Rhodospirillum rubrum Possesses a Variant of the *bchP* Gene, Encoding Geranylgeranyl-Bacteriopheophytin Reductase

Hugh A. Addlesee† and C. Neil Hunter*

*Robert Hill Institute for Photosynthesis and Krebs Institute for Biomolecular Research, Department of Molecular
Biology and Biotechnology, University of Sheffield, Sheffield, United Kingdom*

The *bchP* gene product of *Rhodobacter sphaeroides* is responsible for the reduction of the isoprenoid moiety of bacteriochlorophyll (Bchl) from geranylgeraniol (GG) to phytol; here, we show that this enzyme also catalyzes the reduction of the isoprenoid moiety of bacteriopheophytin (Bphe). In contrast, we demonstrate that a newly identified homolog of this gene in *Rhodospirillum rubrum* encodes an enzyme, GG-Bphe reductase, capable of reducing the isoprenoid moiety of Bphe only. We propose that *Rhodospirillum rubrum* is a naturally occurring *bchP* mutant and that an insertion mutation may have been the initial cause of a partial loss of function. Normal BchP function can be restored to *Rhodospirillum rubrum*, creating a new transconjugant strain possessing Bchl esterified with phytol. We speculate on the requirement of *Rhodospirillum rubrum* for phytylated Bphe and on a potential link between the absence of LH2 and of phytylated Bchl from the wild-type bacterium. The identification of a second role for the fully functional BchP in catalyzing the synthesis of phytylated Bphe strongly suggests that homologs of this enzyme may be similarly responsible for the synthesis of phytylated pheophytin in organisms possessing photosystem 2. In addition to *bchP*, other members of a photosynthesis gene cluster were identified in *Rhodospirillum rubrum*, including a *bchG* gene, demonstrated to encode a functional Bchl synthetase by complementation of a *Rhodobacter sphaeroides* mutant.

Purple photosynthetic bacteria contain two types of antenna complex, responsible for the efficient transfer of light energy to the reaction center (RC), the site of charge separation: light-harvesting complex 1 (LH1) surrounds the RC (35, 36), whereas light-harvesting complex 2 (LH2) is on the periphery of the photosynthetic unit and donates excitation energy to LH1 (12). Both complexes comprise multiple copies of two integral membrane polypeptides, termed α and β, to which bacteriochlorophyll (Bchl) and carotenoid are bound (8). However, not all purple photosynthetic bacteria have the same complement of antenna complexes; for example, *Rhodospirillum rubrum* lacks LH2.

The LH1 of *Rhodospirillum rubrum* is the best characterized of any such complex, with a projection map at 8.5 Å resolution generated from electron microscopy studies (19). This revealed a ring structure composed of 16 αβ heterodimers, the central hole of which is now known to be occupied by the RC (35). The Bchl*a* with which these complexes are assembled is esterified with the C_{20} isoprenoid alcohol geranylgeraniol (GG) (20). In this respect, *Rhodospirillum rubrum* again differs from other purple bacteria, including the well-characterized *Rhodobacter* species *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*. In these species at least, the major alcohol component of the Bchl pigment is phytol (6, 7), which is the commonest esterifying alcohol of chlorophylls and Bchls (32). Like GG, phytol is a C_{20} isoprenoid alcohol, but phytol differs in being more highly saturated. It appears that a GG moiety is a biosynthetic

precursor of a phytol moiety in both of these *Rhodobacter* species and that the product of a single gene is responsible for the three necessary reduction steps (3, 6). This gene, *bchP*, encodes the enzyme GG-Bchl reductase and resides among other photosynthesis-related genes in a photosynthesis gene cluster (PGC) (4, 25).

Our previous work has indicated that although both LH1 and LH2 assemble with GG-esterified Bchl in place of the usual phytylated pigment in a *bchP* mutant of *Rhodobacter sphaeroides*, LH2 is severely affected by this change, while LH1 is not (3). It is possible that the absence of LH2 and phytylated Bchl (Bchl*a*P) from wild-type *Rhodospirillum rubrum* may be linked. Homologs of *bchP* have been found in cyanobacteria and plants, in which the gene product has been identified as the catalyst for the reduction of the isoprenoid moiety of chlorophyll (Chl) (2, 21).

In addition to Bchl, the RC of purple bacteria contains two bacteriopheophytin (Bphe) molecules, one of which is the first clearly resolved acceptor of electrons, following electron transfer from the Bchl dimer (see reference 13 for a review). This situation is mirrored in higher photosynthetic organisms, in which pheophytin serves an identical role within photosystem 2 (PS2) (30). Although the Bchl of *Rhodospirillum rubrum* is esterified with GG and not with phytol, it has previously been reported that the Bphe of this organism is esterified with phytol, as it is in *Rhodobacter sphaeroides* (11). This discrepancy implied that an enzyme capable of reducing the isoprenoid moiety of Bphe*a*GG but not that of Bchl*a*GG existed in *Rhodospirillum rubrum* (Fig. 1); such an enzyme might be expected to show some homology with GG-Bchl reductase.

We searched for and found a *Rhodospirillum rubrum* homolog of *bchP* and demonstrated that the gene product does have the ability to reduce the isoprenoid moiety of Bphe but

* Corresponding author. Mailing address: Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, United Kingdom. Phone: (0114) 222 4191. Fax: (0114) 222 2711. E-mail: c.n.hunter@sheffield.ac.uk.
† Present address: Department of Chemistry, University of Glasgow, Glasgow G12 8QQ, United Kingdom.
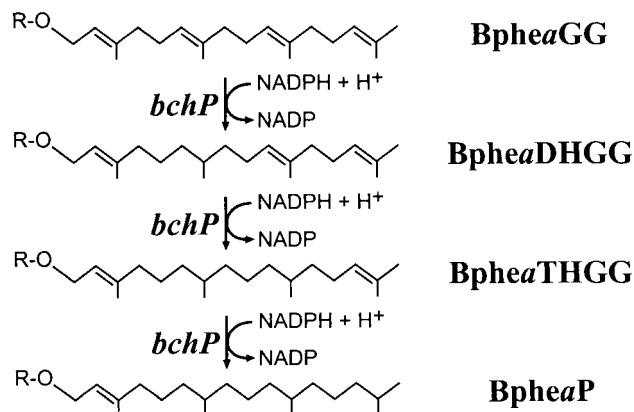
FIG. 1. Proposed pathway for reduction of Bphe*a*GG in *Rhodospirillum rubrum*. The sequence of the hydrogenation reactions is based on that which occurs during Chl biosynthesis in greening plants (33). Reduction proceeds via dihydro-GG-esterified (Bphe*a*DHGG) and tetrahydro-GG-esterified (Bphe*a*THGG) intermediates to the final product, phytylated Bphe (Bphe*a*P). R represents the tetrapyrrole-derived moiety of Bphe.

not that of Bchl. In contrast, it was demonstrated that the *Rhodobacter sphaeroides bchP* gene product catalyzes the reduction of both pigment types. We propose that the homologs of *bchP* in PS2-containing organisms are similarly responsible for the reduction of the isoprenoid moieties of both Chl and pheophytin.

Unequivocal proof of the difference in the activities of the *Rhodobacter sphaeroides* and *Rhodospirillum rubrum bchP* gene products was provided by introducing the *Rhodobacter sphaeroides bchP* gene into *Rhodospirillum rubrum*, generating a novel transconjugant strain of *Rhodospirillum rubrum* in which the Bchls are esterified with phytol. We propose that *Rhodospirillum rubrum* is a naturally occurring *bchP* mutant and that an insertion mutation may have been the initial cause of a partial loss of function. A gene sited close to *bchP* in *Rhodospirillum rubrum* has been demonstrated to encode a functional Bchl synthetase, the enzyme responsible for the addition of the isoprenoid moiety to bacteriochlorophyllide (1, 6, 27).

