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Heterologous Expression of the *bchM* Gene Product from *Rhodobacter capsulatus* and Demonstration that It Encodes *S*-Adenosyl-L-Methionine:Mg-Protoporphyrin IX Methyltransferase

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The bacteriochlorophyll biosynthesis gene, *bchM*, from *Rhodobacter capsulatus* was previously believed to code for a polypeptide involved in formation of the cyclopentone ring of protochlorophyllide from Mg-protoporphyrin IX monomethyl ester. In this study, *R. capsulatus bchM* was expressed in *Escherichia coli* and the gene product was subsequently demonstrated by enzymatic analysis to catalyze methylation of Mg-protoporphyrin IX to form Mg-protoporphyrin IX monomethyl ester. Activity required the substrates Mg-protoporphyrin IX and *S*-adenosyl-L-methionine. ^{14}C-labeled product was formed in incubations containing ^{14}C-methyl-labeled *S*-adenosyl-L-methionine. On the basis of these and previous results, we also conclude that the *bchH* gene, which was previously reported to code for Mg-protoporphyrin IX methyltransferase, is most likely involved in the Mg chelation step.

Biosynthesis of bacteriochlorophyll is a complex process that shares many common steps with chlorophyll synthesis (for a recent review see reference 4). Description of bacteriochlorophyll biosynthetic mutants resulting from nitrosoguanidine mutagenesis (32) and complementation with an R′ plasmid (22) led to the identification of what is termed the photosynthesis gene cluster. The photosynthesis gene cluster is a 46-kb region of the *Rhodobacter capsulatus* genome that contains most if not all of the genes necessary for bacteriochlorophyll and carotenoid biosynthesis as well as genes encoding most of the structural components of the photosystem. Saturation mutagenesis of the photosynthesis gene cluster by transposon insertion gave a more complete understanding of the genes presumed to encode the enzymes of the bacteriochlorophyll biosynthetic pathway (7, 35). Sequence analysis of the photosynthesis gene cluster (2, 9, 11, 12, 31, 34) (EMBL accession no. Z11165) has been followed by directed mutagenesis to clearly define the role of each open reading frame in bacteriochlorophyll biosynthesis (9, 15, 29, 33).

The first committed step of bacteriochlorophyll biosynthesis involves the insertion of Mg into protoporphyrin (Fig. 1), a reaction that is catalyzed by the enzyme Mg-chelatase. Two Mg-chelatase subunits were proposed to be encoded by genes *bchD* and *bchI*, on the basis of the accumulation of protoporphyrin IX in strains containing disruptions of those genes (7, 9, 33, 35). A plant homolog for *bchI*, *ch-42*, has been identified in *Arabidopsis thaliana* (25), and a mutant in that locus accumulates protoporphyrin IX, as would be predicted from the proposed function of these gene products.

The next biosynthetic step in the pathway (Fig. 1) involves transfer of the methyl group from *S*-adenosyl-L-methionine (SAM) to Mg-protoporphyrin IX (MP) to form Mg-protoporphyrin IX monomethyl ester (MPE). *S*-Adenosyl-L-methionine:Mg-protoporphyrin IX methyltransferase (MPMT) (EC 2.1.1.11) catalyzes this reaction, and this reaction has been demonstrated in several plant and bacterial systems (14, 16, 18, 26, 27). Recent reports (4, 8, 9, 17) have suggested that the product of the *bchH* gene in *R. capsulatus* codes for MPMT. This assignment was based on an earlier report that Mg chelation was obligatorily coupled to the methyltransferase reaction (16), and the mutations affecting either enzyme were therefore predicted to accumulate protoporphyrin IX as the primary product, which is the phenotype observed for *bchH* as well as for *bchD* and *bchI* mutations (7, 9, 31, 33). Gorchein et al. (17) also demonstrated that MPMT activity is absent in *bchH*::Tn*5* insertion mutants of *Rhodobacter sphaeroides*, which implicated *bchH* at the methyltransferase step of the pathway. However, recent observations have cast doubt on the hypothesis that *bchH* encodes the MPMT enzyme (8). Both *bchH* and its homolog from the plant *Antirrhinum majus* (19) exhibit extensive sequence similarity with the bacterial gene *cobN*. In vitro studies have indicated that *cobN* encodes a subunit of cobaltochelatase that is involved in chelation of Co into a tetrapyrrole, hydrogenobyrinic acid *a,c* diamide, in the vitamin B_{12} biosynthetic pathway (13). The sequence similarity of *bchH* to *cobN* therefore suggests a role for the *bchH* gene product in the Mg chelation process rather than in the methyltransferase reaction.

