1997

Heterologous Expression of BchP, a Rhodobacter capsulatus Polypeptide Necessary for the Reductive Maturation of Bacteriochlorophyll agg to Bacteriochlorophyll ap

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Heterologous Expression of BchP, a *Rhodobacter capsulatus* Polypeptide Necessary for the Reductive Maturation of Bacteriochlorophyll a_{gg} to Bacteriochlorophyll a_{p}.

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Accepted by the Faculty, Illinois Wesleyan University, in Partial Fulfillment of Research Honors on April 24, 1997

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Abstract

Photosynthetic bacteria contain bacteriochlorophyll (BChl) which has two major portions, a magnesium tetrapyrrole and an esterifying alcohol. BChl plays a key role in photosynthesis which is necessary for converting radiant energy into energy that can be usd in cellular processes. The esterifying alcohol portion affects the function of the BChl in photosynthesis, but its role is not well understood. *Rhodobacter capsulatus* typically produces BChl *a* esyrtified with phytol (BChl a_p), but site directed mutational analysis has shown that a mutation is *bchP* results in the accumulation of a BChl *a* esterified with geranylgeraniol (BChl a_{gg}) indicating that the product of the *bchP* locus (the BchP polypeptide) is necessary for the reductive maturation of BChl a_{gg} to BChl a_p. In order to determine if BchP is sufficient for this process, the gene has been amplified using polymerase chain reaction and restriction endonuclease sites have been created flanking the gene so that it can be cloned into a plasmid known as pT7-7. This construct was then transformed into a strain of E. coli (C600) which contains the pGP1-2 plasmid with the gene for T7 RNA polymerase which is under the control of the λp_L promoter. When the strain containing both plasmids is incubated at 42°C. T7 RNA polymerase is produced which can transcribe *bchP* producing BchP. Future work will include *in vitro* assays to determine if BchP is sufficient for the maturation of BChl a_{gg} to BChl a_p.

Bacteriochlorophyll (BChl) is an essential part of the enzyme complexes in photosynthetic eubacteria responsible for the conversion of radiant energy into energy that can be used in various cellular processes. BChl's as well as plant chlorophylls belong to a family of metallo-organic molecules called tetrapyrroles which are composed of a large, metal containing ring attached to an alcohol by an ester bond (Bauer et al., 1993). All BChl's and chlorophylls contain magnesium and most are esterified with a twenty carbon isoprenoid called phytol, although some species of eubacteria synthesize BChl that contains different esterifying alcohols (Rüdiger & Schoch, 1991 as reported by Bollivar et al., 1994b). Because of the structural similarities between BChl and chlorophyll, it is not surprising that the biosynthetic pathways for the syntheses of these two molecules are similar (Granick, 1948 and Jones, 1978 as reported by Bauer et al., 1993).

Variations in the side groups or hydrogenation state of the ring structure result in a change in the absorption spectrum of the tetrapyrrole (Smith 1991 as reported by Bollivar et al., 1994b). BChl's predominantly absorb wavelengths in the near-infrared region of the spectrum. The effects of variations in the esterifying alcohol portion of BChl, which does not affect the absorption spectrum, are less understood, but several studies indicate that the proper alcohol is necessary for a fully functional photosystem (Henry et al., 1986; Katz et al., 1972; Rüdiger et al., 1976; Rüdiger & Schoch, 1991; Scheer, 1991; Scheer & Struck, 1993; Walter et al., 1979 as reported by Bollivar et

al., 1994b).

Rhodobacter capsulatus synthesizes a version of BChl known as BChl *a*. Approximately 96% of the BChl *a* obtained from *R. capsulatus* is esterified with phytol; the remaining 4% is a mixture of geranylgeraniol, dihydrogeranylgeraniol, and tetrahydrogeranylgeraniol (Shioi & Sasa, 1984 as reported by Bollivar et al., 1994b). This mixture of BChl's suggests a possible pathway for the maturation of the esterifying alcohol portion in which there are sequential hydrations that convert geranylgeranyl into dihydrogeranylgeranyl which is reduced to tetrahydrogeranylgeranyl and ultimately to phytyl (Figure 1) (Bollivar et al., 1994b). This proposed pathway is similar to a pathway believed to be responsible for the equivalent maturation in young plant tissue (Schoch et al., 1977 as reported by Bollivar et al., 1994b), although mature tissue seems to add the alcohol after it has been converted to phytol (Rüdiger, 1987 as reported by Bollivar et al., 1994b).