MATERIALS AND METHODS

Isolation of a *bchP* homolog and other members of a PGC from *Rhodospirillum rubrum*. Degenerate oligonucleotides, designed according to the regions of derived amino acid similarity between the *Rhodobacter capsulatus bchP* gene and an expressed sequence tag from *Arabidopsis thaliana* (2), were used to PCR amplify a 278-bp fragment from *Rhodospirillum rubrum* genomic DNA. This was cloned and sequenced, and the deduced amino acid sequence was found to display significant similarity to each of the original sequences and to other GG-(B)chl reductase genes.

The PCR fragment was radiolabeled and used to probe a partial genomic library of *Rhodospirillum rubrum* constructed in the pBluescript II SK+ phagemid (Stratagene), resulting in the isolation of overlapping *Kpn*I and *Not*I-*Eco*RI fragments spanning 9.3 kb of the genome. DNA was sequenced using an ABI 373A sequencer. Sequence analysis was performed mainly with Lasergene software (DNAStar), with additional use of the European Molecular Biology Open Software Suite (EMBOSS) at the Sanger Centre, Cambridge, United Kingdom. For codon usage analysis, a codon usage table was created using 43 miscellaneous *Rhodospirillum rubrum* gene sequences.

Growth of *Rhodobacter sphaeroides* and *Rhodospirillum rubrum*. *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* strains were grown in M22+ medium (16). *Rhodobacter sphaeroides* was grown semiaerobically in the dark at 34°C; *Rhodospirillum rubrum* was grown photosynthetically at low light intensity (3 W/

m²) at 20°C. Antibiotic concentrations were 20 μg/ml for neomycin, 5 μg/ml for streptomycin, and 1 μg/ml for tetracycline. To overcome problems of phototoxicity, tetracycline was substituted with 250 ng of doxycycline per ml in photosynthetically grown cultures.

Spectroscopic analysis of intracytoplasmic membrane. Cells were disrupted, and cell-free intracytoplasmic membrane fractions were isolated as described previously (26). Absorbance spectra were recorded on a Beckman DU640 spectrophotometer.

Mutagenesis of *bchP* in *Rhodobacter sphaeroides* mutants DBCΩ and DPF2. Plasmid pSCN6-G:T6G5, which carries the Tn5-mutated *Rhodobacter sphaeroides bchP* gene (3), was transferred into the genomes of strains DBCΩ (18) and DPF2 (15) by conjugation and homologous recombination (9, 34), generating mutants DBCΩ:T6G5 and DPF2:T6G5, respectively. DBCΩ carries a streptomycin resistance gene in place of the *pucBA* genes, and so is LH2⁻; DPF2 has LH2 only. Dot blots showing the absence of the parental vector, pSUP202 (34), confirmed that homologous recombination had occurred.

Complementation of *Rhodobacter sphaeroides* Bchl biosynthesis mutants. The oligonucleotides 5′-CATCTAGAGGAGACGACCATATGCAACGGACCGC AGTTCTGC-3′ and 5′-CTGCAAGCTTGGATCCTCATGGGGCCGCCCCG ATC-3′ were used to PCR amplify the *Rhodospirillum rubrum bchG* gene and introduce *Xba*I and *Hin*dIII sites for cloning into pRKSK1 (14), which provides gene expression under the transcriptional control of the *Rhodobacter sphaeroides puc* operon promoter, yielding plasmid pSK1RR*bchG*. Similarly, the oligonucleotides 5′-GGTCTAGAGGAGACGACCATATGGACGCCGTCGTCGCC AC-3′ and 5′-CGCGAAGCTTGGATCCTCAAGACTTGGCGATGCCGA-3′ permitted cloning of the *Rhodospirillum rubrum bchP* homolog into pRKSK1 to give plasmid pSK1RR*bchP*. Additionally, the first *bchP* oligonucleotide was used with the oligonucleotide 5′-TCGCTGCAGCACCGAGCTGGTCGGGGCGCG GATGATTTCGTGA-3′, and the second with 5′-CCCCTGCAGTATGACCC GGCGCGCTGCGA-3′, to separately amplify the 5′ and 3′ ends of *Rhodospirillum rubrum bchP* and to allow the fusion of the two sections via a *Pst*I site encoded within the new oligonucleotides, creating modified gene *bchP*Δ*x*. This was cloned into pRKSK1, yielding plasmid pSK1RR*bchP*Δ*x*.

Another pair of pRKSK1 derivatives was made, each with an insert consisting partly of *Rhodospirillum rubrum bchP* and partly of *Rhodobacter sphaeroides bchP*. The *Rhodospirillum rubrum bchP* fragments were as in plasmid pSK1RR*bchP*Δ*x*. Equivalent fragments of *Rhodobacter sphaeroides bchP* were amplified by PCR using existing oligonucleotides designed for amplification of the entire coding region (3) in conjunction with two new oligonucleotides, 5′-CATCTGCAGCA CCGAGCTGGTCGGCGGCT-3′ and 5′-CACATGCATTACGACCCTACCC GCTGCGAC-3′. The former of these encodes a *Pst*I site; the latter, an *Nsi*I site, which gives *Pst*I-compatible ends. The 5′ end of *Rhodobacter sphaeroides bchP* was fused to the 3′ end of *Rhodospirillum rubrum bchP*, generating plasmid pSK1*bchP*S/*P*R. Similarly, the 5′ end of *Rhodospirillum rubrum bchP* and the 3′ end of *Rhodobacter sphaeroides bchP* yielded pSK1*bchP*R/*P*S. As a positive control, the two *Rhodobacter sphaeroides bchP* fragments were also fused in plasmid pSK1*bchP*S/*P*S. Each pRKSK1 derivative was transformed into *Escherichia coli* strain S17-1 (34) for conjugative transfer into *Rhodobacter sphaeroides*.

HPLC of pigments. The Bchls of *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* strains were extracted and analyzed as described previously (3), which also permitted identification of Bphe pigments. Elution times were approximately 22.6 min for Bphe*a*GG and 27.4 min for phytylated Bphe*a* (Bphe*a*P). High-pressure liquid chromatography (HPLC) standards of these two pigments were prepared from Bchl*a*GG and Bchl*a*P extracted from *Rhodobacter sphaeroides* strains DPF2:T6G5 and DPF2, respectively, by the method of Perkins and Roberts (28). These strains were used because they lack RCs and are thus devoid of native Bphe.

Heterologous expression of *Rhodobacter sphaeroides bchP* in *Rhodospirillum rubrum*. Plasmid pSK1*bchP* (3) is a pRKSK1-based expression plasmid for *Rhodobacter sphaeroides bchP*. Saegesser et al. (31) have demonstrated the suitability of RK2-based vectors, of which pRKSK1 (14) is an example, for the expression of genes in *Rhodospirillum rubrum*. Here, both pSK1*bchP* and pRKSK1 were introduced into *Rhodospirillum rubrum* wild-type strain S1 by conjugation, as for *Rhodobacter sphaeroides*.

Nucleotide sequence accession number. The *Rhodospirillum rubrum* DNA sequence encompassing *bchP* has been assigned EMBL accession number AJ310779.

