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Analysis of *hemF* Gene Function and Expression in *Rhodobacter sphaeroides* 2.4.1

Jill H. Zeilstra-Ryalls* and Kathryn L. Schornberg†

Department of Biological Sciences, Oakland University, Rochester, Michigan 48309

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The *hemF* gene of *Rhodobacter sphaeroides* 2.4.1 is predicted to code for an oxygen-dependent coproporphyrinogen III oxidase. We found that a HemF$^-$ mutant strain is unable to grow under aerobic conditions. We also determined that *hemF* expression is controlled by oxygen, which is mediated, at least in part, by the response regulatory protein PrrA.

The α-Proteobacterium *Rhodobacter sphaeroides* 2.4.1 can obtain energy by photosynthesis as well as by aerobic and anaerobic respiration, which are supported by its ability to synthesize both heme and bacteriochlorophyll. In this organism, both of these compounds are derived from 5-aminolevulinic acid (reviewed in reference 10), and directing the flow of intermediates towards one or the other tetrapyrrole involves transcriptional regulation of genes coding for several enzymes in the branching biosynthesis pathway (15, 18, 27, 29). Here, we report that the *hemF* gene of *R. sphaeroides* 2.4.1, coding for a putative oxygen-dependent coproporphyrinogen III oxidase, is also regulated at the level of transcription.

Bacterial strains and plasmids used in this study are listed in Table 1. Culturing of the bacteria was performed as described previously (4, 6, 22, 24). The *hemF* gene (527 bp upstream of the translation initiation site to 91 bp downstream of the nonsense codon TGA) was amplified from total genomic DNA with the following primers (Integrated DNA Technologies, Inc., Coralville, IA): HemF-UP, 5′-GTCACTTCGTCTCGG GATAGGTCGCGCGGCACTG-3′; HemF-DOWN, 5′-CCT CGCATGAGAGCGTGAGCAAGCGTCAGAGGCC-3′. The 1,496-bp product was cloned into the MscI site of pUI1087 (30), generating pKS7. DNA sequence analysis of both strands confirmed the integrity of the cloned sequences. Construction of the suicide vector pKS13, used to deliver the defective Δ*hemF*::ΩSp^r-St^r allele to *R. sphaeroides* 2.4.1, began with moving the *hemF* sequences contained on an EcoRI-XbaI DNA fragment from pKS7 into pBBR1MCS2 (12), creating pKS9. A 361-bp BamHI DNA fragment was deleted from pKS9 (which deletes 44 bp upstream of the HemF coding sequences through sequences coding for amino acid residues 1 to 105), and an omega Sp-St resistance cassette (20) was inserted at the remaining unique BamHI site. From this plasmid, pKS11, a 3,135-bp SmaI fragment containing Δ*hemF*::ΩSp^r-St^r was moved into pSUP202 (23), replacing the vector sequences contained on a 1,348-bp ScaI fragment and creating pKS13. The *hemF*::*lacZ* transcription reporter plasmid pJZ84 was constructed by first inserting a 717-bp SmaI-NcoI DNA fragment from pKS9, which includes 471 bp of *hemF* sequence upstream of the translation initiation site, into pUI1087 (30), and then, using the flanking PstI-XbaI vector restriction sites, the *hemF* sequences were positioned and correctly oriented in front of a promoterless *lacZ* gene in plasmid vector pCF1010 (14).

