Aerobic and Anaerobic Mg-Protoporphyrin Monomethyl Ester Cyclases in Purple Bacteria

A STRATEGY ADOPTED TO BYPASS THE REPRESSIVE OXYGEN CONTROL SYSTEM*

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Two different mechanisms for Mg-protoporphyrin monomethyl ester (MgPMe) cyclization are shown to coexist in *Rubrivivax gelatinosus* and are proposed to be conserved in all facultative aerobic phototrophs: an aerobic mechanism active under photosynthesis or low oxygenation, and an aerobic mechanism active only under high oxygenation conditions. This was confirmed by analyzing the bacteriochlorophyll accumulation in the wild type and in three mutant strains grown under low or high aeration. A mutant lacking the *aceF* gene is photosynthetic, exhibits normal bacteriochlorophyll accumulation under low oxygenation and anaerobiosis, and accumulates MgPMe under high oxygenation. The photosynthesis-deficient *bchE* mutant produces bacteriochlorophyll only under high oxygenation and accumulates MgPMe under low oxygenation and anaerobiosis. The double knockout mutant is devoid of photosystem and accumulates MgPMe under both conditions indicating the involvement of the two enzymes at the same step of the biosynthesis pathway. Oxygen-mediated expression of *bchE* was studied in the wild type and in a regulatory mutant. The reverse transcriptase-PCR and the *bchE* promoter activity results demonstrate that the expression of the *bchE* gene is oxygen-independent and suggest that it is rather the enzyme activity that should be oxygen-sensitive. No obvious sequence similarities were found between oxygen-dependent *AceF* and the oxygen-independent anaerobic Mg-protoporphyrin monomethylester cyclase (BchE) enzymes. However, common to all BchE proteins is the conserved CXXX-CXCX sequence. This motif is essential for 4Fe-4S cluster formation in many anaerobic enzymes. Expression and purification of BchE were achieved, and the UV-visible spectral analyses confirmed the presence of an active 4Fe-4S cluster in this protein. The use of different classes of enzymes catalyzing the same reaction under different oxygen growth conditions appears to be a common feature of different biosynthetic pathways, and the benefit of possessing both aerobic and anaerobic systems is discussed.

Purple bacteria perform anoxygenic photosynthesis on the basis of a bacteriochlorophyll-mediated process. It takes place within the membrane photosynthetic apparatus, composed of three pigment-protein complexes as follows: two light-harvesting antennae and the reaction center, associated with carotenoids and bacteriochlorophylls. Bacteriochlorophyll *a* is the most widely distributed bacteriochlorin pigment and is found in most photosynthetic bacteria. The early steps in bacteriochlorophyll *a* biosynthesis up to chlorophyllide *a* are common to the biosynthesis pathway of chlorophyll *a*, a pigment present in all organisms capable of oxygenic photosynthesis. The chelation of Mg*2*+ into protoporphyrin IX to form Mg-protoporphyrin IX (MgP)9 is catalyzed by the products of the *bchD*, *bchI*, and *bchH* genes. Methylation of MgP to form MgP monomethyl ester (MgPMe) is then catalyzed by the product of *bchM* for review see Refs. 1 and 2. MgPMe, a key intermediate in the biosynthesis of chlorophylls and bacteriochlorophylls, is the substrate for the oxidative cyclase(s) responsible for the formation of the isocyclic ring V of protochlorophyllide (*Pchlide*) *a* (1, 2). In phototrophic bacteria, this cyclase is encoded by the *bchE* gene. Recently, mutational analyses of selected loci from the photosynthesis gene cluster of the purple nonsulfur bacterium *Rubrivivax gelatinosus* allowed us to identify the *aceF* gene as responsible for the cyclization of MgPMe (3). Pinta et al. (3) generated a photosynthetic competent mutant accumulating a pigment identified as MgPMe only under high oxygenation. The data showed that under high oxygenation, bacteriochlorophyll *a* biosynthesis is controlled by *AceF* at the level of MgPMe oxidative cyclization and raised the question as to how bacteriochlorophyll *a* is synthesized under low oxygenation or anaerobic photosynthetic conditions. We have suggested the co-existence of two pathways leading to the bacteriochlorophyll *a* biosynthesis in this bacterium. This assumption is in agreement with biochemical studies in *Rhodovulum sulfidophilum* claiming that this bacterium incorporates atomic oxygen from either H2O or O2 to form the 13(1)-oxo group of the bacteriochlorophyll *a* isocyclic ring, and suggesting that two different cyclization mechanisms coexist in *R. sulfidophilum* involving both an oxygenase and a hydratase to form the 13(1)-oxo group of the bacteriochlorophyll *a* isocyclic ring (4). However, in *Rhodobacter sphaeroides* cells shifted from respiratory to photosynthetic growth conditions, H2O was shown to be the source of oxygen for the cyclization reaction (5). The derivation of the 13(1)-oxo group from water showed that the formation of the isocyclic ring from the 13-propionic acid methyl ester side chain of MgPMe is an anaerobic process involving a hydratase. This bacterium produces bacteriochlorophyll *a* also under aerobic

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8 The abbreviations used are: MgP, Mg-protoporphyrin IX; MgPMe, Mg-protoporphyrin monomethyl ester; RT, reverse transcriptase; Km, kanamycin; WT, wild type; HPLC, high pressure liquid chromatography; SAM, S-adenosyl-L-methionine; Pchlide, protochlorophyllide; BchE, anaerobic Mg-protoporphyrin monomethylester cyclase.

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conditions, and a homologue of acsF (ORF277) was identified within its photosynthesis gene cluster.

