA comparisons have suggested that *C. aurantiacus* diverged from photosynthetic ancestors much earlier than other phototrophs. However, *C. aurantiacus* shares similarities with green sulfur bacteria and the purple non-sulfur bacteria of *Proetobacteria*. (15)

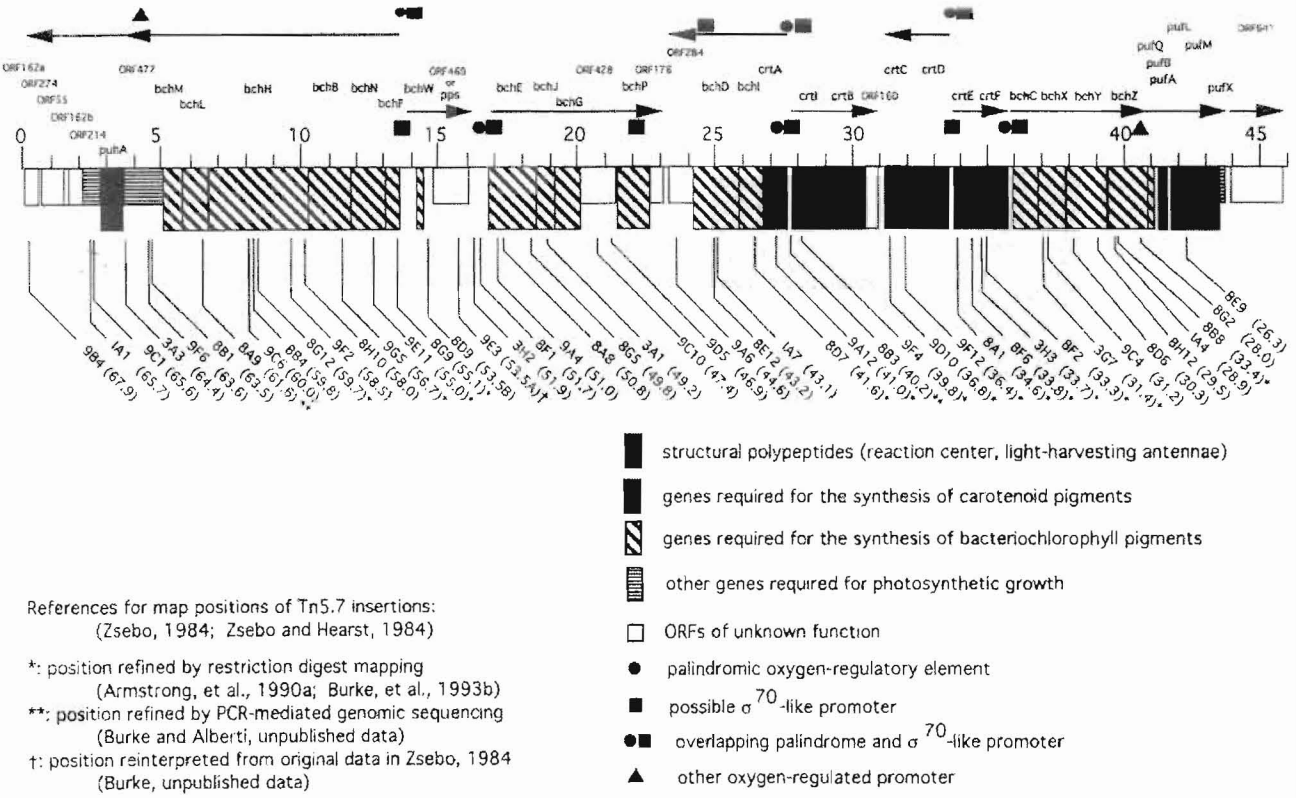

Figure 6. The photosynthesis gene cluster of *Rhodobacter capsulatus* has helped in understanding the genes coding for enzymes and proteins involved in bacterial photosynthesis. It has also contributed to understanding the photosynthesis genes in *C. aurantiacus*. Note that the *bchXYZ* genes are clustered in *R. capsulatus*. (1)