In previous studies involving nitrosoguanidine mutagenesis (Yen & Marrs, 1976 as reported by Bollivar et al., 1994a) and complementation tests (Marrs, 1981 as reported by Bollivar et al., 1994a) using clones of the *R. capsulatus* genome, most of the genes involved in BChl and carotenoid biosynthesis as well as the genes encoding structural proteins of the photosystem were identified and found to lie in a 46-kb region of the genome. Because it contains most of the genes involved in photosynthesis, this region of the genome has been named the photosynthesis gene cluster (PGC). Subsequent studies involving saturation mutagenesis of the

PGC by transposon insertion have given a better understanding of the genes involved in BChl biosynthesis (Biel & Marrs, 1983; Zsebo & Hearst, 1984 as reported by Bollivar et al., 1994a). Sequence analysis (Armstrong et al., 1990; Bollivar et al., 1994c; Burke et al., 1993a; Burke et al., 1993b; Yang & Bauer, 1990; Youvan et al., 1984 as reported by Bollivar et al., 1994a) and site-directed mutagenesis of each of the open reading frames in the PGC have been done to determine the role of each open reading frame involved in BChl biosynthesis (Bollivar et al., 1994c; Giuliano et al., 1988; Taylor et al., 1983; Young et al., 1992 as reported by Bollivar et al., 1994a). An open reading frame is a sequence of DNA that codes for a protein.

Strain DB391 is a mutant strain with decreased photosynthetic growth capabilities that was created by insertion of a kanamycin resistance (Km^R) cassette into the 46th amino acid codon of an open reading frame known as *orf391*, which codes for a 391 amino acid polypeptide. Spectral analysis has shown that the absorption spectrum of the photopigment present in DB391 is the same as the absorption spectrum of BChl found in wild type *R. capsulatus*, phytol-esterified BChl *a* (BChl a_P), suggesting that the ring and its side chains are normal, but the alcohol may be of a variant type. Because the esterifying alcohol does not affect the absorption spectrum of the BChl, HPLC was used to test for variations in this portion of the molecule. The retention time of the pigment obtained from DB391 on the HPLC column was approximately the same as BChl *a* esterified with geranylgeraniol (BChl a_{GG}) indicating that the polypeptide encoded in *orf391* is

necessary for the sequential hydration of BChl a_{gg} to BChl a_p. Because *orf391* is involved in the BChl biosynthetic pathway, it has been renamed *bchP*, and the polypeptide that it encodes is referred to as BchP (Bollivar et al., 1994b).

It is known that BchP is necessary for the hydrogenation of the esterifying alcohol of the tetrapyrrole. The goal of this study is to determine if BchP alone is also sufficient for this process. To test for the function of BchP, the *bchP* gene was cloned into an expression vector (pT7-7) downstream of the T7 RNA polymerase promoter. This construct was then transformed into a strain of *E. coli* (C600) which contains the plasmid pGP1-2. The gene for T7 RNA polymerase is on pGP1-2 and is only transcribed at relatively high temperatures (42°C) due to a temperature sensitive repressor. Production of T7 RNA polymerase results in transcription of *bchP* and ultimately translation of BchP. Expression of BchP has already been demonstrated, and future work will involve determining if BchP is sufficient for completing the terminal steps of BChl a_p biosynthesis.

MATERIAL AND METHODS

Bacterial strains, growth conditions and DNA preparation. *E. coli* strains NM522/pRPS404, NM522/pT7-7 and DH5α/pT7-7::*bchP* were grown in LB broth at 37°C. *E. coli* strains C600/pGP1-2/pT7-7 and C600/pGP1-2/pT7-7::*bchP* were grown in LB broth at 30°C. Ampicillin was used at 60μg/ml for selection of strains NM522/pT7-7 and DH5α/pT7-7::*bchP* and kanamycin was used

at 50μg/ml in strain NM522/pRPS404. Both antibiotics were used to select for *E. coli* strains C600/pGP1-2/pT7-7 and C600/pGP1-2/pT7-7::*bchP*. All DNA was prepared according to miniprep protocols in *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989).

