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Control of *hemA* Expression in *Rhodobacter sphaeroides* 2.4.1: Regulation through Alterations in the Cellular Redox State

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***Rhodobacter sphaeroides* 2.4.1 has the ability to synthesize a variety of tetrapyrroles, reflecting the metabolic versatility of this organism and making it capable of aerobic, anaerobic, photosynthetic, and diazotrophic growth. The *hemA* and *hemT* genes encode isozymes that catalyze the formation of 5-aminolevulinic acid, the first step in the biosynthesis of all tetrapyrroles present in *R. sphaeroides* 2.4.1. As part of our studies of the regulation and expression of these genes, we developed a genetic selection that uses transposon mutagenesis to identify loci affecting the aerobic expression of the *hemA* gene. In developing this selection, we found that sequences constituting an open reading frame immediately upstream of *hemA* positively affect *hemA* transcription. Using a transposon-based selection for increased *hemA* expression in the absence of the upstream open reading frame, we isolated three independent mutants. We have determined that the transposon insertions in these strains map to three different loci located on chromosome I. One of the transposition sites mapped in the vicinity of the recently identified *R. sphaeroides* 2.4.1 homolog of the anaerobic regulatory gene *fnr*. By marker rescue and DNA sequence analysis, we found that the transposition site was located between the first two genes of the *cco* operon in *R. sphaeroides* 2.4.1, which encodes a cytochrome *c* terminal oxidase. Examination of the phenotype of the mutant strain revealed that, in addition to increased aerobic expression of *hemA*, the transposition event also conferred an oxygen-insensitive development of the photosynthetic membranes. We propose that the insertion of the transposon in cells grown in the presence of high oxygen levels has led to the generation of a cellular redox state resembling either reduced oxygen or anaerobiosis, thereby resulting in increased expression of *hemA*, as well as the accumulation of spectral complex formation. Several models are presented to explain these findings.**

Rhodobacter sphaeroides 2.4.1, by virtue of its metabolic versatility, would be expected to possess a diversity of regulatory circuits and mechanisms of gene control that enable it to rapidly adapt to changing environmental conditions. A snapshot of the metabolic versatility inherent to this organism is the presence of the four major physiologically active tetrapyrroles, i.e., hemes, bacteriochlorophylls (Bchls), vitamin B_{12}, and siroheme, which aptly illustrates the variety of growth conditions to which this organism is capable of adapting. Underlying the presence of these important metabolites is the tetrapyrrole biosynthesis pathway which displays a variety of regulatory features, notably oxygen and light control (see reference 20).

The first step in the tetrapyrrole pathway in *R. sphaeroides* 2.4.1 is the condensation of succinyl-coenzyme A and glycine to 5-aminolevulinic acid (ALA), catalyzed by the enzyme ALA synthase (succinyl-coenzyme A:glycine *C*-succinyl transferase [decarboxylating] [EC 2.3.1.37]). Unlike all other prokaryotic organisms in which this enzymatic activity has been identified, *R. sphaeroides* 2.4.1 is the only prokaryote known to possess two genes, *hemA* and *hemT*, that encode ALA synthase isozymes, with *hemA* localized to chromosome I and *hemT* localized to chromosome II.

Several studies from our laboratory have shown that *hemA* is expressed under most standard growth conditions, while *hemT* is transcriptionally off (27, 37). However, *hemT* can be activated by both *cis*- and *trans*-acting mutations which we believe are symbolic of a set of growth conditions that can normally turn on expression of *hemT* (37). Further, we have documented that the DNA sequences 5′ to the coding regions for both *hemA* and *hemT* are completely different (27), and thus we would anticipate that each gene should have its own regulatory controls.

As a first step in understanding the regulatory complexity involved in the control of *hemA* and *hemT* expression, we have focused our attention on the FNR-binding consensus sequence located upstream of *hemA*. As presented elsewhere (36), we have documented the presence of an *fnr* homolog in *R. sphaeroides* 2.4.1 and its role in the anaerobic regulation of *hemA* expression. Adding to the regulatory complexity of *hemA*, we have further documented the existence and role of an open reading frame upstream of *hemA* designated ORFA2.