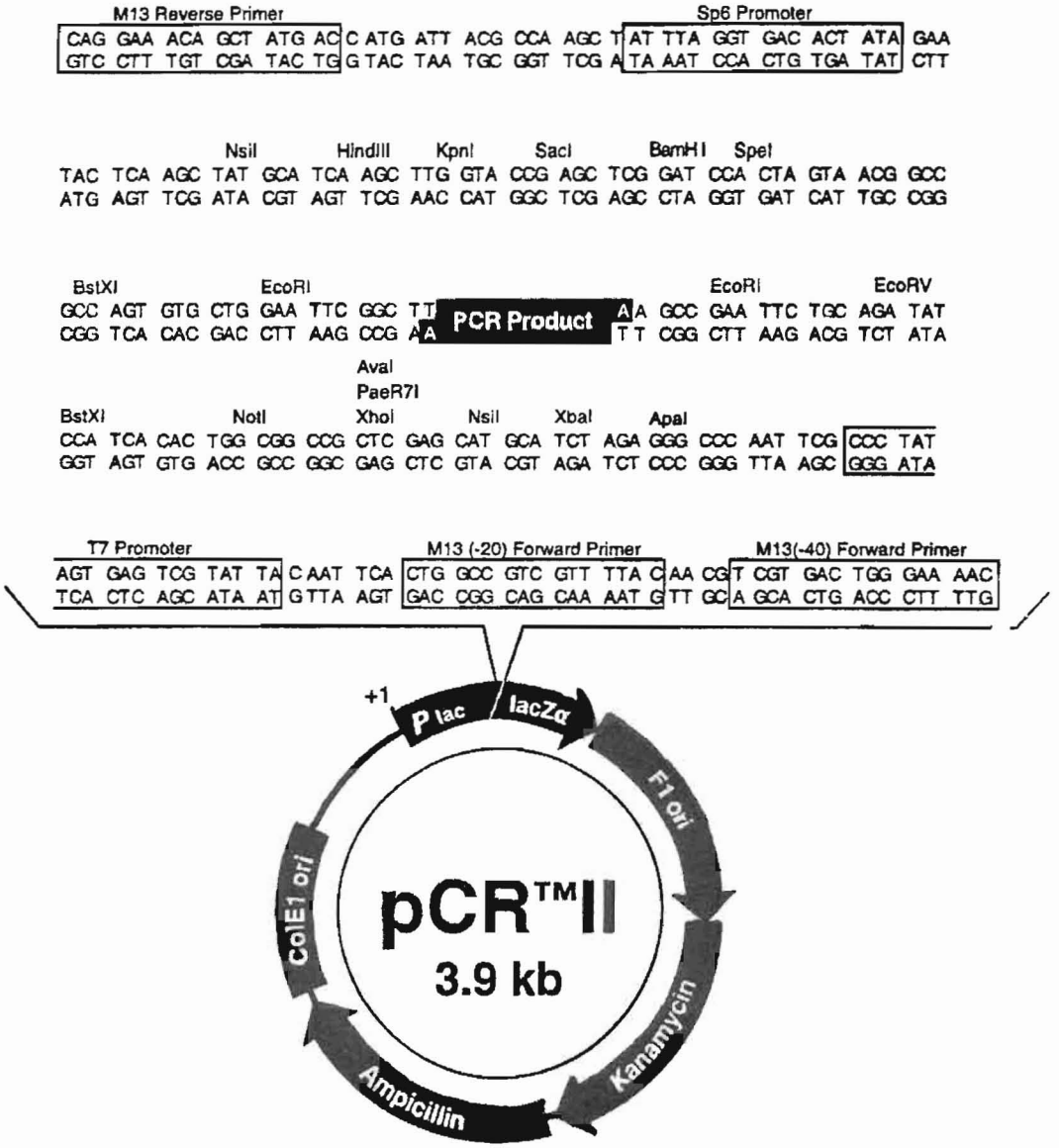

Figure 7. The pCR™ II plasmid vector contains a PCR product insertion site within the *lacZ* gene. The insertion site is surrounded by *Eco*RI restriction endonuclease sites which allows for easy excision of the PCR product after it has been ligated into the plasmid. (Invitrogen® Kit)

