The Characterization of BchF, a Bacteriochlorophyll Biosynthetic Enzyme, in *Rhodobacter capsulatus.*

by

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Abstract

Rhodobacter capsulatus are a purple non-sulfur bacterium capable of photosynthesis. One of the genes identified as being involved in bacteriochlorophyll biosynthesis is *bchF*, which is encoded on the photosynthetic gene cluster in *R. capsulatus*. Molecular cloning techniques were applied to insert the *bchF* gene into an inducible vector, pkk223-3. The gene product, BchF, was subsequently overexpressed in an *Escherichia coli* host, strain TG2. The BchF 19 kDa protein produced was then employed to develop an assay to characterize enzyme activity. The enzyme is proposed to be a hydratase of chlorophyllide *a* forming [2-hydroxyethyl]-chlorophyllide *a*. A zinc analog of chlorophyllide *a*, Zn-pheophorbide *a*, was synthesized for use as the assay substrate. The assay was monitored by HPLC, and under present assay conditions, no hydratase activity was detected.

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1. Introduction

1.1 Bacterial Photosynthesis

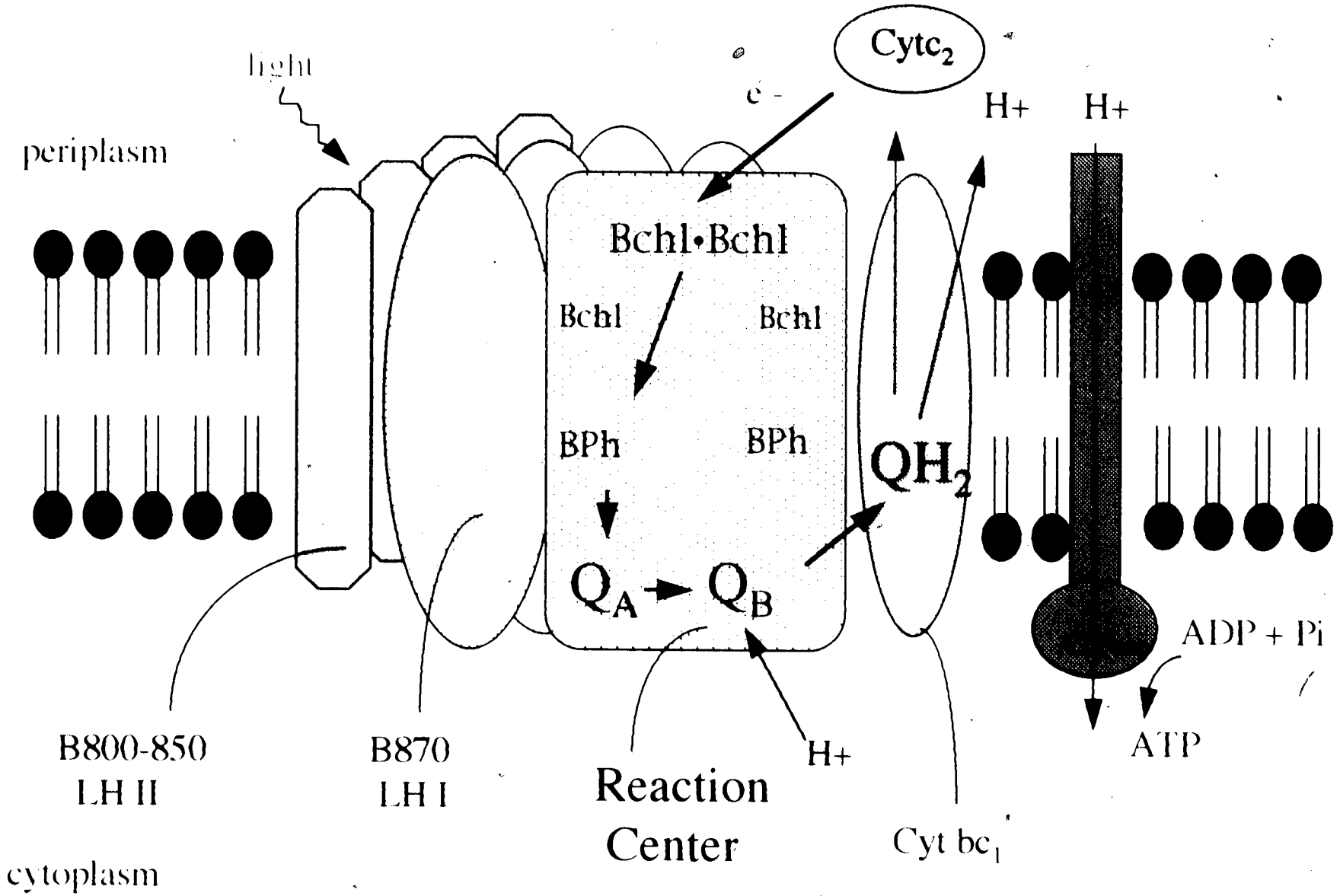
Rhodobacter capsulatus benefits from a diverse metabolism allowing growth through aerobic respiration as well as anaerobic photosynthesis. The ability to grow non-photosynthetically enables researchers to study photosynthetically defective mutants, a difficult task in higher plants. The pathway of electron flow in the purple bacterial reaction centre is very similar to that of Photosystem I I (PSII) of higher plants, algae and cyanobacteria, validating the use of the purple bacterial system as a photosynthetic model.

Bacterial photosynthetic growth is dependent on the synthesis of a specialised intracytoplasmic membrane (ICM) system that is induced in response to a decrease in external oxygen levels. The ICM contains all components of photosynthesis which in *R. capsulatus* include the two light harvesting (LH) complexes (LHI [B870]) and (LHII [B800-850]), the reaction centre (RC) complex, as well as cytochrome bc_1, cytochrome c_2 and ubiquinones. The LH and RC complexes contain non-covalently bound bacteriochlorophyll (Bchl) and carotenoids.

Electron flow (illustrated in Figure 1) in the bacterial photosystem starts when the antenna (light harvesting) pigments absorb a photon and pass on the exciton of light energy to the special pair of Bchl molecules in the reaction centre, this promotes an electron within the special pair to an excited state. The electron is quickly transferred to the bacteriopheophytin (BPh) molecule in the L subunit of the reaction centre. Although there are two seemingly symmetrical prosthetic group spirals, the electrons appear to flow exclusively down the spiral in the L rather than the M subunit. The electron transfer process leaves the special pair with a positive charge which is balanced by an electron from a reduced cytochrome c_2 molecule on the periplasmic side of the bacterial photosynthetic membrane (Rawn,1989).

The pathway continues with the reduced BPh molecule donating an electron to the adjacent quinone molecule (Q_A). As the electron passes from Q_A to Q_B, a quinone at the end of the other prosthetic group spiral, Q_B is converted to a semiquinone radical. A second exciton is absorbed and the flow of a second electron to Q_B is

Figure 1: A schematic representation of bacterial photosynthesis illustrating electron flow.



repeated. Two protons from the cytosol are simultaneously added to Q_B^{2-} forming the fully reduced QH_2 quinol. Finally, the reduced QH_2 is released from the bacterial reaction center into a mobile pool of ubiquinone, which shuttles electrons to the cytochrome bc_1 oxidoreductase complex (Rawn, 1989).

In this way bacterial photosynthesis uses the reaction center, the ubiquinone pool, the cytochrome bc_1 complex and the cytochrome c_2 molecule, which acting together to channel a cyclic flow of electrons that converts light energy into stored chemical energy in the form of an electrochemical gradient. Although the *R. capsulatus* bacterial RC's are related to the PSII RC of higher plants, they cannot oxidize water and thus do not produce molecular oxygen. Instead they maintain their metabolic redox balance by oxidation of organic compounds (Biel, 1995).

1.2 Bacteriochlorophyll

In addition to its role as the primary pigment of photosynthetic bacteria, Bchl is also essential for proper ICM formation. Studies using mutants blocked in Bchl biosynthesis when grown under

inducing conditions for the photosynthetic apparatus, low oxygen, show low steady-state levels of the apoproteins of the RC and LH complexes (Bauer, 1995). Furthermore, these Bchl blocked mutants are impaired in carotenoid accumulation under low oxygen conditions. These studies indicate that the production of Bchl is required for the assembly and correct insertion of many of the components of the photosynthetic apparatus. Due to this essential role, studies on Bchl biosynthesis and regulation can contribute to the overall knowledge of the biogenesis of the photosynthetic apparatus.

R. capsulatus Bchl synthesis and assembly of the photosynthetic, intracytoplasmic membrane is induced during a metabolic switch from aerobic respiration to anaerobic photoheterotrophic growth. However, *R.capsulatus* will accumulate Bchl anaerobically in the dark provided that there is a suitable alternative electron acceptor in the media (such as dimethyl sulfoxide) (Burke *et al.*, 1993).

1.3 Biosynthetic Pathway

Oxygenic photosynthesis, which uses chlorophyll *a* (Chl) as the primary pigment, is shared by higher plant and algal chloroplasts, cyanobacteria and prochlorophytes. Anoxygenic photosynthesis of eubacteria uses a variety of similar pigments, termed bacteriochlorophylls *a-g*. Chl *a* is less reduced than Bchl *a* and chlorophyllide *a* precedes Bchl *a* on its biosynthetic pathway.

The early stages of the biosynthesis of Bchl in purple bacteria shares a pathway with heme synthesis. Starting with the condensation of glycine and succinyl-CoA by δ-aminolevulinic acid (ALA) synthase, the precursor ALA is created (Biel and Marrs, 1983). Through six subsequent enzyme-catalyzed reactions, eight ALAs are combined to build protoporphyrin IX (Figure 2). The genes encoding the well-characterized enzymes involved in the early pathway are referred to as *hemA* through *hemG*. The pathways then diverge with heme biosynthesis accomplished with the insertion of Fe^{+2} into protoporphyrin IX by ferrochelatase (HemH), while the Bchl pathway

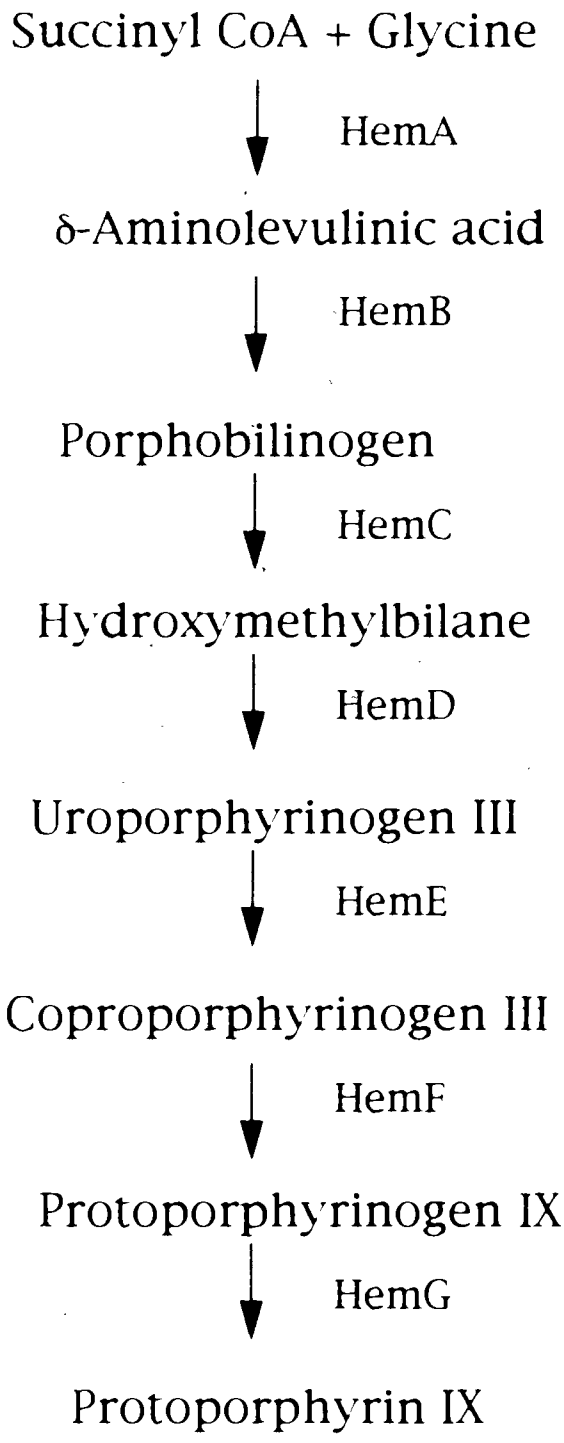


Figure 2: Early pathway of bacteriochlorophyll biosynthesis, and the gene products involved.

requires a magnesium chelatase to insert Mg^{+2} into protoporphyrin IX forming magnesium protoporphyrin (MgProto).

The later stage or "Mg branch" of Bchl (Figure 3) biosynthesis is not as well characterized and enzyme assignment is still incomplete. The enzymes of the Mg branch are encoded by the *bch* genes. Tentative assignments, based primarily on mutant substrate accumulation are shown for the *bch* gene products in Figure 3. There is mounting evidence for these assignments, based on work with cloned gene products, but specific assays for most steps have yet to be developed.

