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Recombinant Expression of the *pufQ* Gene of *Rhodobacter capsulatus*†

SHAFIQUE FIDAI, GABRIEL B. KALMAR, WILLIAM R. RICHARDS,* AND THOR J. BORGFORD

Department of Chemistry and Institute of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

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Genetic studies have shown that the expression of the *pufQ* gene is required for normal levels of bacteriochlorophyll biosynthesis in *Rhodobacter capsulatus*. Yet, the exact function of the *pufQ* gene is unknown, and a *pufQ* gene product has never been isolated. We describe the recombinant overexpression of *pufQ* in *Escherichia coli*, as well as the purification and characterization of its gene product, the 74-amino-acid PufQ protein. Site-directed mutagenesis was used to facilitate the cloning of the *pufQ* gene into various expression vector systems of *E. coli*, including pKK223-3, pLcII-FX, and pMal-c. Although high levels of *pufQ* transcription were evident from constructs of all three vectors, high levels of protein expression were apparent only in the pMal-c system. In vector pMal-c, the recombinant PufQ protein is expressed as a fusion with an amino-terminal maltose-binding domain. After affinity purification on an amylose column, full-length PufQ protein was released from the fusion protein by limited proteolysis with the enzyme factor X_a. The PufQ protein demonstrated a strong tendency to associate with phospholipid vesicles, consistent with the view that it is an integral membrane protein. The PufQ protein was subsequently purified by high-performance liquid chromatography and identified by amino-terminal sequence analysis. A possible role for the PufQ protein in the transport of bacteriochlorophyll biosynthetic intermediates is discussed.

Heme and bacteriochlorophyll (Bchl) share a common biosynthetic pathway up to protoporphyrin IX, after which point the pathway diverges, by either the addition of Fe^{2+} to form heme or the addition of Mg^{2+} to form magnesium protoporphyrin. Enzymes of the magnesium branch catalyze the latter reaction and the subsequent conversion of magnesium protoporphyrin to Bchl and are encoded by genes referred to as *bch* genes (42). Previous work on the mapping of genes in *Rhodobacter capsulatus* has indicated that virtually all of the *bch* genes, as well as *crt* genes encoding enzymes for the biosynthesis of carotenoids, are clustered within a 46-kb region of the genome known as the photosynthetic gene cluster (34, 44). Although some of the *bch* genes have been cloned and sequenced independently (9, 38, 41), Hearst and Alberti have recently reported (2) that the entire 46-kb gene cluster of *R. capsulatus* has now been sequenced. It is important to note that significantly more open reading frames were found than the number of *bch* genes, which had been previously assigned on the basis of phenotypic mapping studies (11).

The photosynthetic gene cluster is bounded at either end by the *puh* and *puf* operons. The former contains a gene (*puhA*) for the H subunit of the photosynthetic reaction center, while the latter contains genes encoding Bchl-binding proteins, including the β- and α-subunits of the B870 light-harvesting complex I and the L and M subunits of the reaction center (*pufB*, *pufA*, *pufL*, and *pufM*, respectively). In addition, there are two open reading frames at either end of the *puf* operon, *pufQ* (or ORFQ) and *pufX* (or ORFX), for which no specific functions have yet been assigned. The organization of the genes of the photosynthetic gene cluster of *R. capsulatus* into operons and superoperons has recently been reviewed (37).

The relative positions of the *puf* and *puh* operons and many of the *bch* and *crt* genes in the closely related bacterium *Rhodobacter sphaeroides* are similar to those found for the equivalent genes of *R. capsulatus* (14). However, the distance between the *puf* and *puh* operons is some 10 kb shorter (40), and the photosynthetic gene cluster extends to include genes encoding other photosynthetic components, including cytochrome c_2 (*cycA*) and the α- and β-subunits of the B800-850 light-harvesting complex II (*pucB* and *pucA*, respectively, in the *puc* operon) (40).

The biosynthesis of Bchl in both *R. capsulatus* and *R. sphaeroides* is known to be inhibited by high light intensities and by high oxygen tension (6, 13, 28, 29). Yet, it has been shown that high oxygen tension does not repress transcription of *bch* genes to the same extent as oxygen represses transcription of the *puc*, *puh*, and *puf* operons (7, 12, 21, 43). Similarily, transcription of *bch* genes is poorly repressed by high light intensities (6). Hence, the repression of *bch* genes by light or oxygen, acting alone or in combination to down-regulate transcription, cannot completely account for the down-regulation of Bchl biosynthesis. Some other factor or factors must be influenced by light and oxygen to down-regulate the biosynthesis of Bchl.

The search for elements involved in the regulation of Bchl biosynthesis caused attention to be focused on the *pufQ* open reading frame located at the extreme 5′ end of the *puf* operon in both *R. capsulatus* (1, 3, 4, 17, 23) and *R. sphaeroides* (15, 22). Transcription of *pufQ* along with other *puf* operon genes is regulated in response to oxygen. The putative *pufQ* gene has a codon usage consistent with other genes of the species (4), and the protein deduced from the DNA sequence (the so-called PufQ protein) contains a centrally located stretch of 25 hydrophobic amino acids long enough to span a membrane (1).

Although the PufQ protein is probably not itself an en-

* Corresponding author.

† Dedicated to Professor Wolfhart Rüdiger, on the occasion of his 60th birthday.