The ability to produce bacteriochlorophyll α both aerobically and anaerobically represents a significant advantage for these bacteria as they become competent to produce the photosystem even under aerobic conditions and to shift rapidly to photosynthetic growth following reduction of oxygen tension and exposure to light.

To elucidate the mechanisms of the bacteriochlorophyll isocyclic ring formation in the photosynthetic bacterium *R. gelatinosus*, we have identified the *bchE* gene and analyzed *bchE* and *acsF* knockout mutants. The observed phenotypes and the characteristics of the bacteriochlorophyll precursors in the mutants strongly support the involvement of the two genes at the same level of the biosynthetic pathway and explain the presence of bacteriochlorophyll α either under aerobic or anaerobic conditions in *R. gelatinosus* and in other facultative aerobic photosynthetic bacteria.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Media—*Escherichia coli* was grown at 37 °C on LB medium. *R. gelatinosus* strain 1 (6) and the mutants were grown in maleate (ML) medium (7) at 30 °C, photosynthetically (anaerobic light) or in the dark in 50-ml flasks filled with 50 ml of medium (low oxygenation conditions), or 250-ml flasks containing 20 ml of medium (high oxygenation conditions). Shaking was at 100 rpm. Antibiotics were used at the following concentrations: chloramphenicol 3 μg/ml, ampicillin 50 μg/ml, kanamycin (Km) 50 μg/ml, spectinomycin 50 μg/ml, streptomycin 50 μg/ml, tetracycline 2 μg/ml. Bacterial strains and plasmids used in this work are listed in Table I.

**Molecular Biology Techniques—**Standard methods were used according to Sambrook et al. (8), unless otherwise indicated. Plasmid DNAs were purified using QiAprep spin miniprep kit (Qiagen) or Quantum prep plasmid midiprep kit (Bio-Rad). DNA was treated with restriction enzymes and other nucleic acid-modifying enzymes (Klenow fragment, T4 DNA polymerase, and T4 DNA ligase) according to the manufacturer's specifications. DNA fragments were analyzed on agarose gels, and different restriction fragments were purified using the GeneClean kit (Bio 101, Inc., Vista, CA).

**Library Screening for bchE—**The PCR was carried out using genomic or plasmid templates in a 50-μl reaction mixture containing PCR buffer, 1.5 μM MgCl₂, 200 μM of each deoxynucleoside triphosphate, 1 μM of each primer, 5% Me₂SO, and 2.5 units of Taq DNA polymerase. 20 cycles were performed in a Hybaid thermal cycler, each cycle comprising 30 s at 92°C, 1 min at 60°C, and 60 s at 72°C. Primers are listed in Table II.

**Gene Transfer and Strain Selection—**Transformation of *R. gelatinosus* cells was carried out by electroporation as described previously (9). Transformants were selected on maleate plates supplemented with the appropriate antibiotic.

**Construction of the bchE-lacZ Reporter Fusion Plasmid—**To construct a bchE-lacZ fusion, the promoter region of bchE containing the putative PsrA-binding site (TGTAATACA) was amplified by PCR (380-bp DNA fragment) from plasmid pSO277. The following primers were designed to create XbaI and BamHI sites of each deoxynucleoside triphosphate, 1 μM of each primer, 5% Me₂SO, and 2.5 units of Taq DNA polymerase. 20 cycles were performed in a Hybaid thermal cycler, each cycle comprising 30 s at 92°C, 40 s at 60°C, and 60 s at 72°C. Primers are listed in Table II.

**RT-PCR Analysis of bchE Gene Expression—**Total RNA from WT cells grown photosynthetically or aerobically under high or low oxygenation was extracted according to Ref. 10, treated with RNase-free DNase I, and then purified by phenol chloroform extraction and ethanol precipitation. A reverse transcription reaction was performed to synthesize the cDNA from 3 μg of total RNA using Superscript II RNase enzyme (Invitrogen) at 60 °C for 1 h with oligonucleotide OI-bchE-R2 (extending from position +1110 to +1091 of the bchE gene). The prod-
ucts were then amplified by PCR and analyzed by electrophoresis on 1% agarose gels. Primers corresponding to the coding region of \textit{bchE} gene were designed using full-length \textit{bchE} gene isolated from position +726 to +748 of the \textit{bchE} gene) and were designed to obtain a 384-bp-long PCR product. For each RT-PCR experiment, a control experiment (RNA template without the reverse transcriptase) was performed to check that there was no trace of genomic DNA.

Construction of Plasmids for High Level Expression of \textit{BchE} in \textit{E. coli}.—\textit{E. coli} BL21(DE3) cells harboring either pSO2775 or pSO2776 were grown in LB medium at 30 °C until they had reached an \textit{OD}_{600} of 0.6. Then isopropyl-\(\beta\)-D-thiogalactopyranoside was added to a final concentration of 1 mM, and growth was continued for 4 h. Isopropyl-\(\beta\)-D-thiogalactopyranoside-induced cells from 200-ml cultures were suspended in 50 ml of binding buffer containing 20 mM Tris/HCl (pH 7.9), 0.5 M NaCl, and 15 mM imidazole. The bound proteins were eluted from the columns with elution buffer containing 500 mM imidazole.