PCR amplification. The *bchP* gene was amplified from pRPS404 (GenBank excession number Z11165, Alberti et al., 1995) by PCR so it could be cloned into the pT7-7 expression vector. PCR reactions contained 0.2mM dNTPs, 1mM of each primer, and approximately 2.2mM pRPS404. PCR was done for 30 cycles in a Thermolyne Amplitron®I thermocycler (Dubuque, IA). Each cycle consisted of a 2 minute melting step at 94°C, a 1.5 minute annealing step at 60°C, and a 5 minute extension step at 72°C. The first cycle was preceded by 5 minutes at 94°C to insure initial melting of pRPS404. The primers for PCR were 5'-GGCGGGTGAATCATATGAAATACGA-3' and 5'-TGGGCCTCAAGCTTCTCGACGGGTT-3' (Cruachem Inc. (Dulles, VA)). Primers were designed to amplify the *bchP* gene and introduce an *Nde*I site at the start codon and a *Hin*dIII site approximately 60 base pairs downstream of the *bchP* stop codon. The *Nde*I site was created by using a 25 base primer that is identical to the sense strand from the -14 base to the +11 base, except that the -1 base was changed from adenine to thymine and the -3 base was changed from guanine to cytosine. These changes resulted in PCR products that contain the sequence 5'-CATATG-3' which is recognized and cleaved by *Nde*I. The region of DNA downstream of the stop codon was scanned for a sequence of DNA that could easily be changed into a restriction site that is also

found in the polylinker sequence of pT7-7 to make subcloning into pT7-7 easier. A region of DNA located 57 to 62 bases downstream of the stop codon was found to differ from a *Hin*dIII restriction site by only one base. This region of DNA was changed into a *Hin*dIII site by using a 25 base primer nearly identical to the nonsense strand from 46 to 70 bases downstream of the stop codon. The only change that was made on the primer was that the base 62 bases from the stop codon was changed from cytosine to adenine, creating the palindromic sequence 5'-AAGCTT-3' which can be digested by *Hin*dIII.

Plasmid construction and verification. Prior to being cloned into the expression vector, the PCR product was cloned directly into the pCR™II vector according to the protocol in the TA Cloning Kit from Invitrogen (San Diego, CA). The PCR product was inserted into the *lacZ* gene on pCR™II allowing for blue-white color selection when transformed cells are grown in the presence of X-Gal (40μg/ml). Proper insertion was confirmed by restriction mapping using *Pst*I which has one site internal to the *bchP* gene and two sites in pCR™II. After confirmation of the proper insert, the *bchP* gene was excised from the pCR™II construct and cloned into pT7-7. The expression vector and the pCR™II construct were digested with *Nde*I and *Hin*dIII. The fragment containing the *bchP* gene and the linearized pT7-7 vector were isolated by running the digestions out on an agrose gel to separate the fragments, then reversing the current and running the appropriate fragments into positively charged DE81 paper from Whatman (Clifton, NJ). The DNA

was then eluted from the paper with a high pH elution buffer (1M NaCl, 50mM argenine pH 11, 5mM EDTA). The two fragments were then ligated with T4 DNA ligase at 15°C resulting in an expression vector for *bchP*. This vector was transformed into *E. coli* DH5α. To be sure the proper construct was made, restriction mapping was used again to test for the proper insert by digesting with *Pst*I (single site internal to *bchP*) and *Bgl*II (one site in pT7-7).

Competent cells and cell transformations. All cells that were transformed were made competent by resuspending midlog cells ($OD_{600}\approx0.5$) grown at 37°C in TSS (LB broth, 10% PEG 3500, 5% DMSO, 50mM $MgCl_2$ pH 6.5). The appropriate plasmid was incubated with 0.1 ml of competent cells for 60 minutes on ice. Next, the cells were incubated for 60 minutes in 1 ml of LB at 37°C to express the appropriate antibiotic resistance protein. All cells that contained the plasmid pGP1-2 were incubated at 30°C instead of 37°C. Transformed cells were then selected for by their resistance to the appropriate antibiotic.

