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# The bacteriochlorophyll biosynthesis gene, *bchM*, of *Rhodobacter sphaeroides* encodes *S*-adenosyl-L-methionine: Mg protoporphyrin IX methyltransferase

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**Abstract** The *bchM* gene of *Rhodobacter sphaeroides* has been sequenced and then overexpressed in *E. coli* producing a protein of $M_r$ approximately 27,500. Cell-free extracts of the transformed *E. coli* strain are able to methylate added Mg protoporphyrin, resulting in the formation of Mg protoporphyrin monomethyl ester. The identity of this product was verified by HPLC. The *bchM* gene product is therefore assigned to the methyltransferase step in bacteriochlorophyll biosynthesis.

*Key words:* Photosynthesis; Bacteriochlorophyll; Chlorophyll; *Rhodobacter sphaeroides*; Methyltransferase

## 1. Introduction

The biosyntheses of bacteriochlorophyll and chlorophyll pigments furnish photosynthetic organisms with the cofactors essential for utilising light energy. The first committed step in each pathway is the chelation of magnesium by protoporphyrin IX, forming Mg protoporphyrin (MgP). This is followed by SAM-dependent methylation of the 6-propionate side chain to form Mg protoporphyrin monomethyl ester (MgPME) (Fig. 1).

The genes for bacteriochlorophyll biosynthesis have been studied largely in the photosynthetic bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, and it is known that in each case the relevant genes are clustered on a region of the genome approximately 46 kb in length [2–7]. Mutational analyses of these clustered genes have produced valuable data on the assignment of the various *bch* genes to each step in the pathway (see for example [5,7]) but complications have arisen from polar effects exerted by the insertional elements used, such as transposon Tn*5*. This has occurred because many *bch* genes are known to be transcriptionally linked [4,8–10]; therefore disruption of a particular *bch* gene can produce additional effects on other genes downstream of the insertion point. In the case of three Tn*5* insertions into the *bchH* gene of *R. sphaeroides*, which produced mutants TB340, TB355 and TB241 [7], measurements of Mg chelatase and methyltransferase activities indicated that both functions were disrupted in these *bchH* insertion mutants, but that only chelatase activity was absent in TB61 and TB55, which have insertions into *bchD* and *bchI*, respectively [11]. Accordingly, *bchI* and *D* were assigned to the Mg chelatase step [11], and in this and other publications *bchH* has been tentatively identified as encoding the methyltransferase since this assignment does explain the observations made at the time [11,12]. This also has relevance to the assignment of function to the *olive* gene of *Antirrhinum majus*, which shows good homology with *bchH* [13].

In this study, we demonstrate that the methyltransferase is encoded by *bchM*, a gene lying transcriptionally downstream of *bchH*. This function is established by overexpression of *bchM* in *E. coli* and the demonstration that cell-free extracts are able to convert exogenous MgP to MgPME. Therefore the insertions TB340, TB355 and TB241 [11] exert their effect by inactivating both *bchH* and *bchM*, producing a chelatase-negative, methyltransferase-negative phenotype. The corollary of this observation is that *bchH* encodes a component of the Mg chelatase along with *bchI* and *D*. Finally, our results imply that the *olive* gene of *A. majus* must also encode a component of the Mg-protoporphyrin chelatase enzyme.

## 2. Materials and methods

### 2.1. Media, strains, plasmids and growth conditions

The oligonucleotides 5′-TAGAGGAGACGACCATATGAACGAATCGACCACCC-3′ and 5′-ACAAAGCTTGGATCCTCACGGCCGGAACTCCAGACA-3′ were used to amplify the *bchM* gene by PCR and introduce *Nde*I and *Bam*HI sites which allowed cloning into pET3a [15], yielding plasmid pETBCHM. Plasmids for overexpression were transformed into *E. coli* BL21(DE3) [16]. *E. coli* BL21(DE3) strains were grown in 50 ml of LB containing 100 $\mu g \cdot ml^{-1}$ of ampicillin at 37°C until the $OD_{600}$ reached 0.6 and proteins were induced by addition of IPTG to 0.4 mM.

