Global Regulation of Photosynthesis and Respiration by Fnrl

The First Two Targets in the Tetrapyrrole Pathway*

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Fnr is a regulator that controls the expression of a variety of genes in response to oxygen limitation in bacteria. To assess the role of Fnr in photosynthesis in Rubrivivax gelatinosus, a strain carrying a null mutation in furL was constructed. It was unable to grow anaerobically in the light, but, intriguingly, it was able to produce photosynthetic complexes under high oxygenation conditions. The mutant lacked all c-type cytochromes normally detectable in microaerobically-grown wild type cells and accumulated coproporphyrin III. These data suggested that the pleiotropic phenotype observed in Fnr is primarily due to the control at the level of the HemN oxygen-independent coproporphyrinogen III dehydrogenase. hemN expression in trans partially suppressed the FNR phenotype, as it rescued heme and cytochrome synthesis. Nevertheless, these cells were photosynthetically deficient, and pigment analyses showed that they were blocked at the level of Mg²⁺-protoporphyrin monomethyl ester. Expression of both hemN and bchE in the FNR mutant restored synthesis of Mg²⁺-protoporphyrilide. We, therefore, conclude that Fnrl controls respiration by regulating hemN expression and controls photosynthesis by regulating both hemN and bchE expression. A comprehensive picture of the control points of microaerobic respiration and photosynthesis by Fnrl is provided, and the prominent role of this factor in activating alternative gene programs after reduction of oxygen tension in facultative aerobes is discussed.

Anoxogenic photosynthetic bacteria can develop the required machinery for growth under aerobic respiration, anaerobic respiration, or photosynthesis under anaerobiosis and light. This facility requires tight control to ensure production of the required complexes and rapid adaptation to changes in environmental conditions. Under anaerobiosis and light, purple bacteria perform anoxogenic photosynthesis on the basis of a bacteriochlorophyll-mediated process. It takes place within the membrane-bound photosynthetic apparatus composed of pigment-protein complexes (reaction center (RC)) and light harvesting (LH)) and involves membrane cytochromes. Synthesis of tetrapyrroles (heme and chlorophyll) is then a crucial process necessary for photosynthesis and respiratory growth of these organisms. Heme synthesis and its regulation have been studied in many organisms (for review, see Refs. 1–4). A key step in this pathway corresponds to the conversion of coproporphyrinogen III to protoporphyrinogen IX. In facultative aerobic prokaryotes, two structurally different enzymes catalyze this reaction depending on the oxygenation level. Under high oxygen, the oxidation is catalyzed in Escherichia coli by the oxygen-dependent coproporphyrinogen III oxidase (HemF). Under anaerobiosis, an oxygen-independent coproporphyrinogen III dehydragenase (HemN) is required (5, 6). Bacteriochlorophyll synthesis and its regulation have also been studied in many phototrophs (for review, see Ref. 7). A key step in the chlorophyll pathway in facultative aerobes corresponds to the cyclization of Mg²⁺-protoporphyrin monomethyl ester (MgPMe). Two structurally unrelated enzymes catalyze this reaction depending again on the oxygen tension. Under high oxygen the oxidation is catalyzed by the oxygen-dependent MgPMe oxidase (AcsF). Under anaerobiosis, an oxygen-independent MgPMe dehydrogenase (BchE) is required (8–10).

In purple bacteria the photosynthesis genes are expressed preferentially under anaerobic photosynthesis conditions. Gene induction was mainly studied in Rhodobacter capsulatus and Rhodobacter sphaeroides species, and it involves many transcriptional regulators (11–13). Among these factors, Fnr was shown to regulate both photosynthesis and respiratory genes (14, 15). In E. coli the global transcriptional regulator Fnr monitors the availability of oxygen in the medium through its sensory domain composed of a labile [4Fe-4S] (16). It binds to its target sequence TTGAN₆TCAA (N is any nucleoside) as a homodimer. The sensing mechanism implies the transformation of the [4Fe-4S] cluster to a [2Fe-2S] in the presence of oxygen, preventing the dimerization process (17, 18).

This study focuses on the Rubrivivax gelatinosus global anaerobic regulator Fnr and the requirement of this factor for photosynthetic and microaerobic growth. We have previously detailed the AcsF/BchE enzyme replacement and suggested a similar HemF to HemN replacement during the shift from an aerobic to an anaerobic environment (10). Other examples of such enzyme replacement have been reported (19). This work further investigates the key targets of FnrL in the tetrapyrrole pathway and elucidates how enzyme replacement during a shift from aerobic to anaerobic growth is affected by FnrL.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Table II and data.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY648960.

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2 The abbreviations used are: RC, reaction center; LH, light harvesting; kb, kilobase; MgPMe, Mg²⁺-protoporphyrin monomethyl ester; kb, kilobase; WT, wild type.