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Reference
E. coli strains		
DH5α	*supE44 ΔlacU169* (φ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	32
TG2	*supE hsdΔ5 thiΔ(lac-proAB) Δ(srl-recA)306*::Tn*10* Tet^r F′ (*traD36 proAB$^+$ lacI^q lacZΔM15*)	32
TB1	*ara Δ(lac proAB) rpsL* (φ80 *lacZΔM15*) *hsdR*	19
QY13	F$^-$ *lac*(Am) *trp*(Am) *BB′bio-256 N$^+$ c*I857 *ΔH* Sm^r *recA*	27
Plasmids		
pΔ4	0.9-kb 5′-end-shortened fragment of *puf* operon containing *pufQ* ligated to *Bam*HI site of promoter-probe vector pXCA601	1
pKK223-3	Expression vector containing the *tac* promoter	10
pSF1	0.25-kb *Nco*I-*Eco*RI fragment of pΔ4 ligated to *Hin*dIII site of pKK223-3	This work
pLcII-FX	Fusion expression vector encoding a portion of the λ *cII* gene fused to a factor X_a recognition site and containing the λ p_L promoter	27
pSF2	0.25-kb *pufQ* gene fragment derived from pSF1 ligated to *Sma*I site of vector pLcII-FX	This work
pMal-c	Fusion expression vector encoding a *malE* gene fused to a factor X_a recognition site and containing the *tac* promoter	19
pSF3	0.25-kb *Bam*HI fragment of pSF2 ligated to *Bam*HI-cleaved pMal-c	This work

zyme, genetic studies indicated that it plays a regulatory role in the Bchl biosynthetic pathway (3, 23). However, the exact nature of this role was unclear, although it is likely that *pufQ* exerts its effect posttranscriptionally. For example, the PufQ protein may activate an enzyme, or enzymes, of the magnesium branch, or it may be involved in the activation or transport of biosynthetic intermediates. The results of *pufQ* deletion studies (3) could be interpreted to mean that the PufQ protein either exerts its effect early in the pathway (for example, as an activator of protoporphyrin IX, Mg^{2+}, or the magnesium chelatase), or at every step of the pathway (for example, as a carrier protein for intermediates of the entire magnesium branch).

Although the significance of *pufQ* in Bchl biosynthesis is well recognized, attempts to isolate a *pufQ* gene product from *Rhodobacter* species have thus far been unsuccessful. As a first step in a study which probes the function of *pufQ*, we report the recombinant expression of the gene product in *Escherichia coli*. We also report the purification and preliminary characterization of the PufQ protein.

MATERIALS AND METHODS

Bacterial strains and growth. The following strains of *E. coli*, the characteristics of which are listed in Table 1, were used for the transformation and/or expression of the vectors indicated: strain DH5α (for pΔ4, pUC-18, and pUC-19), strain TG2 (for pKK223-3 and pSF1 and transfection of phages M13mp18 and M13mp19), strain QY13 (for pLcII-FX and pSF2), and strain TB1 (for pMal-c and pSF3). All strains, with the exception of TG2, were maintained on plates of 2× TY medium, whereas TG2 was maintained on M9 minimal medium plates (32). Liquid cultures of all strains were grown in 2× TY medium at 37°C, except for strain QY13, which was grown at 30°C prior to induction of recombinant gene expression.

Construction of expression plasmids. The relevant characteristics of the plasmids employed or assembled in this study are summarized in Table 1. The *pufQ* gene used in the construction of expression plasmids was derived from a plasmid (pΔ4) originally constructed by Adams et al. (1). The coding region of *pufQ*, plus 26 bp of upstream and 10 bp of downstream DNA, was excised from plasmid pΔ4 by digestion with the restriction enzymes *Nco*I and *Eco*RI. For the purposes of mutagenesis (see below), the 250-bp *pufQ* gene fragment was blunt ended by treatment with T4 DNA polymerase and ligated into the *Sma*I site of pUC-18. The *pufQ* gene was subsequently excised by digestion with *Eco*RI and *Hin*dIII and cloned into the replicative form of phage M13mp19 previously cut with *Eco*RI and *Hin*dIII. The appropriate orientation of the phage construct for mutagenesis was confirmed by dideoxynucleotide sequencing (35). The *pufQ* gene was then altered by site-directed mutagenesis to produce a sequence, 5′ of the coding region, which is a consensus ribosome-binding site (RBS) of genes that are well expressed in *E. coli* (36). An *Xba*I restriction site was also introduced at the extreme 5′ end of the initiation codon to allow facile subcloning of the *pufQ* gene in the fusion vectors. Mutagenesis of the *pufQ* gene was directed by the following 33-mer sequence:

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                                        -----pufQ-----
5′-GGATCGGAAGGAGGTGAATCTAGATG CAA AGC C-3′
          RBS     XbaI Met Gln Ser . . .
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Altered residues used to direct the mutagenesis are indicated in boldface type, the RBS and *Xba*I restriction site are underlined, and the start of the *pufQ* gene is indicated. A uracil-containing single-stranded DNA template was prepared from the phage construct, and site-specific mutagenesis was performed by the oligonucleotide-directed method as described for double priming (32). Single-stranded phage containing the mutation were identified by dideoxynucleotide sequencing (35), and the altered *pufQ* fragment was then cloned into pUC-19, resulting in a vector designated pUC-19(*250-Xba*I). It was subsequently excised from the latter by an *Eco*RI and *Hin*dIII digest and cloned into the *Hin*dIII site of pKK223-3 (10) by using T4 DNA polymerase to fill in the protruding ends, resulting in the expression vector pSF1.

The fusion expression vector pLcII-FX (27) was used for the construction of pSF2. The *pufQ* gene was excised from plasmid pUC-19(*250-Xba*I) by an *Xba*I-*Hin*dIII digest, blunt ended with mung bean nuclease, and cloned into the *Stu*I site of vector pLcII-FX. A spontaneous deletion of an adenosine in the ATG initiation codon of the *pufQ* coding region which occurred during cloning was repaired by site-directed mutagenesis as follows: a 250-bp *pufQ* coding region was removed from the above pLcII-FX construct by digestion at its *Bam*HI sites and inserted into the *Bam*HI site in the polylinker of the replicative form of phage M13mp18. The phage construct with the appropriate orientation for mutagenesis was confirmed by dideoxynucleotide sequencing (35). Site-directed mutagenesis was performed as described above, by