Membrane Protein Preparation and Spectrophotometric Measurements.—Membrane proteins were prepared by cell disruption with a French press and dried pigments were resolubilized in a small volume of acetone/methanol 7:2 (v/v). The membranes were then resuspended in the same buffer. Absorption spectroscopy was performed with a Cary 500 spectrophotometer.



| Primer                  | Sequence                                      | Restriction site |
|------------------------|-----------------------------------------------|------------------|
| Ol-bchE-F              | GGCTTCTTCATCTCCGGCGACG                        | BamHI            |
| Ol-bchE-R              | GAACGGCCAGGCGCTGACA                          | XbaI             |
| Ol-bchE-R2             | CATGCGCCGGTCTCCAGTCCC                        | NdeI             |
| Ol-bchE-FBamHI         | CCACCGGAAATCCAGTGGGCG                        | BamHI            |
| Ol-bchE-RXbaI          | CGGCAGCGCTTCAAGTCGCGG                        | XbaI             |
| Ol-HbchE-FNdeI         | GAGCGGTTTCCATATCGGTGGCGT                    | NdeI             |
| Ol-HbchE-RBamHI        | GATGGCGTTCCGAATCCGCGGC                      | BamHI            |

RESULTS

Sequencing of the entire photosynthesis gene cluster of \textit{R. gelatinosus} followed by mutational analysis allowed the identification of the \textit{acsF} gene as responsible for the aerobic oxidative cyclization of MgPMe (3). In \textit{Rhodobacter} species, this reaction is catalyzed under anaerobiosis by the \textit{BidE} Mg-protoporphyrin IX monomethyl ester oxidative cyclase (11). To elucidate the mechanism of the bacteriochlorophyll isocyclic ring formation in \textit{R. gelatinosus} under anaerobiosis conditions, we have undertaken the study of \textit{bchE} involvement in this process.

Cloning and Sequence Analysis of the \textit{bchE} Genomic Region in \textit{R. gelatinosus}.—The purple photosynthetic bacterium \textit{R. gelatinosus} has within its genome a cluster of 59 kb in length containing most of the photosynthesis related genes (12). Two genes involved in bacteriochlorophyll biosynthesis are missing from this cluster. In an attempt to identify these genes, namely \textit{bchE} and \textit{bchJ} involved in the early stage of bacteriochlorophyll biosynthesis, a \textit{R. gelatinosus} genomic DNA library was screened using a couple of primers (Ol-bchE-F and Ol-bchE-R) based on the published \textit{Rhodobacter} \textit{bchE} gene sequences (AJ010902 and Z11165). A plasmid (pSO2770) containing \textit{bchE} and \textit{bchJ} genes was isolated and sequenced. Sequence similarity searches were performed using the BLAST network service (13). The \textit{bchE} gene is 1746 bp long and encodes a 582- amino acid polypeptide with a predicted molecular mass of about 63 kDa. A putative PsrR-binding motif was identified upstream from the \textit{bchE} gene suggesting an oxygen-dependent expression of \textit{bchE}. BlastP and Clustal analyses indicated that the \textit{BchE} polypeptide from \textit{R. gelatinosus} shows similarities to \textit{BchE} from \textit{R. rubrum} (68% identity and 80% similarity) and to \textit{R. sphaeroides} (61% identity and 74% similarity). Further sequence analyses indicate that \textit{BchE} possesses a vitamin B12-binding motif in its N-terminal domain suggesting that this protein uses vitamin B_{12} as cofactor. A potential S-adenosyl-l-methionine (SAM)-binding motif characteristic of methyltransferases is present in the central region. Moreover, cysteine residues Cys-205, Cys-209, and Cys-212, which are part of a conserved CXXCXXX motif, were found in many \textit{BchE} proteins. This motif was also found and shown to be essential for a 4Fe–4S cluster formation in many oxygen-independent oxidases.

\textit{bchE} Mutant Construction and Characterization.—To gain insight into the role of \textit{bchE} gene in the bacteriochlorophyll isocyclic ring formation, a \textit{bchE} mutant was generated. For that purpose the \textit{bchE} gene fragment cloned in plasmid pSO2771 was disrupted with the kanamycin resistance cartridge inserted at the BassIII site of \textit{bchE} to construct plasmid pSO2772. Wild type cells electroporated with pSO2772 were plated on selective medium under respiratory conditions to select transformants resulting from double crossover events. The \textit{BCHE} mutant colonies showed a brown pigmentation on plates. The \textit{BCHE} mutant was photosynthesis-deficient, and interestingly, when grown aerobically under high oxygenation,
the mutant resembles wild type, i.e. it synthesizes bacteriochlorophyll and assembles the photosystem like a photosynthetic competent strain. Furthermore, the culture medium was pigmented (greenish) only when cells were grown under low oxygenation conditions, suggesting the presence of a pigment secreted by the cells and indicating that they have an altered pigment production.

The involvement of \textit{bchE} in the MgPMe-cyclase reaction was first investigated by absorption spectroscopy. Because the BCHE mutant is photosynthetic deficient, the growth properties of this mutant and its ability to synthesize pigments and photosystem were investigated, in comparison to the WT grown under respiratory conditions, under high or low oxygenation. Under high oxygenation conditions, both strains had the same generation time and assembled photosynthetic complexes as indicated by the absorbance peaks at 800 and 860 nm (Fig. 1A). Nevertheless, under low oxygenation conditions, and unlike the WT, the BCHE mutant assembled no or a very low amount of photosynthetic complexes (Fig. 1B). Moreover, this strain also accumulated an additional compound absorbing at 416 nm characteristic of protochlorophyllide in aqueous solution.