The *hemF* gene is required for aerobic growth. Using plasmid pKS13, we were unable to obtain HemF$^-$ mutants under aerobic conditions, but suitable mutant candidates were obtained when plates of exconjugants were incubated under anaerobic growth conditions (in the dark on medium containing dimethyl sulfoxide as an alternate electron acceptor). Among them, mutant strain JZ3534 was confirmed to be structurally

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
Rhodobacter sphaeroides		
2.4.1	Wild type	W. Sistrom
JZ3534	Δ*hemF*::Ω(Sp^r-St^r)	This study
PRRA2	Δ*prrA*::Ω(Sp^r-St^r)	7
Escherichia coli		
DH5α*phe*	DH5α*phe*::Tn*10d*Cm^r	8
HB101	F$^-$ Δ(*gtp-proA*)*62 leuB6 supE44 ara-14 galK2 lacY1*	1
Plasmids		
pBBR1MCS2	Km^r	12
pBSIISK$^+$	Ap^r	Stratagene
pCF1010	Promoterless *lacZ* vector, Mob$^+$ Tc^r Sp^r-St^r	14
pJZ84	*hemF*::*lacZ* transcription fusion in pCF1010	This study
pKS7	*hemF* in pUI1087	This study
pKS9	*hemF* in pBBR1MCS2	This study
pKS11	Ω(Sp^r-St^r) inserted into (Δ*Bam*HI)pKS9	This study
pKS13	Δ*hemF*::Ω(Sp^r-St^r) in pSUP202	This study
pRK2013	ColEI replicon, Tra$^+$ of RK2, Km^r	5
pSUP202	pBR325 derivative, Mob$^+$ Ap^r Cm^r Tc^r	23
pUI1087	pBSIISK$^+$ with modified polylinker	30
pUI1638	Source of Ω(Sp^r-St^r) cassette	8

* Corresponding author. Mailing address: Department of Biological Sciences, 374 Dodge Hall, Oakland University, Rochester, MI 48309. Phone: (248) 370-4497. Fax: (248) 370-4225. E-mail: zeilstra@oakland.edu.

† Present address: Department of Microbiology, University of Virginia, Charlottesville, VA 22908.

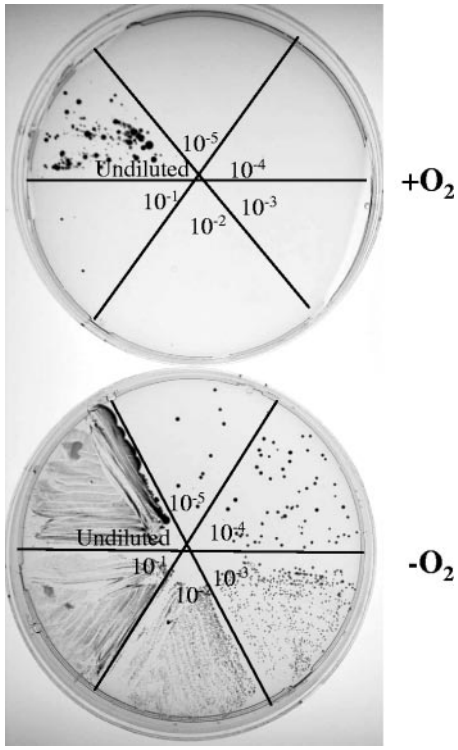

FIG. 1. Plates of serial dilutions from an anaerobic culture of HemF$^-$ mutant strain JZ3534 (see Table 1). The plates spread with 10-μl samples of 10-fold serial dilutions were incubated under the conditions shown.

correct by Southern hybridization (results not shown). We also found that moving plasmid pKS9, containing an intact *hemF* gene, into mutant strain JZ3534 restored growth under aerobic conditions to rates comparable to those of the wild-type strain 2.4.1.

We further evaluated the requirement for an intact *hemF* gene for aerobic growth by first culturing mutant strain JZ3534 under anaerobic conditions and then spreading serial dilutions of the culture on two plates, one of which was incubated aerobically and the other anaerobically. Growth after 5 days on the two plates is shown in Fig. 1, and as listed in Table 2, we found the numbers of CFU differed by approximately 5 orders of magnitude. This suggested that those few colonies that formed on the aerobically incubated plate were from cells

TABLE 2. Viable cell counts of cultures of *R. sphaeroides* mutant strain JZ3534 grown under different conditions

Culture condition[a]	Counts of CFU/ml following culturing on plates	
	Aerobic incubation	Anaerobic incubation[b]
Anaerobic	2×10^3	9×10^7
2% Oxygen	2×10^3	1.7×10^8
21% Oxygen	1×10^6	3.5×10^7

[a] Refers to the culture conditions used prior to plating and counting the colonies.