BchP expression. The expression construct (pT7-7::*bchP*) was transformed into *E. coli* strain C600/pGP1-2. This transformed strain was grown at 30°C in LB with ampicillin and kanamycin to select for cells with both plasmids. Cultures were grown to $OD_{600}\approx0.5$, and transcription of T7 RNA polymerase was induced by raising the temperature to 42°C for 30 minutes, resulting in transcription of *bchP*. The cells were then grown for 90 minutes at 37°C to express BchP. The cultures were centrifuged and

resuspended in 1 ml ddH_2O. A volume of 150µl of 2M NaOH/ 7.5% β-mercaptoethanol was added to lyse the cells. The mixture was held on ice for 10 minutes before 150µl of cold 50% TCA was added to precipitate the proteins. The proteins were pelleted and resuspended in 1 ml acetone to remove excess TCA. The proteins were pelleted again and the acetone removed. The samples were resuspended in 200µl of 2x sample buffer (100mM Tris·Cl (pH6.8), 200mM dithrothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol), boiled for 5 minutes, loaded onto a 12% SDS-polyacrylamide gel and run at 120V. As a control, a similar strain containing the pT7-7 vector without the *bchP* insert was also induced and run on the SDS-PAGE gel. Protein weight was determined by comparison to prestained SDS-PAGE Broad Range Standards from BioRad (Hercules, CA).

Chemicals, reagents enzymes and bacterial strains. All restriction endonucleases were purchased from American Allied Biochemical Company (Aurora, CO) except for *Nde*I which was purchased from Promega (Madison, WI). T4 DNA ligase and *Taq* polymerase were also purchased from Promega. Polyethylene glycol, sodium dodecyl sulfate, β-mercaptoethanol, and lysozyme were from Sigma Chemical Company (St. Louis, MO). Tris, acrylamide, N,N'-methylene-bis-acrylamide, dithrothreitol, and agarose were purchased from BioRad (Hercules, CA) All growth media (yeast extract, peptone, tryptone, and agar) was purchased from Difco Laboratories (Detroit, MI). The dNTP's were purchased from Bethesda Research Laboratories (Bethesda, MD). X-Gal was from

American Biorganics, Inc. (Niagara Falls, NY). Arginine was from Acros Organics (New Jersey, USA). All other chemicals and reagents were purchased from Fisher Biotech (Fair Lawn, NJ). *E. coli* strains C600/pGP1-2, NM522/pRPS404, and NM522/pT7-7 were gifts from Dr. Carl Bauer (Indiana University, Bloomington, IN).

Results

PCR amplification and TA cloning of *bchP*. The bchP gene was amplified by PCR and was cloned into the pCR™II vector (Figure 2). PCR was attempted several times with varying annealing temperatures and template concentrations to optimize amplification. These plasmids were then transformed into DH5α and presence of the insertion was determined by blue-white color selection. The first time this transformation was attempted, there were 3 white colonies and 12 light blue colonies. Minipreps were done of the white colonies and 3 of the light blue colonies for restriction mapping, but the results using several enzyme combinations were inconclusive. The second attempt at transformation using newly ligated constructs resulted in approximately 30 white colonies. Plasmids from 6 of these colonies were miniprepped, and restriction mapping was done with *Pst*I to determine if the proper insert was present. of the six colonies, 5 had bands at approximately 800bp, 1200bp, and 3300bp (Figure 3) which is what is expected if the proper insert is present.

Cloning of *bchP* into expression vector. The *bchP* gene was excised from the pCR™II construct and cloned into the polylinker sequence of pT7-7 under the control of the T7 RNA polymerase promoter (Figure 4). This construct was then transformed into DH5α. Transformed cells were selected for with ampicillin. Restriction mapping of the plasmids from six of the colonies was done with *Pst*I and *Bgl*II. One of the six minipreps gave the expected fragments of 2600bp and 1100bp (Figure 5) indicating that the plasmid contained the *bchP* gene.