Analysis of acetone/methanol pigment extracts from WT and BCHE strains grown under high oxygenation conditions showed identical spectra, with absorption at 770 nm for bacteriochlorophyll \textit{a}, 420–442-472 nm for carotenoids, and 362 nm for the bacteriochlorophyll \textit{a} Soret absorption band (Fig. 2A). Under low oxygenation, the WT spectrum still presented the same characteristics. In contrast, only traces of bacteriochlorophyll \textit{a} could be detected in the low oxygenated BCHE mutant, whereas a majority peak appeared at 416 nm (Fig. 2B). These spectral characteristics of the BCHE mutant grown under low oxygenation recall the spectral characteristics of the SIX9Km (\textit{acsF}\textsubscript{::}Km) mutant grown under high oxygenation (3).

**Functional Complementation of BCHE Phenotype**—pSO2773, a replicative plasmid bearing the \textit{bchE} gene, was introduced into BCHE mutant cells. All the resulting colonies were photosynthetic, produced bacteriochlorophyll \textit{a}, and assembled spectral complexes under photosynthetic as well as under low oxygenation conditions. These experiments confirmed that the loss of bacteriochlorophyll \textit{a} and photosystem in the BCHE mutant grown under low oxygenation was strictly due to the disruption of the \textit{bchE} gene.

**HPLC Analysis of Pigments from High and Low Oxygenated \textit{bchE} Mutant Cells**—Even though the above data suggest that
the BCHE mutant accumulates the MgPMe intermediate of bacteriochlorophyll biosynthesis, like the (acsF::Km) mutant, further analysis of the bacteriochlorophyll intermediates pool accumulated by the bchE disrupted mutant was subsequently undertaken to determine the nature of these pigments. For this analysis, the pigment content of the BCHE mutant grown under either low or high oxygenation was analyzed by HPLC. Isolated pigment fractions were identified on the basis of their UV-visible absorption spectra and of the retention time. In the isolated pigment extract from cells grown under high oxygenation, two fractions were collected (peak 1 and 4) in addition to the carotenoid OH spheroidenone (peak 3) (Fig. 3A). According to their retention time and UV-visible absorption spectra, fraction 1 was identified as Mg-protoporphyrin (416–552-590 nm), whereas fraction 4 was identified as bacteriochlorophyll a (770 nm).

In the extracted pigment from cells grown under low oxygenation, three fractions were collected and analyzed by UV-visible absorbance (Fig. 3B). Fraction 1 elutes after 2.6 min and was identified as Mg-protoporphyrin (MgP) (416–552-590 nm) or its monomethyl ester (MgPMe). Based on the data published previously (3) concerning the retention time of MgPMe and MgP, this compound is proposed to be the MgPMe. Fraction 2 elutes after 4 min and absorbs at 402–504-538–574 and 680 nm, it was identified as protoporphyrin IX. Fraction 4 corresponds to the smallest peak; it elutes after 11 min and exhibits a maximal peak at 770 nm; it was concluded that it corresponds to bacteriochlorophyll a. These results showed that the main accumulated metalloporphyrins in the BCHE mutant grown under low oxygenation are similar to those found in the R. gelatinosus acsF mutant grown under high oxygenation. This is the first direct evidence that both enzymes are involved in MgPMe cyclization and catalyze the same step of bacteriochlorophyll biosynthesis. Nevertheless, AcsF acts strictly under high oxygenation conditions, whereas BchE is involved when the oxygen tension drops. If this assumption is correct, then a double bchE-acsF mutant should be devoid of bacteriochlorophyll a and photosystem irrespective of growth conditions.

**bchE-acsF Double Mutant Construction and Characterization**—To inactivate the acsF gene in the WT and in the BCHE backgrounds, a plasmid (pSO2774) bearing the acsF gene with the /H9024 cartridge inserted at the StuI site was used to electroporate the WT and the BCHE cells. Electroporated cells were plated on selective medium to select transformants resulting from double crossover events. The resulting disrupted strains were named ACSF/H9024 and BCHE-ACSF, respectively.

ACSF/H9024 mutant has almost the same phenotype as SIX9Km (3). It was able to grow under both photosynthetic and respiratory conditions; it accumulated MgPMe only under high oxygenation, and the spectroscopic characterization of the metalloporphyrins was similar to that of MgPMe isolated from the SIX9Km mutant (not shown). ACSF/H9024 mutant however was blue green and devoid of carotenoids because of the polar effect of the /H9024 cartridge on the downstream-localized crtB gene. As expected the BCHE-ACSF double mutant was photosynthesis deficient, and the culture medium was greenish when the cells were grown either under low or high oxygenation. The secreted pigments were extracted from the growing medium using Sep-Pak cartridges, and the collected fraction was analyzed by absorption spectroscopy. The spectrum shows a major peak at 416 nm (data not shown). Spectral analyses of the membranes prepared from cells grown either under low or high oxygenation show the absence of the characteristic absorbance peaks of the photosynthetic complexes, as well as the presence of a pronounced peak at 416 nm.
Acetone/methanol pigment extracts from these membranes showed identical spectra for both growth conditions. A main peak is observed at 416 nm, and neither the peak at 770 nm nor the peak at 362 nm characteristic of the bacteriochlorophyll absorption and its Soret band, respectively, were detected (Fig. 4). These results confirm that the bacteriochlorophyll biosynthesis pathway is fully blocked in the BCHE-ACSF double mutant irrespective of the growth conditions. In other respects, these results show that the \textit{bchE} and \textit{acsF} genes are involved at the same step of the bacteriochlorophyll \textit{a} biosynthesis pathway. This implies that, despite their sequence and probably their structural differences, both enzymes recognize and transform the same substrate (MgPMe) to generate Pchlide. We concluded that \textit{R. gelatinosus} possesses two pathways leading to the MgPMe cyclization, an aerobic pathway controlled by the aerobic MgPMe oxidative cyclase AcsF and the anaerobic MgPMe oxidative cyclase BchE (Fig. 5). The benefit of possessing the two genes as well as the mechanistic differences between the two enzymes will be covered under “Discussion.” Concerning the oxygen-dependent/independent cyclization of MgPMe by AcsF and BchE, respectively, many levels of control might be considered, particularly (i) gene expression control, (ii) enzyme cofactor availability, and (iii) redox potential.