### 2.2. DNA sequencing

This was carried out on an ABI 373A sequencer using an ABI DyeDeoxy reagent kit.

### 2.3. SDS-PAGE

Aliquots of *E. coli* cultures were removed at various times during IPTG induction, harvested, resuspended in solubilisation buffer and incubated at 100°C for 2 min. Samples were separated on a 15% acrylamide gel using the method of Laemmli [17] and stained with Coomassie blue.

### 2.4. Methyltransferase assays, extraction of pigments and high pressure liquid chromatography

Mg protoporphyrin was purchased from Porphyrin Products, Logan, UT. Mg protoporphyrin monomethyl ester was prepared by extracting cells of the *R. sphaeroides* mutant N6 which has been shown to accumulate MgPME [14].

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*Abbreviations:* *R.*, *Rhodobacter*; Bchl, bacteriochlorophyll; SAM, *S*-adenosyl-L-methionine; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; IPTG, isopropyl-β-D-thiogalactosidase; ORF, open reading frame.


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Induced *E. coli* BL21(DE3) strains carrying pET derivatives were harvested after 3 h, washed once in $H_2O$ and resuspended to the same cell density in 50 mM Tris, 5 mM EGTA, pH 8.4. Cell suspensions were sonicated (3 × 30 s) on ice. Methyltransferase assays contained the equivalent of 8 $OD_{600}$ units of sonicated cells, supplemented with 1 mM SAM and 40 nmol of MgP where indicated, in a volume of 0.6 ml. The reactions were incubated at 34°C for 1 h with shaking. Extraction of porphyrins was done according to Gorchein [18].

Porphyrins were analysed by HPLC on a Beckman Ultrasphere ODS column (150 × 4.6 mm, i.d.) The column was run at a flow rate of 1 ml/min with 15% acetonitrile, 85% $H_2O$ containing 0.1% v/v triethylamine for 1 min and the pigments were eluted by changing to 100% acetonitrile over a period of 20 min. Elution of porphyrins was monitored at 405 nm. Using this system, MgP eluted at approximately 8.5 min and MgPME eluted at approximately 12 min. The identity of the pigments eluted as peaks by HPLC was confirmed by recording fluorescence emission spectra on a Spex FluoroMax spectrofluorimeter using an excitation wavelength of 420 nm (results not shown).

## 3. Results and discussion

### 3.1. Sequence of the bchM gene

Fig. 2A. shows the photosynthesis gene cluster of *R. sphaeroides*, with the locations of the genes established by the Tn*5* mutagenesis study of Coomber et al. [7], but amended by more recent work ([10]; H.P. Lang and C.N. Hunter, manuscript in preparation). The region relevant to the present work is shown in an expanded form, together with the locations of the transposon insertions TB340, TB355 and TB241 which are found within *bchH*. In a preliminary study, we established that these three mutations produce strains which are deficient in both Mg chelatase and MgP methyltransferase activities [11]. Bearing in mind the polar effects that can arise from insertional mutagenesis, the region downstream of *bchH* was sequenced, in order to identify other open reading frames. The ORF directly downstream from *bchH*, designated *bchL*, was not investigated further since its strong homology with the *R. capsulatus bchL* sequence and mutational analysis [7] identifies it as encoding a component of the protochlorophyllide reductase enzyme [4,5,19].

The sequence downstream from *bchL* is presented in Fig. 2B. This revealed the presence of an ORF of 668 bp which has typical *R. sphaeroides* codon usage and is preceded by a strong ribosome binding sequence. The predicted product of this gene has 67% identity with the *R. capsulatus bchM* gene product