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**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Media**—E. coli was grown at 37 °C on LB medium. *R. gelatinosus* Strain 1 (20) and the mutants were grown at 30 °C aerobically in the dark (high oxygenation, 250-ml flasks containing 20 ml of medium, or moderate oxygenation, 100-ml flasks containing 50 ml of medium) or microaerobically (low oxygenation, 50-ml flasks filled with 50 ml of medium) or anaerobically in the light (photosynthesis) in malate growth medium. Antibiotics were used at the following concentrations for *E. coli* and *R. gelatinosus*: 50 μg/ml kanamycin, 50 μg/ml ampicillin, 50 μg/ml streptomycin, 50 μg/ml spectinomycin, and 3 μg/ml chloramphenicol. Bacterial strains and plasmids used in this work are listed in Table 1.

**Membrane Protein Preparation**—The membranes were prepared by cell disruption with a French press in 0.1 m sodium phosphate buffer pH 7.4 containing 1 mM phenylmethylsulfonyl fluoride followed by differential ultracentrifugation. The membranes were then resuspended in the same buffer.

**Pigment Extraction and Analysis**—Pigments were extracted from cell suspensions by mixing with 20 volumes of ice-cold acetone/methanol (7:2, v:v) and recovering the supernatant after a low speed centrifugation. The extraction was repeated acetone/methanol (7:2, v:v) and recovering the supernatant from cell suspensions by mixing with 20 volumes of ice-cold water. The membranes were then resuspended in the same buffer. Bchl and carotenoids passed into the hexane upper phase, whereas porphyrins were recovered in the ammonia-acetone sub-phase. This phase was dried; porphyrins were redissolved in ethyl ether or acetone before identification by absorption and fluorescence spectroscopy. In some experiments, the porphyrin extract was analyzed by thin layer chromatography on high performance thin layer chromatography reversed-phase plates (RP18WF254, Merck) following a published protocol (42). Porphyrin spots were revealed by their fluorescence under UV light, scraped off, and extracted in dimethyl-sulfoxide or acetone before spectroscopic characterization.

**Heme Staining**—The purified membrane proteins were separated by SDS-PAGE and stained for covalently bound heme with 3,3′,5,5′-tetratramethylbenzidine as described in Thomas et al. (21).

**Molecular Biology Techniques**—Standard methods were performed if not otherwise indicated according to Sambrook et al. (22). Plasmids were purified using the Qiagen prep plasmid kit. DNA was treated with restriction enzymes and other nucleic acid-modifying enzymes according to the manufacturer’s specifications. DNA fragments were analyzed on agarose gels, and restriction fragments were purified using the Geneclean kit (BIO 101, Inc.). PCR reactions were carried out by using genomic or plasmid templates in a 50-μl reaction mixture containing the DNA, the PCR buffer, 200 μM concentrations of each deoxyribonucleoside triphosphate, 1 μM concentrations of each primer, 5% Me₂SO and 2.5 units of Taq DNA polymerase. All Primers used in this work are listed in Table II (supplemental material). DNA sequencing was performed using a 3100 Genetic Analyzer automatic DNA sequencer. The sequence of fnrL and of hemN2 reported in this paper were deposited in GenBank™ as accession number AY648960.

**Gene Cloning and Plasmid Constructions for Allele Replacement**—To clone the fnrL gene from genomic DNA of *R. gelatinosus*, a 1.4-kb fragment was amplified by PCR using the primers ol-fnr-F and ol-fnr-R. The fragment was cloned into the PCR cloning vector pGEM-T to give pSO7000. The fnrL gene was inactivated by the insertion of the Ω cassette in the unique BstEII site within the fnrL coding sequence. Briefly, plasmid pSO7000 was subjected to BstEII restriction enzyme digestion and treated with Klenow polymerase before ligation with the 2-kb Smal-digested Ω cassette conferring resistance to spectinomycin and streptomycin. The resulting recombinant plasmid was designated pSO7001.

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**TABLE 1**

**Bacterial strains and plasmids**

| Strains and plasmids | Relevant characteristics | Source |
|----------------------|-------------------------|--------|
| E. coli              | supE44, lacB17, recA1, endA1, gyrA6, thi-1, relA1, lac-Δ F [proAB⁺, lacI⁺, lacZΔM15 Tn10 (Te⁺)] | Stratagene |
| R. gelatinosus       | Strain 1 wild type      | Ref. 19 |
| FNR                  | fnrL inactivation strain (fnrL::Δ) | This work |
| Plasmids             |                         |        |
| pGEM-T               | PCR product cloning vector (Ap’) | Promega |
| pDW9                 | Plasmid bearing the W cartridge (Sp’, Sm’) | Ref. 39 |
| pBR1MCS-2            | (mob⁺, K⁺) expression vector | Ref. 40 |
| pSO7000              | 1.4-kb fragment containing the fnrL gene cloned in pGEM-T vector | This work |
| pSO7001              | 2-kb El cartridge inserted at the fnrL BstEII restriction site in the pSO7000 plasmid | This work |
| pSO3001              | pBR1MCS-1 + promoter-less (BamHI-BsaAI) lacZ gene | Ref. 41 |
| pSO3033              | Hem2-lacZ (pSO3001 + promoter region of hemN2) | This work |
| pSO7002              | hemN2 (promoter less) 1.4-kb fragment cloned in pBR1MCS-2 expression vector | This work |
| pSO7003              | bchE (promoter less) 1.7-kb fragment cloned in pSO7002 | This work |

Apr, ampicillin resistant; Km, kanamycin resistant; Sp, spectinomycin; Sm, streptomycin.
Gene Transfer and Strain Selection—Transformation of R. gelatinosus cells was carried out by electroporation as previously described (23). Transformants were selected on Malate plates supplemented with the appropriate antibiotic. Subsequently, after transformant selection, template genomic DNA was prepared from the transformants, and confirmation of the antibiotic resistance marker presence at the desired locus was performed by PCR.