**Effect of Oxygen on \textit{bchE} and \textit{acsF} Genes Expression, Role of \textit{ppsR}**—It has been demonstrated that oxygen regulates bacteriochlorophyll biosynthesis in purple photosynthetic bacteria at the transcriptional level. \textit{Rhodobacter} species control the synthesis of their bacteriochlorophyll in the presence of oxygen by the use of transcriptional factors. PpsR (CrtJ) was shown to repress the expression of many bacteriochlorophyll genes (14, 15). The absence of bacteriochlorophyll \textit{a} in the \textit{acsF} mutant grown under high oxygen tension despite the presence of the \textit{bchE} gene suggests that the expression of \textit{bchE} gene is repressed. The presence of a putative PpsR-binding motif upstream of the \textit{bchE} gene prompted us to examine whether this gene is repressed by the transcriptional regulator PpsR. The gene coding for the transcriptional factor PpsR was isolated from \textit{R. gelatinosus}, and the corresponding disrupted mutant was used to clarify the mechanism by which oxygen regulates the bacteriochlorophyll \textit{a} biosynthesis pathway at the level of MgPMe cyclization. The ACSF\textit{f} mutant and the double mutant ACSF-PPSR were grown under low or high oxygenation, and their pigment contents were compared with the pigments produced in the carotenoid-less control strain SIB1.

Under low oxygenation conditions, acetone/methanol extracts from all strains showed identical spectra with absorbance at 770 nm for bacteriochlorophyll \textit{a} and 362 nm for the bacteriochlorophyll \textit{a} Soret absorption band. However, under high oxygenation, the SIB1 spectrum still presented the same characteristics, whereas the ACSF-PPSR and ACSF\textit{f} mutant showed identical spectra, with absorption maxima at 416 nm. These results suggest that under high oxygenation disruption of \textit{ppsR} does not derepress the \textit{bchE} expression and that this factor is not involved in the control of the expression of the \textit{bchE} gene. These results should be confirmed at the level of gene expression using a reporter fusion plasmid and RT-PCR to assess the \textit{bchE} promoter activity under different conditions.

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2 Steunou, A. S., Astier, C., and Ouchane, S. (2004) \textit{J. Bacteriol.}, in press.
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Fig. 6. bchE promoter activities in the WT (white columns) and in the PPSR mutant (dashed columns) strains, containing the bchE-lacZ transcriptional fusion, grown either under low (LA) or high aeration (HA) conditions. Experiments were performed in triplicate, and error bars denote S.D. Units of β-galactosidase activity represent μmol of o-nitrophenyl-β-D-galactopyranoside hydrolyzed per min per unit of optical density of culture at 680 nm.

Oxygen Regulation of bchE Gene Expression, bchE Promoter Activity—To sustain the obtained genetic results, a bchE-lacZ reporter fusion plasmid pS03027 was introduced into the wild type and the PPSR mutant. Wild type and PPSR mutants containing the bchE-lacZ fusion grown under high or low oxygenation were assayed for β-galactosidase activity. As shown in Fig. 6, under both conditions the level of expression of the bchE-lacZ fusion was not different between the wild type and the PPSR mutant, confirming that the expression of the bchE gene is not regulated by PpsR. It is of note that the promoter activity of bchE is only slightly increased when the oxygen tension drops. In both the WT and the PPSR mutant, the bchE promoter activity was 1.5 times greater when cells were grown under low oxygenation conditions. We conclude that bchE gene is expressed under both high and low oxygenation and independently of the PpsR transcription factor.

Oxygen Regulation of bchE Gene Expression, RT-PCR Analysis—To confirm further the expression of the bchE gene under either high or low oxygenation conditions, Northern hybridization was performed to examine the production of transcripts under these conditions; however, we were unable to detect any transcript. Expression of bchE was then determined by RT-PCR. Total mRNA was extracted from WT cells grown photosynthetically or aerobically under high or low oxygenation and subjected to RT-PCR. The results are shown in Fig. 7. In the positive genomic control a PCR product of the expected size of 384 bp was obtained, and no PCR product was detected in any of the negative control reactions. DNA bands corresponding to the expected size from all RT samples were obtained, demonstrating that in all investigated oxygen conditions, the bchE gene was expressed (Fig. 7). Taken together, the RT-PCR and the bchE promoter activity results revealed that R. gelatinosus bchE gene expression is oxygen-independent and that its transcription is not repressed under aerobic conditions. These findings raised the questions of why the BchE enzyme fails to catalyze the formation of the isocyclic ring V of protochlorophyllide under high oxygenation conditions in the ACSF mutant. Many hypotheses should be considered; the enzyme activity may be controlled either at the translational level, i.e., stability of the protein, or at the post-translational level, i.e., post-translational modifications. BchE sequence analyses revealed the presence of an iron-sulfur cluster-binding motif (CXXXCXXC), suggesting that BchE is an iron-sulfur protein. The assembly of the iron sulfur cluster within the protein may be oxygen-dependent, and the loss of the Fe-S cluster by exposure to oxygen may result in an inactive form of the enzyme. Finally, the redox state of the putative iron-sulfur cluster in BchE and hence the catalytic activity may be oxygen-sensitive, accounting for an enzyme active only under low oxygenation and anaerobic conditions.