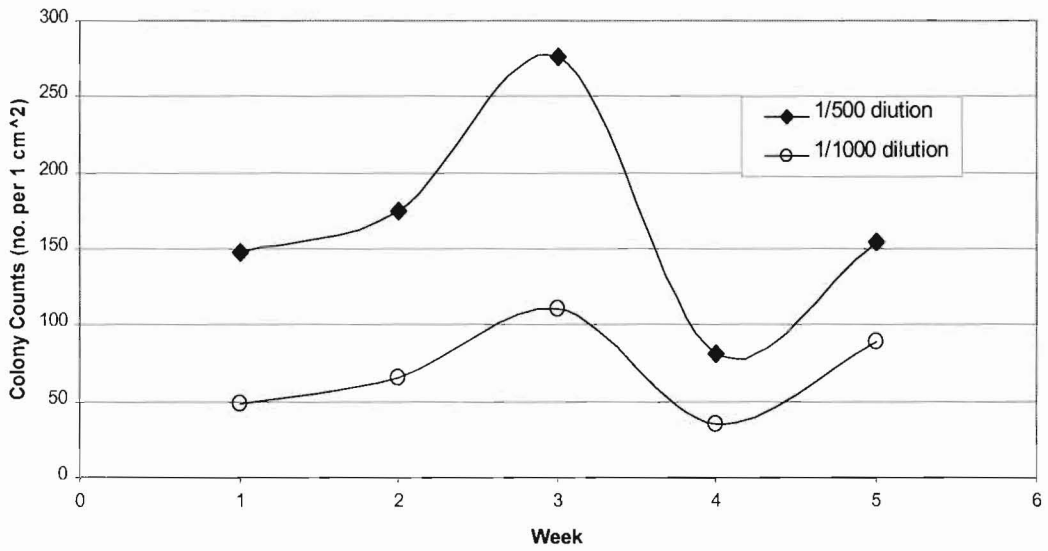

Figure 8. Viability of –80°C stock cultures of *C. aurantiacus* indicates the possibility of long-term storage.

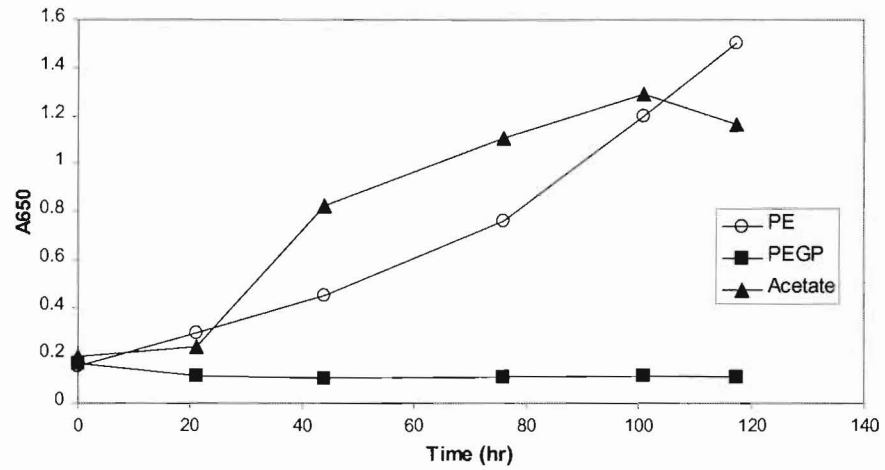

Figure 9. *C. aurantiacus* growth in full tubes (anaerobic) with various media. PE media promoted longer exponential growth, whereas acetate media stimulated more rapid, initial growth. PEGP did not promote growth.