RESULTS

Identification of a *bchP* homolog and other members of a PGC within the genome of *Rhodospirillum rubrum*. A 278-bp stretch of DNA was isolated from *Rhodospirillum rubrum*

A

ppsR | bchG | ORF449 | bchP

1 kb

B

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MQRTAVL---------PYVQLLKPITWFAPMWAFGCGLISSGLPVW--DRWPVIALGVLL 49
MSVNLSLHPRSVPEPRALLELIQPITWFPPIWAYLCGTVSVG--IWPGEKWPLVLLGMVL 58

CGPLVCGTSQAVNDWFDRHVDAINEPDRPIPSGRIPGRVGLYIAIGWTVLSLAVAWVLGP 109
AGPLVCGMSQAANNWCDRHVDAVNEPDRPIPSGRIPGRWGLYIALLMTVLSLAVGWMLGP 118

WVFGAAIFGLALAWAYSAPPFRLKGNGWWGNSAVGLCYEGLPWFTGAAVIAGALPDTRIV 169
WGFGATVFGVLAAWAYSVEPIRLKRSGWWGPGLVALCYEGLPWFTGAAVLSAGAPSFFIV 178

LLAVLYSIGAHGIMTLNDFKAVEGDIRMGVRSLPVQLGVAPAARLACAVMAVPQMVVIGL 229
TVALLYAFGAHGIMTLNDFKALEGDRQHGVRSLPVMLGPEVAAKLACTVMAMAQILVITL 238

VASWDRPYHAGAVGLLLLAQFVLMARLLKRPRELAPWYNATGTTLYVIGMMVSAFALAPL 289
LVIWGKPIHAGIITALLVAQLFAMRVLLRDPAGKCPWYNGTGVTLYVLGMMVAAFAIRGL 298

IGAVELAPLIGAAP                                                303
----------EVLP                                                302
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C

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MDAVVATQTPAEVFDVVVVGGGPAGATAATDLARAGRRVALLDPEGRPKPCGGAIPPRLM 60
M-----------AYDVFVVGGGPSGATAADELARAGKSVALLDRAGRIKPCGGAIPPRLI 49

SEFAIPDDLPCAQVSGARALAPSGRVVDMPITGAVVSMVDRAVFDPWLRARAHQAGACRL 120
EDFDIPDSQIVARIRTARMISPTGRKVDIPIEGGFVGMVDREHFDEWLRRRAAKAGAERL 109

HGRFEGFDRQEDGALVVLWRPEGAAVANRIVARYVVGADGARSKVAALAVAGAAP-PCVA 179
TGTFLRVERDSFHTYVV-WREKATGEEKRSETKLVIGADGAKSDVARAEVPGGDKIPMVI 168

AYHEIIRAPAAPAASAPTPATAAGPAYDPARCDVIYRGDISPDFYGWIFPHGETLSIGSG 239
AYHEIIKPP-----------TSSVIDYDPTRCDVIYDGRVSPDFYGWIFPHGETASVGMG 217

TAVKGFSLREAVALLRREAGLDGLEVVRREGAPIPLRPRKRWDNGRDVVLIGDAAGVVAP 299
TGILGFDLKEATALLRRQAGLDQCETIRKEGAPIPLQPLEKWDNGKDVVLAGDAAGVVAP 277

ASGEGIYYAMASGKMAAEALGEALAGAGPAALAQVRRRFLKAHGKTFWSLGLLQRFWYRS 359
SSGEGIYYAFVGGQVAARACLAALKSGRATDLQLARKMFMADHKTVFKVLRSMQDAYYRD 337

DRWRESFVAICADRDVQRLVWQSYLTKDLARGNMGAYMRIFFKDVAHL---IGIAKS    413
DERRERFVSLCHDTDVQRLTFESYMNKKLTSKQPWAHIKIGFKNVMHLTRLVSPQWT    394
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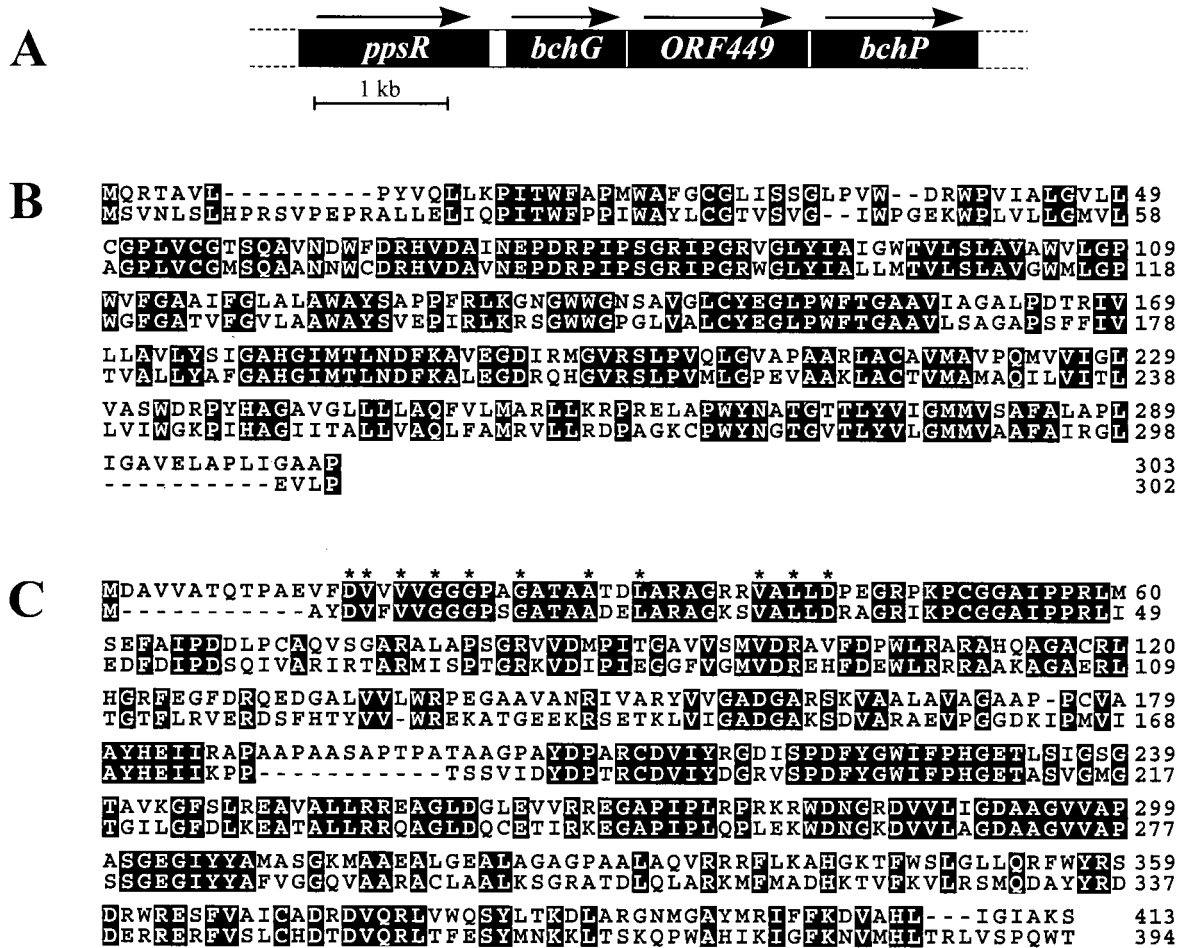
FIG. 2. (A) Physical map of part of a *Rhodospirillum rubrum* PGC. Arrows indicate the direction of transcription of the labeled genes. (B) Deduced amino acid sequence alignment between *Rhodospirillum rubrum* bchG (top) and *Rhodobacter sphaeroides* bchG (bottom). (C) Deduced amino acid sequence alignment between *Rhodospirillum rubrum* bchP (top) and *Rhodobacter sphaeroides* bchP (bottom). Asterisks above the alignment mark the positions of significant amino acids within a putative ADP-binding βαβ fold (37).

genomic DNA by heterologous PCR. This bore strong homology in its deduced amino acid sequence to GG-(b)chl reductases from photosynthetic bacteria and plants. Subsequently, approximately 9 kb of flanking DNA was sequenced; putative open reading frames (ORFs), conforming to a compiled codon usage table for *Rhodospirillum rubrum* genes, were identified through homology with genes present in the PGC of *Rhodobacter sphaeroides* (25).