Overexpression, Purification, and Analysis of a His-tagged BchE Protein—As a first step toward understanding the BchE functioning, we overexpressed the gene encoding bchE and initiated its biochemical studies to check the presence of the iron-sulfur cluster. A full-length recombinant His6-BchE (575 residues) was produced in inclusion bodies in E. coli. The preparation was yellow-brown in color; however, only a small fraction could be solubilized with urea (6 M), and most of the iron-sulfur cluster was degraded upon solubilization as judged by the loss of the color and the loss of the absorption peak around 410 nm, characteristic of oxidized 4Fe-4S (not shown). A truncated form of His6-BchE* (384 residues) was also produced in inclusion bodies (Fig. 8A). Both the inclusion body preparation and the 6 M urea solubilized fraction were yellow-brown in color, indicating that solubilization did not degrade the iron-sulfur cluster. The presence of intact iron-sulfur centers in the solubilized fraction was verified by UV-visible spectroscopy. Fig. 8B shows that the truncated His6-BchE* preparation displays a spectrum between 300 and 600 nm characteristic of iron-sulfur proteins, with a major absorption peak around 410 nm and a weak additional broad absorption around 580 nm. Addition of sodium dithionite resulted in a shift (414–424 nm) of the absorbance in the visible region, demonstrating that the iron-sulfur cluster was redox-active. These features are typical of many proteins containing 4Fe-4S clusters (17–19) but not of a 2Fe-2S cluster. The 2Fe-2S cluster usually shows distinct absorption peaks around 330, 420, 460, and 560 nm (20). The truncated His6-BchE* was purified from the solubilized fraction by column affinity (Fig. 8A, lane 1). The purified protein migrated as a band of ~43 kDa corresponding to the predicted molecular size of the tagged construct, and a second band that could correspond to the dimeric (86 kDa) form.
and structural dissimilarities between AcsF and BchE, both enzymes are very likely able to recognize the same substrate, MgPMe, and catalyze its conversion to Pehlide a. We propose that bacteriochlorophyll a biosynthesis is a branched pathway in *R. gelatinosus* and presumably in other facultative aerobic phototrophs, at the level of MgPMe (Fig. 5).

AcsF homologues were found in many chlorophyll-containing organisms as follows: in purple bacteria (*R. sphaeroides* and *R. palustris*), in green non-sulfur bacteria (*Chloroflexus aurantiacus*), in cyanobacteria (*Prochlorococcus marinus*, *Anabaena* sp. PCC 7120 and *Synechocystis* sp. PCC 6803), in red algae (*Porphyra purpurea*), in green algae (*Chlamydomonas reinhardtii*), and in plants (*Oryza sativa* and *Arabidopsis thaliana*). In all these organisms AcsF and homologues would be required for chlorophyll biosynthesis as shown in *C. reinhardtii* (21), and very likely for MgPMe cyclization to produce protochlorophyllide a under aerobic conditions as recently demonstrated for the CHL27 gene from *Arabidopsis*. The *acsF* homologue was not found in the strict anaerobe *Chlorobium t eidum* (AE006470). Analysis of the deduced amino acid sequence of AcsF and its homologues showed the presence of two metal-center iron ligand motifs (D/E)EXXX. This motif is characteristic of monooxygenases, a class of metalloproteins including the *E. coli* aerobic coproporphyrinogen oxidase (HemF) and the ribonucleotide reductase (NrdB), where two copies of the motif (D/E)XXX, DEXXX provide ligands to bind a binuclear iron cluster (23). It is noteworthy that in the absence of oxygen, *E. coli* activates the anaerobic forms of coproporphyrinogen oxidase (HemN) and the ribonucleotide reductase (NrdG).

Among photosynthetic chlorophyll containing organisms, homologues of the *bchE* gene were found only in photosynthetic bacteria (purple, green, and cyanobacteria). Up to now, *bchE* gene homologues were not found in plants or in the green alga *C. reinhardtii*. By analogy with other anaerobic oxidoreductases (HemN and NrdG), BchE would catalyze the cyclization reaction via radical chemistry requiring the contribution of an iron-sulfur cluster. The iron-sulfur-binding motif including three cysteine residues (CXXX) was found in the BchE sequences, and the presence of the iron-sulfur center in the purified BchE protein was verified by UV-visible spectroscopy. This motif was also found in the amino acid sequences of all HemN proteins as well as in other proteins (Fig. 9) including the anaerobic ribonucleotide reductase-activating enzyme (NrdG), biotin synthase (BioB), and lipoate synthase (LipA) (18, 24). In all the enzymes these three cysteine residues are part of an unusual Fe-S cluster, and all these proteins have in common that they use SAM as a cofactor to form a radical involved in catalysis. A fourth conserved cysteine is found in all aerobic Mg-protoporphyrin monomethyl ester cyclases (Fig. 9); its role in the iron-sulfur binding should be verified by mutagenesis. It was shown by site-directed mutagenesis that the three cysteines of the conserved CXXX motif sequence in the anaerobic ribonucleotide reductase NrdG are sufficient for iron-sulfur binding (17).