**RESULTS**

Fnrl Amino Acid Sequence Analyses—The fnrl gene was located downstream of the ccoNOQPG operon encoding the \( \text{cbb}_2 \) cytochrome oxidase. BlastP and Clustal analyses indicated that the Fnrl polypeptide shares extensive similarity with the Fnr protein from \( \beta \) proteobacteria, R. gelatinosus PM1 (80% identity and 87% similarity), Azoarcus sp. (65% identity and 80% similarity), and Polaromonas sp. (64% identity and 82% similarity). Relatively low similarity was observed with Fnr protein from phototrophic \( \alpha \) proteobacteria including R. sphaeroides (28% identity and 50% similarity) and R. capsulatus (27% identity and 47% similarity).

The Fnrl polypeptide (246 amino acids) contains all characteristics and conserved domains of Fnr protein from numerous bacteria (supplemental data). A helix-turn-helix DNA binding sequence is identified in the \( \text{C} \) terminus of the polypeptide, and the four cysteine residues (Cys-17, Cys-20, Cys-26, and Cys-119), which are part of the conserved sensory domain, are well conserved.

**Anaerobic Induction of Photosynthesis Requires the Fnrl Product; Fnrl Mutant Construction and Characterization**—To gain insight into the role of the fnrl gene in photosynthesis, the fnrl gene was disrupted with the spectinomycin/streptomycin resistance cartridge inserted at the BstEII site of fnrl (Fig. 1). Wild type cells electroporated with pSO7001 plasmid were plated on selective medium under respiratory conditions to select transformants. Colonies resulting from double crossover events were orange compared with the purple color of the wild type colonies. A selected colony was subsequently tested for its aerobic and anaerobic photosynthetic growth characteristics. Interestingly, the mutants carrying insertions in fnrl were able to grow under aerobic conditions but were photosynthesis-deficient. This result indicates that anaerobic photosynthetic growth of R. gelatinosus requires a functional fnrl gene.

To clone the promoter-less hemN2 gene in the pBBR1MCS-2 expression vector, a 1.4-kb PCR fragment was amplified using ol-hemN2-F and ol-hemN2-R. The fragment was cloned first in pGEM-T and then subcloned in pBBR1MCS-2 at the Apal-Spel to give plasmid pSO7002. To clone bchE (promoter-less) in pSO7002, the gene (1.7 kb) was amplified by PCR using primers ol-bchE-F and ol-bchE-R and cloned in pGEM-T. The fragment was digested with SpeI-(Spbl-blunted) and cloned downstream of hemN2 at the Spel-(Xbal-blunted) site of pSO7002. This plasmid was designated pSO7003. The sequence and the orientation of both fragments were confirmed by sequencing.

To construct a hemN2-lacZ fusion, the promoter region of hemN2 containing the putative Fnrl binding site was amplified by PCR (193-bp DNA fragment). The primers (ol-hemN2-BamHI and ol-hemN2-Xbal) were designed to create Xbal and BamHI sites at the ends of the amplified fragment. The sequence and the orientation of the amplified DNA fragment were confirmed by sequencing after cloning into the BamHI and Xbal site of the lacZ transcriptional fusion vector plasmid pSO3001 to generate the fusion plasmid pSO3033 (hemN2-lacZ).

**FIGURE 1. fnrl region map in R. gelatinosus.** fnrl was mapped downstream the cco operon encoding the \( \text{cbb}_2 \) oxidase. The gene organization is different from cco operons in \( \alpha \) proteobacteria (supplemental data). TTGAA, TCAAG, Fnrl binding site found in the promoter region of fnrl. The arrow indicates the putative transcripts. The small arrows indicate the primers used for the PCR; 1, ol-fnr-F; 2, ol-Fnr-R. To disrupt fnrl, \( \Omega \), spectinomycin/streptomycin resistance cartridge was inserted at the BstEII restriction site. For spectral analyses of the wild type and FNR mutant, absorption spectra of membranes from R. gelatinosus (black) and Fnrl mutant (gray) cells were grown under high oxygenation (A) and low oxygenation (B) conditions. Absorption spectra of pigment extracts from R. gelatinosus WT (black) and Fnrl mutant (gray) cells grown under high oxygenation (C) and low oxygenation (D) conditions.
Chlorophyll and Chlorophyll-binding Protein Production in the WT and the FNR Mutant during Aerobic Growth—To assess the effect of mutation in the fnrl gene on the bacteriochlorophyll and bacteriochlorophyll-binding protein (RC-LHII/LHIII) synthesis, the wild type and the FNR mutant were grown under different oxygen conditions, and equal amounts of membrane protein prepared from these cells were analyzed by spectroscopy. As shown in Fig. 1A, spectral analysis of the membranes showed that both wild type and FNR produce comparable amounts of photosynthetic complexes under high oxygenation conditions. However, in the FNR mutant a slight shift of the 870-nm peak and a reduction in the absorbance at 800 nm were observed, indicating a small reduction of the level of LHII. Thus, it seems that the mutation in fnrl slightly affects the antenna size under these conditions. Nevertheless, when grown under low oxygenation conditions, a substantial reduction of the peaks at 860–800 nm was observed in the FNR mutant, indicating that the amount of photosynthetic complexes was drastically reduced in this mutant (Fig. 1B). These results indicate that photosynthetic complex production requires the presence of the Fnrl protein when oxygen tension drops. Pigments were extracted from equal amounts of membrane protein to estimate the amount of bacteriochlorophyll a in these strains. Under high oxygenation conditions, both wild type and FNR produced comparable amounts of bacteriochlorophyll a, as shown by the peak at 770 nm (Fig. 1C). Under low oxygenation conditions, the amount of bacteriochlorophyll a in the FNR mutant is, however, drastically reduced (Fig. 1D). Given the inability of the FNR strain to grow under photosynthetic conditions, we concluded that the FNR mutant cannot produce bacteriochlorophyll a pigments and complexes under photosynthetic anaerobic conditions.