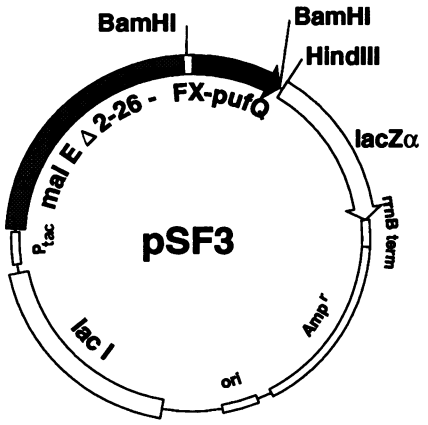

FIG. 1. Partial restriction map and regulatory sequences of plasmid pSF3. The source of the *pufQ* gene was plasmid pΔ4 (1). Expression vector pSF3 was derived from vector pMal-c. The solid and unshaded arrows indicate the coding region and direction of transcription of the *pufQ* gene and the α subunit of the β-galactosidase gene, respectively. The hatched box refers to the coding region of the MBP containing a deletion of its signal sequence. P_{tac} and *rrnB* term, the *tac* promoter and the strong transcription terminator of the *rrnB* operon, respectively.

using a 24-mer oligonucleotide homologous to the template DNA, with the exception of an additional adenosine residue at the ATG initiation codon of *pufQ*. The repaired *pufQ* gene was then removed from the phage and recloned into vector pLcII-FX after digestion of both with *Bam*HI. The orientation of the resulting construct, designated pSF2, was confirmed by dideoxynucleotide sequencing (35) with a 16-mer oligonucleotide homologous to nucleotides encoding amino acids 16 to 21 of the λ*cII* gene.

Vector pSF3 (shown schematically in Fig. 1) was constructed by digestion of vector pSF2 with *Bam*HI and inserting the *pufQ* gene into vector pMal-c (19) similarly cut with *Bam*HI. The orientation and sequence of the pSF3 construct was confirmed by dideoxynucleotide sequencing (35) with a 24-mer *malE* primer.

RNA preparation and Northern blot analysis of mRNA produced during induction of expression vectors. RNA was isolated as described previously (33) from 10-ml cultures of the following strains: TG2(pSF1), QY13(pSF2), and TB1 (pSF3) and control strains TG2(pKK223-3), QY13(pLcII-FX), and TB1(pMal-c). Cultures that were both induced and uninduced for gene expression were prepared. Strains TG2 and TB1 were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) as described below. Strain QY13, grown at 30°C to an A_{600} of 0.6, was heat induced by transferring the cells to a water bath at 42°C and agitated for 15 min. Cultures were then incubated at 37°C for an additional 2 h, following which the cells were lysed three times by rapid freeze-thawing. RNA was resolved on 2% (wt/vol) formaldehyde-agarose gels, transferred to Zeta-probe blotting membrane and processed for Northern (RNA) hybridization as previously described (18). The DNA used to generate a hybridization probe was obtained from a 2.3-kb *Xba*I-*Hin*dIII restriction fragment excised from the plasmid pUC-19(*250-Xba*I) and purified by electroelution from an agarose gel. The DNA was radiolabeled by random primer extension with a Random Priming System I kit.

Expression and purification of the recombinant MBP-FX-PufQ and MBP-FX-β-galactosidase fusion proteins. The recombinant gene product of the *malE-pufQ* gene fusion, carried on vector pSF3, contains sequences encoding maltose-binding protein (MBP), a factor X_a protease recognition sequence, and the PufQ protein and is referred to as the MBP-FX-PufQ fusion protein. The fusion protein was expressed in *E. coli* and purified as follows: 15 liters of 2× TY media containing 100 μg of ampicillin μl^{-1} was inoculated with a 200-ml culture of pSF3-transformed *E. coli* TB1, and the culture was incubated at 37°C in a Chemap fermentor at 535 rpm with bubbling air at a rate of 10 liters min^{-1} until an A_{600} of 0.6 was reached. Gene expression was then induced by the addition of 0.3 mM IPTG, and the cells were grown an additional 2 h. After incubation, the culture was cooled to 18°C and the cells concentrated to a final volume of 2 liters with a Pellicon tangential flow filtration unit equipped with a 0.45-mm-pore-size filter cassette. The concentrated cells were then harvested by centrifugation at 4,000 × *g* for 20 min in a Sorvall GS3 centrifugation rotor at 4°C. Cell pellets were resuspended in a total volume of 500 ml of buffer A [10 mM sodium phosphate (pH 7.0), 30 mM NaCl, 0.25% (vol/vol) Tween 20, 10 mM β-mercaptoethanol, 10 mM EDTA, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride]. Cell suspensions were incubated with lysozyme (1 mg ml^{-1}) for 30 min at 4°C, and then sonicated with three 20-s bursts at maximum power from a FisherSonic-Dismembrator model 300 sonicator fitted with a medium-size probe. Finally, NaCl was added to lysates to a final concentration of 0.5 M, and the lysed cells were centrifuged at 9,000 × *g* for 30 min in a Sorval SS34 rotor. The supernatant, containing the crude cell extract, was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before and after purification by affinity chromatography. The method of Laemmli (24), with 15% (wt/vol) polyacrylamide gels, was employed for this and all subsequent SDS-PAGE analyses (except when otherwise indicated), visualizing the bands with Coomassie brilliant blue. Affinity chromatography was accomplished after dilution of the supernatant fivefold with buffer B (10 mM sodium phosphate [pH 7.0], 0.5 M NaCl, 0.25% [vol/vol] Tween 20, 10 mM β-mercaptoethanol, 1 mM EGTA, 1 mM sodium azide) and loading onto a 50-ml amylose column which had been previously washed with 50 ml of buffer B. The column was eluted at a flow rate of 1 ml min^{-1} with 150 ml of buffer B followed by 250 ml of buffer B minus Tween 20. Fusion protein was recovered in a single fraction by elution with 10 mM maltose. The fusion protein (in 30 ml) was then dialyzed against 4 liters of buffer C (20 mM Tris-Cl [pH 8.0], 0.1 M NaCl) with dialysis membranes having a molecular weight cutoff of 12,000 to 14,000. Identical methods were used to express and purify the gene product of the unaltered pMal-c vector (*malE-lacZα* gene fusion), the MBP-FX-β-galactosidase fusion protein.