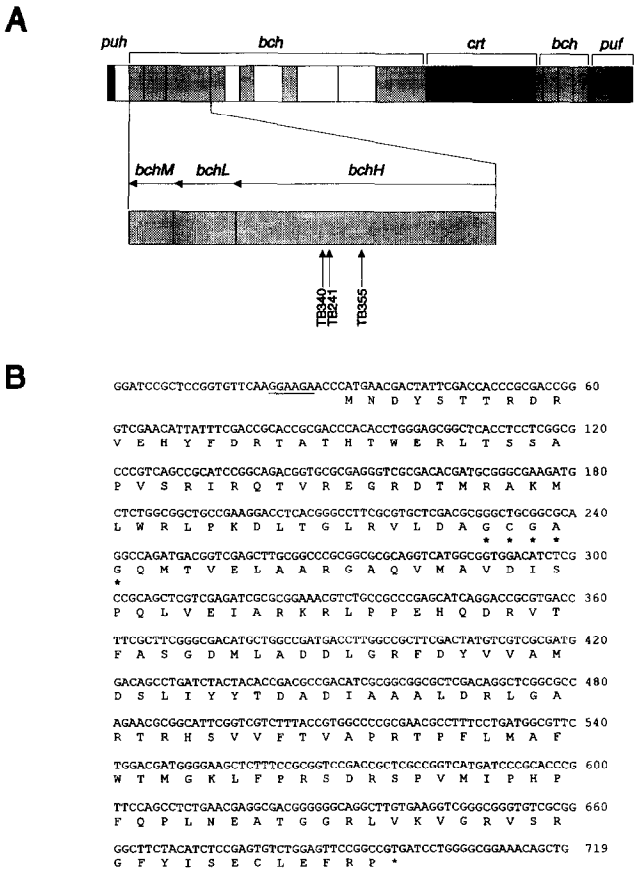


Fig. 2. (A) A map of the photosynthesis gene cluster of *R. sphaeroides*, together with an expanded map of the region encoding *bchH, L* and *M* indicating the position of the *bchH* transposon insertions used in the previous study [11]. (B) DNA sequence of the *bchM* gene and the deduced protein sequence. A potential ribosome binding sequence is underlined and a potential SAM-binding consensus is indicated by asterisks.

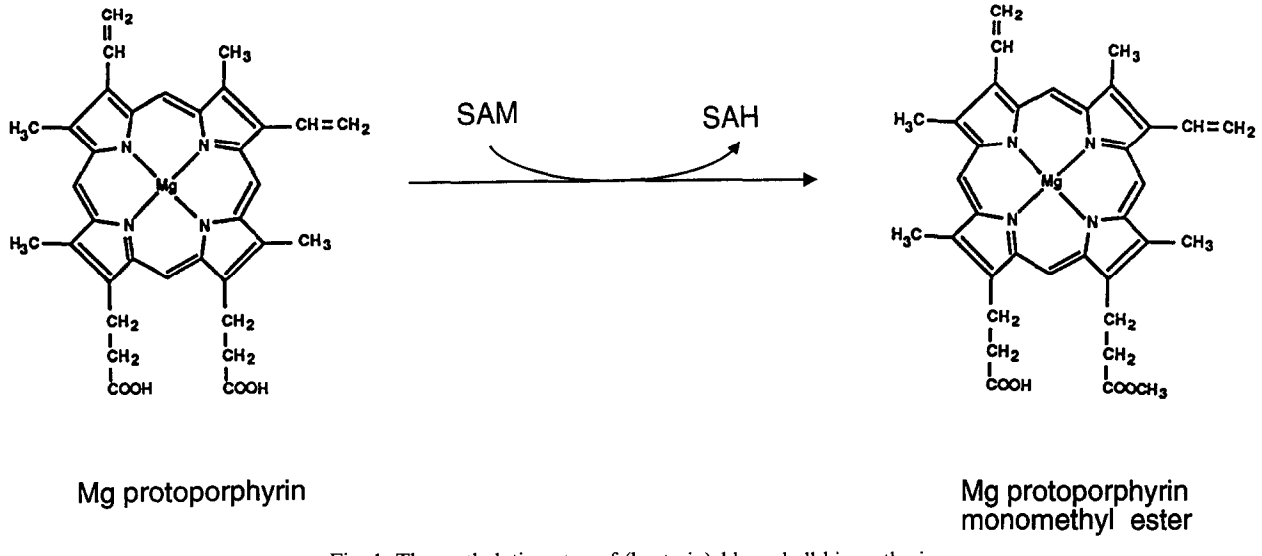


Fig. 1. The methylation step of (bacterio)chlorophyll biosynthesis.