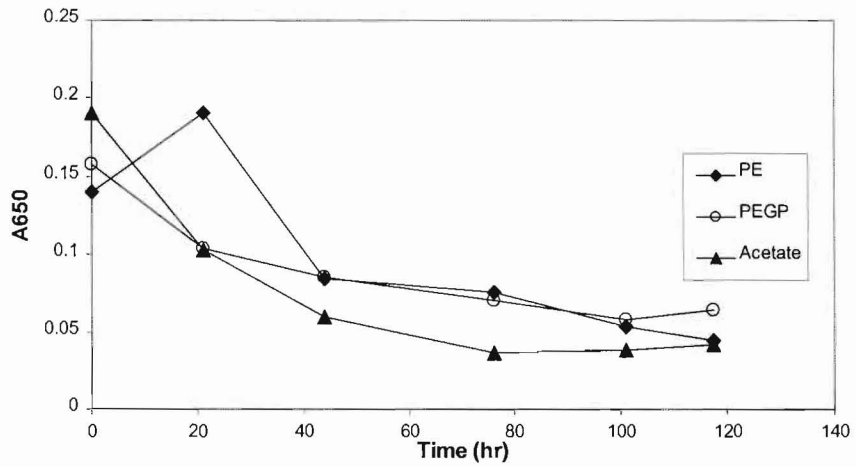

Figure 10. *C. aurantiacus* growth in half filled tubes (aerobic) with various media. None of the three types of media promoted growth.

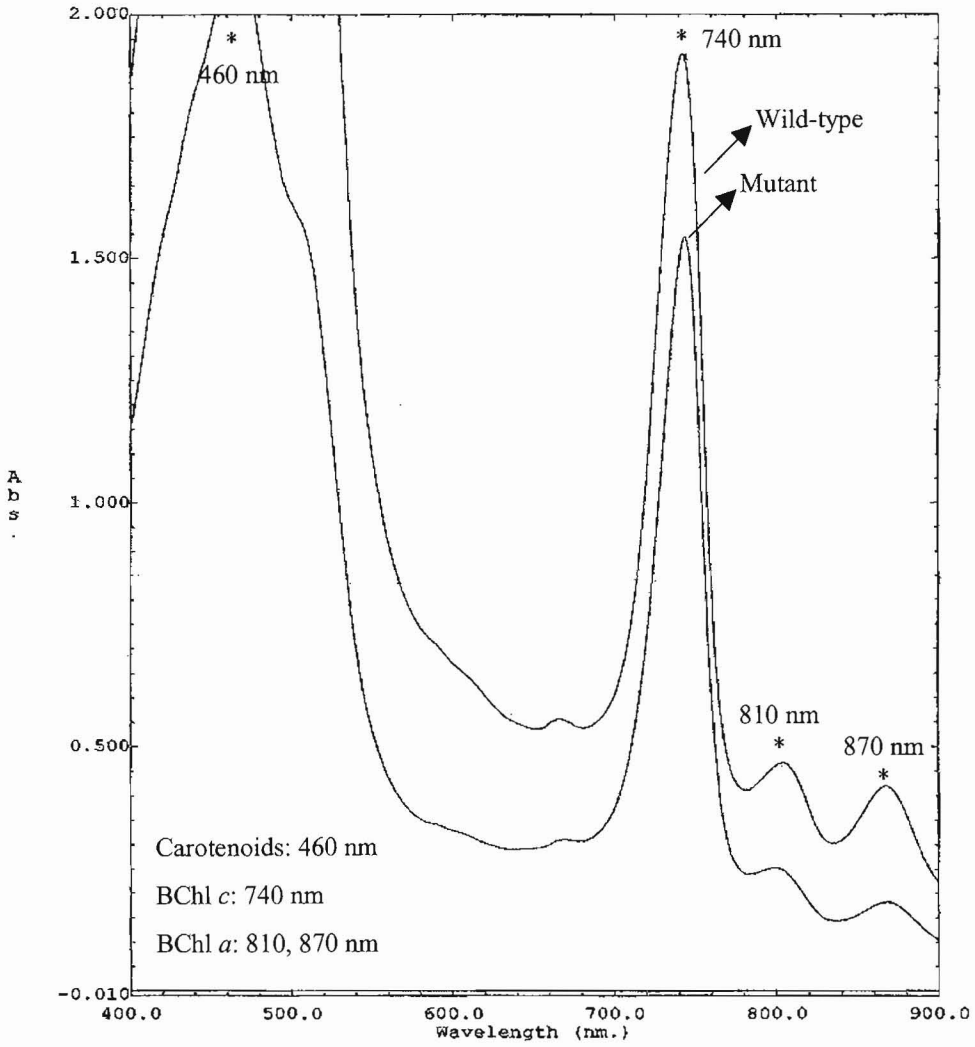

Figure 11. Absorbance spectra of whole cell cultures. Mutant and wild-type spectra do not differ indicating that mutant samples were not deficient in bacteriochlorophyll synthesis as expected.