Transformation of pT7-7::*bchP* into C600/pGP1-2. The expression vector was transformed into *E. coli* strain C600 which already contains pGP1-2 (Figure 6). The pGP1-2 plasmid contains the kanamycin resistance gene and the expression vector contains the ampicillin resistance gene, therefore cells with both plasmids were selected for by their resistance to both of these antibiotics. This transformation was attempted six times before it was successful. The first time all incubations were done at 37°C. I initially thought that the cells were not competent, but after two more unsuccessful trials using 37°C incubating temperatures, I believed that the transformations were successful, but 37°C incubations were inducing T7 RNA polymerase and therefore BchP production. Because BchP is believed to be involved in hydrogenating organic structures, it seems possible that BchP is lethal to *E. coli*, because it may modify molecules that are necessary for the survival of this organism. For the fourth and fifth attempts, all incubations after the transformation were done

at 30°C, but it was still unsuccessful. These cells were grown at 37°C prior to making them competent, and there was probably enough T7 RNA polymerase present to transcribe BchP. For the sixth attempt at transformation, all of the incubations, including those prior to making the cells competent, were done at 30°C. Approximately 50 colonies were present when the transformed cells were grown on selective media containing both ampicillin and kanamycin.

Heterologous expression of BchP. The BchP polypeptide was expressed in *E. coli* strain C600/pGP1-2/pT7-7::*bchP*. Proteins of this strain were separated by size on an SDS-PAGE gel (Figure 7) and compared to proteins from a similar strain which lacks the *bchP* insert (C600/pGP1-2/pT7-7). The colonies that contained the insert had a single band that was more intense than the strain without the insert. The size of this protein was determined to be approximately 44.7 kiloDaltons which is very close to the expected value of 43 kD.

Discussion

Expression system. In this expression system, *bchP* is placed under the control of bacteriophage T7 RNA polymerase. There are two reasons that this approach is used. First, T7 RNA polymerase transcribes several times faster than *E. coli* RNA polymerase, and it is also less likely to terminate transcription and can circumnavigate a plasmid several times, so more copies of

transcripts are made. Second, T7 RNA polymerase is very selective for initiation at its own promoter and does not initiate transcription from any *E. coli* sequences, therefore only genes downstream of the T7 promoter will be expressed when T7 RNA polymerase is induced. Furthermore, genes under its control will not be transcribed by *E. coli* RNA polymerase.

There are two plasmids that are necessary for this expression system. The pGP1-2 plasmid contains the gene for T7 RNA polymerase which is under control of the λp_L promoter. The λp_L promoter can be repressed by a temperature sensitive λ repressor (cI857) which is also coded for on pGP1-2. At low temperatures (30°C) cI857 represses T7 RNA polymerase, but at high temperatures (42°C) the repressor is inactive allowing for transcription of T7 RNA polymerase. The pGP1-2 vector also contains a gene for kanamycin resistance.

The other plasmid is the expression vector pT7-7 with the desired gene (*bchP*) subcloned into a polylinker sequence under control of the T7 promoter. When both of these plasmids are maintained in the same *E. coli* cell, expression of *bchP* can be very tightly regulated. At 30°C there is no T7 RNA polymerase and therefore no *bchP* transcribed, but at 42°C T7 RNA polymerase is transcribed resulting in expression of *bchP*. A gene for ampicillin resistance is also located on pT7-7 allowing for double selection of cells with both plasmids.

The importance of using a highly regulated expression system was demonstrated by the fact that cells that were grown at high temperatures (37°C) during the transformation did not survive.

This is because T7 RNA polymerase and subsequently *bchP* were expressed. It is likely that BchP was having some effect on the cells that was lethal. Because BchP is lethal to *E. coli*, it is very important that expression of this polypeptide is under very tight control.

TA cloning. Because the *bchP* gene could not be efficiently cloned directly from the PCR product to the expression vector, an intermediate vector was made by cloning the PCR product into the pCR™II vector. PCR products synthesized with *Taq* polymerase tend to have a single adenine added to each 3' end giving it sticky ends. The pCR™II vector is a linear piece of DNA that has a single thymine overhang at both of its 3' ends. Hydrogen bond interactions between the thymine and adenine overhangs on these two pieces of DNA allow for efficient ligation with T4 DNA ligase.