The biosynthetic step following MPE formation is the formation of Mg-2,4-divinylphaeoporphyrin a_5 (divinylprotochlorophyllide) from MPE. This is a complex reaction involving addition of oxygen to the 6-propionate followed by oxidation to form a keto group and closure of a five-membered ring (Fig. 1). The genes proposed to be involved in this reaction, *bchE* and *bchM*, were previously defined by mutational analysis (9, 29, 31, 35). On the basis of fluorescence emission analysis and thin-layer chromatography, both *bchE* and *bchM* mutants were described as accumulating primarily MPE (7, 9, 31). It was therefore concluded that both genes are involved in the cyclase reaction.

In an attempt to further define the early steps of the Mg-tetrapyrrole biosynthetic pathway, we have reexamined the role of the *bchM* gene and have found that the *bchM* disruption mutant, ZY4, accumulates primarily MP rather than MPE as previously reported (8). Enzyme assays with extracts of an *Escherichia coli* strain expressing the *R. capsulatus bchM* gene

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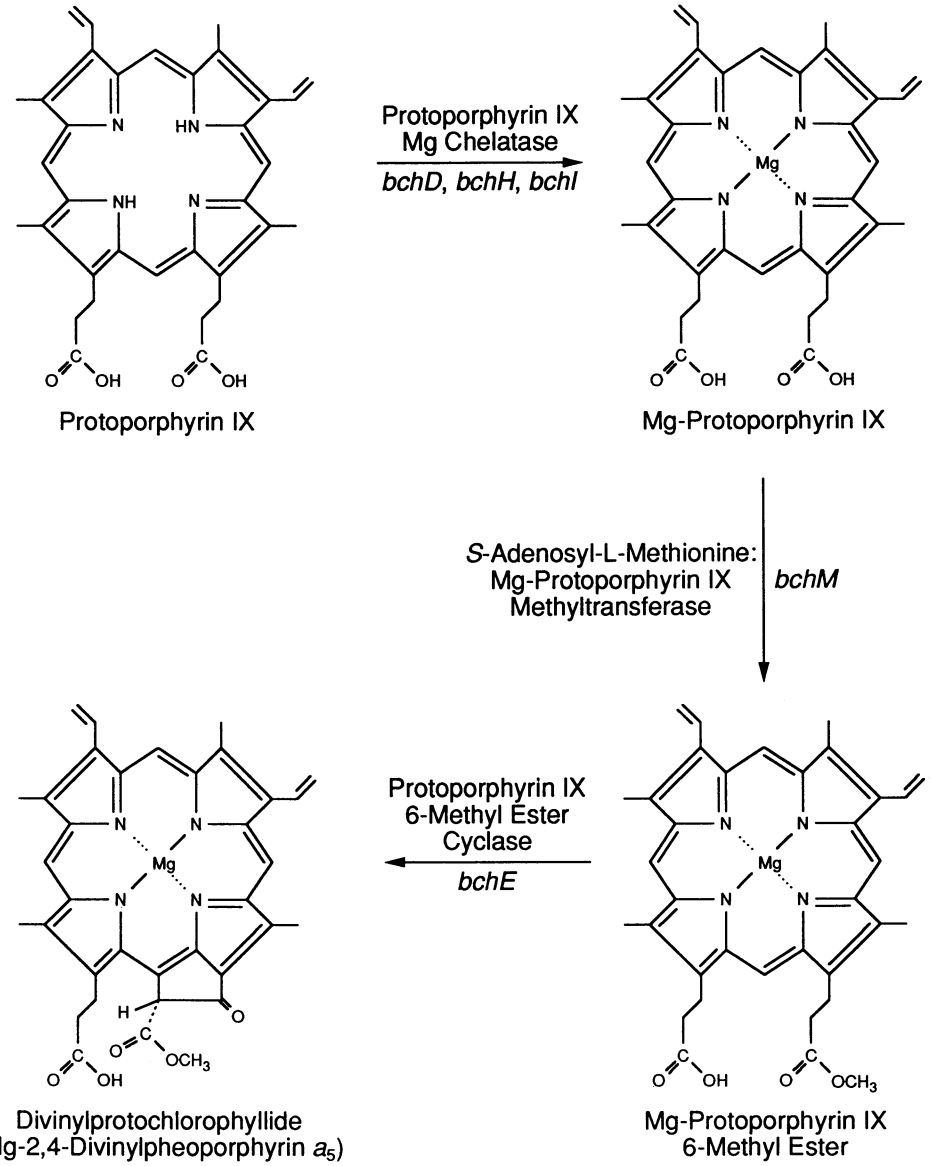