1.4 The Photosynthetic Gene Cluster

The first photosynthetic genes to be mapped in a photosynthetic bacterium were those of *R. capsulatus* (Zsebo and Hearst, 1984). Some of the essential photosynthetic genes in *R. capsulatus*, including the genes for the Mg branch of Bchl biosynthesis and carotenoid (spheroidene and spheriodenone)

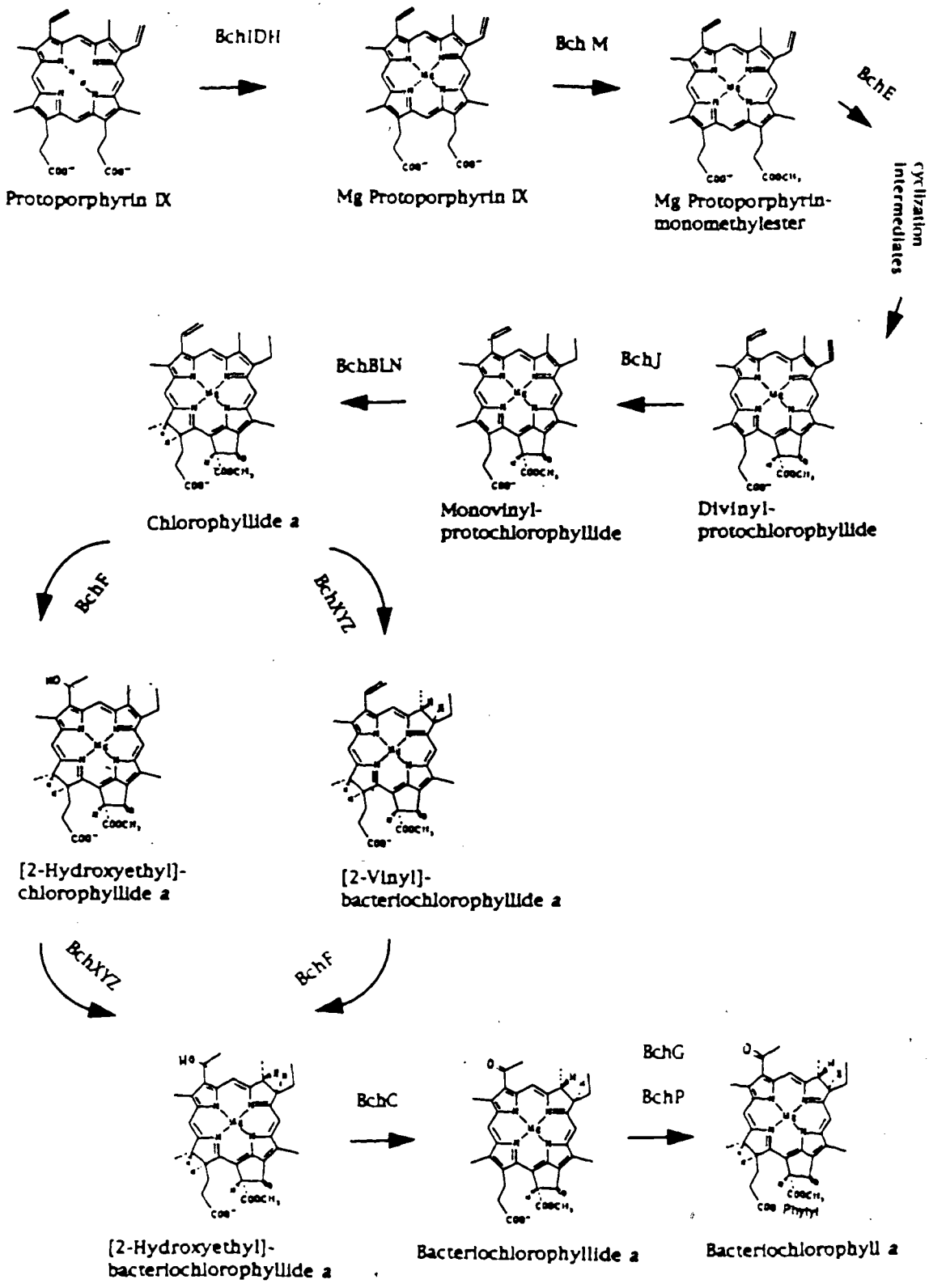


Figure 3: The Magnesium branch of bacteriochlorophyll *a* biosynthesis pathway. The gene products associated with each step are shown above the arrows.

biosynthesis, are contained within an approximately 46 kb region of the chromosome termed the photosynthetic gene cluster (Figure 4). This encompasses the *pufL*, *pufM* and *puhA* genes which encode the Reaction Center L-, M- and H- polypeptides and the *pufA* and *pufB* genes encoding the LHI α- and β-polypeptides. The auxiliary LHII peripheral antenna polypeptides are encoded in the *puc* operon which is not included in the gene cluster (Alberti *et al.*, 1995). The various *bch* and *crt* operons are centrally grouped surrounded by the *puf* and *puh* operons. Some of these pigment biosynthetic operons have read through transcription into downstream *puf* and *puh* operons encoding the protein complexes. This transcriptional read-through has been termed a superoperon. One example of superoperon activity comes from Wellington *et al.* (1992) who showed read-through transcription from *bchFNBHLM* to *puhA*. The promoter sequence of the *bchFNBHLM* is not yet characterized. An *E.coli* σ^{70} promoter-like sequence -40 bp upstream of the *bchF* gene has been identified but it is not yet clear if this sequence functions as a promoter in *R.capsulatus* (Beatty, 1995).

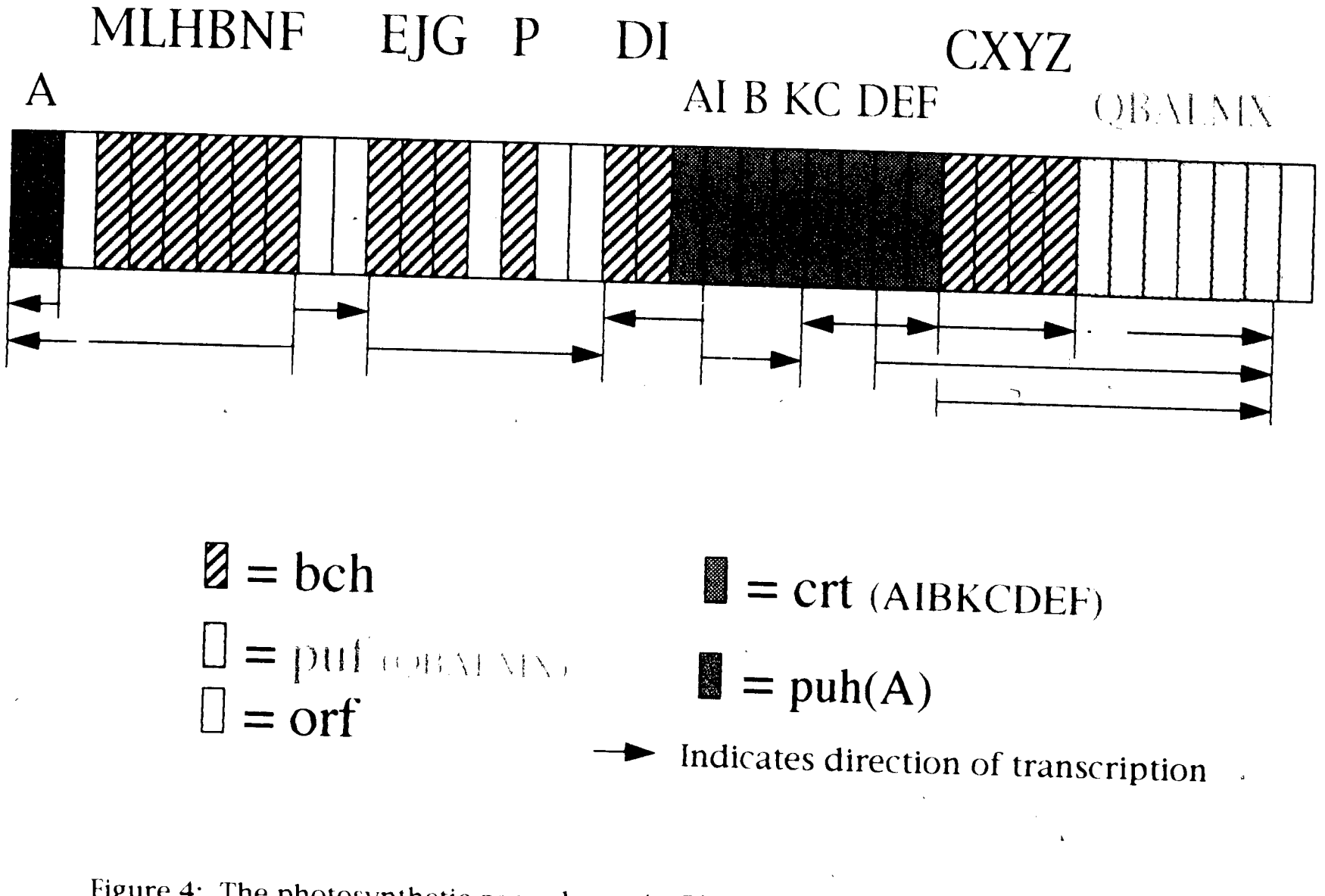


Figure 4: The photosynthetic gene cluster in *Rhodobacter capsulatus* (not drawn to scale).

A further example of superoperon organization is shown by the overlapping transcripts of *crtEF, bchCXYZ* and *pufQBALMX* operons in *R. capsulatus* . Wellington *et al.* (1991) demonstrated that though each of the operons can be expressed from its own promoter, read-through transcription is possible starting at the *crtEF* promoter and continuing through the *bchCXYZ* and *pufQBALMX* operons. In a system where the balance between production of Bchl and synthesis of apoproteins is essential, superoperon organization is an efficient mechanism of regulation. The superoperon read-through transcription allows for direct transcriptional control of Bchl and the structural polypeptide production.

1.5 The bch Genes

Based on early mapping studies by Zsebo and Hearst (1984) of bacteriochlorophyll biosynthetic genes, as well as studies of the accumulation of substrates in mutant strains, the eight *bch* genes (*bchA-H*) had been originally tentatively assigned as follows: *bchD* - "magnesium chelatase", *bchH* - "methyl transferase", *bchE* -

"oxidative cyclase", *bchB* - "protochlorophyllide (Pchlide) reductase", *bchF* - "2-vinyl hydratase", *bchA* - "chlorin reductase", *bchC* - "2-hydroxyethyl oxidase" and *bchG* - "Bchl synthetase" by Yen and Marrs (1976) with further work by Taylor and coworkers in 1983. At the time, none of these assignments was confirmed by the cloning and overexpression of gene products.

The first *bch* gene to be cloned and sequenced was *bchC*. Substrate accumulation studies had suggested that it may have encoded [2-hydroxyethyl]-bacteriochlorophyllide *a* dehydrogenase (Wellington and Beatty, 1989). Next two new genes, *bchL* and *bchM*, were sequenced and assigned as encoding additional enzymes involved in "Pchlide reductase" and "oxidative cyclase" respectively(Yang, and Bauer, 1990).

A major progression in the study of bacteriochlorophyll synthesis came when Hearst and co-workers sequenced the entire 46 kilobase photosynthetic gene cluster (EMBL DNA sequence databank, accession number Z11165) and many more ORF's were visible in regions assigned to *bch* genes. In addition to genes attributed to *bchB* and *bchF*, a *bchK* was proposed to exist just upstream from

bchH , and was thought to be part of a large operon *bchBFKHLM* (Yang and Bauer, 1990). This was later revised to *bchFNBHLM* with the inclusion of *bchN* and the designation of *bchK* being dropped (Burke *et al.*,1993 b).

Further studies revealed that *bchL*, *bchB* and *bchN* are all required for the *trans*-reduction of ring D of PChlide to form chlorophyllide *a*, although no activity has been reported with the cloned gene products. These three genes show high homology with the chloroplast genes of *Marchantia polymorpha* and *Chlamydomonas reinhardtii* and therefore are thought to encode the light-independent PChlide reductase of *R.capsulatus* (Burke *et al.*, 1993 b).

The *bchA* region was now seen to include three ORF's named *bchXYZ*. The reduction of ring B of [2-hydroxyethyl]-chlorophyllide *a* to [2-hydroxyethyl]-bacteriochlorophyllide *a* is now assigned to the enzyme referred to as chlorin reductase, which consists of the three subunits *bchXYZ* (Burke *et al.*, 1993 b).

In addition, several ORF's were seen in the region of *bchE* now known as *bchJ*, *bchG* and *bchP* and another ORF, now identified as *bchI* was seen in the region of *bchD*. In *R. capsulatus*, the mutation

of *bchJ* affected the ratio of monovinyl- and divinylprotochlorophyllide present; since the *bchJ* mutated strains showed accumulation of 2,4-divinylprotochlorophyllide. Thus, *bchJ* appeared to encode a polypeptide that is involved in the reduction of the 4-vinyl group of 2,4-divinylprotochlorophyllide (Suzuki and Bauer, 1995). A recent study has characterised the *bchG* gene product as the bacteriochlorophyll *a* synthase. Development of a BchG assay has clarified its role as adding the geranylgeranyl tail in the final steps of the biosynthesis, followed by the action of *bchP* gene product which is thought to reduce the geranylgeranyl tail to phytyl (Oster *et al.*, 1997).

The methyl transferase (MT) enzyme was initially misassigned as being encoded by *bchH.* Since the MT and magnesium chelatase appeared to be tightly coupled, the fact that a *bchH* mutant accumulated protoporphyrin IX (proto) was interpreted as a deficiency in both enzymes, leading to the misassignment. However, based on work with overexpressed gene products, it is now clear that *bchM* encodes the MT (Bollivar *et al.*, 1994), while the *BchH, -D* and *-I* proteins are subunits of the magnesium chelatase enzyme complex

in *Rhodobacter* (Gibson *et al.*, 1995). *BchH* probably provides a proto binding site in the complex as proto remained bound to the BchH protein throughout three chromatographic steps (Willows *et al.*, 1996)). In addition, BchH appèars to act as an activator and stabiliser of BchM, as BchH has been reported to increase BchM's MT activity approximately seven-fold (Hinchigeri *et al.* ,1997).

Early work on the extraction of intermediates from a mutant of *R. sphaeroides* revealed the presence of a [2-vinyl]-bacteriopheophorbide (Mg lost in purification). This did not fit the proposed biosynthetic pathway and led to the possibility of an alternate pathway involving a branch point where the reduction from a chlorin to a bacteriochlorin could occur before or after the hydration of the 2-vinyl group to an α-hydroxyethyl group (Pudek and Richards, 1975).

Tentative assignment of *bchF* as the 2-vinylhydratase comes from substrate accumulation evidence from mutant strains such as MB1003 and KZR8G9. The MB1003 *bchF* point mutant and the KZR8G9 transposon insertion mutant both show accumulation of the same compound, [2-vinyl]-bacteriochlorophyllide *a* (Burke *et al.*

1993 a). The mutants show accumulation of chlorophyllide *a* as well due to the putative branch point position (Figure 3) of *bchF*. The *bchF* gene is thought to encode an enzyme (BchF) which catalyses the hydration of either of two substrates, chlorophyllide *a* or [2-vinyl]-bacteriochlorophyllide *a*. The hydration reaction of the 2-vinyl group of these substrates by the *bchF* gene product, BchF, results in [2-hydroxyethyl]-chlorophyllide *a* or [2-hydroxyethyl]-bacteriochlorophyllide *a* respectively (Figure 3).