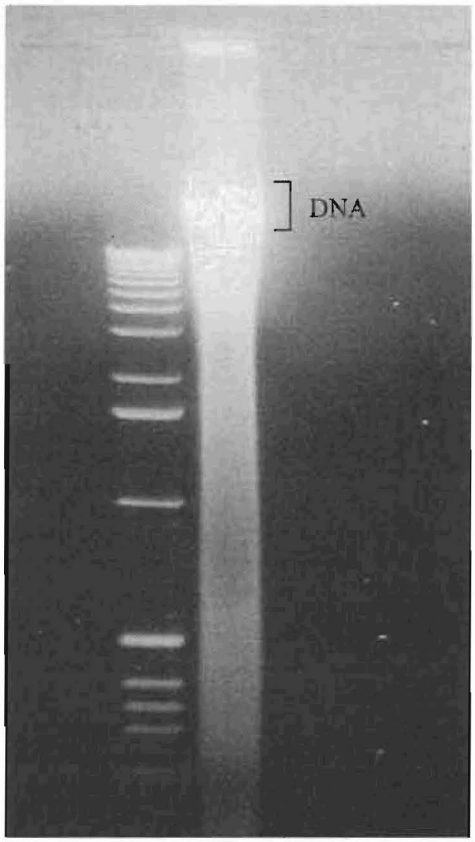

Figure 12. DNA isolated from *C. aurantiacus*. Smearing down the lane indicates the presence of RNA and/or sheared DNA.

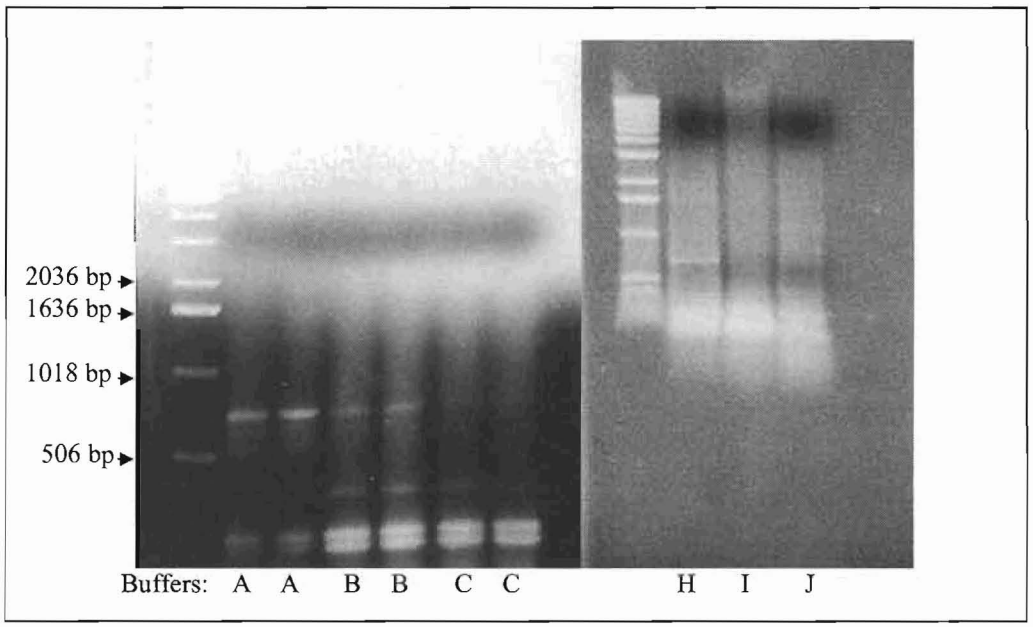

Figure 13. PCR reaction mixtures using buffers A, B, C, and H yielded a distinct band around 700 bp. The ~700 bp band corresponds to the approximate size of *bchZ* gene that the PCR primers were designed to amplify.