The Fnrl Protein Is Also Required for Cytochrome Induction—The FNR mutant had a doubling time (2.5 h) comparable with that of the wild type when grown under high oxygenation conditions, whereas under low oxygenation conditions the FNR mutant growth was slower (4 h doubling time). It was established that a drop in oxygen tension induces expression of the cbb₃-type cytochrome oxidase in R. gelatinosus WT. To assess the impact of fnrl disruption on cbb₃ oxidase, staining of WT and the FNR mutant membranes for covalently bound heme was performed to check the presence of cytochrome c subunits CcoP (32 kDa) and CcoO (23 kDa) of cbb₃. As shown on the heme staining gel (Fig. 2A), both subunits were detected in wild type and FNR membranes grown under high oxygenation conditions. The band at 43 kDa present in the WT and in the mutant corresponds to the reaction center-attached tetra-heme cytochrome c (PufC). As oxygen tension drops, all c cytochromes were decreased in the FNR mutant. Under low oxygenation conditions, the membranes from the WT contained all these c-type cytochromes, with increased amounts of the cbb₃ c-type subunits. In the FNR mutant membranes, only traces of c cytochrome could be detected. The same gel was further stained with Coomassie Blue to check the total amount of loaded proteins (Fig. 2B). We concluded that as a result of fnrl inactivation, synthesis of heme was significantly weaker, and consequently, this inactivation had a pleiotropic effect on cytochrome production. Consistent with this, the FNR mutant grows slower under low oxygenation conditions.

Tetrapyrrole Analyses in the FNR Mutant—Decrease of all c-type cytochromes, normally detectable by heme staining in microaerobically grown wild type cells, in the FNR mutant prompted us to analyze the porphyrins in this mutant. Indeed, microaerobically grown WT cells, in the FNR mutant secreted a red pigment into the medium (Fig. 2C1). This pigment was UV-light fluorescent (Fig. 2C2), presumably due to the accumulation and secretion of heme biosynthesis pathway intermediates. Therefore, porphyrins were extracted from the growth medium by ammonia/acetone/hexane treatment and analyzed by spectroscopy (Fig. 3A). The major absorption band at 396 nm and the minor ones at 498-530-565-617 nm as well as the fluorescence emission bands (Fig. 3B) observed in the (622–687 nm) range indicated that the extract contained coproporphyrin III (oxidized coproporphyrinogen III) (24). However, the emission spectrum also showed a 578-nm band that could not be attributed to coproporphyrin III. This minor component (which was present in
variable amounts from one culture to another) could be distin-
guished from coproporphyrin III by its absorption at 410 nm, as
shown by the excitation spectra (Fig. 3B). It could be tentatively
identified as a metallo-coproporphyrin III (possibly zinc), a
derivative already found to be excreted together with copropor-
phyrin in other cultures (25–27). Given that coproporphyrino-
gen III is converted to protoporphyrinogen IX under low oxy-
gen tension by $\text{hemN}$, we concluded that the $\text{hemN}$ gene is
not functional in the absence of FnrL.

Analysis of hemN Expression; Micro-aerobic Induction of
hemN2 Requires the FnrL Product—To elucidate the role of
FnrL in hemN gene expression, we have cloned a hemN gene
that mapped in the photosynthetic gene cluster of $R$. gelatino-
sus. But in the course of this study we discovered that $R$. gelati-
nosus possesses two hemN genes, hemN1 and hemN2. Both
genes contain an FNR consensus sequence in their respective
promoters, suggesting that regulation of both hemN genes is
mediated by the FnrL regulator. However, only hemN2 encodes
a functional oxygen-independent coproporphyrinogen III
dehydrogenase.$^3$ To quantify hemN2 promoter activity and the
impact of FnrL, lacZ fusion to hemN2 promoter was con-

$^3$ S. Ouchane, unpublished information.

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**FIGURE 3.** Characterization of the pigment extracted from the culture
medium of FNR cells grown under low oxygenation condition. A, absorp-
tion (solid line) and fluorescence emission excited at 400 nm (gray dotted line).
B, fluorescence excitation spectra excited. The solvent was acetone.