1.6 BchF Hydration

Until recently the source for the hydroxyl group oxygen involved in the BchF reaction was not known. Though most probably water via a hydratase, it was still possible that a monooxygenase reaction involving molecular oxygen was involved. In higher plants and green algae the 9-oxo group of chlorophylls *a* and *b* are derived from molecular oxygen (via a monooxygenase mechanism). The 3-formyl oxygen of Chl *b* is also derived from molecular oxygen in both higher plants and green algae. Since facultative photosynthetic bacteria (such as *R. sphaeroides*) increase Bchl biosynthesis in

decreasing oxygen environments and can even form Bchl under anaerobic conditions, an alternative origin to O_2 such as water for the 9-oxo group was considered probable. However, aerobic photosynthetic bacteria (like *Roseobacter denitrificans*) accumulate Bchl only under aerobic conditions and only in the dark. Porra *et al.* (1996) showed that *R. denitrificans* obtained the 9-oxo group oxygen from molecular oxygen, whereas in *R. sphaeroides* the source was water. However they also showed that both bacteria (*R. sphaeroides* and *R. denitrificans*) share a common hydratase mechanism for the formation of the 2-acetyl group. Therefore, it is assumed that *R. capsulatus* will follow suit and also use water in the hydratase reaction mediated by BchF forming a 2-α-hydroxyethyl group which is subsequently oxidized to 2-acetyl by BchC.

1.7 Chlorophyll versus Bacteriochlorophyll

During the synthesis of both chlorophyll *a* and Bchl *a* the reduction of the tetrapyrrole ring system converts Pchlide into chlorophyllide *a*. The addition of a geranylgeranyl tail to chlorophyllide *a* produces Chl a_{GG}, while additional Bchl specific

reactions, catalyzed by BchXYZ, BchF and BchC, occur before the addition of the geranylgeranyl tail to bacteriochlorophyllide by BchG and its reduction to phytyl by BchP (Figure 5). In eubacteria such as *R.capsulatus,* a second reduction (by the enzyme complex BchXYZ) converts the chlorin into a bacteriochlorin, a step unique to the synthesis of Bchl. It is also before or after this point that BchF plays its role, whereby the chlorin could first undergo hydration by BchF and then proceed to be reduced by BchXYZ (or the reverse order can occur, reduction followed by hydration).

1.8 Zinc Analogs

Zinc has substituted for magnesium as the central atom in various of metallo tetrapyrroles, including being applied successfully in many photosynthetic systems. As early as 1963, Gibson *et al.* used Zn-protoporphyrin as a substrate in a methyl transferase study. The zinc analog was accepted as a substrate for the methyl transferase reaction, showing a very high activity in *R. sphaeroides.*

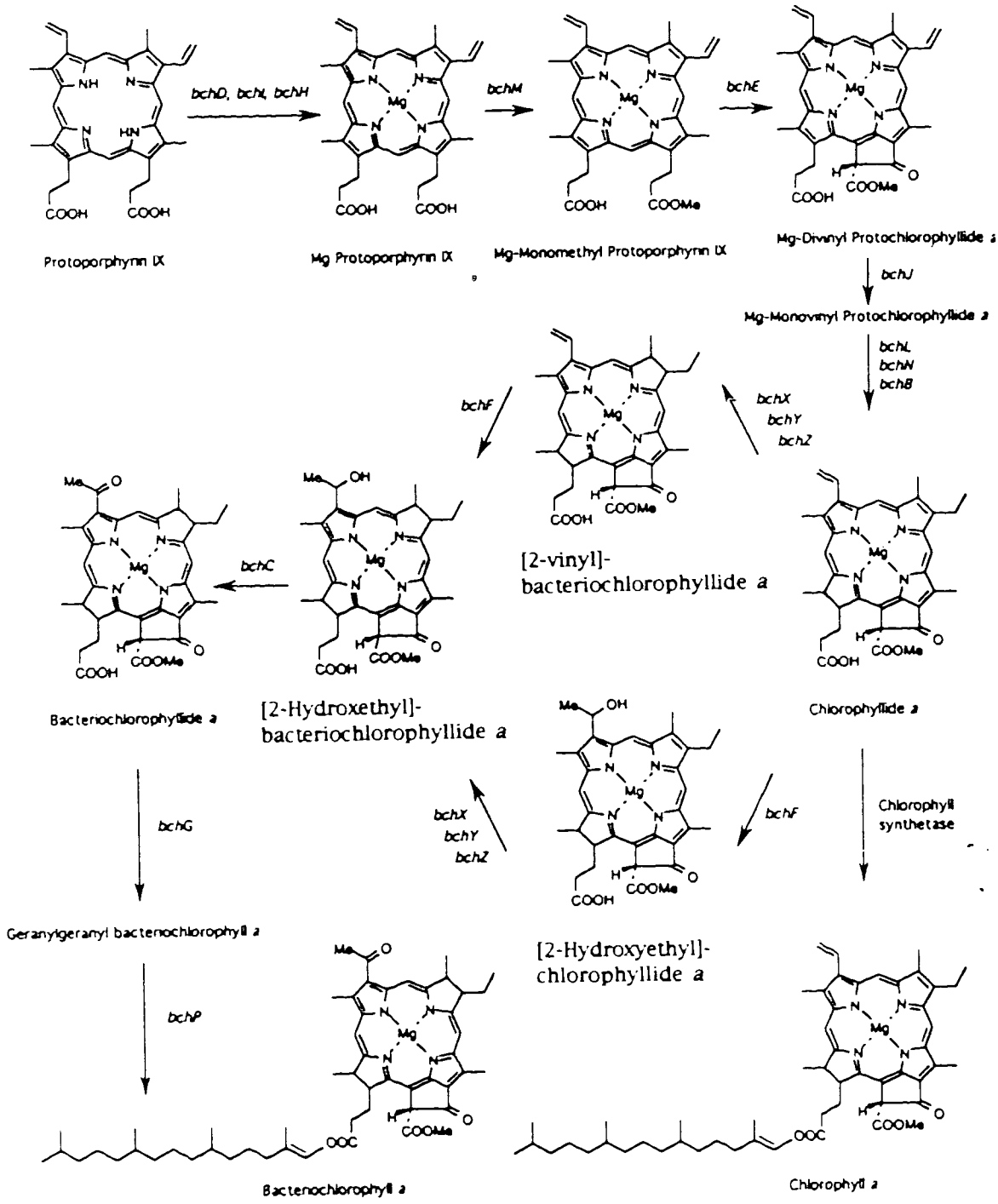


Figure 5: Chlorophyll *a* versus bacteriochlorophyll *a* biosynthesis.
Reproduced with permission from Bauer, 1995.

In 1987, Zn protoporphyrin IX monomethyl ester gave oxidative cyclase and reductase activities comparable to the magnesium counterpart. A continuous assay that monitored both reactions in wheat etioplasts was applied to various magnesium metal substitutes. This study revealed that while zinc and calcium were successful analogs, nickel, copper and metal-free center substitutes were completely inactive under the assay conditions (Nasrulhag-Boyce, *et al.* 1987).

Helfrich *et al.* (1994) applied a Zn-pheophorbide *a* analog in the assay for chlorophyll synthetase, with HPLC and NMR detection. They chose zinc analogs because of difficulties with the stability of the magnesium chelate in their harsh chemical synthesis reactions. The study demonstrated a successful assay for chlorophyll synthetase acting on Zn-pheophorbide *a* as a substrate.

Zn-pheophytin was used in a study examining pigment stabilization of the chlorophyll binding apoproteins in barley etioplasts by Eichacker *et al.* (1996). They found that Zn-pheophytin was an excellent binding substitute for chlorophyll. Similarly, when studying the reconstitution of the LHI complex and its structural

subunit complex with various bacteriochlorophyll analogs, Davis *et al.* (1996), demonstrated that Zn-bacteriopheophytin was a successful Bchl analog. The Zn-bacteriopheophytin substitute reconstituted both complexes in *Rhodospirillum rubrum* and *R. sphaeroides.*

Interesting collaborative evidence that zinc is successful as a magnesium replacement in photosynthetic pigments is the recent discovery of a naturally occurring zinc chlorophyll analog. The aerobic bacterium *Acidiphillum rubrum* was found to have a Zn-containing bacteriochlorophyll (Wakao, *et al.* 1996).

1.9 *Sequence Analysis*

The sequence of *bchF* is available on GENEBANK as part of the photosynthetic gene cluster entered by Hearst and coworkers as EMBL DNA sequence databank, accession number Z1165. The 516 base pair *bchF* sequence translates into a 171 amino acid protein, illustrated in Figure 6. Three programs were run to predict the presence of transmembrane α-helices using the methods of Rao and Argos, Eisenberg and SOAP (PCGene,1993). Computer analysis of the

BchF

N-Terminus

MPNSPQNPSRKALYTEEERARRDATPWTLVQ

AILAPLQFLAFGVSLVLVVRFLFTGEGYEAAT

ISILIKTLLLYTIMVTGAIWEKVVFGQYLFAPA

FFWEDVFSFGVIALHTAYLWALFTGQPDNMQ

MFIALAAYATYVINAGQFLWKLRQARLQAAS

EDAGTLVMERGTR

C-terminus

Figure 6: The protein sequence of BchF, with predicted transmembrane α-helices underlined in red.

BchF protein sequence gave a hydropathy index plot shown in Figure 7. The Rao and Argos, and Eisenberg programs predicted four transmembrane α-helices; the SOAP method indicated three transmembrane spans, and classified BchF as an integral protein. In addition GGBSM and Garnier programs (PCGene, 1993) re-enforced the prediction of α-helical content, but were not designed to analyze membrane-spanning probability. The position of these putative membrane spans are indicated by underlines in the sequence diagram. The solid red line shows transmembrane regions predicted by all three programs, while the dotted red line marks the additional transmembrane α-helix listed by the Rao and Argos and Eisenberg methods.

Further computer analysis compared the sequence of the whole protein and regions such as the N-terminus, potential membrane-spanning domains and a sequence from the C-terminus were run through a BLAST/GENEBANK comparative search through all other proteins of known sequence. The only significant homology was found with *R. sphaeroides* BchF sequence. The first forty amino acids of the *bchF R. sphaeroides* sequence have been entered into

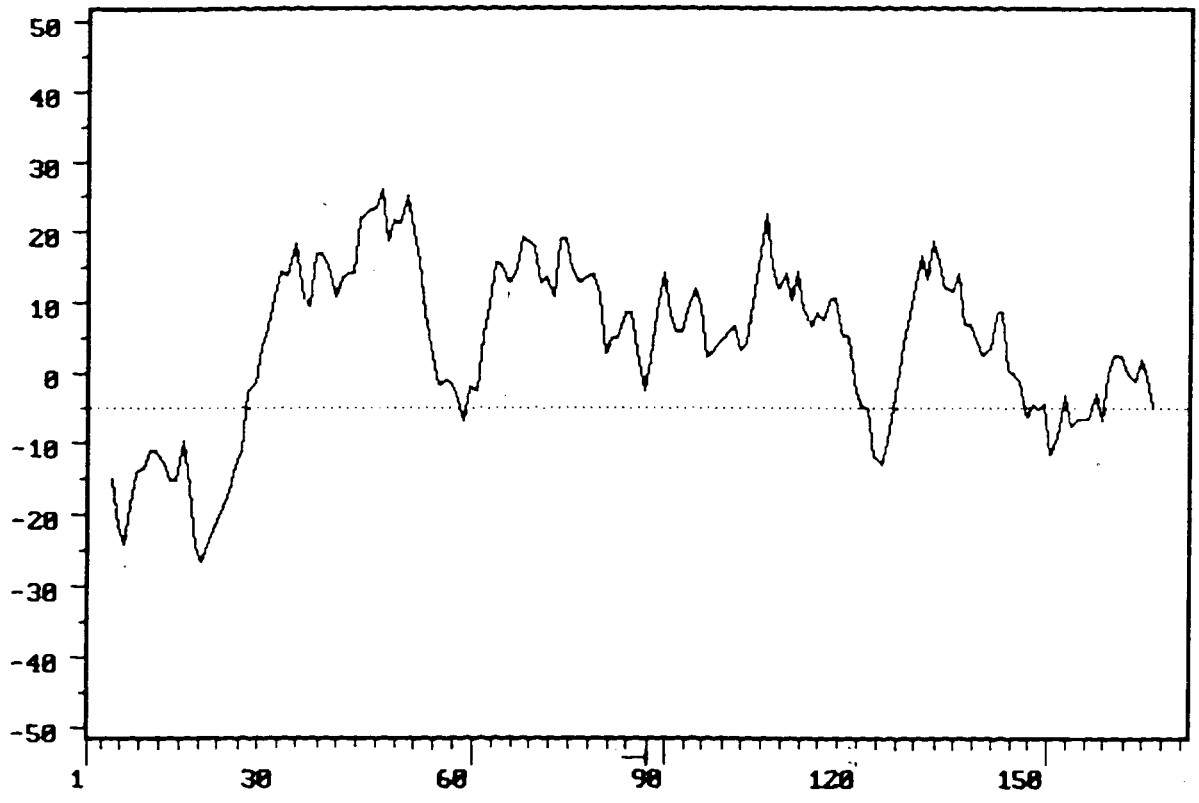


Figure 7 : BchF hydropathy index plot derived from the program SOAP, computed using an interval of 9 amino acids.

GENEBANK and showed a high degree of identity with the BchF *R. capsulatus* sequence. The *R. capsulatus* BchF protein does not show homology to other known, sequenced hydratases. Nor does the sequence reveal any potential for the metal binding motifs found in some other hydratases such as fumarase.