Site-specific proteolysis of the MBP-FX-PufQ fusion protein. Factor X_a was prepared from bovine serum as described by Esnouf and Williams (16). Conditions for the digestion of each preparation of the MBP-FX-PufQ fusion protein by factor X_a were determined empirically. Two sets of buffers were used to prepare the PufQ protein for further analysis. Digestions were performed either in buffer C with an 800:1 (wt/wt) ratio of fusion protein to factor X_a or in buffer C containing 10 mM sodium cholate with an 80:1 (wt/wt) ratio of fusion protein to factor X_a. The sodium cholate was necessary in order to maintain solubility of the PufQ protein. Typically, complete digestion was achieved in 12 h at 23°C in both buffers. The digests were terminated by

the addition of phenylmethylsulfonyl fluoride (from a 100 mM stock solution in 2-propanol) to a final concentration of 1 mM. Following digestion in buffer C, the unconjugated MBP was purified by affinity chromatography on an amylose column as described above for the MBP-FX-PufQ fusion protein.

Purification of the PufQ-protein by HPLC and N-terminal sequence analyses. Fusion protein digested in buffer C with factor X_a (in the absence of sodium cholate) was solubilized in 60% (vol/vol) acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid. The sample was then applied to a Vydac C4 reversed-phase high-performance liquid chromatography (HPLC) column (10 mm by 25 cm) previously equilibrated in 40% (vol/vol) acetonitrile–0.1% (vol/vol) trifluoroacetic acid. The HPLC system was a Spectra-Physics model 8810 equipped with an Applied Biosystems model 757 absorbance detector set at 280 nm. The column was eluted for 60 min at room temperature with a gradient of 40 to 60% (vol/vol) acetonitrile–0.1% (vol/vol) trifluoroacetic acid at a flow rate of 1.0 ml min^{-1} and then for an additional 20 min with isocratic 60% (vol/vol) acetonitrile–0.1% (vol/vol) trifluoroacetic acid. For analysis of the peak fractions by SDS-PAGE, samples were concentrated under vacuum, washed with 0.5 ml of water to remove any traces of trifluoroacetic acid, and concentrated again under vacuum. For N-terminal sequence analysis, two fractions, both of which had M_r values similar to that of the PufQ protein, were concentrated under vacuum to a final volume of 20 μl and sequenced in the Microsequencing Center at the University of Victoria, Victoria, British Columbia, Canada, with an Applied Biosystems model 473 protein sequencer.

Immunoblotting of proteins. Western immunoblot assays were performed by standard procedures (20). Samples were separated by SDS-PAGE in 12% (wt/vol) polyacrylamide gels (24) and electroeluted onto Immobilon P filters, composed of polyvinylidene diflouride, with an LKB multipore transfer apparatus at 1.2 mA cm^{-2} for 2 h. The filters were blocked in 3% (wt/vol) fetal calf serum (FCS) in buffer D (20 mM Tris-Cl [pH 7.4], 0.15 M NaCl), shaking either for 1 h at room temperature or overnight at 4°C. The filters were then washed for 30 min in buffer D containing 0.1% (wt/vol) FCS and incubated by shaking gently at room temperature with a 10^5-fold dilution of rabbit anti-MBP serum in buffer D containing 1.0% (wt/vol) FCS and 0.05% Tween 20. The wash with buffer D containing 0.1% FCS was repeated, and the filters were then incubated at room temperature with a 10^5-fold dilution of goat anti-rabbit serum conjugated to horseradish peroxidase in buffer D containing 1.0% (wt/vol) FCS and 0.05% Tween 20. After a final wash in buffer D containing 0.1% FCS, the filters were developed with 1.0 mM diaminobenzidine in 50 mM Tris-Cl, pH 7.6, containing 0.01% (vol/vol) hydrogen peroxide.

Reconstitution of the PufQ protein into phospholipid vesicles. Liposomes were prepared by sonicating 175 mg of Asolectin soybean phospholipids (39% phosphatidylcholine, 23% phosphatidylethanolamine, 20% phosphatidylinositol, 5% phosphatidic acid, and 13% unidentified lipids) plus 0.8 μCi of dipalmitoylphosphatidyl[*methyl*-^{3}H]choline, in 2.5 ml of buffer C at 80% output until the turbid solution was clear. Aliquots containing 0.54 ml of the liposomes (38 mg of phospholipids) were then added to 1.4 mg of the fusion protein digested in buffer C (as described above) in the presence of 10 mM sodium cholate. The digestion mixture was then incubated for 15 min at 37°C and dialyzed for 24 h against 2 liters of buffer C (with two changes of the same volume of buffer) to remove the sodium cholate. Dialyzed samples (1.2 ml) were layered at the top of centrifuge tubes containing discontinuous sucrose gradients formed by successive layers (bottom to top) of 3.0 ml of 50%, 3.0 ml of 35%, and 4.5 ml of 15% (wt/vol) sucrose and centrifuged in a Beckman L8-80 ultracentrifuge with a SW40Ti swinging bucket rotor for 20 h at 30,000 × *g* and 13°C. For comparison, a control sample containing radiolabeled liposomes, formed as described above, was added to solutions of 10 mM sodium cholate containing 1.4 mg of MBP and centrifuged as above. Following centrifugation, the gradients were divided into equal 1-ml fractions and 50-μl aliquots of each were analyzed in a Beckman LS6000 series liquid scintillation counter for the presence of ^{3}H-labeled phospholipids. Protein determination on each of the fractions was performed by a modified Lowry assay (26).