In addition to the *bchP* homolog, there are equivalents of *ppsR*, which is involved in the repression of pigment biosynthesis; *bchG*, which encodes GG-Bchl synthetase; and *ORF427*, which may encode an assembly factor for photosynthetic complexes. The *Rhodospirillum rubrum* homolog of *ORF427* is *ORF449* (designated *ORF1* in the sequence accession) (Fig. 2A). The sequence identity between the predicted *bchG* gene products of *Rhodospirillum rubrum* and *Rhodobacter sphaeroides* is 58.8% (Fig. 2B). In both *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, *bchG*, *ORF427* (*ORF428* in *Rhodobacter capsulatus*), and *bchP* are members of a more extensive operon (4); upstream of *bchG*, this operon includes two further Bchl biosynthesis genes, *bchE* and *bchJ*, while

immediately downstream of *bchP* lies an isopentenyl diphosphate isomerase gene (25).

In each of these species, *ppsR* (*ORF469* in *Rhodobacter capsulatus*) is sited upstream of *bchE*. Downstream of *bchP* in *Rhodospirillum rubrum* we have identified a partial ORF, putatively encoding a component of the sensory apparatus of this organism. The product of this ORF shows strong homology to Ptr, a component of the photosensory apparatus of *Rhodospirillum centenum*, but only in a putative cytoplasmic output domain (17); the likely periplasmic sensory domain is more homologous to those of chemoreceptors, such as *Rhodobacter capsulatus* McpA (24). Between *bchP* and this partial ORF lies a complete ORF of unknown function, transcribed in the opposite direction.

The predicted gene product of *Rhodospirillum rubrum* bchP has 52.5% sequence identity with *Rhodobacter sphaeroides* BchP (Fig. 2C) and shows strong homology throughout virtually its entire length to this and other known GG-(b)chl reductase enzymes. In common with these enzymes, close to the N terminus it aligns with an amino acid fingerprint predicting the occurrence of an ADP-binding βαβ fold, which may bind the

ADP moiety of a flavin adenine dinucleotide, NADH, or NADPH cofactor (37). *Rhodospirillum rubrum* BchP has identical amino acids with *Rhodobacter sphaeroides* BchP at all significant positions within this fingerprint, suggesting that its cofactor requirement is likely to be identical.

One difference is, however, immediately apparent upon comparison with the GG-(b)chl reductase sequences. Near the center of each there are two highly conserved residues, a proline and a tyrosine (P188 and Y206 of the *Rhodospirillum rubrum* sequence). There are only five to six amino acids between these residues in each of the sequences, with the single exception being that from *Rhodospirillum rubrum*, in which 17 amino acids are present (Fig. 3A). The DNA encoding these amino acids displays atypical codon usage for *Rhodospirillum rubrum* genes, but in another reading frame, typical codon usage is found (Fig. 3B).

Functional analysis shows that the *bchP* homolog from *Rhodospirillum rubrum* encodes a modified BchP protein identified as GG-Bphe reductase. It was possible that the *Rhodospirillum rubrum bchP* homolog encoded a nonfunctional product, which would explain the preponderance of GG-esterified pigments in this organism. Complementation of a *Rhodobacter sphaeroides bchP* mutant was used to test for this possibility. Plasmid pSK1RR*bchP*, containing *Rhodospirillum rubrum bchP*, was transferred into *Rhodobacter sphaeroides* transposon insertion mutant T6G5 (3) by conjugation. This mutant, which accumulates only GG-esterified Bchl, has now also been found to possess GG-esterified Bphe instead of the phytylated Bphe normally found in *Rhodobacter sphaeroides* (11), indicating that the *bchP* gene product is also responsible for the synthesis of this pigment in *Rhodobacter sphaeroides*. In order to provide an increased ratio of Bphe to Bchl as an aid to pigment analysis, the complementations were actually carried out in an LH1-RC-only background in mutant DBCΩ:T6G5, thereby elevating the Bphe-containing RCs as a proportion of the total photosynthetic complexes.

HPLC analysis of a DBCΩ:T6G5[pSK1RR*bchP*] cell extract (Fig. 4Aiii) failed to reveal any reduction of GG-esterified Bchl*a* to dihydro-GG-esterified, tetrahydro-GG-esterified, or phytylated Bchl*a*; see Fig. 4Ciii for a comparison, in which these pigments are resolved. Significantly, however, the Bphe*a*GG had been replaced with Bphe*a*P as a result of transferring *Rhodospirillum rubrum bchP* into *Rhodobacter sphaeroides*, showing that the encoded protein catalyzes the reduction of Bphe*a*GG but not of Bchl*a*GG. This time, comparisons can be made with a Bphe*a*P standard (Fig. 4Av) and with a *Rhodospirillum rubrum* control which contains Bphe*a*P (Fig. 4Di). In contrast, *Rhodobacter sphaeroides* BchP performs the conversion of both Bchl*a*GG to Bchl*a*P and Bphe*a*GG to Bphe*a*P; this is shown in Fig. 4Aiv, in which the native *bchP* gene is used to complement the *bchP* mutant of *Rhodobacter sphaeroides*.

Modification of the central region of *Rhodospirillum rubrum* BchP and interchange of the N- and C-terminal domains of the *Rhodospirillum rubrum* and *Rhodobacter sphaeroides* BchP proteins. As has already been noted, *Rhodospirillum rubrum* BchP differs from other similar polypeptides in the presence of an abnormal stretch of 17 amino acids towards the center of the sequence. We speculated that this difference may be responsible for the discrepancy in substrate specificity between the enzymes, and so the central region of the gene was modified to

make it more homologous to the central region of the *Rhodobacter sphaeroides* gene by replacing codons encoding the sequence AAPAASAPTPATAAGPA with those coding for TSS VLQ; see Fig 3A. The modified *Rhodospirillum rubrum* gene, *bchP*Δ*x*, was also cloned into vector pRKSK1, yielding plasmid pSK1RR*bchP*Δ*x*.

Complementation of *Rhodobacter sphaeroides* mutant DBCΩ:T6G5 was again attempted (Fig. 4Bi), demonstrating that the modified gene still encoded a GG-Bphe reductase but that no GG-Bchl reductase activity had been acquired. Plasmid pSK1*bchP_R/P_S*, which contained a hybrid gene consisting of the 5′ half of *Rhodospirillum rubrum bchP* and the 3′ half of *Rhodobacter sphaeroides bchP*, with the fragments fused as for *bchP*Δ*x*, was found to possess neither GG-Bphe reductase nor GG-Bchl reductase activity (Fig. 4Bii). However, when complementation of the mutant was attempted with a similar fusion between the 5′ half of *Rhodobacter sphaeroides bchP* and the 3′ half of *Rhodospirillum rubrum bchP*, expressed in plasmid pSK1*bchP_S/P_R*, not only was Bphe*a*P biosynthesis restored, but approximately 10% of the Bchl present was found to be esterified with dihydro-GG (Fig. 4Biii). Complete restoration of both Bchl*a*P and Bphe*a*P synthesis was achieved by using the positive control plasmid, pSK1*bchP_S/P_S*, in which the two relevant *Rhodobacter sphaeroides bchP* fragments were reunited (Fig. 4Biv).