**FIGURE 4.** hemN2 promoter activity in the WT and in the FNR mutant
strains containing the hemN2-lacZ transcriptional fusion grown either
under low (LO) or high oxygenation (HO) conditions. Experiments were
performed in triplicate, and error bars denote S.D. Units of $\beta$-galactosidase
activity represent $\mu$mol of o-nitrophenyl-$\beta$-D galactopyranoside hydrolyzed/
min/unit of optical density of culture at 680 nm.

The resulting strains were cultivated under high and low oxygenation
conditions, and hemN2 expression was monitored by assaying $\beta$-galactosidase activity. As shown in Fig. 4, expression of the hemN2 gene in highly oxygenated WT and FNR cultures was very low and independent of FnrL. The expression was strongly induced (5-fold) under oxygen limitation conditions in
the wild type (1560 units). However, induction of hemN2 gene
under oxygen limitation was dependent on FnrL, since the pro-
moter activity was still low in the FNR mutant (320 units).

Furthermore, hemN2 expression was also strongly induced
under anaerobic photosynthetic conditions in the WT (1600
units; not shown). Because the FNR mutant does not grow
anaerobically, it was not possible to assess the predicted role of
FnrL in anaerobic hemN2 expression in the mutant.

**Phenotypic Consequences of hemN Expression in trans in the
FNR Mutant**—The data obtained from porphyrin analyses of
the FNR mutant grown under microaerobic conditions in combi-
nation with hemN mutant analyses suggest that hemN2 is the
first crucial tetrapyrrole gene controlled by Fnr. Therefore, we
suggest that Fnr controls respiration and photosynthesis first at
the level of hemN2. To test this model, a plasmid carrying a
promoter-less hemN2 gene under the control of a lacZ pro-
moter was introduced in the FNR mutant (Fig. 5). Cells bearing
either the empty vector (FNR-0) or the vector carrying hemN2
under the lacZ promoter (FNR-N2) were grown under low oxy-
gen conditions and tested for red fluorescence upon illumina-
tion with UV light. This test was positive for cells bearing the
vector plasmid (control) as they secreted coproporphyrin III
into the medium. For the FNR mutant bearing the extra copies of
hemN2, fluorescence was considerably reduced in the culture
(Fig. 5A). Porphyrin content analyses from the medium and the
cells showed that there was no coproporphyrin III (see below).
Regulation of Photosynthesis and Respiration by FnrL

Thus, it appears that constitutive expression of hemN2 is sufficient to rescue the heme synthesis in the cells grown under low oxygen conditions despite the absence of FnrL. Similar experiments were performed with hemN1; however, extra copies of hemN1 did not restore the heme synthesis pathway.

Suppression of the FNR heme-deficient phenotype by hemN2 suggested that these cells may contain a functional photosystem and the cytochrome cbb₃ oxidase. To test these hypotheses, cytochrome c type content in the membranes from these strains was analyzed by heme staining. As shown in Fig. 5B, the FNR-0 mutant strain bearing the empty vector had a cytochrome c profile similar to that of the FNR mutant, whereas the FNR-N2 mutant strain bearing the hemN2 gene retrieved a cytochrome c profile comparable with that of the WT. These results clearly demonstrate that heme and cytochrome synthesis necessitate a functional FnrL under low oxygen tension. Yet the FNR-N2 mutant bearing the extra copies of hemN2 remained photosynthesis-deficient and incapable of photosystem production, indicating that there must be at least a photosynthesis-involved gene that requires FnrL-mediated expression under the photosynthetic growth lifestyle. What then is the target gene of FnrL in photosynthetic growth in addition to hemN2?

BchE, the Key Step in Chlorophyll Synthesis, Is Controlled by FnrL—If FnrL regulates the expression of other photosynthesis genes, then the promoter of these genes is expected to have an FNR consensus binding sequence. No FNR binding motif was found in the photosynthetic gene cluster (genes involved in chlorophyll synthesis, bchCXYZ, bchFNBHLM, bchGP, and the pufBALMC operon encoding the LH/RC subunits). However, inspection of the bchE gene promoter revealed the presence of a TTGAC₁₀CAC sequence that may serve for FNR-mediated induction of these chlorophyll biosynthesis genes under low oxygen tension and anaerobic conditions. Under these conditions, in the absence of bchE expression, no chlorophyll synthesis could be achieved, and no functional photosynthetic complexes could assemble in the membrane leading to photosynthetically incompetent cells.

If FnrL is required for the expression of bchE, then the FNR mutant expressing hemN2 in trans should accumulate MgPMe (10). To test this hypothesis, FNR and FNR-hemN2 cells were grown under high oxygenation and low oxygenation conditions, and their membranes and tetrapyrrole content were analyzed.

As shown in Fig. 6, spectral analyses of the membranes showed that both FNR and FNR-N2 produced comparable and very low amounts of Bchl complexes under low oxygenation conditions. But whereas FNR excreted coproporphyrin, the FNR-N2 mutant excreted another pigment that absorbs at 418 nm, with spectral characteristics and chromatographic behavior identical to those of Mg²⁺-protoporphyrin monomethyl ester, an intermediate already observed in BchE mutant grown under low oxygenation (10). Under high oxygenation conditions, no differences were observed between the membranes of these strains (not shown). These results demonstrate that in the presence of the hemN2 gene, the FNR mutant recovers heme synthesis but still accumulates the bacteriochlorophyll intermediate MgPMe.