Here, we exploit the power of transposon mutagenesis to reveal yet another *trans*-acting region, linked to *frrL*, that plays a role in *hemA* expression and in photosynthetic membrane development. The results of these studies suggest the existence of a relationship between the redox state of the cell and gene expression. On the basis of these results, we present a model that incorporates many of our recent findings.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains of *R. sphaeroides* 2.4.1 and *Escherichia coli* and the plasmids used in this study are listed in Table 1. Growth of both *R. sphaeroides* (3, 4) and *E. coli* (24) were described previously. Cell densities of *R. sphaeroides* were determined at 660 nm with a Lambda 4C spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.). To provide plasmid selection and maintenance, media were supplemented with antibiotics where appropriate. Final concentrations for *R. sphaeroides* were 0.8 μg/ml for tetracycline and 50 μg/ml (each) for spectinomycin, streptomycin, trimethoprim (TMP), and kanamycin (KAN), unless otherwise noted. For *E. coli*,

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference(s)
R. sphaeroides		
2.4.1	Wild type	W. Sistrom
JZ722	*har-1*; Fig. 2	This study
JZ723	*har-2*; Fig. 2	This study
JZ724	*har-3*; Fig. 2	This study
E. coli		
DH5α	(ϕ80d*lacZ*ΔM15) Δ*lacU169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*	16
DH5α*phe*	Same as DH5α but with *phe*::Tn*10d*Cm	7
Plasmids		
pRK415	Tc^r	18
pLA2917	Cosmid vector; Tc^r	1
pUI8180	Cosmid spanning region of *har-1*	5, 36
RSF1010	Tc^r	15
pUI1957	*Bam*HI subclone of pUI8180, bearing the *ccoNOQP* and *rdxBHIS* operons in pRK415	36
pUI1989	Recombinant JZ722 × pUI8180	This study
pUI1063	$\Omega Sp^r/St^r$-*hemA*::*lacZYA′* in RSF1010	36
pUI1088	$\Omega Sp^r/St^r$-ORFA2-*hemA*::*lacZYA′* in RSF1010	36
pUI1080	$\Omega Sp^r/St^r$-*hemA*::*aph* in pRK415	This study
pSUPTn*5*TpMCS	Suicide vector bearing Tn*5*Tp	2
pUI1927	$\Omega Sp^r/St^r$-*hemA*::*aph* in pRK415; Fig. 1	This study
pUI1902	$\Omega Sp^r/St^r$-ORFA2-*hemA*::*aph* in pRK415; Fig. 1	This study
pCF200	$\Omega Sp^r/St^r$-*puc*::*lacZYA′* in RSF1010	22
pUI1830	$\Omega Sp^r/St^r$-*puf*::*lacZYA′* in RSF1010	L. Gong

the final concentrations were 15 μg/ml for tetracycline and 50 μg/ml (each) for KAN, spectinomycin, streptomycin, and TMP; for TMP selection, minimal M63 medium was used (24). Reagent-grade antibiotics were used and were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Conjugation. Mobilization of plasmids into *R. sphaeroides* was performed by previously described protocols (3). K_2TeO_2 was incorporated into the media when necessary for the counterselection of *E. coli* donor strain(s).

Transposon mutagenesis. Mutagenesis with the Tn*5* transposon bearing TMP resistance was performed by conjugation of *R. sphaeroides* with the suicide vector plasmid pSUPTn*5*TpMCS (2), followed by selection of exconjugants on media containing TMP.

DNA manipulations and DNA sequence analysis. For the isolation of plasmid DNA, restriction endonuclease treatment, and other enzymatic treatment of DNA fragments and plasmids, standard protocols or manufacturers' instructions were followed, with enzymes purchased from New England BioLabs, Inc. (Beverly, Mass.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), Bethesda Research Laboratories Life Technologies, Inc. (Gaithersburg, Md.), and Promega Corp. (Madison, Wis.). DNA analysis was performed by standard electrophoretic techniques, and pulsed-field agarose gel electrophoresis was performed as previously described (31). DNA transfer and Southern hybridization analysis were performed according to previously described protocols (36), or manufacturers' instructions with the NEBlot Phototope Kit (New England Biolabs, Inc.).

DNA sequencing was performed at the DNA Core Facility of the Department of Microbiology and Molecular Genetics, University of Texas Health Science Center, Houston, with an ABI 373A automatic DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.). Oligonucleotides used for priming standard sequencing reactions were synthesized at the DNA Core Facility of the Department of Microbiology and Molecular Genetics.