Functional analysis of *Rhodospirillum rubrum bchG*. The BchG proteins of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* attach either geranylgeranyl pyrophosphate (GGPP) or phytyl pyrophosphate (PPP) to bacteriochlorophyllide (1, 6, 27). In view of the novel properties of *Rhodospirillum rubrum* BchP, *bchG* from this organism was transferred to *Rhodobacter sphaeroides* in order to verify that it encoded a functional Bchl synthetase. Plasmid pSK1RR*bchG* was introduced into T6G1, a mutant of *Rhodobacter sphaeroides* deficient in GG-Bchl synthetase activity as a result of transposon insertion into the native *bchG* gene (1). While the negative control, T6G1[pRKSK1], remained devoid of Bchl (Fig. 4Ci), HPLC analysis of an acetone-methanol extract of T6G1[pSK1RR*bchG*] cells demonstrated that Bchl was now present and 63% of this Bchl was esterified with GG; the remainder was esterified with tetrahydro-GG, dihydro-GG, or phytol (Fig. 4Cii).

This profile is similar to that observed previously for *Rhodobacter sphaeroides* strain T6G1[pEB *bchG*] (Fig. 4Ciii), in which complementation of the same mutant was attempted with the native *Rhodobacter sphaeroides* gene in a related expression vector (1). Partial restoration of pigment protein complex levels was also evident in T6G1[pSK1RR*bchG*], but not in T6G1[pRKSK1] (data not shown). Complete restoration of a wild-type phenotype to mutant T6G1 cannot be achieved with a *bchG* gene alone, since the transposon insertion also causes disruption of downstream *bchP* gene expression (1). The presence of substantial quantities of Bchl in T6G1[pSK1RR*bchG*] demonstrated that the introduced *Rhodospirillum rubrum* gene did encode a functional Bchl synthetase.

Generation of a novel mutant strain of *Rhodospirillum rubrum* possessing Bchl esterified with phytol. The data obtained show that *Rhodospirillum rubrum* can be thought of as a partially disabled *bchP* mutant, in which the encoded enzyme possesses one of the two activities present in the *Rhodobacter sphaeroides* homolog. Plasmid pSK1*bchP*, bearing the *Rhodo-*
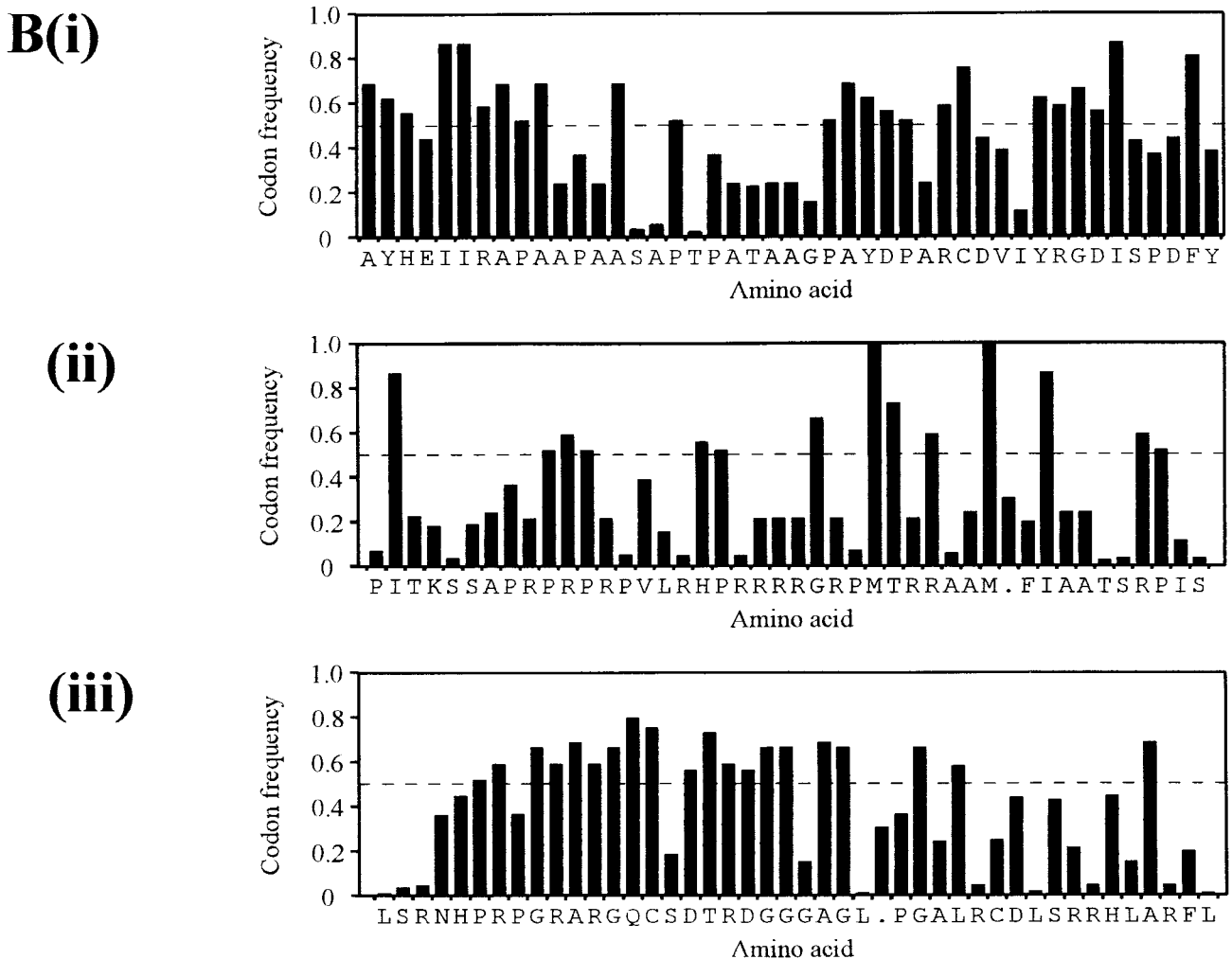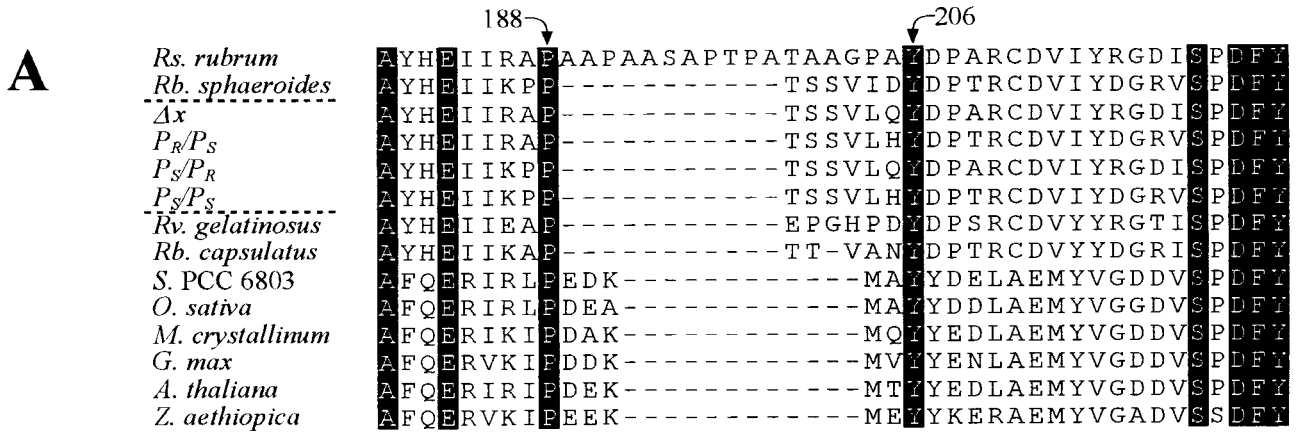
A