Detailed analysis of heme metabolism, deoxyribonucleotide synthesis, and now the bacteriochlorophyll biosynthesis pathways revealed that the oxidative reaction step proceeds via different mechanisms depending on the oxygen growth conditions (Fig. 10). Oxygen-dependent reactions requiring molecular oxygen are catalyzed with di-iron-containing enzymes, whereas anaerobic enzymes requiring water contain SAM and iron-sulfur. The protein sequences derived from the genes of the aerobic enzymes are completely different from the se-

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**Fig. 8. Purification and spectroscopic characterization of recombinant R. gelatinosus His$_6$-BchE**. A, SDS-PAGE analysis of proteins contained in whole cell extract prepared from induced *E. coli* BL21 carrying pSO2776 (lane 3), inclusion bodies (lane 2), and after chromatography on nickel nitrilotriacetic acid-agarose affinity column (lane 1). Lane M shows the molecular weight markers. B, UV-visible light absorption spectra of urea-solubilized inclusion bodies containing recombinant His$_6$-BchE$^\ast$ proteins. In gray is the spectrum of the oxidized form. Addition of sodium dithionite leads to the reduced form shown in black. Inset, UV-visible light absorption spectra of purified recombinant His$_6$-BchE$^\ast$ protein. In gray is the spectrum of the oxidized form. Addition of sodium dithionite leads to the reduced form of the protein shown in black.

**DISCUSSION**

By using H$_2^{18}$O and $^{18}$O labeling in vitro assays and mass spectrometry, Porra et al. (4) examined bacteriochlorophyll a formation in *R. sulfidophilum*, a strain that produces bacteriochlorophyll a both aerobically and anaerobically. Labeling experiments showed that under anaerobic conditions, the oxygen atom incorporated into the keto group on ring V of the MgPMe molecule is derived from H$_2$O, whereas under aerobic conditions, the oxygen is derived from molecular oxygen (4). These data suggested two different mechanisms for MgPMe cyclization in this bacterium. Pinta et al. (3) have identified in *R. gelatinosus* AcsF as the aerobic Mg-protoporphyrin monomethyl ester cyclase and showed its involvement in the cyclization of MgPMe. In this report we have identified the anaerobic Mg-protoporphyrin monomethyl ester cyclase encoded by the *bchE* gene, and we showed that BchE is active only under low oxygenation or anaerobiosis. On the other hand, analysis of the mutants allowed the confirmation of the proposed two mechanism model for the cyclization and the identification of the involved *acsF* and *bchE* genes. Despite considerable sequence was also revealed. Similar UV-visible spectra were observed for the purified protein, with a peak around 410 nm for the oxidized form and a peak around 418 nm for the reduced form (Fig. 8B, inset) indicating the presence of a redox-active iron-sulfur cluster.
quences derived from genes specifying anaerobic enzymes. The use of different classes of enzymes catalyzing the same reaction appears therefore to be a common feature of different biosynthesis pathways (Fig. 10). For instance, there have been reports of two coproporphyrinogen oxidase systems, one oxygen-requiring type (HemF) found in aerobic organisms using a binuclear iron center and another type found in anaerobic systems (HemN) requiring H₂O as electron acceptor and functioning with an iron-sulfur cluster (19). In *E. coli*, three classes of ribonucleotide reductases have been classified according to the radical generator and oxygen growth conditions. Class I enzyme (NrdB) produces a tyrosyl radical through the action of a binuclear iron center, and class III enzyme (NrdG) produces a glycyl radical through the action of an iron-sulfur cluster and SAM (25, 26).

In the case of bacteriochlorophyll biosynthesis, it was proposed that under anaerobiosis BchE catalyzes the cyclization of MgPMe via radical chemistry (27). The reaction should be SAM and vitamin B₁₂-dependent, consistent with the reported phenotype for the *R. capsulatus* vitamin B₁₂ biosynthesis-deficient mutant accumulating MgPMe (27). Under aerobiciosis, the aerobic enzyme AcsF is a di-iron-containing enzyme requiring molecular oxygen for the catalysis. A three-step model in which the Pchlide α is produced after hydroxylation and oxidations of MgPMe was proposed by Porra et al. (28).

Oxygen regulation and expression of genes encoding the aerobic or the anaerobic enzymes were studied in many bacteria. In *Bradyrhizobium japonicum*, no significant expression of the *hemN* gene was detected in aerobically grown cells; however, the gene expression was strongly induced under microaerobic or anaerobic conditions (29). In *Pseudomonas aeruginosa* expression of both *hemF* and *hemN* was induced during anaerobic growth (30). In *E. coli*, expression of the *nrdB* gene was also shown to decrease under anaerobiosis to a lower basal level, but the gene is still expressed under both conditions (31). Expression of the *bchE* gene was studied by assessing the promoter activity and the mRNA production under both aerobic and anaerobic conditions. However, the BchE Mg-protoporphyrin monomethyl ester cyclase is inactive under high oxygenation. This is probably due to the sensitivity of the iron-sulfur cluster to oxygen. Further biochemical studies should be performed to identify the post-transcriptional steps that control the enzyme activity under aerobic conditions. To bypass this undesirable oxygen...
effect, the aerobic organisms have acquired or maintained the aerobic form of the enzyme.