FIG. 1. Biosynthetic pathway from protoporphyrin IX to divinylprotochlorophyllide. This portion of the bacteriochlorophyll *a* biosynthetic pathway is shown with the genetic loci ascribed to each step.

product demonstrate that *bchM* encodes MPMT and the in vitro enzyme activity is dependent upon the presence of SAM and MP. The predicted BchM polypeptide was also found to have an amino acid sequence motif that is conserved with other enzymes that utilize SAM as a substrate.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmid construction. *R. capsulatus* ZY4 was grown in RCV^+ liquid medium at 34°C as previously described (31). *E. coli* C600/pGP1-2/pT7-7 and C600/pGP1-2/pT7-7::bchM were routinely grown at 30°C in LB medium (5). Ampicillin was used at 100 μg/ml, and kanamycin was used at 50 μg/ml. An *E. coli* T7 RNA polymerase-based expression system was used to express the *bchM* gene product (28). A *bchM* expression plasmid was constructed by cloning a PCR-amplified (23) *bchM* gene fragment into the expression plasmid T7-7 (3). The primers used for PCR (5′-CCGACTGCATATGCCCTCCGATTAC and 5′-GCATC TGCAGTATCATGGCCGATAC) were synthesized to introduce an *Nde*I restriction site at the *bchM* methionine initiation codon and a *Pst*I restriction site just downstream of the *bchM* TGA stop codon. Cloning of the PCR-amplified *bchM* gene into respective *Nde*I and *Pst*I sites of plasmid T7-7 resulted in the placement of *bchM* expression under control of T7 RNA polymerase. In addition, the plasmid provides an *E. coli* consensus ribosome binding site for efficient translation.

Extraction of pigments from ZY4. All reagents used in incubations and extractions were obtained from Fisher Scientific (Pittsburgh, Pa.) unless otherwise noted. Actively growing ZY4 cells were harvested by centrifugation at 8,000 × *g* for 10 min at 4°C. The cells were resuspended in 1 ml of 0.1 M NH_4OH, and then 9 ml of ice-cold acetone was added. Insoluble material was removed by centrifugation at 8,000 × *g*.

The supernatant fraction was extracted with an equal volume of hexanes followed by 1/3 volume of hexanes. The volume of the remaining acetone fraction was measured, and 1/17 volume of saturated NaCl and 1/70 volume of 0.5 M KH_2PO_4 were added. The pigments were extracted into peroxide-free diethyl ether by two successive partitions with 1/2 volume of ether.

BchM expression and enzymatic assay conditions. Heat-induced expression in *E. coli* was performed essentially as described by Ausubel et al. (3). Cells were harvested by centrifugation at 4,080 × *g* for 10 min at 4°C. The cell pellets were frozen at −80°C until used. As described more fully in the Results and Discussion section, induction of *bchM* expression was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (21) to detect the expression of a peptide with an apparent molecular weight of 22,300.