Materials. Dipalmitoylphosphatidyl[*methyl*-^{3}H]choline (50 Ci mol^{-1}) was supplied by DuPont NEN Research Products, Lachine, Quebec. Soybean phospholipids (Asolectin) were supplied by Associated Concentrates, Woodside, N.Y. All restriction enzymes, T4 DNA ligase, T4 DNA polymerase, mung bean nuclease, FCS, and IPTG were purchased from Bethesda Research Laboratories, Gaithersburg, Md. The pKK223-3 vector was purchased from Pharmacia Biotech Inc., Baie d'Urfé, Quebec, Canada; T7 DNA polymerase (Sequenase) was purchased from United States Biochemicals Corp., Cleveland, Ohio; calf intestinal phosphatase was from Boehringer Mannheim, Laval, Quebec, Canada; and goat anti-rabbit serum conjugated to horseradish peroxidase was from Mandel Scientific, Rockwood, Ontario, Canada. The Random Priming System I kit and Protein Fusion and Purification kit (containing the pMal-c vector, amylose affinity column, rabbit anti-MBP serum, and 24-mer *malE* sequencing primer) were purchased from New England Biolabs, Beverly, Mass. Other oligonucleotides for mutagenesis and sequencing were purchased from the Oligonucleotide Synthesis Laboratories of either the University of British Columbia, Vancouver, British Columbia, Canada (33-mer), or the Institute of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia, Canada (16-mer and 24-mer). Zeta-probe blotting membranes and Immobilon P filters were purchased from Bio-Rad, Richmond, Calif., and Millipore, Bedford, Mass., respectively. All other chemicals and reagents were of the highest grade commercially available.

RESULTS

Expression of vectors containing the *pufQ* gene. Transcription of *pufQ* genes in the recombinant constructs pSF1, pSF2, and pSF3 was evaluated by Northern blot analysis. In each of the three constructs, *pufQ*-specific transcripts were undetectable prior to induction (data not shown) but were detected following induction (Fig. 2). The probe specificity was determined by performing Northern blots on each of the expression vectors lacking the *pufQ* gene. In these control vectors, no *pufQ*-specific RNA species were detected either before or after induction (data not shown).

During induction of vector pSF1, transcripts of approximately 400 and 660 bases were detected (Fig. 2, lane 1). Transcripts of these lengths are consistent with the anticipated 410- and 580-base lengths which correspond to termination of transcription at either the *rrnB* T_1 and *rrnB* T_2 sites on the vector pKK223-3. SDS-PAGE analysis of lysates of *E. coli* TG2(pSF1) were performed, but there was no indication of PufQ protein expression either before or after IPTG induction (data not shown).

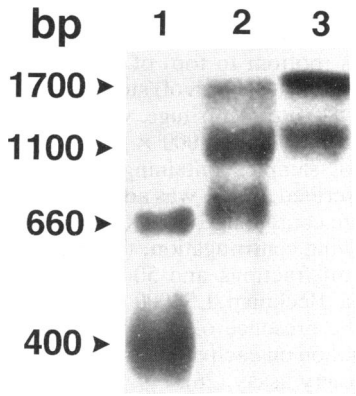

FIG. 2. Northern hybridization analysis of *E. coli* strains harboring expression vectors containing the *pufQ* gene. Total RNA (100 μg) from the following induced strains of *E. coli* was run on a 2% (wt/vol) formaldehyde-agarose gel and blotted with a *pufQ* specific probe as described in Materials and Methods. Lane 1, TG2(pSF1); lane 2, QY13(pSF2); lane 3, TB1(pSF3). Uninduced strains expressed negligible amounts of transcript, while no transcripts were detected in expression vectors lacking inserts containing the *pufQ* gene (data not shown).

During induction of vector pSF2, three distinct *pufQ*-specific mRNA species having lengths of approximately 740, 1,100, and 1,500 bases were detected (Fig. 2, lane 2). Since the transcriptional termination sites of the vector pLcII-FX are not known (27), distances from the end of the *pufQ* gene to transcription termination sites on the vector cannot be calculated. However, the minimum *λcII-FX-pufQ* mRNA length of 720 bases for vector pSF2 is consistent with the observed transcript length of 740 bases, suggesting that the 1,100- and 1,500-base mRNA species also observed are poorly terminated read-through transcripts. Protein expression in cell lysates of *E. coli* QY13(pSF2) was analyzed by SDS-PAGE. The results indicated that a protein with an M_r value of approximately 15,000 Da was present in the crude cell extract of induced, but not uninduced, cultures of *E. coli* QY13 infected with pSF2 (data not shown). A polypeptide of approximately 12,000 Da corresponding to the cII-FX-PufQ fusion protein is expected from a fusion of the gene sequences. Although expression was observed, the levels of expression were not high enough to warrant protein purification from this clone.

During induction of vector pSF3, two *pufQ*-specific transcripts of 1,260 and 1,700 bases were detected (Fig. 2, lane 3), the latter of which corresponded to the anticipated transcript length for the *MBP-FX-pufQ* mRNA. Analysis of *E. coli* TB1(pSF3) lysates by SDS-PAGE following IPTG induction (19) indicated the presence of a protein with an M_r value of 50,000 Da in crude cell extracts (Fig. 3, lane 1). Expression of the *pufQ* gene fusion in vector pSF3 was expected to yield a MBP-FX-PufQ fusion protein with a molecular weight of 50,500.

Isolation and purification of the PufQ protein. A large scale (15-liter) growth of *E. coli* TB1(pSF3) was carried out, and the overexpressed fusion protein was purified by affinity chromatography on an amylose column as described in Materials and Methods. Only a single band with a M_r value of 50,000 was observed by SDS-PAGE (Fig. 3, lane 2). Western blot (immunoblot) analysis with rabbit anti-MBP serum demonstrated that the overexpressed fusion protein contained the maltose-binding domain (Fig. 4). The MBP-

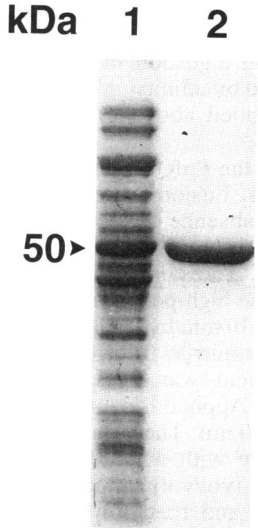

FIG. 3. SDS-PAGE of crude cell extracts of *E. coli* TB1 transformed with plasmid pSF3 before and after affinity chromatography. Following induction with IPTG, crude cell lysates were analyzed before (lane 1) and after (lane 2) purification on an amylose affinity column as described in Materials and Methods. A band with a M_r of 50,000 (50) (corresponding to the MBP-FX-PufQ fusion protein) is marked in both fractions.