DNA sequence analysis. DNA sequences were analyzed by using the program manual for the Wisconsin Package, version 8.0 (11a). Sequence alignment was done with the GAP program.

Spectral analysis of membrane fractions. Crude cell-free lysates were prepared by sonication for 2 min in ICM buffer (10 mM KPO_4, 1 mM EDTA; pH 7.2) with a Sonifier Cell Disruptor (Branson Sonic Power Co., Danbury, Conn.), followed by centrifugation at top speed (15,400 × *g*) in a microcentrifuge at 4°C to remove unbroken cells and cell debris. Spectra were recorded with a Lambda 4C spectrophotometer (Perkin-Elmer Corp.). The B875 and B800-850 complex levels were determined by the method of Meinhardt et al. (25) from the spectral data.

Bchl and Crt analyses. Bchl and carotenoid (Crt) levels were determined in extracts of cells grown in Sistrom's minimal medium with sparging of compressed air. The extraction and quantitation of pigments were performed as previously described (35).

β-Galactosidase assays. Assays of β-galactosidase activity were performed on preparations of crude cell extracts as described elsewhere (32). Reagent-grade *o*-nitrophenyl-β-D-galactopyranoside, purchased from Sigma Chemical Co., was used as the substrate.

Protein determinations. The concentration of protein in extracts subjected to spectral analysis or assayed for β-galactosidase activity was determined with the Pierce BCA Protein Assay Reagent (Pierce, Rockford, Ill.). In all cases, bovine serum albumin was used as a reference.

RESULTS

Defining the region upstream of *hemA* required for aerobically regulated expression. Prior to the construction of a genetic selection allowing us to identify *trans*-acting loci that affect *hemA* expression, it was essential to define what DNA sequences upstream of *hemA* were required for the regulated expression of *hemA*. While the apparent start sites of *hemA* transcription have been defined (27), no sequences upstream of *hemA* that are similar to the typical −35 and −10 consensus promoter sequences have been identified. Thus, it was not obvious what DNA sequences were required for the full range of *hemA* expression. An intriguing dissimilarity between the *hemA* genes of *R. sphaeroides* 2.4.1 and *Rhodobacter capsulatus* is the presence in *R. sphaeroides* 2.4.1 of an open reading frame (ORFA2) immediately upstream of *hemA* (27) which is absent from *R. capsulatus*. While the DNA sequences upstream of ORFA2 have approximately 75% similarity (with one gap) over 892 nucleotides with those sequences found upstream of the *hemA* gene of *R. capsulatus*, and the *hemA* sequences of the two organisms are themselves 77% identical, there is no DNA sequence homology between *R. capsulatus* and *R. sphaeroides* 2.4.1 in the region of the ORFA2 sequence. We have not identified sequences in GenBank, either at the nucleotide or protein sequence level, that bear any resemblance to ORFA2 sequences.

Studies of the mRNAs transcribed from this region of the genome by Neidle and Kaplan (27) suggest that ORFA2 and *hemA* are not cotranscribed. Primer extension studies identified a transcription initiation site for the ORFA2 message 24 bp upstream of the start codon for translation (27). Sequences at −35 and −10 indicate the presence of a promoter upstream of ORFA2 on the basis of their similarity to the promoter

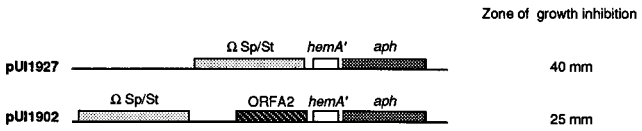

FIG. 1. Schematic diagram of plasmids used to evaluate expression levels of *hemA* with and without ORFA2. Plasmids pUI1927 and pUI1902 are identical, except pUI1902 includes ORFA2 while pUI1927 does not contain ORFA2. Zones of growth inhibition of wild-type 2.4.1 exconjugants bearing either pUI1927 or pUI1902 were determined as described in footnote *a* to Table 2.