E. coli cells were resuspended in buffer {20 mM TES [*N*-Tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; Research Organics, Cleveland, Ohio], 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Research Organics), 1 mM $MgCl_2$, 1 mM EDTA, pH 7.7} and lysed by two passes through a French pressure cell (SLM Aminco, Urbana, Ill.) at 20,000 lb/in^2. Assays (500 μl) were done in the same buffer with the addition of 2 mM SAM (Sigma, St. Louis, Mo.), 40 μM MP (Porphyrin Products, Logan, Utah), and a total protein concentration of 0.12 mg/ml as determined by the method of Bradford (10). Incubations were for 1 h at 30°C. Products were isolated by an extraction procedure based on that given in reference 30: addition of 500 μl of buffer and 3 ml of ice-cold acetone to the incubations was followed by mixing and centrifugation at 7,710 × *g* for 5 min at 4°C. The supernatant was retained and the pellet was resuspended in 500 μl of 0.125 M NH_4OH, and 1.5 ml of acetone was added. Centrifugation was performed as described above, and the supernatants were combined and extracted with 7.5 ml of hexanes (Kodak, Rochester, N.Y.), then with 2.5 ml of hexanes, and finally with 3 ml of 2-methylbutane, followed by brief exposure to a nitrogen stream to remove residual 2-methylbutane. Saturated NaCl (1.7 ml) was added to the acetone fraction, followed by sufficient 0.25 M maleic acid (pH 5.0) to bring the pH of the solution to 6.8. The tetrapyrroles were then extracted into peroxide-free diethyl ether by two successive partitions with 3-ml portions of ether.

HPLC conditions. High-performance liquid chromatography (HPLC) was performed as previously described (30). Samples (20 μl) of the ether extract were diluted to 100 μl with methanol:5 mM aqueous tetrabutylammonium phosphate (Rainin, Woburn, Mass.) 7:3 [vol/vol]), and aliquots were injected onto a Zorbax octadecylsilane column (Du Pont, Wilmington, Del.) (4.6 by 25 mm) preequilibrated with methanol:5 mM aqueous tetrabutylammonium phosphate (7:3 [vol/vol]). Elution with this solvent continued for 3 min; then the solvent was changed to methanol:water (7:3 [vol/vol]) (flow rate 1.0 ml/min). Elution was monitored with a Fluorichrom fluorescence detector (Varian, Sunnyvale, Calif.).

Thin-layer chromatography. Thin-layer chromatography of the ether extracts was done on silica gel G plates. Development was with toluene:ethyl acetate:ethanol (4:1:1 [vol/vol/vol]). Migration of tetrapyrroles was monitored by fluorescence under long-wavelength UV light as previously described (7).

Fluorescence spectroscopy. Fluorescence emission spectra of fractions collected from the HPLC were taken with a fully corrected fluorescence spectrophotometer (Spex, Metuchen, N.J.) with an excitation wavelength of 420 nm.

RESULTS AND DISCUSSION

Accumulation of MP in the ZY4 mutant. An HPLC elution profile of ether extracts from a *bchM* mutant strain of *R. capsulatus*, ZY4, is shown in Fig. 2A. The major accumulating compound in ZY4 extracts eluted at approximately 9 min. This compound had a fluorescence emission maximum at 596 nm, which is characteristic of an early intermediate (MP or MPE) in the Mg-tetrapyrrole pathway (Fig. 3C). This compound coeluted with standard MP on this chromatography system and does not move from the origin in the thin-layer chromatography system used (data not shown), thereby indicating that the Mg-tetrapyrrole intermediate accumulated by strain ZY4 is MP rather than MPE as previously reported (31). A second, smaller peak eluting at 15 min exhibits a fluorescence emission maximum at 627 nm (Fig. 3D), which is similar to that of divinylprotochlorophyllide and yet does not coelute with divinylprotochlorophyllide or MPE, and therefore its structure remains unidentified. A third small peak eluting at 20 min also does not coelute with any known intermediates, and its fluorescence emission maximum of 603 nm does not correspond to any known intermediates (Fig. 3E). It is presumed that both of the unidentified compounds accumulated by ZY4 are either derivatives of MP formed as a result of abnormal accumulation of this intermediate or are possibly early degradation products. As noted above, absence of evidence for the accumulation of MPE in strain ZY4 conflicts with earlier reports (9, 31). This conflict can be explained by the similar fluorescence emission maxima of these compounds which could result in the misidentification of MP as MPE. Additionally, the Mg-tetrapyrrole intermediates, when accumulated to abnormally high levels by stationary-phase cultures, appear to form aberrant derivatives of MP, some of which can be mistaken for MPE.