The intermediate vector was created for two reasons. First, by maintaining this construct in *E. coli*, the plasmid and therefore the *bchP* gene was amplified many times. Second, the restriction sites that were introduced by PCR are very near the ends of the PCR product, and probably not very suitable for efficient endonuclease activity. By inserting the PCR product into the pCR™II vector, the sites are further from the ends, so the enzymes can more easily cleave at these sites.

Heterologous expression of BchP. *E. coli* strain C600/pGP1-2/pT7-7::*bchP* was induced to express the BchP polypeptide. Expression was verified by SDS-PAGE. The size of

the expressed polypeptide was determined to be approximately 44.7kD, but considering the amount of error due to the fact that the protein bands of the standards have a significant width preventing accurate measurements of protein migration, this value is very close to the expected size of 43kD. Because the 44.7kD band is not present in the control strain, and because it is very close to the accepted size for the polypeptide, we can be confident that the expressed polypeptide is BchP.

Future Work. An expression system for BchP has been created, and BchP expression has been demonstrated. BChl a_{gg} can be isolated from a different photosynthetic eubacterium, *Rhodospirillum rubrum,* which uses BChl a_{gg} as its primary BChl. BChl a_{gg}, which is the substrate of the reaction that BchP is believed to be involved in, can then be incubated with BchP and $NADH+H^+$. The resulting product of this incubation can then be analyzed by HPLC to determine the structure of the esterifying alcohol. If, upon incubation, BChl a_p is the primary BChl present, we conclude that BchP is sufficient for the reductive maturation of BChl a_{gg} to BChl a_p. If BChl a_p is not present, it is possible that another polypeptide is also necessary for this reaction, but it is also possible that other conditions necessary for this reaction are not met, such as the appropriate buffer conditions, electron donors, or pH.

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Figure Captions

Figure 1. BchP is necessary for the sequential hydration of the esterifying alcohol in the terminal step of BChl biosynthesis. In the proposed pathway, geranylgeranyl is reduced to dihydrogeranylgeranyl, then to tetrahydrogeranylgeranyl and finally to phytyl. $NADPH+H^+$ is believed to donate the hydrogen to the alcohol (Bollivar et al., 1994b).

Figure 2. The pCR™II::*bchP* vector contains genes for ampicillin and kanamycin resistance, and has the PCR product containing the *bchP* gene inserted into the *LacZ* gene allowing for blue-white color selection when cells are grown in the presence of X-Gal.

Figure 3. This gel has wells at the top and in the middle, allowing to visualize DNA from twice as many samples. In lane 1 of the top and bottom is the standard. Lanes 2, 4, and 6 contain undigested DNA from six colonies of cells transformed with pCR™II::*bchP*. Lanes 3, 5, and 7 contain DNA from the same colonies digested with *Pst*I. All of the digestions gave the expected fragment sizes for the proper insert, except for lane 5 on the bottom.

Figure 4. The expression vector, pT7-7::*bchP*, contains a gene for ampicillin resistance and also has the bchP gene under

the control of the T7 RNA polymerase promoter (pT7).

Figure 5. Restriction mapping of 6 colonies transformed with pT7-7::*bchP* using *Pst*I and *Bgl*II was done to determine if the correct insertion was made. Only the plasmid DNA in lane 4 gave the expected fragment sizes of 2.6kb and 1.1kb.

Figure 6. The pGP1-2 plasmid contains the gene for T7 RNA polymerase which can be repressed by the temperature sensitive λ repressor (cI857). At low temperatures (30°C) cI857 is active, and no T7 RNA polymerase is transcribed, but at high temperatures (42°C) cI857 is inactive and transcription occurs.

Figure 7. Strain C600/pGP1-2/pT7-7::bchP was induced to express T7 RNA polymerase and in turn BchP. A similar strain (without the bchP gene insert) was also induced as a control. Lane #1 is an SDS-PAGE standard. Lane #2 is the control lane, and lanes #3-#10 are different stocks of the bchP expression strain. Lanes 3-10 have a thicker band at approximately 44.7 kiloDaltons which is very close to the expected weight of the BchP polypeptide (43kD).