region upstream of the *R. sphaeroides* 2.4.1 *rrnB* operon (6, 27). These studies also identified a transcription initiation site for the *hemA* message 79 bp upstream of the start codon for translation under aerobic conditions, while two initiation sites are observed under anaerobic conditions, with the major start site corresponding to the initiation site observed under aerobic conditions and the second site located 37 bp upstream of the start codon for translation. Given the lack of identifiable promoter sequences upstream of *hemA*, the presence of ORFA2, and the absence of an obvious terminator sequence between ORFA2 and *hemA*, it was necessary to evaluate the contribution, if any, of ORFA2 to the expression of *hemA*. We constructed two plasmids bearing transcriptional fusions between *hemA*, with and without ORFA2, and the *aph* gene from Tn*903* encoding KAN resistance (Fig. 1). Each plasmid was introduced into wild-type 2.4.1, and the exconjugants were compared with respect to the level of KAN resistance conferred by each plasmid. Those exconjugants containing the plasmid which included ORFA2 had significantly higher levels of KAN resistance, reflected in the smaller diameter of the zone of inhibited growth around a filter disk containing KAN, as shown in Fig. 1. Two parallel plasmid constructs positioned the same sequences upstream of the *sacB* gene of *Bacillus subtilis* (29). When these plasmids were introduced into wild-type 2.4.1, sucrose sensitivity was increased for those exconjugants containing ORFA2 relative to those lacking ORFA2. In the former, no growth was observed on media containing 1% sucrose, while growth was observed on 1% sucrose for those exconjugants containing plasmids that did not include ORFA2. Thus, two independent analyses confirmed the increased expression from *hemA* when ORFA2 sequences linked to *hemA* were present. These results also indicated, however, that the fusion lacking ORFA2 was still capable of *hemA* expression, which has been confirmed in other studies employing *lacZ* fusions to constructs similar to those employed here (36). Furthermore, the sequences present upstream of *hemA* but excluding ORFA2 are also sufficient for oxygen-regulated expression of *hemA* (36). Therefore, in order to select for *trans*-acting mutations leading to increased expression of *hemA*, we excluded from the plasmid construction ORFA2 but included 246 bp upstream of the start site for translation of *hemA*. This sequence also includes the FNR-binding consensus sequence and was fused to the *aph* gene. The plasmid construction was then used in 2.4.1 to select, under aerobic conditions, for increased resistance to KAN. This construction lacking ORFA2 gives us a greater range when selecting for increased KAN resistance, since the baseline resistance is very low in strains lacking ORFA2.

Transposon-induced *trans*-acting mutations affecting the expression of *hemA*. Plasmid pUI1080 (Table 1), containing a transcriptional fusion between 306 bp of *hemA* sequences, beginning 246 bp upstream of the translational start of *hemA* and ending at the 20th codon of *hemA*, and the gene encoding KAN resistance from Tn*903*, was first introduced into wild-type 2.4.1. In a second step, the suicide plasmid pSUP Tn*5*TpMCS (Table 1), bearing a Tn*5* transposon encoding TMP resistance was introduced into strain 2.4.1 (pUI1080), and the exconjugants were plated on media containing 50 μg (each) of KAN and TMP per ml. Thus, we selected for both the transposition event (Tp^r) and increased transcription from sequences upstream of *hemA*, elevating the level of KAN resistance. From this selection, eight initial isolates were obtained. Among these, the isolates with the highest level of KAN resistance, JZ722, JZ723, and JZ724, were analyzed further.

TABLE 2. Relative resistance to KAN of *R. sphaeroides* strains

Strain	Diam (mm) of zone of inhibition[a]	Growth[b] on KAN (10 μg/ml)
JZ722(pUI1080)	25	+++
JZ723(pUI1080)	30	+++
JZ724(pUI1080)	28	+++
2.4.1(pUI1080)	40	−−−

[a] Zone of inhibition determined with 25 μl of a 50-mg/ml solution of KAN placed on a filter disk positioned in the center of a lawn of cells.

[b] +++, growth equivalent to that of wild-type 2.4.1. in the absence of KAN; −−−, no growth observed after 4 days of incubation at 30°C.

Loss of the resident pUI1080 plasmid and reintroduction of the same plasmid from *E. coli* with retention of the mutant phenotype demonstrated that each isolate showing elevated Kn^r contained a *trans*-acting mutation. Each of the three independent transposon-bearing strains was compared with wild-type 2.4.1 with regard to the level of KAN resistance following loss and reintroduction of pUI1080, and the results are presented in Table 2. Both by zone of inhibition and growth on 10 μg of KAN per ml, each of the three mutant strains showed increased aerobic expression of *hemA*::*aph*. The loci thus identified were designated *har-1* (in JZ722), *har-2* (in JZ723), and *har-3* (in JZ724) (*har* stands for *hemA* regulator), and each was mapped to a precise location on chromosome I of *R. sphaeroides* 2.4.1, with the exception of *har-2*, as shown in Fig. 2