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                      188                           206
Rs. rubrum      AYHEIIRAPAAPAASAPTPATAAGPAYDPARCDVIYRGDISPDFY
Rb. sphaeroides AYHEIIKPP-------------TSSVIDYDPTRCDVIYDGRVSPDFY
Δx              AYHEIIRAP-------------TSSVLQYDPARCDVIYRGDISPDFY
PR/PS           AYHEIIRAP-------------TSSVLHYDPTRCDVIYDGRVSPDFY
PS/PR           AYHEIIKPP-------------TSSVLQYDPARCDVIYRGDISPDFY
PS/PS           AYHEIIKPP-------------TSSVLHYDPTRCDVIYDGRVSPDFY
Rv. gelatinosus AYHEIIEAP-------------EPGHPDYDPSRCDVYYRGTISPDFY
Rb. capsulatus  AYHEIIKAP-------------TT-VANYDPTRCDVYYDGRISPDFY
S. PCC 6803     AFQERIRLPEDK----------MAYYDELAEMYVGDDVSPDFY
O. sativa       AFQERIRLPDEA----------MAYYDDLAEMYVGDDVSPDFY
M. crystallinum AFQERIKIPDAK----------MQYYEDLAEMYVGDDVSPDFY
G. max          AFQERVKIPDDK----------MVYYENLAEMYVGDDVSPDFY
A. thaliana     AFQERIRIPDEK----------MTYYEDLAEMYVGDDVSPDFY
Z. aethiopica   AFQERVKIPEEK----------MEYYKERAEMYVGADVSSDFY
```

B(i)

(ii)

(iii)

FIG. 3. (A) Partial alignment of the *Rhodospirillum rubrum bchP* deduced amino acid sequence (*Rs. rubrum*) with sequences of known and putative GG-(b)chl reductases from the following species (sequence accession numbers in parentheses): *Rubrivivax gelatinosus* (EMBL AB034704); *Rhodobacter sphaeroides* (EMBL AJ010302); *Rhodobacter capsulatus* (Swissprot P26172); *Synechocystis* sp. strain PCC 6803 (Swissprot Q55087); *Oryza sativa* (EMBL AP001080); *Mesembryanthemum crystallinum* (PIR T12299); *Glycine max* (EMBL AF068686); *Arabidopsis thaliana* (EMBL Y14044); and *Zantedeschia aethiopica* (EMBL AF055296). The numbers above the alignment are amino acid positions within *Rhodospirillum rubrum* BchP. The sequences labeled Δx, P_R/P_S, P_S/P_R, and P_S/P_S represent the predicted products of the modified and hybrid *bchP* genes present in plasmids pSK1RR*bchP*Δx, pSK1*bchP*$_R$/P_S, pSK1*bchP*$_S$/P_R, and pSK1*bchP*$_S$/P_S, respectively. (B) Codon usage analysis for the multialigned region of *Rhodospirillum rubrum* BchP. Each of the three forward reading frames is presented, with frame i being the true reading frame. Codon frequencies add up to 1.0 for each amino acid species. Average codon frequency for the entire *bchP* gene (0.497) is indicated by a dashed line. It can be seen that the codon frequency drops substantially for the extra amino acids in the correct frame, but that it is more typical in frame iii in this region.
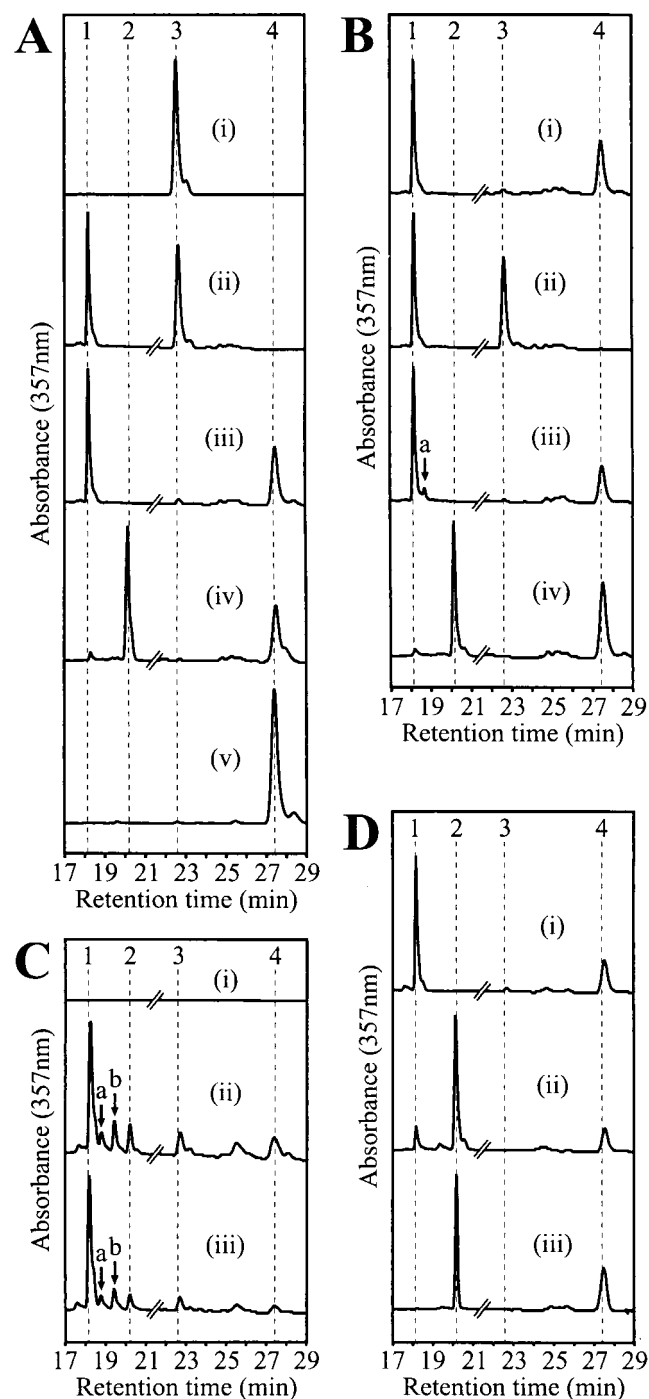
FIG. 4. HPLC traces of acetone-methanol extracts of *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* strains. (A) Functional analysis of *Rhodospirillum rubrum bchP*. (i) Bphe*a*GG standard; (ii) *Rhodobacter sphaeroides bchP* mutant DBCΩ:T6G5; (iii) DBCΩ:T6G5 [pSK1RR*bchP*], the *Rhodobacter sphaeroides bchP* mutant complemented with *Rhodospirillum rubrum bchP*; (iv) DBCΩ:T6G5[pSK1*bchP*], the *Rhodobacter sphaeroides bchP* mutant complemented with *Rhodobacter sphaeroides bchP*; (v) Bphe*a*P standard. (B) Functional analysis of modified versions of *Rhodospirillum rubrum bchP* via complementation of *Rhodobacter sphaeroides bchP* mutant DBCΩ:T6G5. (i) DBCΩ:T6G5[pSK1RR*bchPΔx*]; (ii) DBCΩ:T6G5[pSK1*bchP$_R$/P$_S$*]; (iii) DBCΩ:T6G5[pSK1*bchP$_S$/P$_R$*]; and (iv) DBCΩ:T6G5[pSK1*bchP$_S$/ P$_S$*]. (C) Functional analysis of *Rhodospirillum rubrum bchG*. (i) *Rhodobacter sphaeroides bchG* mutant T6G1[pRKSK1]; (ii) T6G1[pSK1RR*bchG*],

the *Rhodobacter sphaeroides bchG* mutant complemented with *Rhodospirillum rubrum bchG*; (iii) T6G1[pEB *bchG*], the *Rhodobacter sphaeroides bchG* mutant complemented with *Rhodobacter sphaeroides bchG*. (D) Analysis of a novel mutant strain of *Rhodospirillum rubrum*. (i) Wild-type *Rhodospirillum rubrum*[pRKSK1]; (ii) *Rhodospirillum rubrum* [pSK1*bchP*], the new, Bchl*a*P-containing strain; (iii) *Rhodobacter sphaeroides* wild-type strain DBCΩ. The retention times of the four principal pigments are indicated by vertical dashed lines: 1, Bchl*a*GG; 2, Bchl*a*P; 3, Bphe*a*GG; and 4, Bphe*a*P. Other known peaks are individually labeled: a, dihydro-GG-esterified Bchl*a*; b, tetrahydro-GG-esterified Bchl*a*. Traces are normalized according to the maximum Bchl peak, with the exception of the trace for strain T6G1[pRKSK1], displayed at the same scale as that of strain T6G1[pSK1RR*bchG*]. The Bphe region of most traces is expanded vertically sixfold; this is indicated by a break in the trace at a retention time of 21.5 min.

bacter sphaeroides bchP gene, was introduced into *Rhodospirillum rubrum*, as was the insert-free plasmid pRKSK1 as a negative control. HPLC analysis of the resulting strains showed that, while *Rhodospirillum rubrum* [pRKSK1] had only the Bchl*a*GG normally found in *Rhodospirillum rubrum* (Fig. 4Di), this pigment was present at only a low level in the strain containing pSK1*bchP*; instead, there was a predominance of Bchl*a*P (Fig. 4Dii). The biosynthesis of this pigment demonstrated that the only impediment to its formation in *Rhodospirillum rubrum* had been a mutation in the native gene.

Preliminary analysis of the novel, Bchl*a*P-containing strain by absorbance spectroscopy showed that the photosynthetic complexes were present in approximately wild-type levels, although a perturbation of the LH1 structure was indicated by a blue shift in the position of the 880-nm peak by approximately 3.5 nm (Fig. 5). As in *Rhodospirillum rubrum*[pRKSK1], the Bphe species present was Bphe*a*P (Fig. 4D), and the strain retained the ability to grow photosynthetically.

DISCUSSION