SAM-dependent conversion of MP to MPE by extracts of *E. coli* cells expressing *bchM*. The identification of MP as the primary accumulating tetrapyrrole in ZY4 suggests that the insertion mutation in this strain causes a block in the conversion of MP to MPE, a reaction that is catalyzed by MPMT. To confirm this possibility, a T7 polymerase-based expression construct was created to assay for MPMT activity in *E. coli* extracts that contain the expressed *bchM* gene product. As shown in Fig. 4, SDS-PAGE analysis of the polypeptide profile of the *bchM* expression strain demonstrates the presence of an expressed peptide with an apparent molecular weight of 22,300, which is slightly less than the predicted molecular weight of 25,000 for the *bchM* gene product (Fig. 4). The protein is found only in the strain with the plasmid containing the *bchM* insert (lane 2) and is not present in the strain with the control plasmid lacking the insert (lane 1).

E. coli extract containing *R. capsulatus* BchM was incubated with SAM and MP at 30°C and then extracted and analyzed by HPLC. As shown in Fig. 2, the MP substrate peak elutes at approximately 9.0 min (Fig. 2E). There is no change in the MP elution profile upon incubation with an extract prepared from cells containing plasmid without insert (Fig. 2C). In contrast, a 1-h incubation of MP with the *E. coli* extract prepared from cells expressing BchM resulted in a reduction of the substrate peak concomitant with the appearance of an additional peak eluting at approximately 16.5 min (Fig. 2B). The fluorescence emission spectrum of the 16.5-min HPLC fraction (Fig. 3B) is nearly identical with the MP spectrum (Fig. 3A), but the increased retention time indicates a more hydrophobic molecule, presumably MPE.

To verify the presence of a SAM-derived methyl group in the incubation product, an incubation was done in an assay mixture containing ^{14}C-methyl-labeled SAM. The assay was