FX-PufQ fusion protein was hydrolyzed by the sequence-specific factor X_a protease (16), which was expected to cleave exactly at the N-terminal methionine residue of the PufQ protein. SDS-PAGE analysis of the hydrolysate indicated that the hydrolysis had gone essentially to completion, since no unhydrolyzed fusion protein was visible with the

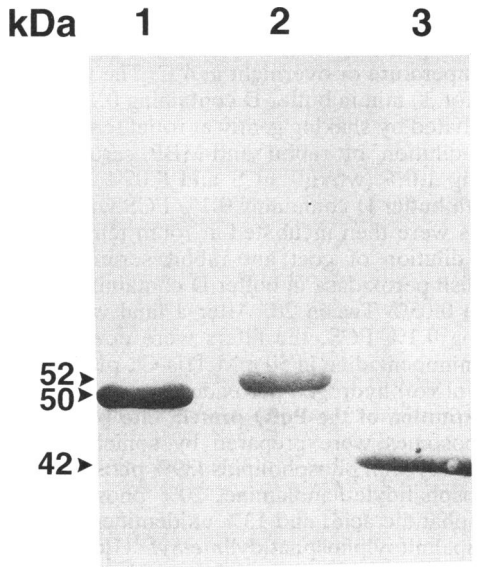

FIG. 4. Western immunoblot analysis of the maltose-binding domain of MBP and MBP fusion proteins. The following proteins were analyzed with rabbit anti-MBP serum. Lane 1, MBP-FX-PufQ fusion protein; lane 2, MBP-FX–β-galactosidase fusion protein; lane 3, MBP. Unconjugated MBP was prepared by digestion of MBP-FX-PufQ fusion protein with factor X_a protease, and it and the two fusion proteins were purified by affinity chromatography as described in Materials and Methods.

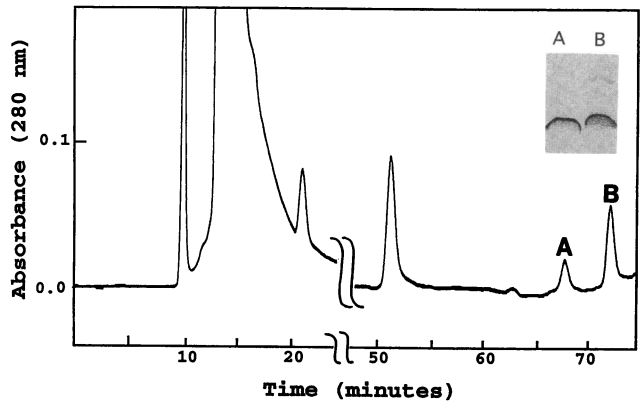

FIG. 5. Purification of MBP, PufQ protein, and Δ_{1-7}PufQ by HPLC. Samples of MBP-FX-PufQ fusion protein digested with factor X_a protease were solubilized and run on a Vydac C4 reversed-phase HPLC column as described in Materials and Methods. Peaks which were identified contained MBP (14.3 min) and either PufQ protein (68 min) (A) or Δ_{1-7}PufQ (73 min) (B). The inset shows the analysis of the two latter fractions by SDS-PAGE.

Coomassie blue stain (data not shown). The digested fusion protein was purified on a C4 reversed-phase HPLC column as described in Materials and Methods (Fig. 5). The MBP eluted from the HPLC column in the 40 to 60% acetonitrile gradient at 14.3 min. Peak fractions collected at 68 and 73 min during elution with isocratic 60% acetonitrile contained small polypeptides exhibiting similar, although not identical, M_r values of approximately 7,600 and 8,700, respectively, as determined by SDS-PAGE (Fig. 5, inset). Five cycles of N-terminal sequence analysis were performed with each polypeptide. The sequence of the 68-min peak fraction was identical to the first five amino acids of the PufQ protein, while the 73-min peak fraction contained a polypeptide derived from the PufQ protein by the deletion of the first seven amino acids (referred to as Δ_{1-7}PufQ).

Binding of the PufQ protein to liposomes. Although the MBP-FX-PufQ fusion protein was soluble in aqueous buffers, the isolated PufQ protein was completely insoluble unless sodium cholate was present. It was also seen to be soluble in phospholipid vesicles after digestion of the fusion protein under conditions whereby the fusion protein and soybean phospholipids were first sonicated together. However, complete digestion of the fusion protein could not be achieved under these conditions, presumably because some of the protease hydrolysis sites were on the inside of the vesicles. If the fusion protein was added to preformed liposomes prior to digestion with factor X_a protease, the PufQ protein aggregated during digestion because of poor incorporation into liposomes. In order to maintain the solubility of the PufQ protein while ensuring complete proteolytic digestion, preformed ^{3}H-labeled liposomes were added to cholate micelles of the digested fusion protein. Following dialysis of the cholate, the liposomes and protein were centrifuged on discontinuous gradients of sucrose as described in Materials and Methods. The protein and phospholipid profiles of fractions from this sucrose gradient were compared with a control gradient containing radiolabeled liposomes and unconjugated MBP only (Fig. 6). When gradient fractions were compared, only the gradient containing the hydrolyzed MBP-FX-PufQ fusion protein contained a band at the 35% sucrose interface (Fig. 6B, fraction 7). This band was found to contain a significant amount of both