In both strains only traces of bacteriochlorophyll a could be detected, presumably due to residual activity of the (yet unidentified) aerobic coproporphyrinogen III oxidase HemF and the aerobic Mg²⁺-protoporphyrin monomethyl ester AcsF, which are partially functional under the low-oxygen conditions. The FNR mutant expressing hemN2 behaves exactly as a BCHE null mutant under limited oxygen conditions. We, therefore, concluded that FnrL is required for bchE expression induction under low oxygen tension.

Additional Level of Regulatory Control That Requires FnrL—We have shown that the FnrL requirement for heme synthesis can be bypassed via the presence of the hemN2 gene in trans. According to the heme and chlorophyll analyses in FNR-0 and FNR-hemN2 mutants, to bypass the FnrL requirement for chlorophyll synthesis, both hemN2 and bchE should be provided in trans. For that purpose, the bchE gene was cloned downstream hemN2 and introduced into the FNR mutant. The colonies obtained (FNR-N2E) could not grow photosynthetically. Furthermore, these cultures were greenish, and they exhibited a
bright red fluorescence under UV light, indicating the presence of porphyrin(s). Pigments extracted from microaerobically grown cells were separated into a hexane-soluble fraction and an acetone-soluble one (see “Experimental Procedures”). Absorption spectroscopy of the hexane fraction indicated that it contained a low amount of Bchl (Qy band peaking at 773 nm) and carotenoids, as expected (Fig. 7A). In the absorption spectrum of the acetone phase (Fig. 7A), the main Soret band peaking at 439 nm and the weaker 529-, 578-, and 629-nm bands indicated that the cells accumulated mostly Mg²⁺ protochlorophyllide. The 750-nm band together with the 358- and 389-nm bands were indicative of the presence of bacteriopheophorbide or of bacteriopheophytin, but because this pigment was soluble in acetone and not in hexane, it was bacteriopheophorbide. The shoulder observed near 420 nm could come from a Mg²⁺-protoporphyrin derivative, possibly its monomethyl ester. These assignments were confirmed by fluorescence emission spectra recorded with excitations at 390, 420, and 440 nm (Fig. 7B). Mg²⁺ protochlorophyllide emission bands at 630, 684, and 702 nm were observed for the three excitations. The emission at 593 nm attributed to Mg²⁺-protoporphyrin (monomethyl ester) was observed for excitations at 390 and particularly at 420 nm. The emission at 761 nm coming from bacteriopheophorbide
(28) was preferentially excited at 390 nm. The presence of these intermediates was otherwise confirmed by thin layer chromatography (not shown). The same intermediates were identified when the cells were grown semi-aerobically and also in the growth medium from these cultures. It should be noticed that traces of bacteriopheophorbide were also observed in dark-grown wild type cells (not shown).

The phenotype and the pigment content of the FNR-N2E strain were identical to that of a dark grown \textit{bchJ}-disrupted mutant, which was able to assemble RC-LH1 complexes and also accumulated the same intermediates. However, this \textit{BchJ} mutant was still able to grow photosynthetically, unlike FNR-N2E. These data suggest that the \textit{Fnr} target is not \textit{bchJ}.

We, therefore, concluded that there should be additional photosynthesis or regulatory genes that require expression of \textit{FnrL} to assemble the photosystem under anaerobic and light conditions.

**DISCUSSION**

Microorganisms have to cope with drastic changes in environmental conditions such as oxygen availability. Therefore, they have developed different systems to respond rapidly to transitory changes in conditions by adapting their gene expression. One way to adapt is to activate alternative gene programs to take advantage of the appropriate and most effective metabolic routes and to shut down the undesirable ineffective pathways. For instance, purple bacteria are among the most versatile microorganisms thanks to their capacity to adapt their growth machinery to the availability of oxygen and light (11, 12, 29). They can grow by aerobic, microaerobic, and anaerobic respiration as well as by anaerobic photosynthesis. Electron transfer associated with respiratory and photosynthesis processes requires heme and chlorophyll biosyntheses for cytochrome and photosystem functioning. Both microaerobic respiration and photosynthesis rely on the \textit{FnrL} regulator. In \textit{E. coli}, \textit{Fnr} controls the expression of more than 100 genes (30). In the purple bacterium \textit{R. capsulatus}, \textit{Fnr} regulates a set of heme synthesis genes (\textit{hemA}, \textit{hemB}, \textit{hemC}, \textit{hemH}, and \textit{heme}) (3). A model derived from early studies mainly based on analyses of the \textit{Fnr} mutants and promoter activity measurements in \textit{R. sphaeroides} was proposed (31, 32). From these studies it was inferred that \textit{Fnr} acts as a positive regulator of genes for heme biosynthesis, 5-aminolevulinic acid synthase (\textit{hemA}) and the coprophorphyrinogen III oxidases (\textit{hemZ} and \textit{hemN}), for bacteriochlorophyll synthesis \textit{bchEJG} operon, and for the \textit{pucBA} operon encoding the polypeptides of the light-harvesting complex LHII (11, 14, 32–34). In this organism the \textit{fnrL} gene was shown to regulate respiratory genes including the \textit{ccoNOQP} operon encoding the microaerobic \textit{cbb}$_3$ cytochrome oxidase and the \textit{rdxBHIS} genes involved in cytochrome \textit{cbb}$_3$ biogenesis (35, 36). These results suggested that coordination of the expression of all these genes is neces-

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4 S. Ouchane, unpublished information.