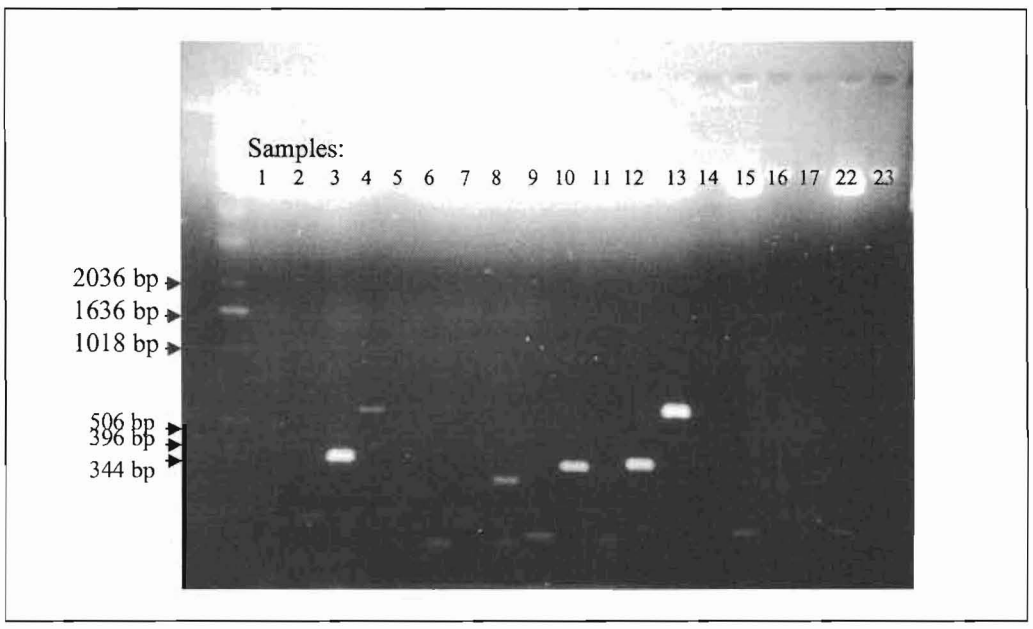

Figure 14. White colony DNA minipreps digested with *Eco*RI. Plasmid DNA from samples 4 and 13 contained a cloned insert of ~700 bp. Another cloned insert of ~340 bp was present in samples 3, 10, and 12.

REFERENCES

1. Alberti, M., D.H. Burke, and J.E. Hearst. 1995. Structure and sequence of the photosynthesis gene cluster. p. 1083-1106. *In* R.E. Blankenship, M.T. Madigan, and C.E. Bauer (ed), *Anoxygenic photosynthetic bacteria.* Kluwer Academic Publishers, Dordrecht, The Netherlands.

2. Beale, S.I. 1995. Biosynthesis and structures of porphyrins and hemes. p. 153-157. *In* R.E. Blankenship, M.T. Madigan, and C.E. Bauer (ed), *Anoxygenic photosynthetic bacteria.* Kluwer Academic Publishers, Dordrecht, The Netherlands.

3. Castenholz, R.W. and B.K. Pierson. 1995. Ecology of thermophilic anoxygenic phototrophs, p. 87-103. *In* R.E. Blankenship, M.T. Madigan, and C.E. Bauer (ed), *Anoxygenic photosynthetic bacteria.* Kluwer Academic Publishers, Dordrecht, The Netherlands.

4. Feiler, U., J.A. Shiozawa, and A. Ertlmaier. 1995. Biochemical and spectroscopic properties of the reaction center of the green filamentous bacterium, *Chloroflexus aurantiacus*, p. 699-708. *In* R.E. Blankenship, M.T. Madigan, and C.E. Bauer (ed), *Anoxygenic photosynthetic bacteria.* Kluwer Academic Publishers, Dordrecht, The Netherlands.