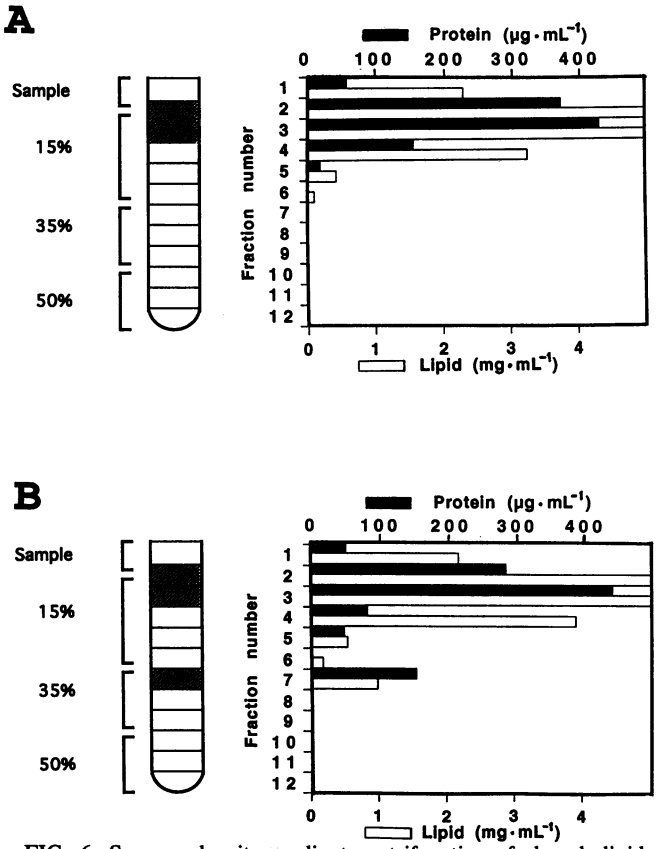

FIG. 6. Sucrose density gradient centrifugation of phospholipid vesicles reconstituted with either unconjugated MBP (A) or hydrolyzed MBP-FX-PufQ fusion protein (B). Sucrose gradient fractions were analyzed for protein (shaded bars) and phospholipid (open bars) as described in Materials and Methods. The protein content of protein-containing fractions of the tube containing the hydrolyzed fusion protein was analyzed by SDS-PAGE (see Fig. 7).

phospholipid and protein. SDS-PAGE analysis of this fraction indicated the presence of only the PufQ protein and Δ_{1-7}PufQ (Fig. 7, lane 7).

DISCUSSION

Overexpression of the *pufQ* gene. In order to explore the function of the *pufQ* gene product, it was necessary to generate quantities of the protein from nonnative sources. The putative PufQ protein, deduced from the sequence of the *pufQ* gene, is 74 amino acids in length and relatively hydrophobic. With a molecular weight of 8,556, the protein was considered too large to be produced economically by peptide synthesis but perhaps large enough to be made by recombinant DNA methods. We examined three different vector systems of *E. coli* for their ability to overexpress *pufQ* and were, therefore, able to provide a direct comparison of each of the systems. The recombinant expression and purification of PufQ protein was made difficult by the lack of a ready assay. Consequently, our only initial criterion for overexpression of PufQ protein was the appearance of a protein in extracts exhibiting a M_r value similar to the anticipated molecular weight. Amino-terminal analysis of isolated peptides was used as the final identification of the products.

The pKK223-3 vector, used to construct pSF1 which

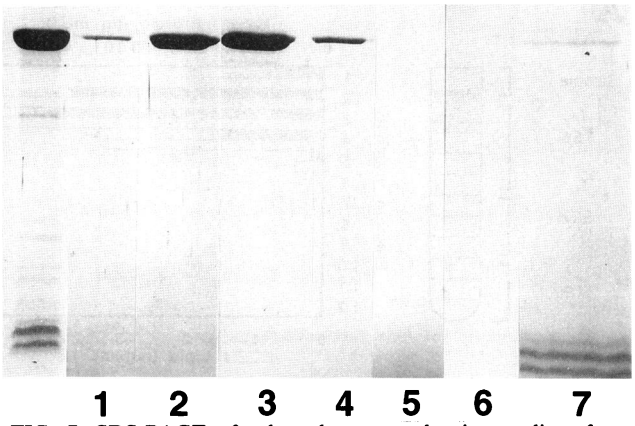

FIG. 7. SDS-PAGE of selected sucrose density gradient fractions. Protein-containing fractions from a sucrose gradient tube containing hydrolyzed MBP-FX-PufQ fusion protein and phospholipid vesicles (Fig. 6B) were prepared for SDS-PAGE as described in Materials and Methods. Lanes 1 to 7 correspond to fractions 1 to 7 in Fig. 6B. The top fractions (lanes 2 to 4) located near the 15% sucrose boundary are seen to contain the MBP only, while the PufQ protein and Δ_{1-7}PufQ appear in the fraction (lane 7) located at the 35% sucrose boundary. A control lane containing the unfractionated hydrolyzed fusion protein is also shown on the far left.

contains the *pufQ* gene behind a genetically engineered RBS, is designed such that transcription of a recombinant gene is driven by a strong IPTG-inducible promoter. The expression vector pLcII-FX, used to construct pSF2, differs in several respects from pKK223-3. It possesses a promoter that is regulated by the temperature-sensitive repressor *c*I857 (27) and provides better repression of uninduced genes. Moreover, the gene products of pLcII-FX-derived clones, unlike pKK223-3, are produced as fusions with a 31-amino-acid portion of the lambda cII protein. A factor X_a protease recognition sequence is present in the fusion at the junction between the recombinant peptide and the cII peptide. Hence, the fusion protein may be processed with factor X_a to separate the two peptide components. Expression studies were performed with both pSF1 and pSF2. Only in the latter case was there any evidence for the presence of an inducible protein with the expected M_r value in cell extracts; however, the levels of expression were too low to facilitate purification, and useful quantities of PufQ protein were never obtained from this system.