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**FIGURE 8. Schematic overview of the oxygen regulation of tetrapyrrole synthesis in \textit{R. gelatinosus} and other phototrophs.** \textit{FnrL} is essential during microaerobic and anaerobic photosynthesis growth. The main target genes of \textit{FnrL} are \textit{hemN} and \textit{bchE}. Transcription of both genes requires \textit{FnrL} and is induced under microaerobic and PS conditions by \textit{FnrL}. Strict aerobes possess and make use exclusively of oxygen-independent enzymes \textit{HemN} and \textit{BchE}.
sary for photosynthesis in *R. sphaeroides*. The present work indicates that cytochrome synthesis and microaerobic respiration rely on the expression of only one Fnr-dependent gene. In fact, only the HemN product is missing to supply cells with heme for the cytochrome production in response to the lowering of the oxygen tension in the FNR mutant. Constitutive expression of *hemN* in the mutant restores the heme synthesis, whereas expression of both *hemN* and *bchE* did not re-establish photosynthetic growth of this mutant, indicating that other Fnr-regulated genes are required for photosynthesis.

Because the FNR mutant behaves like the wild type under high aeration conditions, we concluded that cells should possess and activate an alternative gene program under high oxygenation to produce cytochromes and chlorophylls. It is tempting to speculate that HemF and AcsF are the first actors of this alternative program.

The Two First Key Steps of the Tetrapyrrole Pathway Controlled by FnrL.—The interpretation of the data obtained from porphyrin analysis in FNR and HemN mutants, in combination with previous results on the regulation of chlorophyll synthesis in *R. gelatinosus* (9, 10) permits us to draw a comprehensive picture of the control targets of microaerobic respiration and chlorophyll synthesis by Fnr. This picture is summarized in the model in Fig. 8. Depending on the oxygen tension, two pathways leading to heme and bacteriochlorophyll biosynthesis exist in this bacterium and probably in other phototrophic bacteria. It is well known that the bacteriochlorophyll branch subsequently diverges from the heme branch at the intermediate, protoporphyrin IX step. Under high oxygenation in the heme synthesis pathway, conversion of coproporphyrinogen III to protoporphyrinogen IX is catalyzed by the oxygen-dependent coproporphyrinogen III oxidase HemF. Similarly, in the chlorophyll pathway, the cyclization of Mg$_2^{2+}$-protoporphyrin monomethyl ester is catalyzed by the oxygen-dependent MgPMe oxidase AcsF (9, 10). Both heme and chlorophyll synthesis are Fnr-independent under high oxygenation conditions (Fig. 8). Under low oxygenation and anaerobiosis, this bacterium activates an alternative gene program triggered by FnrL to supply cells with heme and bacteriochlorophyll. The conversion of coproporphyrinogen III to protoporphyrinogen IX is then catalyzed by the alternative oxygen-independent coproporphyrinogen III dehydrogenase HemN. In the chlorophyll pathway the cyclization of Mg$_2^{2+}$-protoporphyrin monomethyl ester is catalyzed by the alternative oxygen-independent MgPMe dehydrogenase BchE (10). In this case the expression of *hemN* and *bchE* requires the presence of FnrL, so that both heme and chlorophyll syntheses became Fnr-dependent (Fig. 8). In this alternative pathway changes in oxygen tension are sensed by Fnr, which then activates the expression of *hemN* for the heme synthesis and *bchE* required for bacteriochlorophyll biosynthesis. Thus, as a consequence of the absence of FnrL, heme and chlorophyll, essential for photosynthesis, are not provided, resulting in a photosynthesis-deficient strain.

The first tetrapyrrole precursor, aminolevulinic acid, is synthesized either from succinyl-CoA and glycine via aminolevulinic acid synthase (*hemA*) or from glutamate by the C5 pathway via glutamyl-tRNA reductase (*gtrA*). In *R. sphaeroides*, induction of *hemA* also requires FnrL.

*hemA* has been found only in the α-proteobacteria, and the C5 pathway involving *gtrA* was found in the other proteobacteria groups (2). So far in *R. gelatinosus* only the C5 pathway (*gtrA*) was described, and the genome analysis of *R. gelatinosus* PM1 strain revealed the presence of only *gtrA* gene. In agreement with our results, activation of *gtrA* in *R. gelatinosos* mediated by FnrL is very unlikely, since the Fnr binding site is missing in the *gtrA* promoter region, and the FNR mutant is not affected at the level of aminolevulinic acid synthesis. It is of note that under oxygen-limited conditions, *fnrL* is auto-activated (not shown), resulting in a self-amplifying cascade. This auto-regulation also reflects the importance of this regulator and the key role of its target genes in adaptation to changes in oxygen tension.