Figure 1

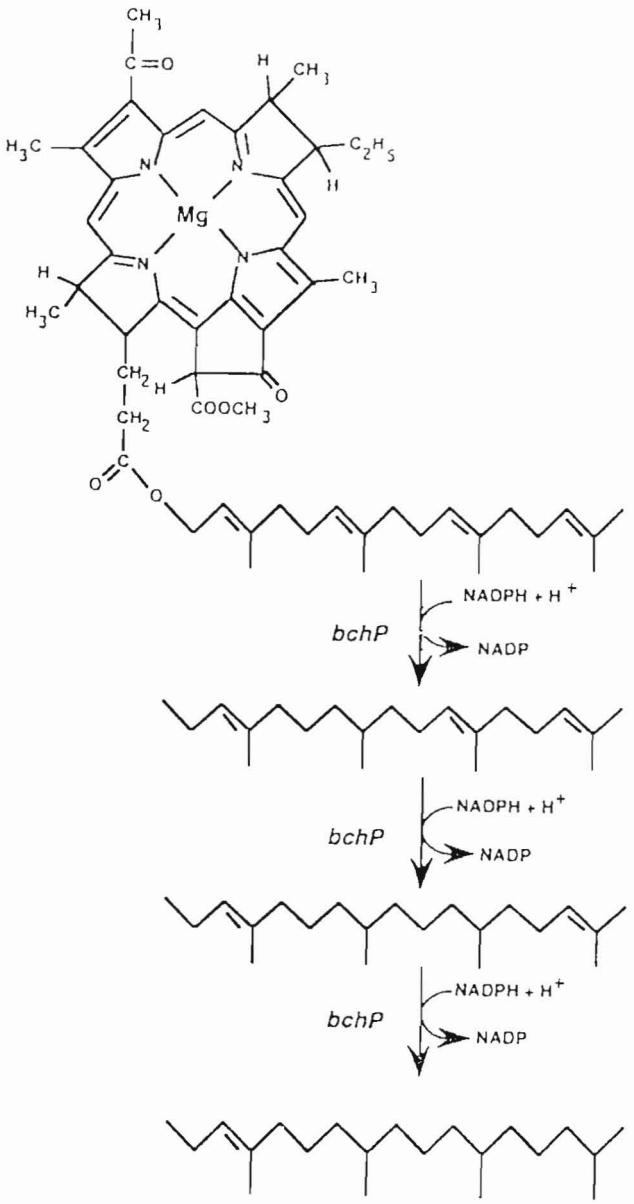
CH3
C=O
H
CH3
H3C
C2H5
N
N
Mg
N
N
H
H3C
CH3
CH2
H
COOCH3
O
CH2
C
O
O
NADPH + H+
bchP
NADP
NADPH + H+
bchP
NADP
NADPH + H+
bchP
NADP