The pMal-c expression vector system, used to construct pSF3 (Fig. 1), shares feature of both the pKK223-3 and pLcII-FX systems. The vector has a strong IPTG-inducible promoter, and recombinant proteins are produced as fusions which can be processed with factor X_a. An added benefit to this system is that recombinant proteins are also fusions with a maltose-binding domain rather than the cII peptide. Therefore, fusion proteins can be purified from cell extracts in one step by chromatography on an amylose column. Expression of vector pSF3 produced high levels of a MBP-FX-PufQ fusion protein which was readily purified by amylose column chromatography (Fig. 3) and was shown to possess the maltose-binding domain as determined by Western blot analysis with anti-MBP antisera (Fig. 4). The PufQ protein was then separated from the maltose-binding domain by limited proteolysis with factor X_a as described in Materials and Methods. Peptides produced in the protease digest were resolved by reversed-phase HPLC (Fig. 5), and the identity of the PufQ protein was verified by amino-terminal sequence analysis. An unexpected product of the factor X_a digestion was the truncated PufQ protein (Δ_{1-7}PufQ), seven amino acids shorter than the full-length PufQ protein. The amino acid sequence in this region of the protein does not resemble a factor X_a site; however, it does have in common with a factor X_a site an arginine residue preceding the point of cleavage. Despite the loss of seven amino acids, the truncated protein behaved in a manner similar to the full-length protein in terms of its membrane-binding properties (see below).

It was of some interest to establish reasons for differences in the success of the three expression vector systems. Northern blot analysis of mRNA transcripts (Fig. 2) indicated that in every case the *pufQ*-specific mRNA was abundant and the anticipated lengths of transcripts were found; complementary transcripts with apparent lengths consistent with mRNA of 410 and 580 bases (pSF1), 740 bases (pSF2), and 1,700 bases (pSF3) were all observed. The results of this analysis suggested that differences in the levels of *pufQ* expression resulted not from significant differences in the level of gene transcription or the stability of *pufQ* mRNA but from differences in the translation and/or stability of the protein. The fusion expression systems pSF2 and pSF3 were successful in producing quantities of inducible fusion proteins, whereas the pKK223-3-derived vector pSF1 was not successful in producing detectable amounts of the PufQ protein. The small PufQ protein may have been more susceptible to degradation than the larger fusion proteins. There are few examples of small proteins (i.e., less than 80 amino acids) having been successfully expressed recombinantly unless as fusions. Another explanation is also possible. As discussed below, the PufQ protein is expected to be quite hydrophobic and, therefore, may associate with membranes when expressed in *E. coli*, leading to the lysis of the cell at an early stage of expression. Of the two fusion systems, pSF3 was clearly the superior. The success of the pSF3 vector may be accounted for by the sheer size of the maltose-binding portion of the fusion protein, i.e., the MBP is simply large enough to compensate for the PufQ protein's unfavorable solubility.

Properties of the PufQ protein. The isolated PufQ protein showed a marked tendency to aggregate in the absence of detergents. This observation was consistent with predictions that the hydrophobic PufQ protein is an intrinsic membrane protein. This possibility was examined by more direct methods as follows: the fusion protein was digested with factor X_a in the presence of sodium cholate, and the digest was combined with preformed membrane vesicles. Following removal of the detergent by dialysis, the mixture was fractionated on a discontinuous sucrose density gradient (Fig. 6B). In mixtures containing the PufQ protein, there was a visible banding of lipid and protein at the interface between 15 and 35% sucrose in a location similar to that observed for disrupted bacterial membranes. The protein component of the interface fraction was composed almost exclusively of the PufQ protein (together with the truncated Δ_{1-7}PufQ) as determined by SDS-PAGE (Fig. 7). A similar banding behavior was not evident in control mixtures containing lipid and the MBP only (Fig. 6A).

The likelihood of a membrane location for the PufQ protein supports the hypothesis that it may be involved in the binding or transport of magnesium tetrapyrrole intermediates. The idea of a carrier protein of intermediates during Bchl synthesis was originally proposed by Lascelles (25). Pigment-protein complexes excreted by certain *bch* mutants

of *R. sphaeroides* were considered to be possible carrier proteins of Bchl synthesis (31). However, Bollivar and Bauer (8) have recently determined that intermediates excreted by mutants of *R. capsulatus* were bound to the major outer membrane porin protein rather than to a putative carrier protein. The existence of a carrier protein for Bchl synthesis is still an attractive hypothesis, however, because of the fact that, while magnesium tetrapyrrole intermediates are only poorly soluble in water, they are able to freely pass across the cell membrane during excretion. A portion of the intermediate being excreted always remains behind in the cell membrane of the *bch* mutant, however. In order for enzymes to be able to carry out modifications on side chains attached to the porphyrin ring, these intermediates would need to be prevented from entering the membrane, perhaps by being bound to the PufQ protein by chelation of the magnesium atom to histidine and/or glutamine residues present in its N-terminal region.

In mutant strains of *R. capsulatus* in which both the *puf* and *puc* operons were either missing or defective, Bchl itself could not be detected; however, one such mutant was found to be able to form a small amount of the magnesium-free derivative of Bchl (either bacteriopheophytin or bacteriopheophorbide), and the amount of the latter could be increased by the addition of a ferrochelatase inhibitor, *N*-methylprotoporphyrin (30). Hence, it does not seem that expression of the *pufQ* gene is absolutely required for Bchl synthesis, but it may be required for its optimum synthesis. It should also be noted that other purple bacteria (e.g., *Rhodospirillum rubrum* [5] and *Rhodopseudomonas viridis* [39]) lack a *pufQ* gene at an equivalent location in the *puf* operon. Hence, if an important universal function exists for the *pufQ* gene in Bchl synthesis, a homolog for this gene must be located at a different site in the chromosomes of these bacteria.

In summary, we have been able to express the small and very hydrophobic PufQ protein and have shown that it specifically associates with membrane vesicles. The methodology described in this paper should be appropriate to the expression of other small hydrophobic proteins. The properties of the overexpressed PufQ protein are consistent with the hypothesis that it acts to bind or transport magnesium tetrapyrrole intermediates during Bchl synthesis. Experiments to further test this hypothesis are currently in progress.

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