Identification of part of a PGC of *Rhodospirillum rubrum*. In common with other purple bacteria and heliobacteria, such as *Rhodobacter capsulatus*, *Rhodospirillum centenum*, and *Heliobacillus mobilis*, photosynthesis-related genes of *Rhodobacter sphaeroides* are clustered on the genome within a region of approximately 40 kb (4, 25, 38, 39). Within the *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* PGCs, the *bchP* and *bchG* genes are found in close proximity (1, 3), and a similar situation has now been found to exist in *Rhodospirillum rubrum*. The DNA flanking the newly identified *bchP* homolog included a *bchG* gene, demonstrated to encode a functional GG-Bchl synthetase by successful complementation of a *bchG* mutant of *Rhodobacter sphaeroides*; previously published data placed a *bchG* gene within a *Rhodospirillum rubrum* PGC (5). Also present in the newly sequenced region were homologs of the *Rhodobacter sphaeroides* PGC genes *ppsR* and *ORF427*, the *Rhodospirillum rubrum* homolog of the latter being *ORF449*. The gene order *bchG-ORF449-bchP* present in *Rhodospirillum rubrum* is identical to that in both *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*.

BchP of *Rhodobacter sphaeroides* converts Bphe*a*GG to Bphe*a*P, as well as Bchl*a*GG to Bchl*a*P, whereas BchP of *Rhodospirillum rubrum* is restricted to the former of these
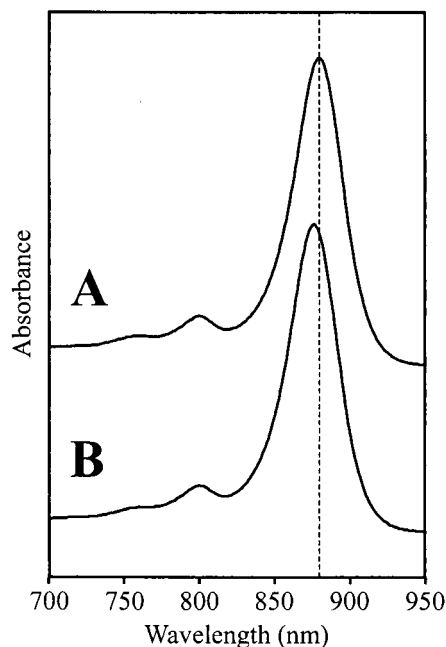
FIG. 5. Absorbance spectra of intracytoplasmic membranes from (A) wild-type *Rhodospirillum rubrum*[pRKSK1] and (B) *Rhodospirillum rubrum* complemented with *bchP* in plasmid pSK1*bchP*. Spectra are normalized at their LH1 absorbance maxima. It can be seen that the 880-nm peak is slightly blue-shifted in *Rhodospirillum rubrum* [pSK1*bchP*].

activities. Both the Bchl and Bphe components of the *Rhodobacter sphaeroides* photosynthetic apparatus are principally esterified with phytol. In contrast, the isoprenoid moieties of these two pigments in *Rhodospirillum rubrum* differ; that of Bphe is again phytol, but that of Bchl is the less-reduced alcohol GG. GG-esterified Bchl appears to be a precursor of Bchl*a*P in both *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, although direct esterification with phytol and the intermediates dihydro-GG and tetrahydro-GG may also occur.

The gene responsible for the hydrogenation of the isoprenoid moiety of Bchl in these species is *bchP* (3, 6). The present work shows that BchP of *Rhodobacter sphaeroides* has, in effect, two substrates, converting Bphe*a*GG to Bphe*a*P as well as Bchl*a*GG to Bchl*a*P. In contrast, the BchP of *Rhodospirillum rubrum* is restricted to the former of these activities. *Rhodobacter sphaeroides bchP* was heterologously expressed in wild-type *Rhodospirillum rubrum*; the resultant strain possessed Bchl*a*P, further demonstrating that the enzymes encoded by the *bchP* genes differ in their biosynthetic abilities.

From the available data, certain deductions can be made concerning the activity of the GG-Bphe reductase enzyme of *Rhodospirillum rubrum* in relation to the dual-function enzyme of the *Rhodobacter* species. It appears that GG-Bphe reductase must be highly specific, in being able only to reduce attached isoprenoid moieties of Bphe, and not free GG or GGPP. If GG-Bphe reductase was able to reduce free GG or GGPP, then any PPP formed would be available to the Bchl synthetase present. Not only is the Bchl synthetase of *Rhodobacter capsulatus* able to utilize PPP as a substrate, giving Bchl*a*P, but it is preferred over GGPP in vitro (27). Given that the *Rhodobacter sphaeroides* Bchl synthetase is likely to have similar sub-

strate requirements, it would be expected that Bchl*a*P would be detectable in *Rhodobacter sphaeroides* strain DBCΩ:T6G5 [pSK1RR*bchP*], a *bchP* mutant complemented with *Rhodospirillum rubrum bchP*; however, no Bchl*a*P was found.

On the basis of the observed substrate preference of the *Rhodobacter capsulatus* Bchl synthetase, Oster et al. (27) proposed that the *bchP* gene product was able to reduce GG or GGPP prior to esterification. Interestingly, Keller et al. (21) have demonstrated that a homolog of *bchP* from *Arabidopsis thaliana* encodes a multifunctional GG reductase, catalyzing the reduction of GGPP to PPP as well as that of Chl*a*GG to Chl*a*P. We conclude that the GG-Bphe reductase of *Rhodospirillum rubrum* differs in two respects from previously studied BchP homologs. First, it requires attached isoprenoid moieties of Bphe as substrates, and second, it can reduce Bphe*a*GG to Bphe*a*P, but it cannot reduce Bchl*a*GG to Bchl*a*P.

The cofactor requirement of *Rhodospirillum rubrum* BchP is likely to be identical to that of *Rhodobacter sphaeroides* BchP; this has been putatively identified as NADPH (3). The origin of the proposed substrate of GG-Bphe reductase, Bphe*a*GG, is not certain, but loss of the Mg^{2+} ion from Bchl is the most likely source of the Bphe that *Rhodospirillum rubrum* and other species of photosynthetic bacteria require. In their experiments with the *Rhodobacter capsulatus* Bchl synthetase enzyme, Oster et al. (27) found bacteriopheophorbide *a*, the nonesterified equivalent of Bphe*a*, to be an unsuitable substrate for esterification with either GGPP or PPP, and yet no separate Bphe synthetase gene appears to be present within the known PGCs. Magnesium is lost from chlorophylls relatively easily; this may be nonenzymatic, or may perhaps be catalyzed by a dechelatase enzyme, similar to those postulated to exist in plants (see reference 22 for a review).