Figure 2

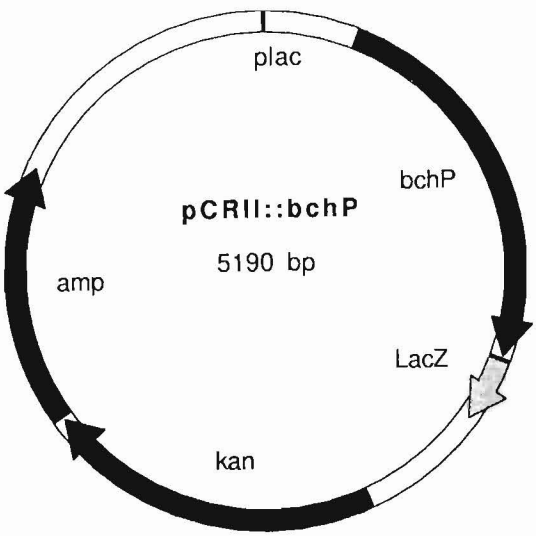

Figure 3

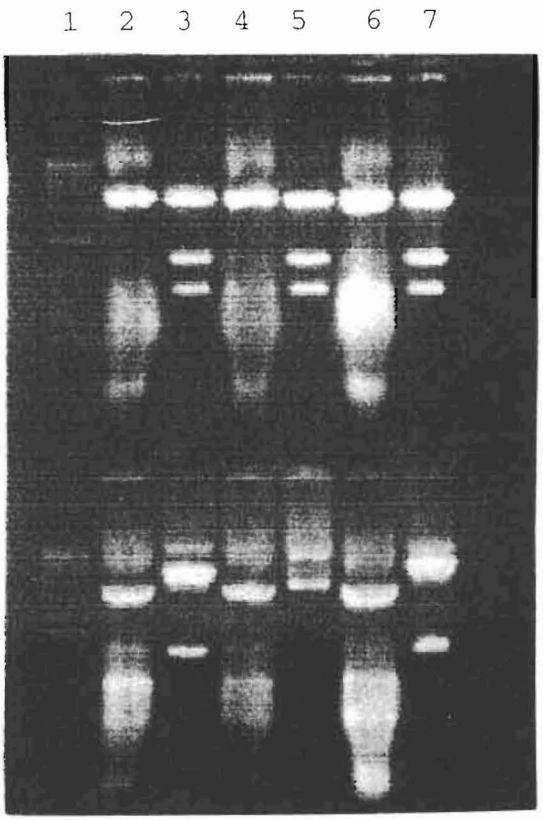

Figure 4

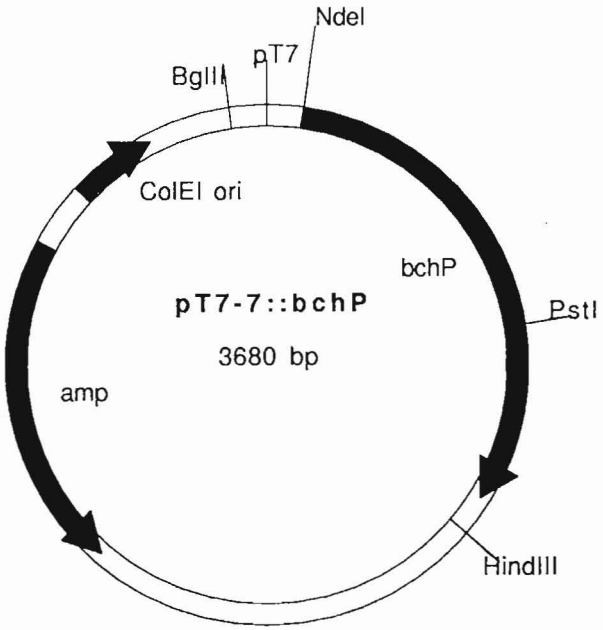
NdeI
pT7
BglII
ColEI ori
bchP
pT7-7::bchP
3680 bp
PstI
amp
HindIII

Figure 5

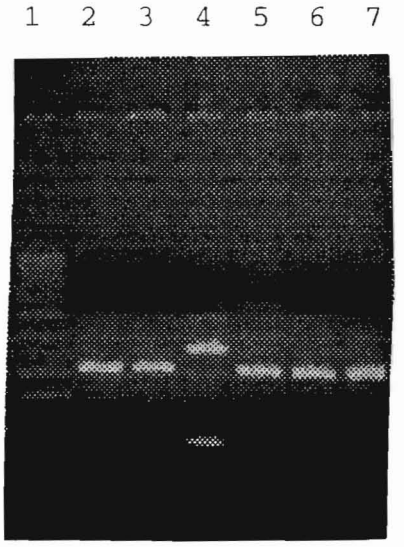

Figure 6

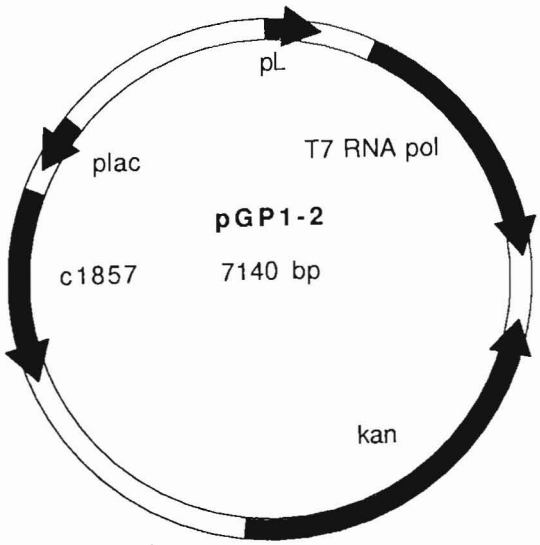

Figure 7