The *Rhodobacter* Case—It was previously shown that *R. sphaeroides* requires the *fnrL* gene for growth under anaerobic photosynthetic growth conditions (14). In *R. capsulatus*, the close relative of *R. sphaeroides*, FnrL is not required for photosynthetic growth. Two plausible explanations for the different photosynthetic growth properties of *R. capsulatus* and *R. sphaeroides* fnrL mutants can be proposed. First, a second functional FnrL gene might be present in *R. capsulatus*. Alternatively, key target genes such as *hemN* and *bchE* might escape from the absolute Fnr requirement in *R. capsulatus*. As a matter of fact, promoter sequence analyses showed that *bchE* displays the FNR consensus sequence in *R. sphaeroides* but not in *R. capsulatus*. Consequently, in *R. capsulatus*, bacteriochlorophylls will be synthesized in the FNR mutant as long as the heme synthesis pathway is not blocked.

The *hemA* gene promoter of *R. capsulatus* does not contain the FNR site, whereas an Fnr binding site is present in the promoter region of *hemN* (*hemZ*). Nevertheless, a recent study in *R. capsulatus* showed that Fnr has a relatively moderate role in controlling the expression of the *hemN* gene (3). Taken together, these data may explain the photosynthesis-competent phenotype of FNR mutant in *R. capsulatus*. Zeilstra-Ryalls and Kaplan (34) proposed that the tetrapyrrole and bacteriochlorophyll biosynthetic genes *hemA, hemZ, hemN,* and *bchE* may require increased expression under photosynthetic conditions in *R. sphaeroides*. Later, Oh *et al.* (31) have shown that *bchE* and *hemN* expression requires FnrL. Constitutive expression of these genes in *R. sphaeroides* FNR mutant should provide new data on the precise role of Fnr in photosynthesis in this bacterium. Overall, both *R. gelatinosus* and *R. sphaeroides* possess the two routes leading to heme and bacteriochlorophyll synthesis under high oxygenation (*hemF* and *acsF* homologue ORF277) and low oxygenation (*hemN* and *bchE*) conditions. In both organisms, FnrL regulator plays a prominent role throughout the life cycle of these microorganisms and allows their adaptation to microaerobic respiration and photosynthesis following a drop in oxygen tension by controlling the tetrapyrrole synthesis pathway. We should point out that *acsF* was not identified in the available partial genome sequence of *R. capsulatus*.

Aerobic and Anaerobic Tetrapyrrole Branches—Coexistence of both aerobic and anaerobic tetrapyrrole branches in the facultative aerobic phototrophic bacteria (*R. gelatinosus, R. sphaeroides, Rhodopseudomonas palustris, Synechocystis sp.*) urged
us to further check heme and chlorophyll synthesis in strict aerobes and strict anaerobes. It was suggested that in the strict anaerobic bacterium *Chlorobium tepidum* chlorophyll synthesis is supplied only by the oxygen-independent MgPMe dehydrogenase BchE (10). A homologue of the MgPMe cyclase AcsF encoding gene was not found in its genome. Similarly, our searches for coproporphyrinogen III dehydrogenase (*hemN*) and oxidase (*hemF*) in this bacterium revealed the presence of oxygen-independent HemN dehydrogenase and the absence of any oxygen-dependent coproporphyrinogen III oxidase (HemF). This suggests that, in this strict anaerobe, only the anaerobic tetrapyrrole branch is active. Because of its lifestyle *C. tepidum* has no need of the aerobic HemF and AcsF enzymes and has solely retained the oxygen-independent enzymes.

In the case of the strict aerobe *Chlamydomonas reinhardtii*, although this organism can grow and produce photosystems under oxygen limited conditions, no homologue of the *bchE* gene was found in its genome (10). This alga possesses, however, two AcsF homologue isoforms (Crd1 and Cth1) (37, 38). The *crd1* gene was shown to be induced under low oxygen tension, and a CRD1 mutant is chlorotic under such conditions, which indicates that chlorophyll is supplied via Crd1. Surprisingly, our searches for oxygen-independent coproporphyrinogen III dehydrogenase HemN and oxygen-dependent coproporphyrinogen III oxidase HemF in the genome of this organism revealed that *C. reinhardtii* lacks a homologue of the *hemN* gene but possesses two HemF homologues, Cpx1 and Cpx2. It is tempting to speculate that this alga takes the advantage of redundant oxygen-dependent coproporphyrinogen III oxidases (Cpx1 and Cpx2) and Mg$^{2+}$-protoporphyrin monomethyl ester cyclases (Crd1 and Cth1) to cope with oxygen limitation and to produce functional photosystems under low oxygen conditions. Further analyses and experiments should be performed to examine this hypothesis.

Facultative aerobes like *R. gelatinosus* enjoy the selective advantage of having both aerobic and anaerobic tetrapyrrole branches. This allows bacteriochlorophyll production under both aerobic and anaerobic conditions (Fig. 8). Bacteriochlorophyll reserve produced under aerobic conditions will then be a substantial asset to rapidly build up the photosystem when cells face changes in oxygen availability, resulting in cells qualified to immediately start photosynthetic growth after exposure to light.

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