[b] Plates were incubated anaerobically in the dark, and dimethyl sulfoxide was added to the medium as an alternate electron acceptor.

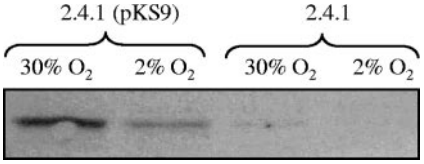

FIG. 2. Immunoblots of protein samples probed with anti-HemF antiserum. Samples examined are identified in the figure and are derived from cells grown under the designated conditions. In all cases, equivalent amounts of total protein were used (25 μg).

containing a second mutation that suppresses the growth defect conferred by the absence of a functional *hemF* gene. To explore this possibility we sparged liquid cultures, inoculated from the anaerobically grown preculture, with low oxygen (2%) and with air (approximately 21% oxygen). The results, listed in Table 2, revealed that the aerobic versus anaerobic viable cell counts of the low-oxygen culture differed by approximately 5 orders of magnitude, but the counts of the culture that had been sparged with air differed by only approximately 10-fold. Finally, we performed a comparative (aerobic versus anaerobic) viable cell count of four isolated colonies from the air-sparged culture that had formed on the aerobically incubated plate. Now the numbers of CFU were of the same order of magnitude on both sets of plates, each being 1×10^7 to 4×10^7 CFU/ml. Apparently, whereas low or no oxygen permits growth of the HemF$^-$ mutant strain in liquid culture, high concentrations of oxygen are nonpermissive and suppressor mutations are selected for.

Oxygen controls *hemF* expression. Using methods previously described (9), immunoblots prepared from extracts of wild-type 2.4.1 cells with and without pKS9 carrying the *hemF* gene, grown either aerobically or anaerobically, were probed with rabbit polyclonal anti-HemF antisera (Cocalico Biologicals, Inc., Reamstown, PA). As shown in Fig. 2, the levels of HemF protein detected were much higher in cells grown aerobically than in anaerobically grown cells, and the amount correlated with gene dosage. These results suggest that *hemF* expression is controlled by oxygen.

Transcription of *hemF* is regulated by PrrA. In *R. sphaeroides* 2.4.1, established regulators of gene expression that respond to changes in oxygen tensions include FnrL, the AppA-PpsR regu-

TABLE 3. β-Galactosidase activities in cell extracts of *R. sphaeroides* bearing the *hemF*::*lacZ* transcriptional fusion plasmid pJZ84

Strain	β-Galactosidase activity[a]			
	30% oxygen		Anaerobic	
	$\bar{x}$	σ_n	$\bar{x}$	σ_n
Wild type 2.4.1(pJZ84)	908	27	296	7
PRRA2(pJZ84)	510	11	795	13

[a] Activities are expressed in units of β-galactosidase activity, defined as micromoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein extract. Values with means ($\bar{x}$) and standard deviations (σ_n) indicated are from duplicate assays of activities in extracts from three independent isolates. The strains were grown in Sistrom's minimal succinate (24) with tetracycline (final concentration, 0.8 μg ml^{-1}) either aerobically; by sparging liquid cultures with a mixture of 30% oxygen, 5% carbon dioxide, and 65% nitrogen; or anaerobically in the dark with dimethyl sulfoxide as alternate electron acceptor.