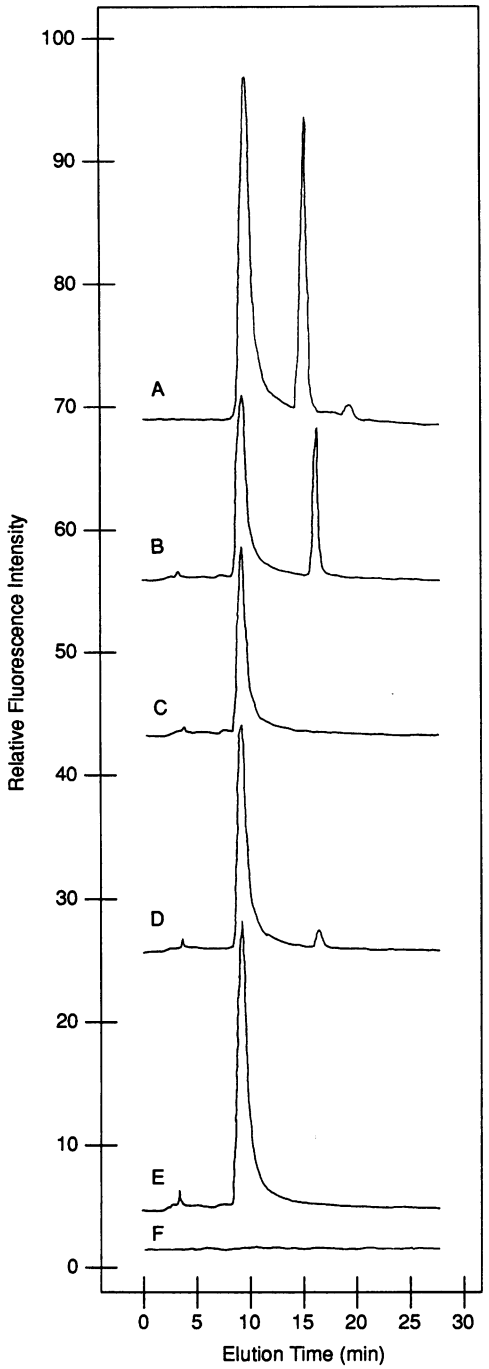

FIG. 2. HPLC trace of ether extracts. HPLC was done as previously described (16). Trace A is the ether extract from the *R. capsulatus bchM* mutant strain ZY4. Trace B is of tetrapyrroles extracted from a 1-h 30°C-incubation of an *E. coli* extract containing BchM and all required MPMT components (buffer, SAM, and MP). Trace C is of tetrapyrroles extracted from a similar 1-h incubation with all required MPMT components with an *E. coli* extract obtained from a strain containing plasmid with no insert. Trace D is of tetrapyrroles extracted from a 1-h incubation of *E. coli* extracts containing BchM with MPMT components lacking SAM substrate. Trace E is of tetrapyrroles extracted from the assay mixture as in trace B with the exception that there was no 30°C incubation prior to extraction. Trace F is of tetrapyrroles extracted from an incubation of *E. coli* extracts containing BchM with MPMT components lacking MP substrate.

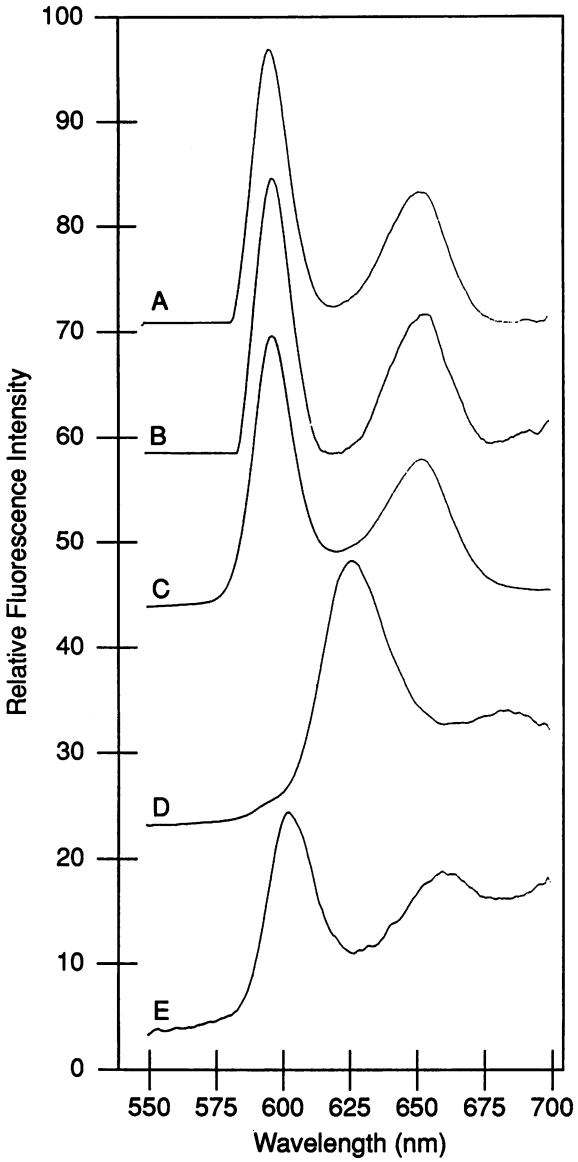

FIG. 3. Fluorescence emission analysis of HPLC fractions. Fluorescence emission spectra of tetrapyrroles were taken in 70% methanol. Excitation was at 420 nm. Trace A is a fraction containing standard MP, eluting at 9.0 min. Trace B is MPE produced in enzyme incubations, eluting at 16.5 min. Trace C is MP extracted from *R. capsulatus* ZY4, eluting at 9.0 min. Trace D is material extracted from ZY4, eluting at 15 min. Trace E is material collected from ZY4, eluting at 20 min.