1.10 Summary

The putative hydratase enzyme BchF acts at a crucial point in the Bchl biosynthetic pathway: at the point of divergence between higher plant chlorophyll *a* biosynthesis and the bacterial pathway. Considering the debate concerning the evolutionary order of Chl and Bchl biosynthetic pathways, then BchF and BchXYZ will be interesting to study enzymologically as they control the juncture between the two pigment systems. The possibility of a branch point and alternate substrates for both BchF and BchXYZ hold potential for interesting comparative kinetic studies measuring the K_m and V_{max} values for each potential substrate. The possible substrate preferences, or preferred "route" through the branch point would further define the biosynthetic pathway.

The goal of this project is the expression of the BchF enzyme in *Escherichia coli* as a tool for the development of a specific assay and future kinetic studies. The production of an active BchF enzyme is a necessary step in the functional assignment of this significant protein.

2. Materials and Methods

2.1 Media

All the following weights are per litre of ddH_2O unless indicated otherwise.

2X Ty Amp broth : 16 g tryptone, 10 g yeast extract, 5 g NaCl with 200 *u*g Ampicillin.

2X Ty Amp plates : 16 g tryptone, 10 g yeast extract, 5 g NaCl and 14g agar.

Ty Mg broth : 20 g tryptone, 5 g yeast extract, 5 g NaCl and 10 mM $MgSO_4$.

5X Minimal A Salt Solution : 52.5 g K_2HPO_4, 22.5 g KH_2PO_4, 5 g $(NH_4)_2SO_4$ and 2.5 g sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)

1X Minimal A Media Broth : 10 ml 5X Minimal A salt solution plus 39.5 ml ddH2O, autoclaved and cooled to 60°C.

Then add 0.5 ml 20% (w/v) glucose (previously autoclaved), 50 *u*l 20% (w/v) $MgSO_4$ (previously autoclaved) and 25 *u*l 1% (w/v) Vitamin B1 (filter sterilised) and 50*u*l of 0.2g/ml stock Ampicillin.

2.2 Bacterial Strains

2.2.1 Escherichia coli Strain TG2

The genotype is supE hsd Δ 5 thi Δ (lac-proAB) Δ (srl-recA) 306:: Tn 10 (tet^r) F' [tra D 36 pro AB^+ lac I^q laZ Δ M15]. The strain TG2 must be grown on Minimal media to maintain $lacI^q$. Otherwise the strain will grow on any rich broth, such as 2xTY at 37°C. TG2 overproduces the lac repressor (LacIq) and so makes a suitable host for vectors containing IPTG inducible promoters.

2.2.2 Escherichia coli Strain DH5×

Sup E44 Δ lac U 169 (ϕ 80 lac Z Δ M15) hsd R17 rec A1 end A1 gyr A96 thi-1 rel A1 is the genotype of DH5α. The strain DH5× (Sambrook *et al.* ,1989) was maintained and grown on 2 x Ty plates and 2 x Ty broth at 37°C. This strain was used occasionally for transformations of the plasmid pUC18.

2.3 Plasmids

The plasmid pKK223-3 (Brosius and Holy, 1984) is a transcription expression vector and contains a tac promoter (IPTG inducible) which drives expression of recombinant genes, and ribosomal termination sequences, as well as ampicillin resistance.

The pUC18 (Sambrook *et al.*, 1989) is a high copy number *E.coli* plasmid, with ampicillin resistance, commonly used for sequencing because of the availability of primers.

The plasmid pDB32 (Bauer, personal communication) obtained from Dr. Carl Bauer of Indiana University was constructed by inserted a 1080bp fragment containing *bchF* flanked by approximately 200 bp of the upstream region, 300 bp of truncated *bchN* downstream. This fragment was cloned into the vector pSP72, which is a non-inducible *E. coli* plasmid. The pDB32 plasmid was the source of *bchF* for subsequent cloning.

2.4 Plasmid Preparation Solutions

Solution I: 50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl(pH 8.0) and 0.004 g/ml lysozyme prepared fresh.

Solution II: 0.2N NaOH and 1%(w/v) SDS

Solution III: 60 ml of 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml ddH_2O, pH 4.8, giving a solution 3 M potassium and 5 M acetate.

2.5 Buffers

TAE : 40 mM Tris-acetate and 1 mM EDTA

TE8 : 10 mM Tris-Cl, pH 8.0 and 1 mM EDTA, pH 8.0.

5XTBE : 54 g Tris base, 27.5 g boric acid, 2.93 g EDTA per litre ddH_2O, pH 8.1-8.3

TfBI : 30 mM potassium acetate, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$, 15% (v/v) glycerol, pH adjusted to 7.0 and filter sterilised, made up to 40 ml with ddH_2O.

TfBII : 10 mM Na-MOPS, pH 7.5, 75 mM $CaCl_2$, 10 mM KCl and 15% glycerol, solution pH adjusted to 7.0 and filter sterilised.

5x Electrode SDS buffer: 15 g Tris-Cl, 72 g glycine, 5 g SDS, pH 8.3 per litre ddH_2O.

6x SDS Sample buffer: 7 ml 0.5 M Tris-Cl, pH 6.8, 1.0 g SDS, 3.8 g glycerol, 0.93 g dithiothreitol, 1.2 mg bromophenol blue, add ddH_2O to a final volume of 10 ml.

Coomassie Blue Stain: 0.1500 g Coomassie blue in 500 ml Fast. Fast Destain is 40% (v/v) methanol, 10%(v/v) glacial acetic acid in ddH_2O.

2.6 Molecular Biological Methods

Plasmid preparation, preparation of competent *E. coli* cells and transformation of *E. coli* protocols were performed as outlined in Sambrook *et al.*, 1989.

2.6.1 Polymerase Chain Reaction

The *bchF* gene was PCR-amplified using engineered primers (Gibco Co.) to incorporate *XbaI* sites adjacent to the start and stop codons. The PCR conditions were as follows: 0.25mM dNTP, 5 *ul* Gibco Vent DNA polymerase buffer, 300 ng of start and stop end primers, 100 ng template DNA, 5 ng BSA, 2.5 *ul* of DMSO and 1 unit of Vent DNA polymerase (Gibco Co.). The PCR was run in a programmable thermal controller with a 30 cycle pattern of 98 °C for 30 seconds, 42 °C for 60 seconds, and 72 °C for 60 seconds. A final 10 minutes at 72 °C completed the reaction. The PCR-amplified *bchF* gene was purified by agarose gel electrophoresis and subsequently digested with *XbaI*.

2.6.2 Mung Bean Nuclease

Mung bean nuclease was used to blunt end the 5' overhang resulting from digestion with the restriction enzyme *XbaI* through limited single strand hydrolysis. For removal of the single stranded extensions, the DNA was suspended (0.1 *ug/ul*) in 1X mung bean nuclease buffer. Upon the addition of 1.0 unit of mung bean nuclease per ug of DNA, the digest was incubated at 30°C for 30 minutes. The

enzyme was inactivated by the addition of 0.01% (w/v) SDS. The DNA was recovered by ethanol precipitation.

2.6.3 T4 DNA Polymerase

T4 DNA polymerase fills in protruding ends with a 5' to 3' polymerase activity. The DNA which was previously digested with *XbaI* was purified and resuspended in 10 *u*l of ddH_2O. 1 *u*l of T4 DNA polymerase buffer was then added and combined with 100 *u*M (each) dNTP and 50 *u*g/ml BSA. 1 unit of T4 DNA polymerase per *u*g of DNA was added. The polymerase reaction was incubated for 20 minutes at 12 °C. The enzyme was then heat inactivated at 75 °C for 10 minutes.

2.6.4 Dephosphorylation of Plasmids

The 5' phosphate of linearized plasmid DNA was removed by treatment with calf intestinal alkaline phosphatase (CIP). 1 unit of CIP per *u*g of DNA was added directly to a completed restriction digest reaction or to precipitated DNA resuspended in React buffers. The reaction was incubated at 37°C for 1 hour and then the sample was gel purified. The DNA was excised from the gel, recovered

through spin columns and alcohol precipitation and used in subsequent ligations.

2.6.5 Ligation of DNA Fragments into Plasmids

DNA fragments were ligated to linearized and dephosphorylated plasmids using the enzyme T4 DNA ligase. The concentration of insert and vector was estimated by visualising small aliquots of each on agarose gels. For most cases a 2:1 molar ratio of insert to vector was used in the ligation reaction. The ligation mixture consisted of 2 *ul* of 5x ligation buffer and 1 unit of enzyme diluted to a final volume of 10 *ul*. Incubations were carried out at room temperature, overnight for blunt-ended ligations.

2.7 Test Expression Protocol

To determine overexpression in a small scale, 1 ml of overnight culture was taken into 4 ml of Min A media and grown until A_{600nm} reached approximately 0.3 at 37 °C. The culture was then induced with 0.4 mM IPTG (final concentration) and 1 ml aliquots were taken every 30 minutes to follow the time course of the protein expression.

The cells were pelleted and resuspended in 200 ul of TE8 with 2mg/ml lysozyme and incubated for 30 minutes. The cells were subsequently sonicated (3 x 30 sec. bursts) and centrifuged for 5 minutes at 15,000 rpm. The supernatant was removed and ultracentrifuged, 45,000 K for 1.5 hours at 4 °C. The result was a division into soluble and membrane fractions which were run on SDS-PAGE gels.

2.8 Large Scale Expression

To produce larger quantities of protein for the assay procedure a volume scale-up of the test expression protocol was applied. The expression was carried out in 100 ml of MinA media, and the induction time was selected as one hour, shown to maximal by the previous time trials.

2.9 Separation of Proteins by Gel Electrophoresis

The appropriate percentage (12% - 20%) polyacrylamide gels were poured in Bio-Rad mini-protein casting apparatus and were electrophoresed using the SDS-buffer system of Laemmli (Laemmli, 1970). Protein samples were denatured and solubilized prior to

electrophoresis by heating at 95 °C for 5 minutes in SDS sample buffer. Electrophoresis was performed at 200 Volts in SDS electrophoresis buffer for approximately 45 minutes, until the bromophenol blue marker dye had reached the bottom of the gel. Gels were stained in Coomassie blue stain and destained in Fast Destain solution. Destained gels were stored by first immersing them in 50% glycerol for 2 minutes, then sealing them in gel wrap, or drying on a gel dryer for 1 hour.

2.10 Sequencing

Selected constructs were inserted into pUC18 and the DNA purified for commercial sequencing. The Biotechnology lab in Nucleic Acid Protein Service Unit of the University of British Columbia performed four-dye, one lane fluorescence labeling automated sequencing with Taq polymerase. Both forward and reverse pUC18 primers (M13 and reverse M13) were used to sequence the 550 bp BchF region of the construct.

2.11 Zinc Derivatives

To produce the substrate and control pigments for the hydratase assay zinc analogs of chlorophyllide *a* were produced. The zinc analogs were synthesized from the precursor pheophorbide *a* , supplied by Porphyrin Products Inc. The zinc insertion protocol is outlined in Richards *et al.*, 1987. The method for preparation of [2-hydroxyethyl]-pheophorbide *a* was adapted from the procedure of Richards and Lascelles (1969). The production of HBr/glacial acetic acid was omitted, instead 30% HBr/glacial acetic acid was purchased from Aldrich Co.

2.12 High Pressure Liquid Chromatography (HPLC)

The method of pigment detection for the hydratase reaction was HPLC. The HPLC was carried out with a Waters 991 Photodiode Array Detector and Waters 600 controller with Millenium software. The main solvent system applied was 70% methanol / 30% water with an initial five minute flow interval of the 70% methanol / 30% water / 5mM PicA for each run. PicA is a commercial ion-pairing reagent. The ODS column was run at a flow rate of 0.8 ml/min with resulting pressures ranging from 1424 psi to 1560 psi. The elution

was monitored at 407nm and 416nm, for zinc-containing and zinc-free pigments.

2.13 Assay Methods

The hydratase reaction to be assayed involved the conversion of the 2-vinyl group of chlorophyllide *a* to a hydroxyethyl group. The successful reaction would produce the product Zn-[2-hydroxy-ethyl]-chlorophyllide *a* from the substrate of Zn-pheophorbide *a*. The hydratase assay was carried out at pH 8.0, 37 °C for 1 and 3 hours in the dark. The assay mixture contained 4mls of 0.5 mg/ml soluble protein fraction and 1ml of 0.15 mg/ml Zn-pheophorbide *a* dissolved in 1% (v/v) Triton X-100 in TE8. The assay mixture was frequently purged with nitrogen in an attempt to limit substrate and product degradative oxidation The pigments were extracted for analysis by first precipitating the protein with basic acetone and centrifuged. The supernatant was then brought to pH 1 with 10% (v/v) hydrochloric acid which would force the removal of the zinc. The pigments were subsequently extracted into ethyl acetate, dried over nitrogen, and dissolved into 100% HPLC grade methanol. Aliquots of 20 *ul* were injected into the HPLC.