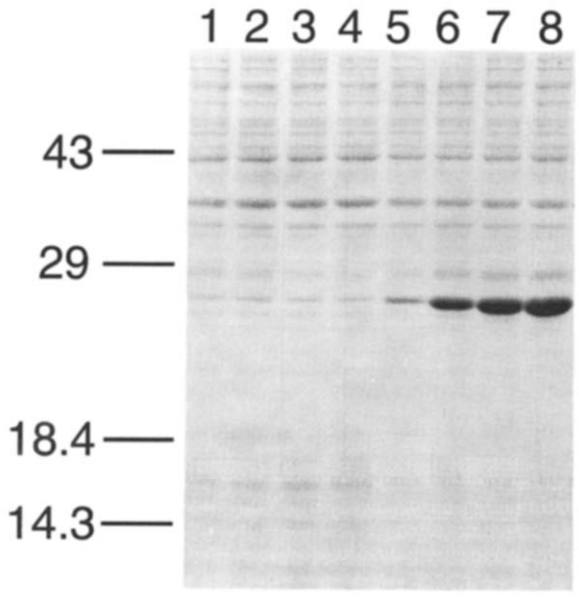


Fig. 3. SDS-PAGE of *E. coli* BL21(DE3) cells containing the plasmids pET3a (lanes 1–4) and pETBCHM (lanes 5–8), at 0 (lanes 1 and 5), 1 (lanes 2 and 6), 2 (lanes 3 and 7) and 3 h (lanes 4 and 8) after IPTG addition. The sizes of the molecular weight markers in kDa are indicated.

(J.E. Hearst, EMBL sequence submission, accession no. Z11165), therefore we have designated the *R. sphaeroides* gene *bchM*. The predicted protein has a molecular weight of 25,681 and has a sequence motif (GCGAG) which is similar to those conserved among SAM methyltransferases and is proposed to be involved in SAM binding [20].

### 3.2. *Overexpression of the* bchM *gene in* E. coli *and determination of methyltransferase activity*

The PCR amplified *bchM* gene was cloned into pET3a, yielding plasmid pETBCHM. Fig. 3 shows a comparison of the proteins produced by *E. coli* BL21(DE3) containing either pET3a or pETBCHM after induction with IPTG. In *E. coli* cells containing pET3a, no inducible protein is present, whereas in cells containing pETBCHM a protein of approximate $M_r$ 27,500 is induced. This apparent molecular weight agrees well with that predicted from the deduced amino acid sequence.

Sonicated extracts of IPTG induced *E. coli* cells containing either pET3a or pETBCHM were analysed for MgP methyltransferase activity in the presence of SAM alone, MgP alone and MgP and SAM together. Following incubation at 34°C, porphyrins were extracted and analysed by HPLC (Fig. 4). This method of extraction removes all the MgP substrate, so no peak attributable to this pigment can been seen in Fig. 4; (MgP standard elutes at approximately 8.5 min) (Fig. 4, trace 7). The expected product of the methyltransferase step, MgPME, elutes at 12 min (Fig. 4, trace 8).

Fig. 4 shows the result of HPLC analysis of porphyins produced by incubation mixtures containing sonicated *E. coli* cells and substrates as indicated in the figure legend. Only cells containing the *bchM* gene product produce MgPME in the presence of added MgP (traces 2 and 4). Surprisingly, a significant amount of MgPME was formed without the addition of exogenous SAM (trace 4), which is probably due to an endogenous SAM pool in the *E. coli* cells. However, the amount of product was slightly increased by addition of exogenous SAM (trace 2). No product was formed in the presence of SAM alone (trace 6) or in any of the control (pET3a) incubations (traces 1,3 and 5)indicating that formation of MgPME was dependent on the presence of the *bchM* gene product, MgP and, to some extent, SAM. Therefore we conclude that *bchM* encodes Mg protoporphyrin methyltransferase.