done as described above except that the incubation mixture contained 10^7 cpm of ^{14}C-methyl-SAM (Du Pont NEN, Wilmington, Del.) (the final specific radioactivity was 10^7 cpm/μmol). The final ether extract was concentrated to 100 μl, and 20 μl was injected onto the HPLC column. Eluate fractions (1 ml) were collected at 1-min intervals, and the radioactivity was determined by liquid scintillation counting. The HPLC elution profile shows a major peak of retained radioactivity that coeluted with MPE at 16.5 min (Fig. 5). The early-eluting radioactive peak corresponds to unretained material, which presumably includes degraded product and residual SAM.

Because Mg-protoporphyrin IX dimethyl ester coelutes with

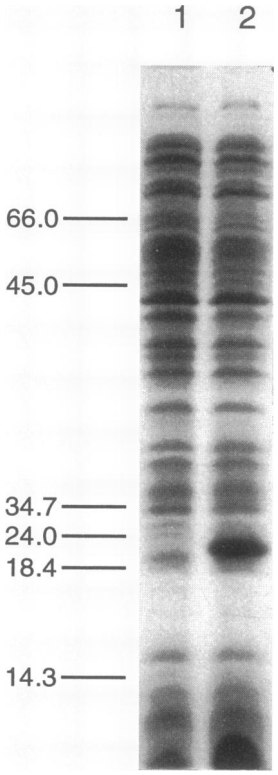

FIG. 4. SDS-PAGE of *E. coli* extracts. Coomassie blue-stained SDS-PAGE gel (15% polyacrylamide) showing the polypeptide profile of extracts from strains C600/pGP1-2/pT7-7 (control, lane 1) and C600/pGP1-2/pT7-7::bchM (BchM expressing strain, lane 2). An equal amount of total protein (10 μg) was loaded into each lane. Relative migration of molecular weight markers and their respective sizes (in kilodaltons) are shown in the left margin.

MPE on the HPLC system used, thin-layer chromatography was required to test for the presence of a single methyl group on the incubation product. Analysis by thin-layer chromatography confirmed the presence of a single methyl group on the Mg-tetrapyrrole product eluting at 16.5 min (R_f = 0.55 compared with R_f = 0.87 for standard Mg-protoporphyrin IX dimethyl ester; unesterified MP does not migrate from the origin in this system). It is therefore concluded that the product eluting at 16.5 min is indeed MPE. A small amount of MPE production was also observed in a reaction lacking added SAM (Fig. 2D), which can be attributed to the presence of endogenous SAM in the unfractionated *E. coli* cell lysates. No product was formed without the addition of MP (Fig. 2F).

Homology of BchM to known methyltransferases. A search of the GenBank database (1) resulted in alignments of the predicted BchM protein with deduced peptides of several methyltransferase genes and other gene products which utilize SAM as a substrate. The most highly conserved region is centered around the predicted BchM sequence beginning at amino acid residue 67, Asp-Ala-Gly-Cys-Gly-Thr-Gly (31). This region aligns quite well with the methyltransferase consensus sequence, Leu(Asp/Glu)-(Val/Leu/Ile)-Gly-X-Gly-X-Gly (20). Longer stretches of sequence similarity were also observed between BchM and specific methyltransferases. For example, BchM has 46% identity and 69% similarity in a 39-residue region encompassing the methyltransferase consensus sequence with the *ermA* gene product from Tn*554* (24).