3. Results

3.1 Molecular Cloning

The molecular cloning of *bchF* started with the plasmid pDB32 supplied by Dr. Carl Bauer of Indiana University. This vector, pictured in Figure 8, contained the entire *bchF* sequence flanked by approximately 300 base pairs of *bchN* sequence downstream and about 200 base pairs of an open reading frame upstream creating a 1080 bp fragment in a non-expressing vector. There is an upstream "*E. coli*-like σ^{70} promoter region" which may be a promoter for *bchFNBHLM* superoperon in *R. capsulatus*. The first construct inserted the entire 1080 bp fragment into the pKK223-3 expression vector in an attempt to see if *E. coli* would accept this possible promoter region and express *bchF*. The construct, termed pBC1 (Figure 9), was digested with *SmaI*, *HindIII* and *BglI* restriction enzymes to verify the proper insertion direction. The test expression protocol (described in Chapter 2) was performed on TG2 containing pBC1 and samples were analyzed by gel electrophoresis. The pBC1

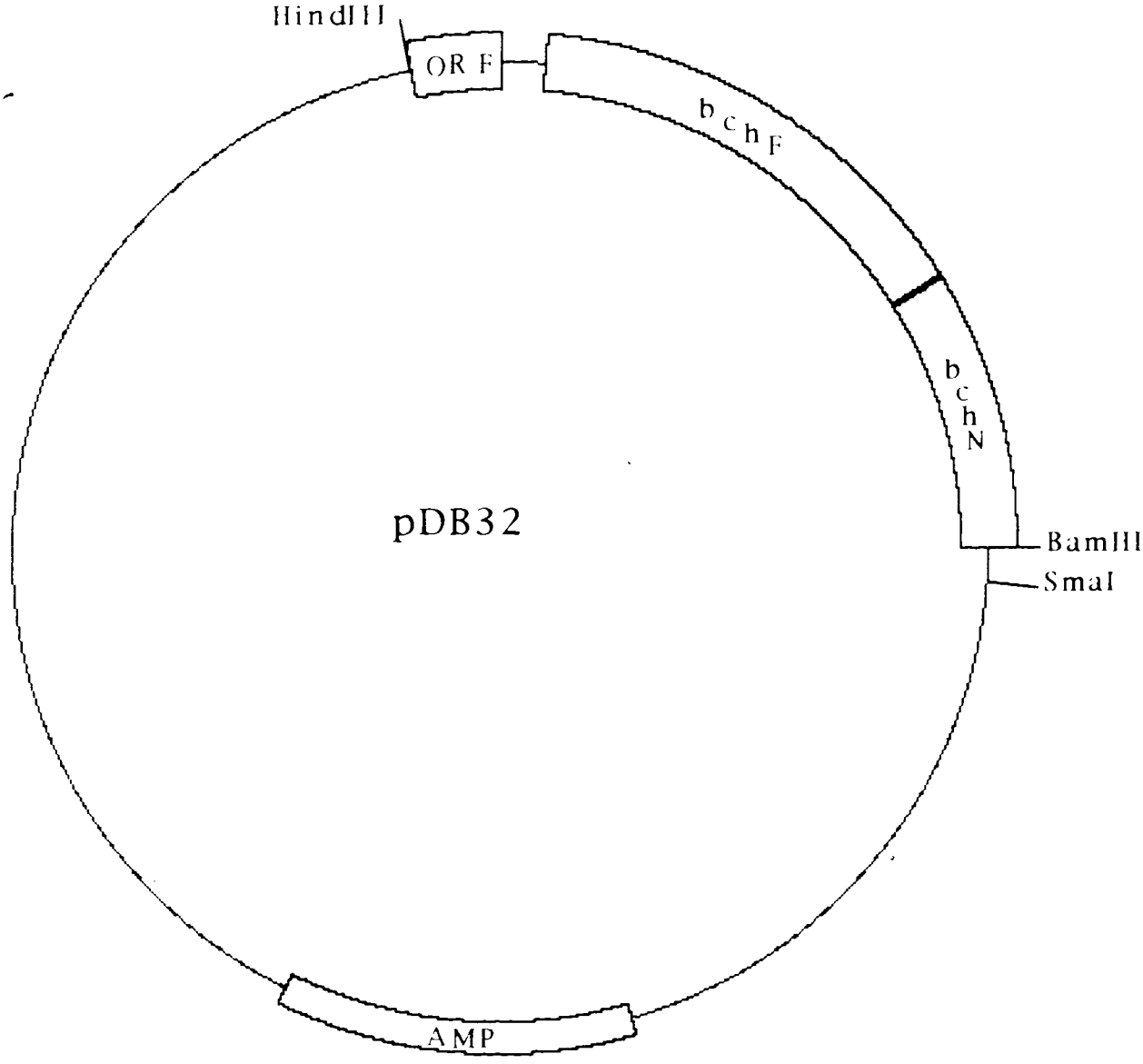


Figure 8 : The plasmid pDB32 (from Dr. C. Bauer, Indiana University) the source of the *bchF* gene. The *bchF* insert is flanked by a partial ORF and a truncated *bchN* creating a 1080 bp fragment.

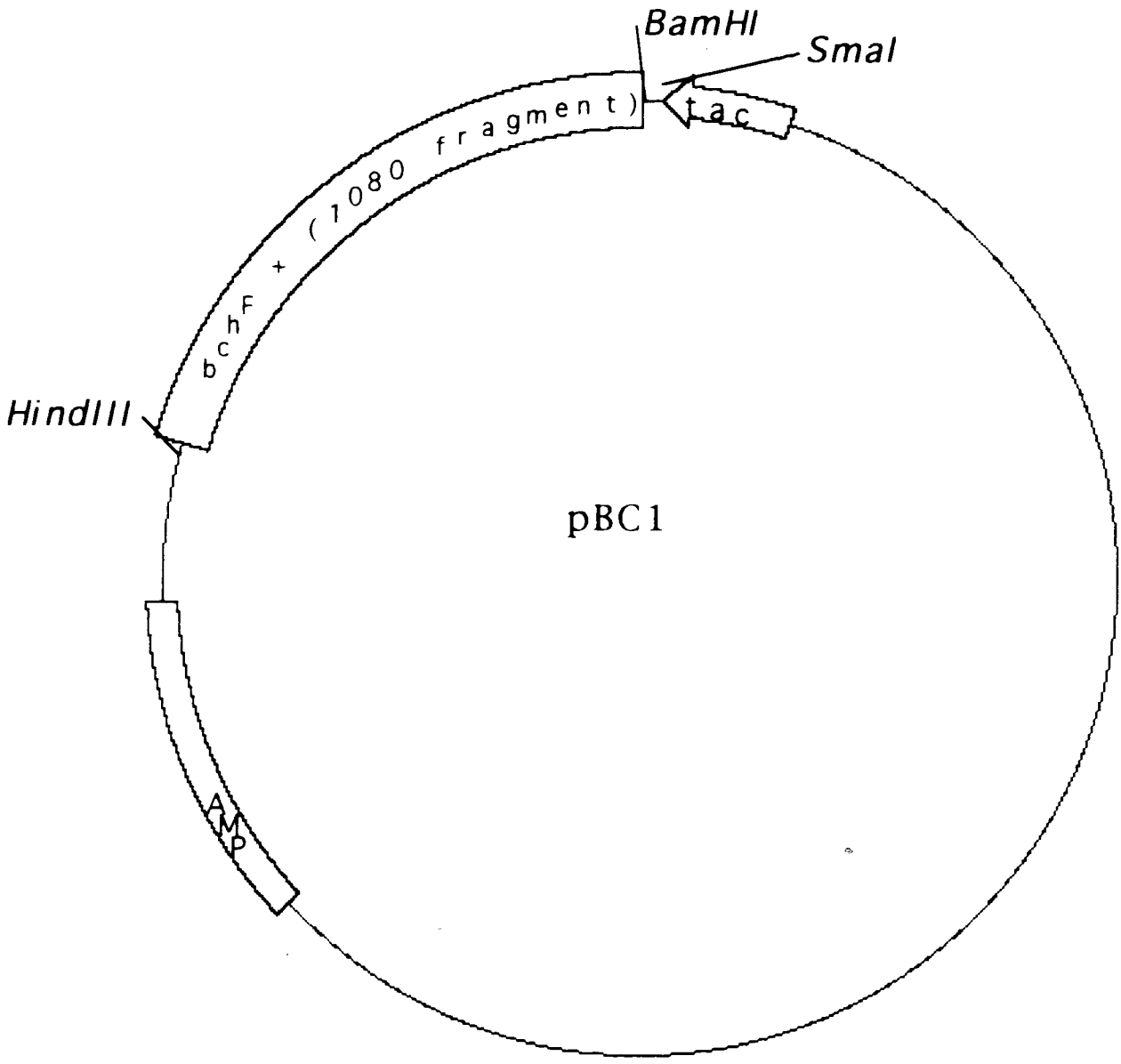


Figure 9 : The plasmid pBC1, containing the 1080 insert with *bchF* under its own promoter control.

construct did not result in the detectable expression of *bchF*. The construct's flanking regions were probably much too large for the intrinsic pKK223-3 promoter to express *bchF*. Therefore, the host TG2 *E. coli* did not accept the putative "*E. coli* like" promoter sequence which has still not yet been demonstrated to be the *bchF* promoter in *R. capsulatus* (Alberti, 1995).

Since the *bchF* sequence did not contain strategically placed restrictions sites for direct digest and insertion, further molecular manipulations were needed. To engineer a *bchF* fragment that could be inserted adjacent to the pKK223-3 promoter, PCR was used to create the 516bp *bchF* insert with *XbaI* sites on either end. This was achieved by specific PCR primers which were designed containing the *XbaI* sites adjacent to complementary sequence for the start and stop regions of *bchF*. When applied in PCR, these primers created a *bchF* insert with start and stop regions flanked by *XbaI* sites. *XbaI* was chosen as a restriction site which would be digested to leave the ATG start of *bchF* intact. An *XbaI* site (TCTAGA) was engineered into the PCR primers to make a *bchF* fragment flanked by *XbaI* sites. This fragment could then be digested by *XbaI* leaving sticky ends. As

shown in Diagram 1 for the upstream end, the ends were then blunted with mung bean nuclease for insertion into the *SmaI* site of pKK223-3. This insertion placed the ATG start an optimal 18 bp from the promoter. This construct, pBC2 (Figure 10), was digested by *BglI* to verify insertion direction and a test expression was completed and analyzed on gel electrophoresis. The TG2 host with pBC2 did not express *bchF*. Despite attempts to control the reaction, the mung bean nuclease may have continued to digest away the start codon, leaving a blunted insert that could not be expressed.

Diagram 1: *XbaI* digested upstream and downstream ends of the *bchF* PCR fragment and then blunted with mung bean nuclease.

A. Upstream containing start sequence:

CTAGATGCCG
TACGGC

after mung bean nuclease digest →

ATGCCG
TACGGC

B. Downstream containing stop sequence:

CGATGAT
GCT ACTAGATC

after mung bean nuclease digest →

CGATGAT
GCTACTA

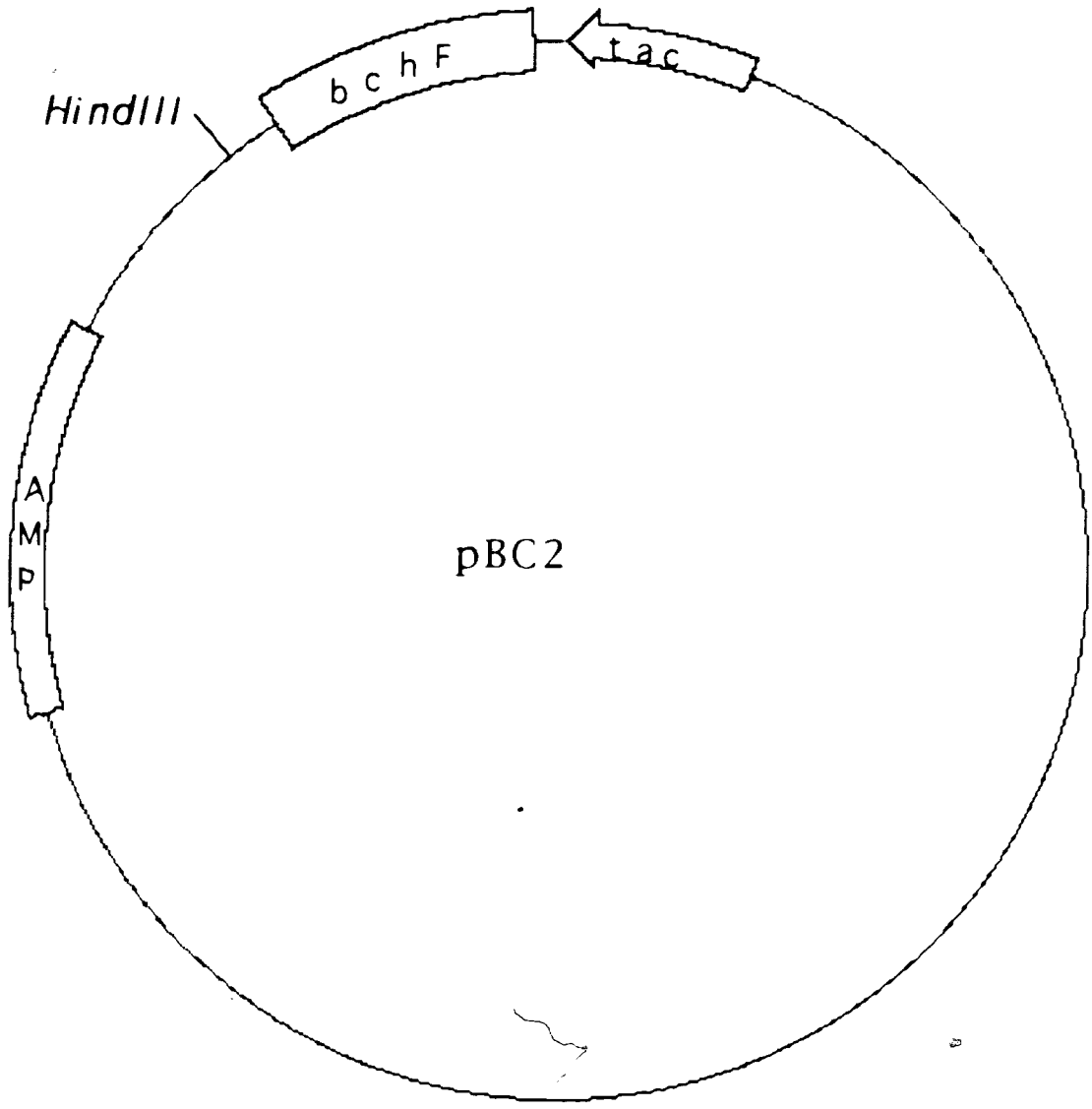


Figure 10 : The plasmid pBC2 containing the mung bean nuclease-blunted *bchF* insert.

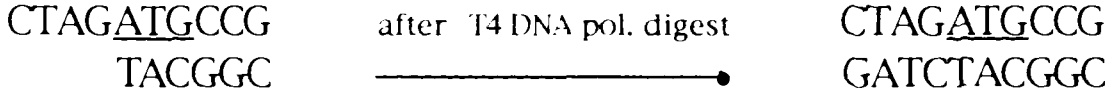
As an alternative to using Mung Bean Nuclease to blunt the *XbaI*/516bp fragment, T4 DNA polymerase was used to fill in the sticky ends rather than remove them. The filled-in T4 DNA polymerase blunted ends are illustrated in Diagram 2.