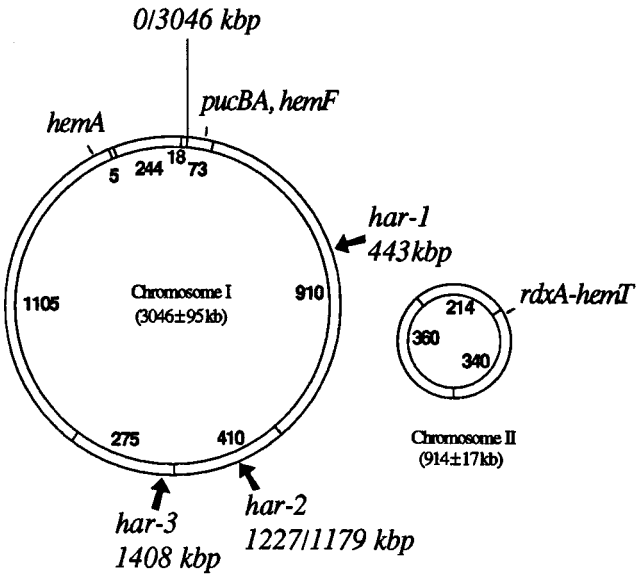

FIG. 2. Positions of transposon insertions on the physical map of *R. sphaeroides* 2.4.1. Approximate coordinates are provided in kilobase pairs relative to the zero position indicated. Numbers within the circles depicting chromosomes I and II of the *R. sphaeroides* 2.4.1 genome refer to the approximate sizes of the DNA fragments generated when chromosomal DNA is digested with *Ase*I.