Coexistence of both aerobic and anaerobic cyclases in the facultative aerobic phototrophic bacteria raises an interesting question regarding the mechanism of chlorophyll biosynthesis in strict aerobes and strict anaerobes. The wide distribution of *aces*F homologues among the strict aerobic phototrophs concomitant to the lack of *bchE* homologues in the genomes analyzed so far leads us to suggest a simple scheme whereby strict aerobes possess and use only the aerobic Mg-protoporphyrin monomethyl ester cyclase, whereas the strict anaerobes devoid of *aces*F homologues possess and use only the anaerobic Mg-protoporphyrin monomethyl ester cyclase to produce chlorophyll (Fig. 5). That way, aerobic restricted plants do not need the anaerobic form of the Mg-protoporphyrin monomethyl ester cyclase and will make use of their aerobic cyclase; the strict anaerobic bacterium *C. tepidum* has no need of the aerobic *AcsF* enzyme and will exclusively retain the anaerobic cyclase form and make use of it. As for facultative aerobes, they enjoy the selective benefit of having both aerobic and anaerobic cyclases providing the competitive advantage of producing chlorophylls under both aerobic and anaerobic conditions. Surprisingly, no homologue of *bchE* gene was found in the *C. reinhardtii*, although this organism can grow and produce *C. reinhardtii*, even in a low amount under anaerobic conditions, will then be a significant advantage to rap-

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REFERENCES

1. Suzuki, J. Y., Bolivar, D. W., and Bauer, C. E. (1997) *Annu. Rev. Genet. 31*, 81–98
2. Beale, S. I. (1999) *Photosynth. Res. 60*, 43–79
3. Pinta, V., Picaud, M., Reiss-Husson, F., and Astier, C. (2002) *J. Bacteriol. 184*, 746–753
4. Porra, R. J., Urzinger, M., Winkler, J., Bubenzer, C., and Scheer, H. (1998) *Eur. J. Biochem. 257*, 185–191
5. Porra, R. J., Schafer, W., Katheder, A., and Scheer, H. (1995) *FEBS Lett. 371*, 21–24
6. Uffen, R. L. (1976) *Proc. Natl. Acad. Sci. U. S. A. 73*, 3298–3302
7. Agalidis, I., Rivas, E., and Reiss-Husson, F. (1990) *Photosynth. Res. 23*, 249–255
8. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
9. Ouchane, S., Picaud, M., Reiss-Husson, F., Vernotte, C., and Astier, C. (1996) *Mol. Gen. Genet. 252*, 379–385
10. Heck, C., Rothfuchs, R., Jager, A., Rauhut, R., and Klug, G. (1996) *Mol. Microbiol. 20*, 1165–1178
11. Yang, Z. M., and Bauer, C. E. (1990) *J. Bacteriol. 172*, 5001–5010
12. Igarashi, N., Harada, J., Nagashima, S., Matsuura, K., Shimada, K., and Nagashima, K. V. (2001) *J. Mol. Biol. 31*, 333–341
13. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res. 25*, 3389–3402
14. Bauer, C. E., and Bird, T. H. (1996) *Cell 85*, 5–8
15. Zeilstra-Ryalls, J. H., Gomesky, M., Yeliseev, A. A., Eraso, J. M., and Kaplan, S. (1998) *Methods Enzymol. 297*, 151–186
16. Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M., II, and Peterson, K. M. (1994) *BioTechniques 16*, 800–802
17. Tamarit, J., Gerez, C., Meier, C., Mulliez, E., Trautwein, A., and Fontecave, M. (2000) *J. Biol. Chem. 275*, 15669–15675
18. Ollagnier-De Choudens, S., Sanakis, Y., Hewitson, K. S., Roach, P., Baldwin, J. E., Munck, E., and Fontecave, M. (2000) *Biochemistry 39*, 4165–4173
19. Layer, G., Verfurth, K., Mahlitz, E., and Jahn, D. (2002) *J. Biol. Chem. 277*, 34136–34142
20. Orme-Johnson, W. H., and Orme-Johnson, N. R. (1982) in *Iron-Sulfur Proteins* (Spiro, T. G., ed) pp. 67–96, Wiley-Interscience, New York
21. Moseley, J., Quinn, J., Eriksson, M., and Merchant, S. (2000) *EMBO J. 19*, 2139–2151
22. Prentki, P., and Krisch, H. M. (1984) *Gene 33*, 303–313
23. Jordan, A., Pontis, E., Atta, M., Krook, M., Gibert, I., Barbe, J., and Reichard, P. (1994) *Proc. Natl. Acad. Sci. U. S. A. 91*, 12892–12896
24. Ollagnier, S., Mulliez, E., Guillaud, J., Elasson, F., Fontecave, M., and Reichard, P. (1996) *J. Biol. Chem. 271*, 9410–9416
25. Jordan, A., and Reichard, P. (1998) *Annu. Rev. Biochem. 67*, 71–98
26. Poole, A. M., Logan, D. T., and Sjoberg, B. M. (2002) *J. Mol. Evol. 55*, 180–196
27. Gough, S. P., Petersen, B. O., and Duus, J. O. (2000) *Proc. Natl. Acad. Sci. U. S. A. 97*, 6908–6913
28. Porra, R. J., Schafer, W., Guadón, N., Katheder, I., Drews, G., and Scheer, H. (1996) *Eur. J. Biochem. 239*, 85–92
29. Fischer, H. M., Velasco, L., Delgado, M. J., Bedmar, E. J., Scharen, S., Zingg, D., Gettlfert, M., and Hennecke, H. (2001) *J. Bacteriol. 183*, 1300–1311
30. Rompf, A., Hungerer, C., Hoffmann, T., Lindenmeyer, M., Romling, U., Gross, U., Deus, M. O., Arti, H., Igarashi, Y., and Jahn, D. (1998) *Mol. Microbiol. 29*, 985–997
31. Casado, C., Lagostera, M., and Barbe, J. (1991) *FEMS Microbiol. Lett. 67*, 153–157
32. Quinn, J. M., Eriksson, M., Moseley, J. L., and Merchant, S. (2002) *Plant Physiol. 128*, 463–471
33. Ouchane, S., Picaud, M., Vernotte, C., and Astier, C. (1997) *EMBO J. 16*, 4777–4787
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