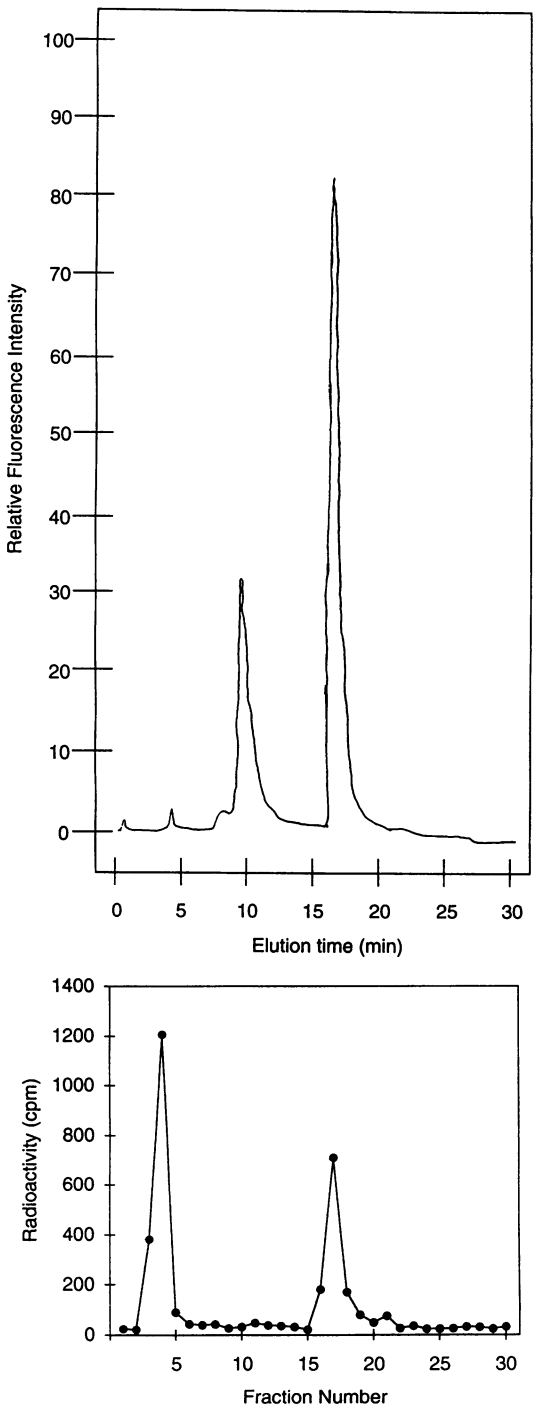

FIG. 5. HPLC of product derived from incubation in assay mixture containing ^{14}C-methyl-labeled SAM. The top panel is the fluorescence elution profile, and the lower panel is the radioactivity elution profile.

The *ermA* gene product is known to utilize SAM as a substrate to methylate specific adenine residues of 23S rRNA conferring erythromycin resistance in *Staphylococcus aureus*.

Conclusions. The experiments described here clearly demonstrate that *bchM* encodes MPMT that catalyzes transfer of a methyl group from SAM to MP to form MPE. The results appear to conflict directly with those of Gorchein et al. (17),

who reported that a Tn*5* insertion mutant in the *bchH* gene lacked methyltransferase activity. However, the *bchH* gene is located upstream of the *bchM* gene, and the two genes are thought to be cotranscribed (31). Thus, the results of Gorchein et al. can most likely be explained by well-documented Tn*5*-mediated polarity effects (5).

On the basis of the results of this study, the observed accumulation of protoporphyrin IX in *bchH* mutants, and the homology of *bchH* with a cobaltochelatase subunit (13), we propose that the *bchH* gene encodes a subunit of the Mg-chelatase which catalyzes the insertion of Mg^{2+} into protoporphyrin IX to form MP. Direct support of this proposal will however require similar expression of all three genes believed to be involved in Mg chelation (*bchD*, *bchH*, and *bchI*) and an enzymatic assay to establish Mg-chelatase activity.

The demonstration that *bchM* encodes the methyltransferase also suggests that only one gene, *bchE*, is involved in the isocyclic ring closure reaction that results in the formation of divinylprotochlorophyllide from MPE, despite the complexity of the reaction. Alternatively, it is possible that additional, unidentified genes are involved in the cyclase reaction. The experiments reported here serve to emphasize the importance of creating expression constructs that allow the clear demonstration of biosynthetic function.

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