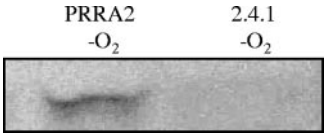

FIG. 3. Immunoblots of protein samples probed with anti-HemF antiserum. Samples examined are from cultures of *R. sphaeroides* wild-type strain 2.4.1 or mutant strain PRRA2 cells grown in parallel under anaerobic dark (with dimethyl sulfoxide) conditions. Equivalent amounts of total protein were used (53 μg).

latory system, and the PrrBA two-component system (reviewed in reference 28). While all genes known to be regulated by FnrL have an easily discernible FNR consensus-like sequence within their upstream sequences (16–18, 27, 29, 31), *hemF* does not, making it unlikely that FnrL regulates *hemF* transcription. Also, transcriptome profiling demonstrated that *hemF* is not a member of the PpsR regulon (15). Putative DNA binding sites of the response regulatory protein PrrA are difficult to identify by DNA sequence inspection alone, as there is low conservation of the recognition elements and because the distance between half sites is variable (13). Therefore, we evaluated the role of PrrA in *hemF* expression using the *hemF*::*lacZ* transcriptional reporter plasmid pJZ84 (Table 1). β-Galactosidase activities in extracts of wild-type 2.4.1 versus PrrA$^-$ mutant strain PRRA2 cells having the reporter plasmid were assayed according to previously described methods (9). As indicated in Table 3, enzyme activity levels are approximately threefold lower in extracts of anaerobically versus aerobically grown wild-type 2.4.1 cells. By contrast, we found β-galactosidase activity levels are approximately 1.6-fold higher in extracts of anaerobically cultured PRRA2 mutant cells than in extracts of cells grown aerobically.

We also probed membranes of total protein from anaerobically grown wild-type 2.4.1 and PRRA2 mutant cells with anti-HemF antiserum. As shown in Fig. 3, while no HemF protein could be detected in the wild-type 2.4.1 lysate, it is clearly discernible in the lysate of the PrrA$^-$ mutant cells.

Conclusions. Based on our results, we conclude that the *hemF* gene of *Rhodobacter sphaeroides* 2.4.1 is required for colony formation in the presence of air or for growth of liquid cultures sparged with air. These phenotypes are consistent with the *hemF* expression pattern we discerned at the protein level using immunoblot analysis; namely, HemF levels are reduced in cells grown under anaerobic conditions compared to levels present in aerobically grown cells.

Our measurements of transcription using a *hemF*::*lacZ* reporter plasmid indicate that *hemF* is regulated at the level of transcription, which is consistent with transcript measurements reported by Moskvin et al. (15). We also found that downregulation of *hemF* when oxygen tensions are reduced requires an intact *prrA* gene. A recent description of PrrA regulation in *R. sphaeroides* indicates that although PrrA positively affects transcription for many genes belonging to its regulon, it can also function as a negative effector of transcription (11). Based on our results, this is true for the *hemF* gene. The data also suggest there may be further complexities associated with regulated expression of *hemF*, since β-galactosidase activities measured in extracts of cells grown aerobically differ by approximately 56% in the PrrA$^-$ strain PRRA2 versus wild-type 2.4.1.

Panek and O'Brian (19) found that, unlike *hemN* genes, which code for enzymes that catalyze oxygen-independent formation of protoporphyrinogen IX, *hemF* genes are not widely represented among bacteria. Further, all bacteria having *hemF* genes also have *hemN* genes. For several species of bacteria that have *hemF*, the *hemN* gene alone is capable of meeting the cellular needs for protoporphyrinogen IX regardless of the presence or absence of oxygen (21, 25, 26), and measurements of *hemN* and *hemF* transcript levels in *E. coli* K-12 indicate that those genes are not greatly affected by varying oxygen tensions (3). By contrast, in *R. sphaeroides* 2.4.1, transcription of *hemN* and *hemZ*, coding for putative oxygen-independent isoenzymes (2, 29), are induced by lowering oxygen tensions in an FnrL-dependent manner (18, 27). Now we have found that *hemF* expression is low in the presence of low or no oxygen and high when oxygen tensions are high. We suggest that, for *R. sphaeroides* 2.4.1, oxygen availability not only dictates what catalytic mechanism can be used for protoporphyrinogen IX formation but also which enzyme type is present in concentrations that are sufficient to support growth.

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