5. Feiler, U. and G. Hauska. 1995. The reaction center from green sulfur bacteria, p. 665-685. *In* R.E. Blankenship, M.T. Madigan, and C.E. Bauer (ed), *Anoxygenic photosynthetic bacteria.* Kluwer Academic Publishers, Dordrecht, The Netherlands.

6. Gruber, T.M., J.A Eisen, K. Gish, and D.A. Bryant. 1998. The phylogenetic relationships of *Chlorobium tepidum* and *Chloroflexus aurantiacus* based on their RecA sequences. *FEMS Micro. Lett.* **162:** 53-60.

7. Hanada S, A. Hiraishi, K. Shimada, and K. Matsuura. 1995. Isolation of *Chloroflexus aurantiacus* and related thermophilic phototrophic bacteria from Japanese hot springs using an improved isolation procedure. *J. Gen. Appl. Microbiol.*. **41:** 119-130.

8. Henderson, E., B.K. Pierson, and J.A. Lake. 1983. *Chloroflexus aurantiacus* has 30s ribosomal subunits of the eubacterial type. *J. Bacteriol.* **155:** 900-902.

9. Imhoff, J.F. 1995. Taxonomy and physiology of phototrophic purple bacteria and green sulfur bacteria, p. 1-15. *In* R.E. Blankenship, M.T. Madigan, and C.E. Bauer (ed), *Anoxygenic photosynthetic bacteria.* Kluwer Academic Publishers, Dordrecht, The Netherlands.

10. National Center for Biotechnology Information: GenBank. http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html

11. Oleze J. 1992. Light and oxygen regulation of the synthesis of bacteriochlorophylls a and c in *Chloroflexus aurantiacus*. *J. Bacteriol.* **174:** 5021-5026.

12. Pierson, B.K. and R.W. Castenholz. 1974. A phototrophic gliding filamentous bacterium of hot springs, *Chloroflexus aurantiacus* , gen. and sp. nov. *Arch. Microbiol.* **100:** 5-24.

13. Pierson, B.K. and R.W. Castenholz. 1995. Taxonomy and physiology of filamentous anoxygenic phototrophs, p. 31-47. *In* R.E. Blankenship, M.T. Madigan, and C.E. Bauer (ed), *Anoxygenic photosynthetic bacteria.* Kluwer Academic Publishers, Dordrecht, The Netherlands.

14. Pierson, B.K., L.M. Keith, and J.G. Leovy. 1984. Isolation of pigmentation mutants of the green filamentous photosynthetic bacterium *Chloroflexus aurantiacus*. *J. Bacteriol.* **159:** 222-227.

15. Prescott, L.M, J.P. Harley, and D.A. Klein. *Microbiology*, 4th edition. WCB/McGraw-Hill: Boston. 1999.

16. Robinson, S.J. and T.E. Redlinger. 1987. Isolation of genes encoding the photosynthetic apparatus of *Chloroflexus*. *Progress in Photo. Res.* **4**: 741-744.

17. Sambrook, J., E.F. Fritsch, and T. Maniatis. *Molecular cloning: a laboratory manual.* 2nd edition. Cold Spring Harbor Laboratory Press, New York. 1989.

18. Senge, M.O. and K.M Smith. 1995. Biosynthesis and structures of the bacteriochlorophylls, p. 137-151. *In* R.E. Blankenship, M.T. Madigan, and C.E. Bauer (ed), *Anoxygenic photosynthetic bacteria.* Kluwer Academic Publishers, Dordrecht, The Netherlands.

19. Sprague, S.G., L.A. Staehelin, and R.C. Fuller. 1981. Semiaerobic induction of bacteriochlorophyll synthesis in the green bacterium *Chloroflexus aurantiacus*. *J. Baceriol.* **147**: 1032-1039.

20. Weckesser, J., J. Mayer, and G. Schulz. 1995. Anoxygenic phototrophic bacteria: model organisms for studies on cell wall macromolecules, pg. 207-230. *In* R.E. Blankenship, M.T. Madigan, and C.E. Bauer (ed), *Anoxygenic photosynthetic bacteria.* Kluwer Academic Publishers, Dordrecht, The Netherlands.