The resulting blunted fragment was then inserted into the *SmaI* site of pKK223-3 (thereby destroying the *SmaI* site) with the start of *bchF* 22 bp from the promoter region. As with the other constructs, this third construct, pBC3 (Figure 11) was digested with *BglI* and analyzed on agarose gel electrophoresis to verify insertion direction.

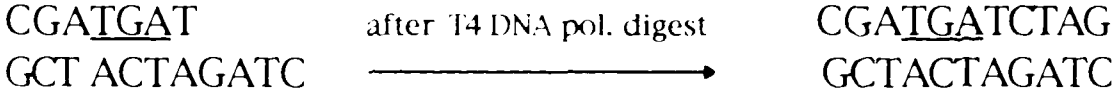
The T4 DNA polymerase blunted *bchF* fragment was also inserted into the plasmid pUC18 for commercial sequencing. The construct was sequenced with forward and reverse primers (M13 and M13 reverse). The reverse primer resulted in a clear sequence that was able to be analyzed right through from the start to stop regions of *bchF* (approximately 550 bp). The first 400 bp of this sequence was then confirmed by the forward M13 primed sequence. When the four-dye fluorescence label sequencing results from the Nucleic Acid Service Unit were analyzed, they matched the DNA

Diagram 2: *XbaI* digested upstream and downstream ends of the *bchF* PCR fragment and then blunted with T4 DNA polymerase.

A. Upstream containing start sequence:



B. Downstream containing stop sequence:



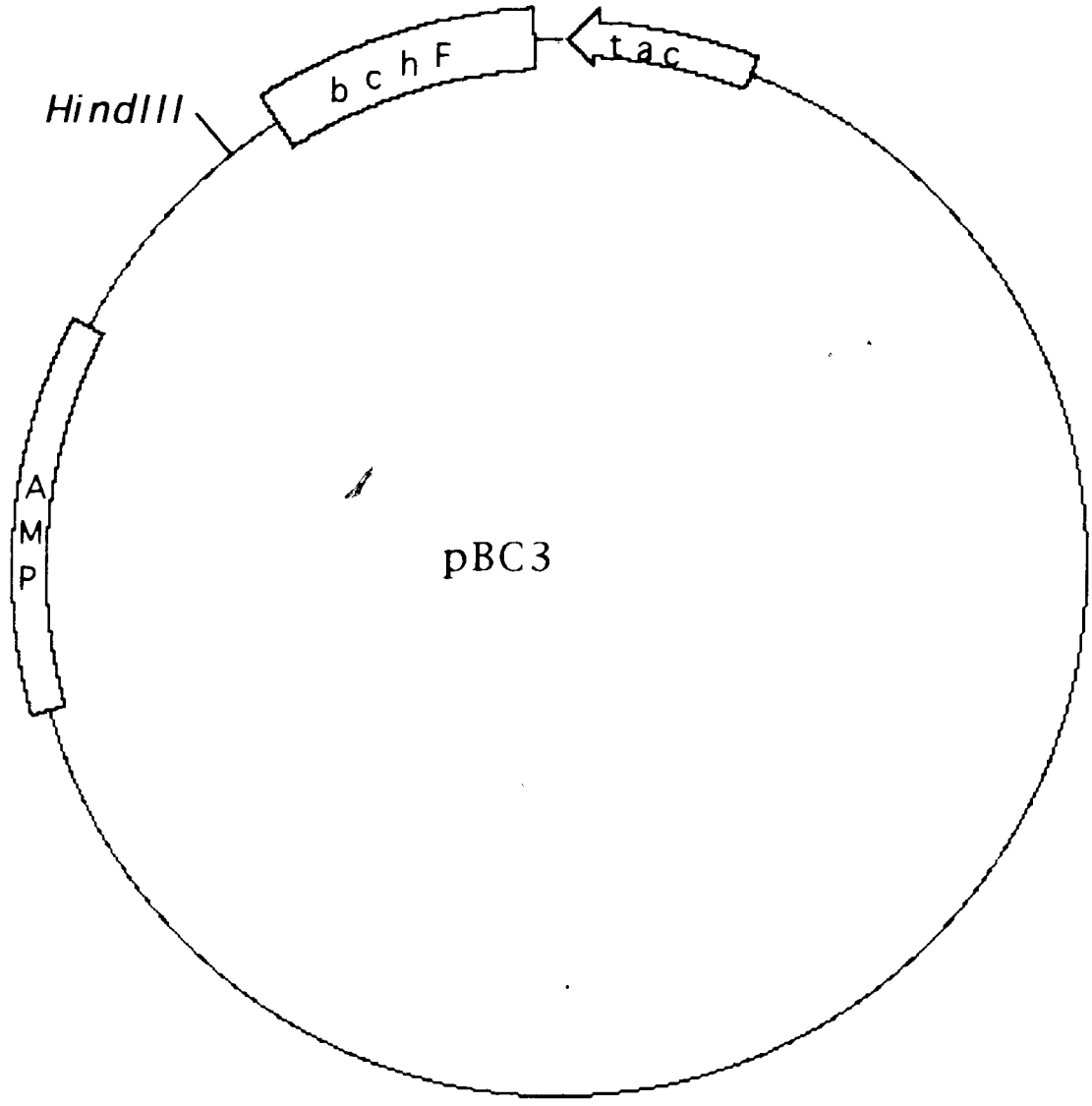


Figure 11 : The plasmid pBC3 containing the T4 DNA polymerase-blunted *bchF* insert.

sequence of an intact *bchF* gene with the appropriate flanking regions.

When the test expression of TG2 containing pBC3 was analyzed with gel electrophoresis it showed over-expression of a 19,000 Dalton protein, the correct size for BchF.

3.2 Production of BchF

The construct pBC3, when IPTG induced, produced an approximately 19 kDa protein which corresponds to the size of the 171 amino acid BchF enzyme. A time course trial was applied as described in Chapter 2 to follow the production of this 19 kDa protein. Samples were taken at half hour intervals for the first two hours and then hourly up to five hours. There was some basal expression in the zero time sample but the protein appeared in much greater amounts at thirty minutes with maximal amounts at one hour. After two hours of induction the protein was degraded by the host *E.coli*. This time course expression pattern can be viewed in Figure 12 A.

After an initial low-speed centrifugation to remove cellular debris, the over-expression samples were ultracentrifuged to separate the soluble protein and membrane fractions. The 19 kDa protein was found solely in the soluble fraction, as there was no induced protein present in the membrane fraction after only one of two washes. The membrane fraction is pictured in Figure 12B. Controls of TG2 containing pKK223-3 (empty plasmid, no insert) and host TG2 *E.coli* were run in all time trials and none demonstrated any significant protein induction.

Figure 12 A: Soluble fraction of time scale expression.

Lane 1 - broad range standard, lane 2 - 0 min., lane 3 - 30 min., lane 4 - 1 hour, lane 5 - 2 hours, lane 6 - 3 hours lane 7 - pKK223-3 control sample at 1 hour.

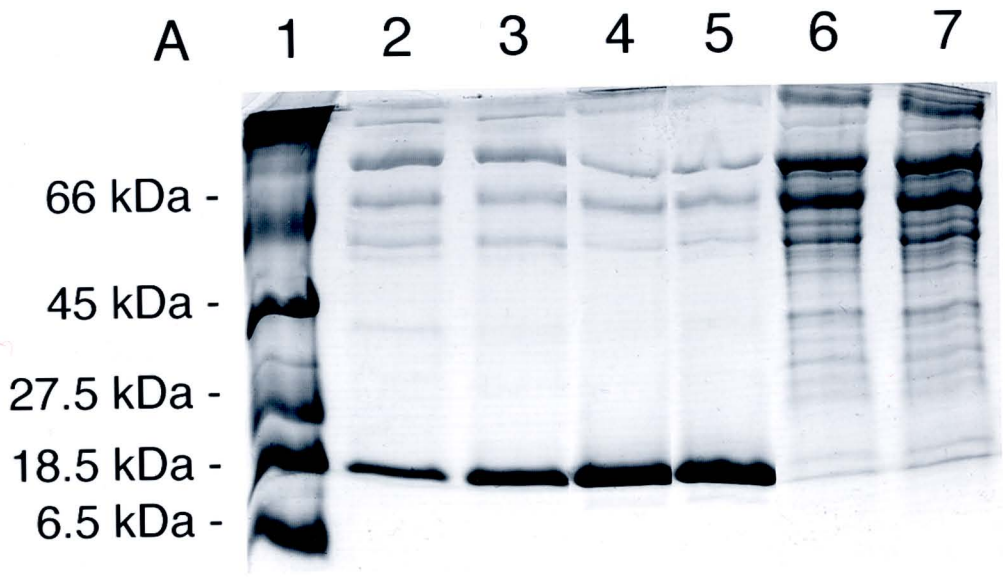
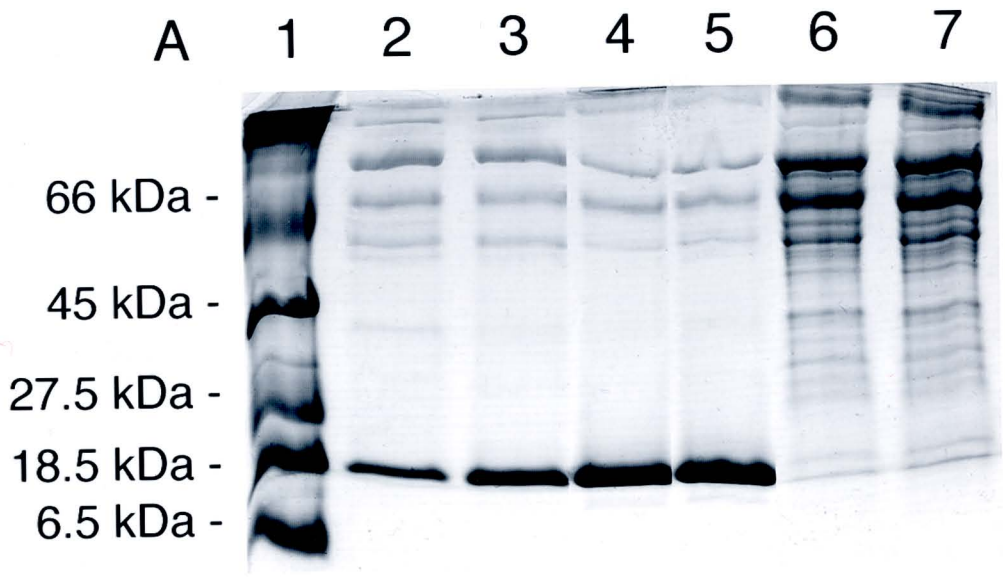

A
1
2
3
4
5
6
7
66 kDa -
45 kDa -
27.5 kDa -
18.5 kDa -
6.5 kDa -

Figure 12 B : Membrane fraction of time scale expression.

Lane 1 - broad range standard, lane 2 - 0 min., lane 3 - 30 min., lane 4 - 1 hour, lane 5 - 1.5 hours, lane 6 - 2 hours, lane 7 - 3 hours, lane 8 - pKK223-3 control sample at 1 hour, lane 9 - TG2 control sample at 1 hour.