### 3.3. *Concluding remarks*

We have demonstrated that *bchM* encodes Mg protoporphyrin methyltransferase. This is in agreement with the results of Bollivar et al [21] who have overexpressed the *bchM* gene of *R. capsulatus* in *E. coli*. This assignment of function to *bchM* allows us to place a different interpretation on our previous study which reported the enzymic activities measured in some *R. sphaeroides bch* mutants [11]. The absence of methyltransferase activity in the *bchH* insertion mutants TB340, TB355 and TB241 must have arisen from the polar effects exerted on *bchM*; thus the insertions must have effectively produced multiple *bchHLM* mutations. This has been verified by complementation tests involving constructs containing only *bchH*

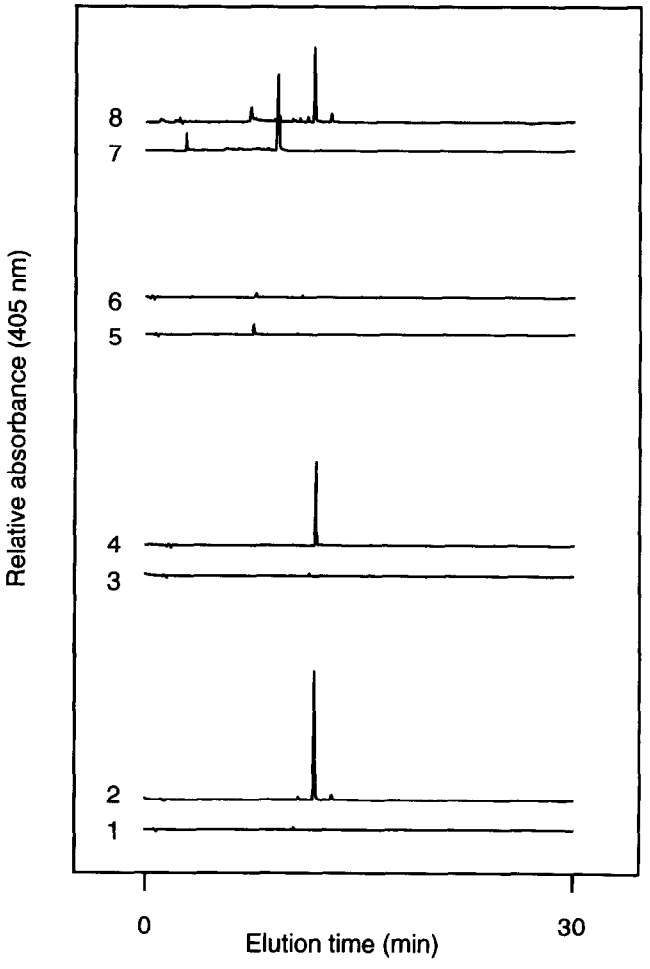


Fig. 4. HPLC traces of the products formed from incubation of *E. coli* extracts. The strains contained pET3a (traces 1,3 and 5), or pETBCHM (traces 2, 4 and 6). Substrates were: MgP + SAM (traces 1 and 2), MgP (traces 3 and 4), SAM (traces 5 and 6). The elution of MgP and MgPME standards is also indicated (traces 7 and 8).

(L.C.D. Gibson and C.N. Hunter, unpublished). The additional absence of Mg chelatase activity from these strains [11] demonstrates that this lesion is the primary effect of insertion into *bchH*, and that *bchH*, along with *bchI* and *D* encode components of the Mg chelatase complex. Hudson et al [13] noted the strong homology between *bchH* and the *Antirrhinum majus olive* gene but were unable to assign a definite function to this gene, despite its likely role in chlorophyll biosynthesis. The implication of the present study is that *olive* also encodes a component of the Mg chelatase complex; this would be consistent with one of the suggestions made by Hudson et al. [13] when they noted an homology between *olive* and the *cobN* gene product from *Pseudomonas denitrificans* which also encodes a subunit of an enzyme with the ability to chelate a metal (cobaltous) ion into a tetrapyrrole [22].

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