Attempts to identify the basis for differences in activity between the BchP homologs. Sequence analysis shows that *bchP* from *Rhodospirillum rubrum* differs from *Rhodobacter sphaeroides bchP* and other known genes encoding GG-(b)chl reductases in the presence of an additional stretch of 11 to 12 codons close to the center of the coding region. Insertion of a fragment of *Rhodospirillum rubrum* DNA at some stage could have disrupted a once fully functional *bchP* gene, resulting in partial loss of function. The observation that the DNA encoding the extra amino acids displays atypical codon usage for *Rhodospirillum rubrum* genes but typical codon usage in another reading frame supports the occurrence of such an insertion event. Manipulation of this region to eliminate the extra codons and to make it resemble that present in *Rhodobacter sphaeroides bchP* failed to produce a gene capable of restoring Bchl*a*P biosynthesis to *Rhodobacter sphaeroides*. However, further mutations may have occurred over time, reinforcing the enzyme's inability to utilize substrates other than Bphe*a*GG.

In an attempt to localize such changes, two complementary hybrid genes were constructed and tested for their ability to complement a *Rhodobacter sphaeroides bchP* mutant. Synthesis of Bchl*a*P was not restored in either case; indeed, when the 5' half of *Rhodospirillum rubrum bchP* was fused to the 3' half of *Rhodobacter sphaeroides bchP*, no activity of either kind resulted. However, a fusion between the 5' half of *Rhodobacter sphaeroides bchP* and the 3' half of *Rhodospirillum rubrum bchP* resulted in a functional GG-Bphe reductase and the presence of a small amount of dihydro-GG-Bchl*a*, demonstrat-

ing that the hybrid enzyme was at least capable of catalyzing the first step in the reduction of the GG moiety of Bchl. Interestingly, a recently described purple bacterium, *Roseospirillum parvum*, has been found to possess tetrahydro-GG-esterified Bchl*a* as 20% of its Bchl content, with the remainder being almost entirely Bchl*a*P; no Bphe species other than Bphe*a*P were identified (29). This pigment composition may indicate a related defect in a GG-Bchl reductase enzyme.

Relationship between GG-Bphe reductase activity and assembly and function of the photosynthetic complexes. Although the Bchl of *Rhodospirillum rubrum* is esterified with GG, the Bphe within the RC is, in contrast, esterified with phytol. We infer a preference for phytylated Bphe over Bphe*a*GG during the assembly of the RC and a requirement for the phytylated pigment for optimal functioning of the complex. The RC of *Rhodobacter sphaeroides* can assemble with Bphe*a*GG instead of the usual Bphe*a*P, for example, in the *bchP* mutant DBCΩ:T6G5, but upon expression of *Rhodospirillum rubrum bchP* from plasmid pSK1RR*bchP*, the only pigment to become phytylated is the Bphe*a* of the RC. Mutation of *bchP* in *Rhodospirillum rubrum* may result in a strain possessing RCs assembled with Bphe*a*GG, but the survival of such a strain under laboratory conditions may not be representative of the situation in the natural environment.

Although a requirement for Bphe*a*P in *Rhodospirillum rubrum* has not been proven, this pigment must engender some selective advantage for a GG-Bphe reductase gene to exist at all; the source of this advantage is, however, uncertain. Interestingly, it has been inferred from crystallographic data for the RC that the phytol tails of the Bchl and Bphe molecules are more tightly packed in the active branch than in the inactive branch (10).

Phytol tails are predicted to be less rigid than GG tails and may therefore be important for this tight packing. However, published data on *bchP* mutants of both *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* appear to be directly contradictory to the importance of phytol over GG as the esterifying alcohol of the Bphe molecules of the RC. In the *Rhodobacter sphaeroides* mutant, reduction of the photosynthetic growth rate in relation to the wild type was found to be in almost direct proportion to the reduction in the total level of Bchl present in the photosynthetic complexes, suggesting that it was the formation or stability of the complexes that was affected, rather than their electron transfer capability (3). Bollivar et al. (6) found that neither RC function nor energy transfer from light-harvesting to RC complexes was impaired in a *bchP* mutant of *Rhodobacter capsulatus*, which presumably also possessed Bphe*a*GG in place of the usual Bphe*a*P. All of these findings may, however, simply reflect limitations in the experimental procedures performed. Further detailed analyses of mutant and wild-type RCs from *Rhodospirillum rubrum* and *Rhodobacter sphaeroides* may lead to an answer. Particularly useful may be *Rhodobacter sphaeroides* strain DBCΩ:T6G5[pSK1RR*bchP*], the porphyrin pigment and photosynthetic complex compositions of which mirror those found in wild-type *Rhodospirillum rubrum*.

Higher plants, green algae, and cyanobacteria possess a pigment-protein complex, PS2, the RC of which is believed to originate from the purple bacterial RC (23). Among the structural and functional similarities, they each have a related series of redox components arranged within their core proteins (30, 40). In place of the Bphe present in the bacterial RC, PS2 contains pheophytin. Phytol is the most common esterifying alcohol of Chls, and it is likely that this is also the case for this pheophytin, essentially the only pheophytin found in chloroplasts. The identification of a second role for the fully functional BchP, in catalyzing the synthesis of Bphe*a*P as well as that of Bchl*a*P, strongly suggests that homologs of this enzyme in PS2-containing organisms may be similarly responsible for the synthesis of phytylated pheophytin.

Finally, if, as we suggest in the introduction, the absence of LH2 and Bchl*a*P from wild-type *Rhodospirillum rubrum* is linked, this raises the intriguing possibility that ancestors of this bacterium may have possessed an LH2-type complex and that it was the loss of this complex which eased the pressure to maintain GG-Bchl reductase activity.

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