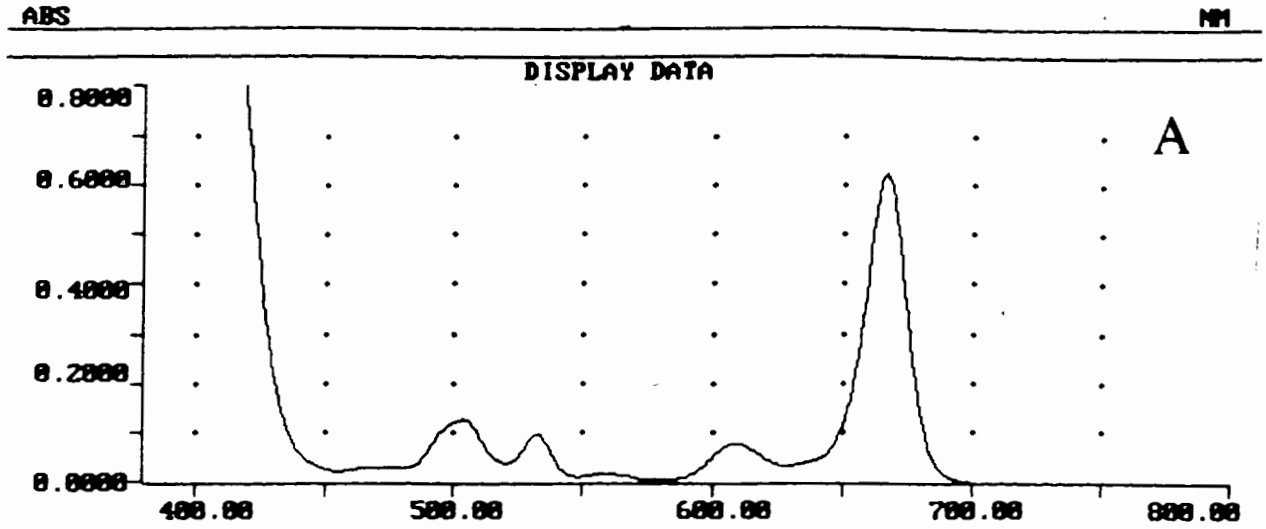

A
1
2
3
4
5
6
7
66 kDa -
45 kDa -
27.5 kDa -
18.5 kDa -
6.5 kDa -

3.3 Substrate Production

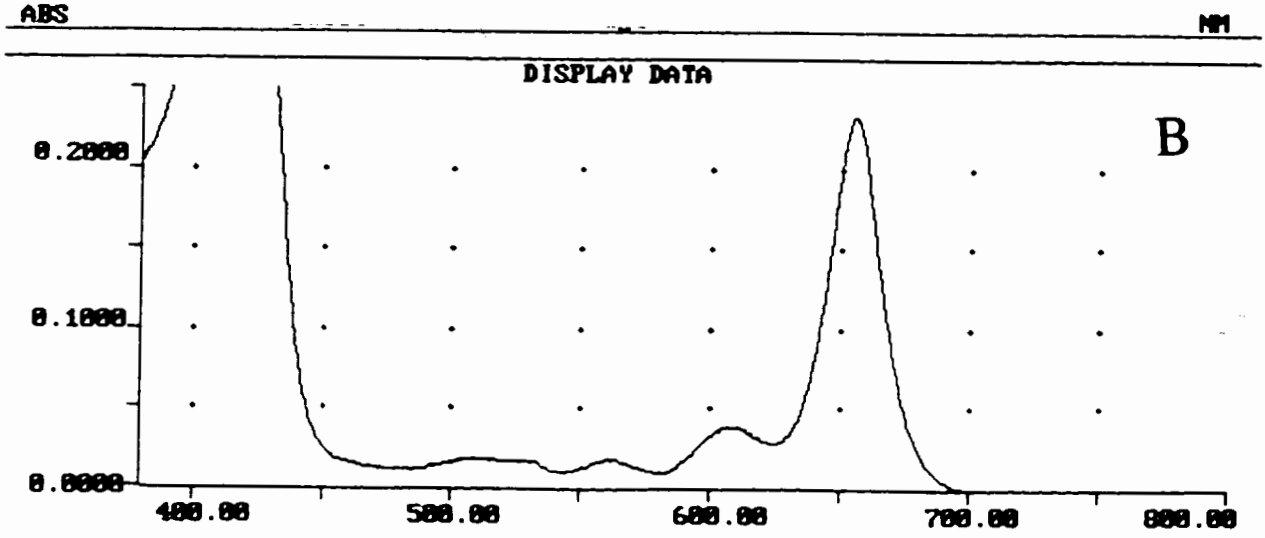
There are two methods of substrate production available for application in this project. The first possible method is substrate accumulation in the bchF$^-$ mutant (DB171) and the bchF$^-$, bchXYZ$^-$ double mutant (CB1200) strains of *R. capsulatus.* These mutant strains could provide a source of chlorophyllide *a* as well as [2-vinyl]-bacteriochlorophyllide *a*, the two putative substrates for the hydratase reaction. Due to difficulties culturing the mutant strains, an alternative substrate production method was applied. This second method of substrate production was derived from commercially obtained pheophorbide *a* , the metal free version of chlorophyllide *a*. Zinc was inserted into the pheophorbide *a* (as described in Chapter 2) creating a zinc analog to the substrate chlorophyllide *a*. As discussed in Chapter 1, zinc analogs are a viable alternative to the less stable magnesium pigments.

3.4 Spectral Analysis

As displayed in Figure 13 A, the pheophorbide *a* spectrum in ether exhibits absorption maxima at 407 nm and 667 nm. The spectrum of the Zn-pheophorbide *a* after zinc insertion has maxima shifted to 416 nm and 655 nm (Figure 13 B). The pheophorbide has characteristic intermediary peaks at 504, 533 and 609 nm. With zinc inserted these peaks diminish, though there remains a small peak at 608 nm. The [2-hydroxyethyl]- pheophorbide *a* (Figure 13 C) shows maxima at 408 nm and 671 nm, as well as smaller peaks 506, 544, 616 nm, shifted slightly from the pheophorbide spectrum. The Zn-[2-hydroxyethyl]- pheophorbide *a* (Figure 13 D) displayed peaks at 416 nm and 656 nm but not enough of this product was obtained for a clear spectrum. The shift in the spectra between the substrate Zn-pheophorbide *a* and the hydratase reaction product Zn-[2-hydroxyethyl]-pheophorbide *a* is negligible. Similarly, there is no significant shift between the spectra of pheophorbide *a* and the [2-hydroxyethyl]-pheophorbide *a*. Therefore, spectrophotometric detection cannot be applied to the hydratase assay. The mode of

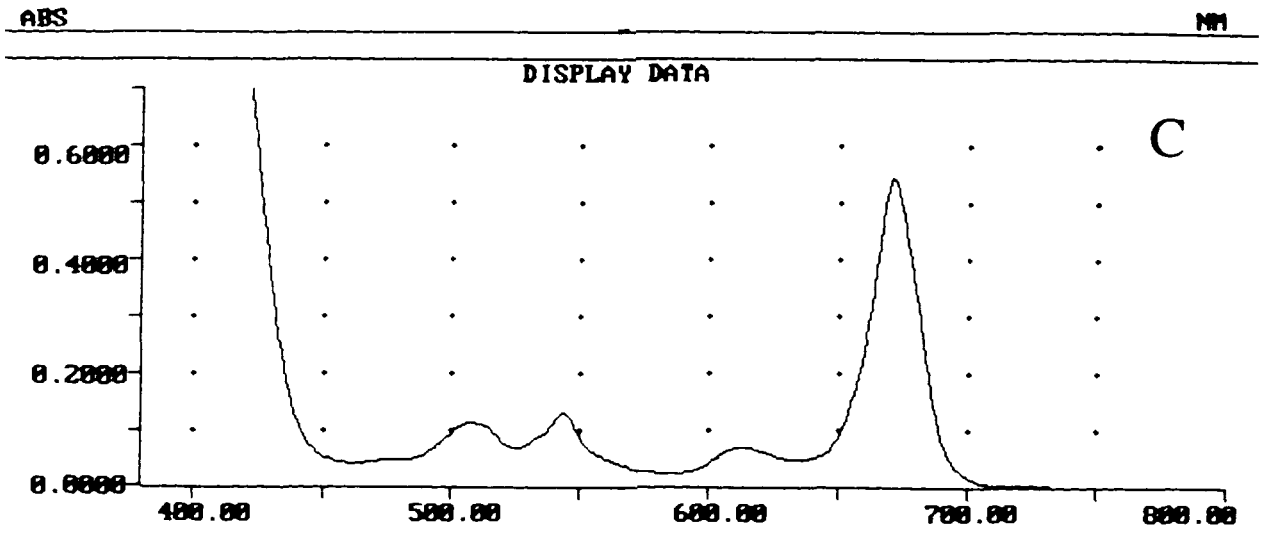


A. Pheophorbide *a* , maxima at 407, 504, 533, 609 and 667nm.

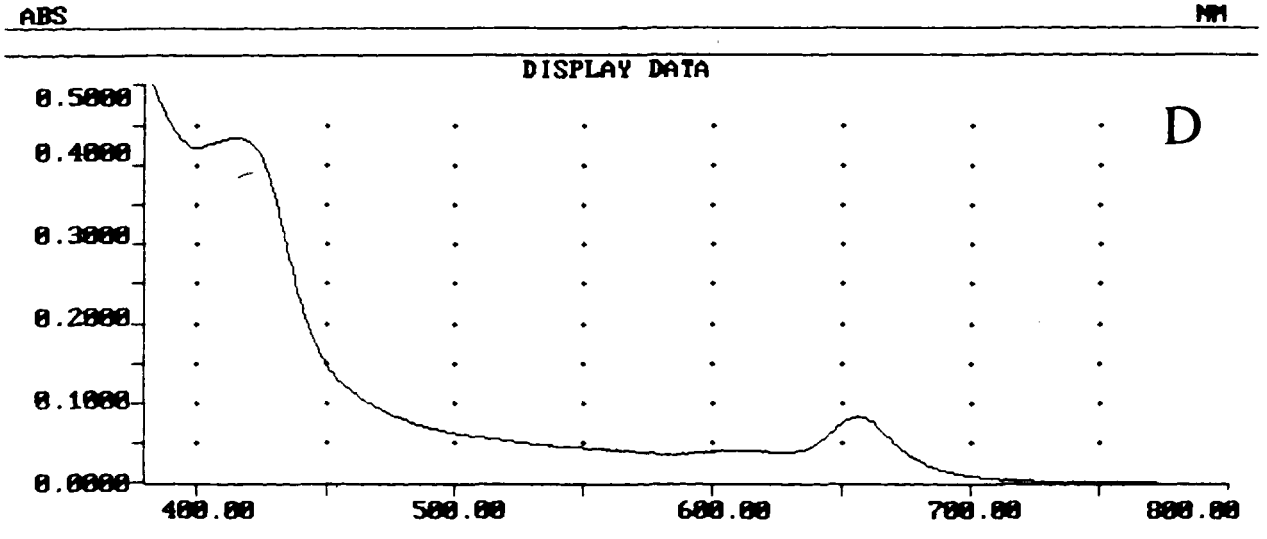


B. Zn-pheophorbide *a*, maxima at 416, 608 and 655nm.

Figure 13 A, B : Spectra of pigments in ether.



C. [2-Hydroxyethyl]-pheophorbide *a*, maxima at 408, 506, 544, 616 and 671 nm.



D. Zn-[2-hydroxyethyl]-pheophorbide *a*, maxima at 416 and 656 nm

Figure 13 C, D: Spectra of pigments in ether.

detection selected for the BchF hydratase assay capable of distinguishing between the substrate and the product was HPLC.

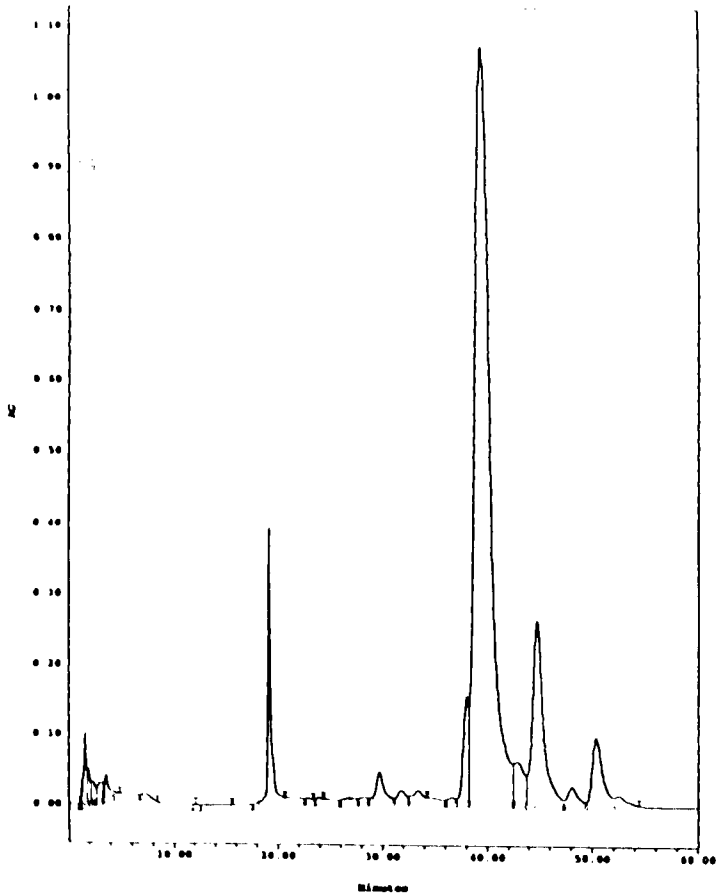
3.5 HPLC Assay Analysis

Standards of pheophorbide *a*, Zn-pheophorbide *a*, and [2-hydroxyethyl]-pheophorbide *a*, were all run prior to the assay samples. The metal free pigments have a greater intensity of signal when monitored at their Soret maximum of 407 nm. The zinc-containing samples show enhancement of signal when monitored at 416 nm. The samples had broad enough Soret bands to be analyzed at either 407 nm or 416 nm. Since the extraction procedure (outlined in Chapter 2) removed the zinc, the samples were monitored at 407 nm. The scans of the standards monitored at 407 nm can be viewed in Figure 14 A, B and C. Pheophorbide *a* shows a major peak at 40 minute elution time, whereas Zn-pheophorbide elutes earlier at 30 minutes viewed as the small central peak in the assay extracts. The [2-hydroxyethyl]-pheophorbide shows a series of peaks proposed to include oxidation byproducts from its production

as well as residual pheophorbide clusters. All samples show a 20 minute elution peak, which is classified as a major oxidation by-product, present even in the commercially-obtained precursor pheophorbide and is constant throughout standards and assay samples. This 20 minute peak has a greater intensity when monitored at 407 nm than at 416 nm, suggesting it is a metal-free pigment.

An assay sample of a three-hour incubation is shown in Figure 15 along with a TG2 protein control sample. There is no discernible difference between the BchF containing sample and the TG2 soluble-protein control. No activity was detected under the present assay conditions. The primary peak detected is at 40 minutes, assigned to be pheophorbide. The smaller peak at 30 minutes may be residual Zn-pheophorbide that did not loose the metal in the acid extraction step. Also present is the 20 minute by-product peak As with the standard samples, the 20 minute contaminate peak has a greater relative absorbance at 407 nm than 416 nm indicating that it is a zinc-free by-product.

Pheophorbide *a*



[2-Hydroxyethyl]-pheophorbide *a*

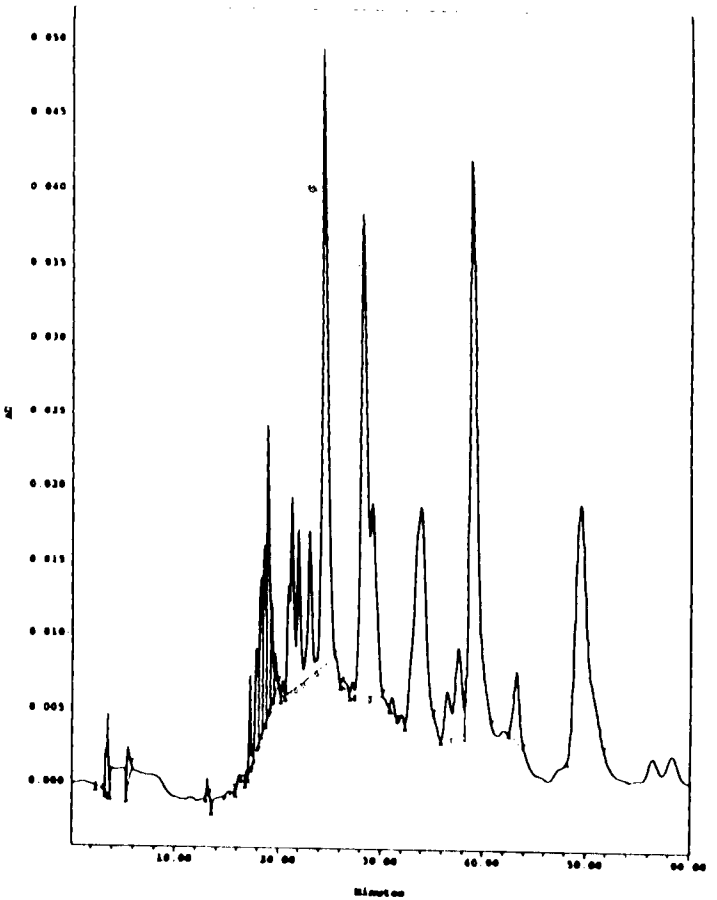


Figure14 A : HPLC standards: Pheophorbide *a* and [2-Hydroxyethyl]-pheophorbide *a*. Flow rate at 0.8 ml/min, monitoring wavelength 407 nm.

Zn-Pheophorbide *a*

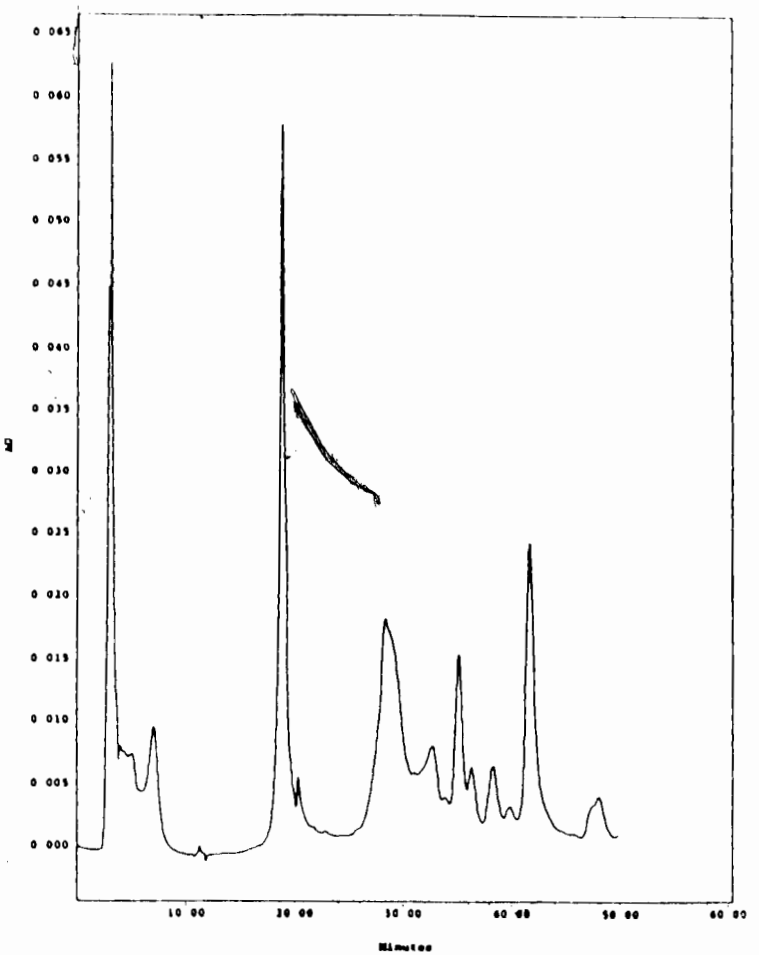
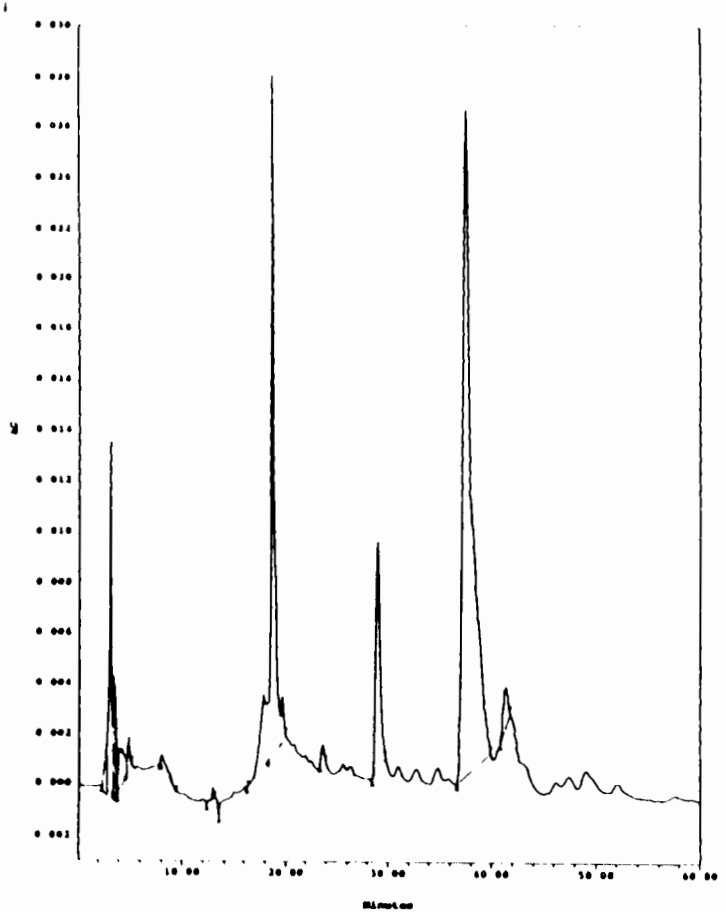


Figure14 B : HPLC standard: Zn-pheophorbide *a*.
Flow rate at 0.8 ml/min, monitoring wavelength 407 nm.

TG2 protein control sample



Assay Sample 1

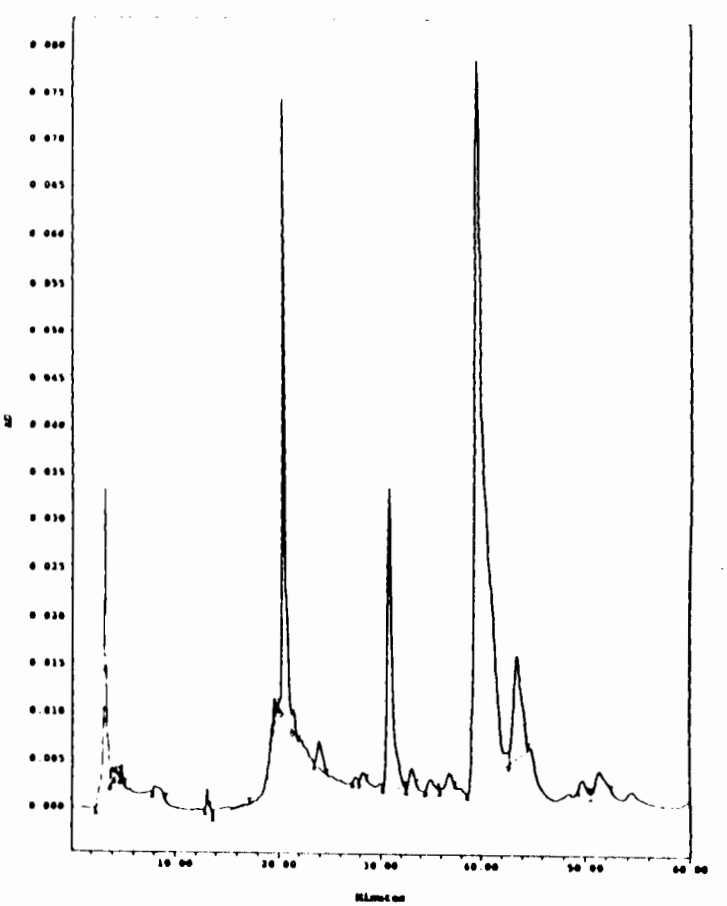


Figure15 : HPLC samples: Control (TG2 protein) and Assay Sample 1 extracts. Flow rate at 0.8 ml/min, monitoring wavelength 407 nm.

4. Discussion

4.1 BchF Expression

Despite the evidence suggesting BchF is an integral membrane protein presented in Chapter 3, the *E. coli* over-expression system applied here produced protein in the soluble fraction after ultracentrifugation. No portion of the over-expressed protein remained in the membrane fraction after even one wash. The *E. coli* host cell not only failed to insert the BchF enzyme into its membranes, but after the first two hours of induction the host cell rapidly degraded the product of over-expression. By the third hour of induction the protein was completely reabsorbed. The large production of a foreign protein may overwhelm the host system, even to levels of toxicity. It would appear from the initial molecular construct (pBC1) that the *bchF* promoter, suggested to be "*E. coli-like* σ^{70}" promoter located upstream of *bchF* (Alberti *et al.*, 1995), is not recognized as such by *E. coli*.

4.2 BchF Assay

The present assay conditions described in Chapter 3 did not result in measurable hydratase activity. The BchF protein may very well require reconstitution into a membrane environment to show enzyme activity, or may simply be over-expressed in a non-active form which cannot be restored by reconstitution. As a result of *E. coli's* inability to insert BchF into the membrane, production of enzymatically active BchF may call for expression in a *R. capsulatus* mutant, rather than *E. coli.*

The assay was applied to only one of the two possible substrates, Zn-pheophorbide, the analog to chlorophyllide, while [2-vinyl]-bacteriochlorophyll remained unexamined. If a reliable source for this pigment or its zinc analog could be found, further assays of BchF and this alternate substrate could be performed. It is possible that chlorophyllide is not the preferred substrate and 2-[vinyl]-bacteriochlorophyll would show greater activity with BchF,

however, the mutant studies of the substrate accumulation suggested that both possibilities should yield some activity.

The mechanism of the hydratase reaction is unknown. BchF lacks the metal binding motifs that some other hydratases have so likely does not mimic their mechanisms of hydration. The substrate active binding site is still undiscovered.

4.3 Zinc Analogs

Zinc insertion, as described in Chapter 3, is a quantitative procedure easily applied when precursor supplies are limited. However the application is limited by the availability of metal free pigments. For complete assay analysis with both possible substrates, extraction of pigment from the mutant strains CB1200 and DB171 still needs to be further explored. CB1200 is a double mutant of *bchF* and *bchXYZ* that accumulates chlorophyllide *a*, usually as pheophorbide *a*. The DB171 mutant is a *bchF* - strain that builds up both chlorophyllide *a* and [2-vinyl]-bacteriochlorophyllide *a*. If the pigment extraction of these strains yields Mg-free substrates, the zinc insertion technique could then be applied. The presence of

contaminants in the commercially-obtained pheophorbide *a* is further indication that mutant substrate accumulation would be a preferred pigment production. The extraction process could then be tightly controlled and attempts at curtailing destructive oxidation could be made.

4.4 Future Direction

Future efforts at assaying the BchF will have to include reconstitution of the protein into phospholipid vesicles since the soluble form of BchF produced by *E. coli* did not show discernible activity. The reconstitution could follow solubilzation of BchF in a detergent, which would be subsequently removed by dialysis. There are other alternative methods to produce a membrane bound BchF. For example, a signal targeting sequence for *E. coli* membrane insertion could be fused to the *bchF* gene. The fusion product could then be expressed in an *E. coli* host where the targeting sequence would may insure proper membrane insertion.

An additional area to be explored is the substrate production method, which will have to be modified; hopefully a purer source of pheophorbide *a* could be found. The substrate could be further purified on preparative HPLC. Alternatively, the substrate could by produced through mutant-strain pigment accumulation and subsequent purification. Certainly, extensive efforts will have to be applied to exclude air from further pigment extraction methods. The assay conditions such as pH, temperature, reaction time and substrate concentration could be maximized when an active reconstituted protein is produced. Once the objectives of an active form of BchF, through reconstitution or other method, and the purification of substrates are achieved, extensive kinetic analysis and substrate studies for BchF could be performed.

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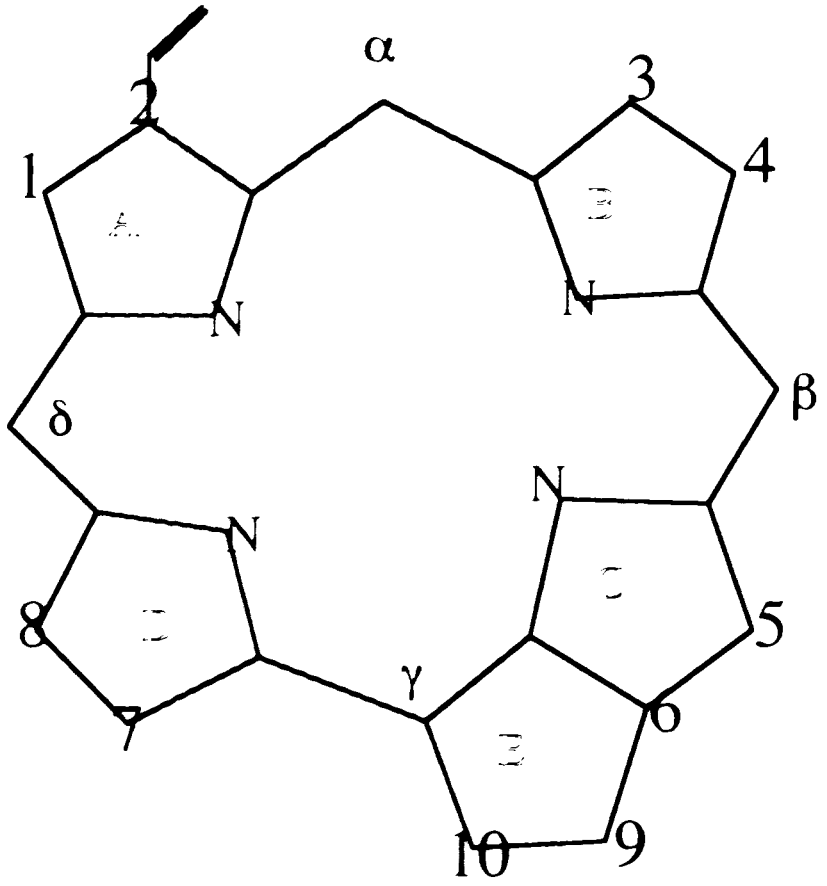

Appendix A : Schematic of the Fischer Ring Numbering System applied in the text.