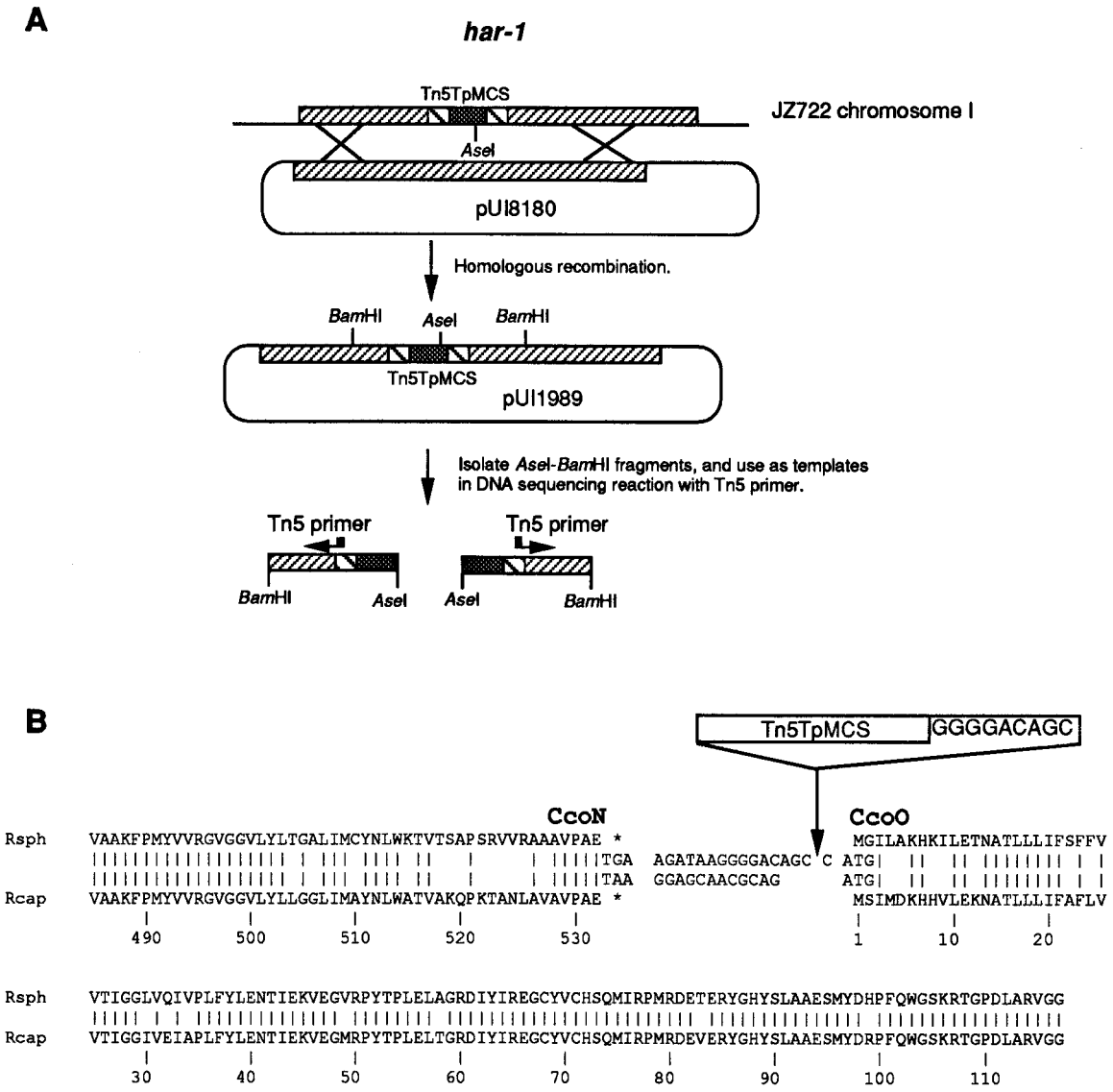

FIG. 3. Isolation of recombinant cosmid containing the transposon and precise location of the transposon insertion in strain JZ722. (A) Schematic diagram of steps involved in obtaining the recombinant cosmid DNA containing the transposon and the DNA sequence analysis of the transposon junctions. (B) Sequence alignment and location of the transposon insertion in JZ722. Rsph, predicted CcoN and CcoO protein sequences and DNA sequence of the residues between the open reading frames for JZ722, together with the location of the transposon. Rcap, predicted protein sequences for CcoN and CcoO of *R. capsulatus* (33).

(data not shown); the *har-2* locus could not be unambiguously localized between the two positions indicated.

Defining the *har-1* locus. One of the transposon insertion sites, *har-1*, mapped to a region of chromosome I that we had in a parallel study determined to contain the *rdxB* gene present in cosmid pUI8180 (see reference 36). By Southern hybridization analysis of JZ722 chromosomal DNA using restriction fragments derived from cosmid pUI8180, we determined the presence of DNA sequences spanning the new *Ase*I junction created by insertion of the transposon at the *har-1* locus (results not shown). We identified by Southern hybridization an approximately 13-kbp *Bam*HI fragment of JZ722 chromosomal DNA, using the transposon DNA as a probe. This finding was consistent with insertion of the 5-kbp transposon into a previously identified 8-kbp *Bam*HI fragment that was present among the *Bam*HI fragments generated from pUI8180 DNA (results not shown). By introducing cosmid pUI8180 into JZ722, we were able to rescue the transposon and flanking DNA from the chromosome onto the cosmid by homologous recombination (Fig. 3A). The plasmid containing the marker-rescued DNA fragment was identified among pools of plasmid DNA isolated from JZ722 by transforming *E. coli* and selecting for TMP resistance. Restriction analysis of the recombinant transposon-bearing cosmid with *Bam*HI endonuclease revealed that the transposon resided in a DNA fragment corresponding to the same 8-kbp fragment present in the wild-type cosmid DNA. We isolated and purified the two unique *Ase*I-*Bam*HI restriction fragments (created by the insertion of a unique *Ase*I site resident within the transposon DNA) from the recombinant cosmid and used each as a template for DNA sequencing with a primer corresponding to the sequence at either end of the transposon, as shown schematically in Fig. 3A. This analysis revealed the precise location of the transposon in the original *har-1* mutation. As confirmation of the DNA se-

TABLE 3. β-Galactosidase activity from cell extracts of *R. sphaeroides* 2.4.1 strains bearing *hemA*::*lacZ* transcriptional fusion plasmids[a]

Strain	β-Galactosidase activity[b]		Relative activity
	$\bar{x}$	σ_n	
2.4.1(pUI1063)	20	1	1
JZ722(pUI1063)	77	2	4
2.4.1(pUI1088)	802	85	40
JZ722(pUI1088)	1,311	141	66

[a] The strains were grown in Sistrom's minimal medium in 20% O_2, supplemented with antibiotics as appropriate.

[b] Equal to micromoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein extract. Average values ($\bar{x}$), with the standard deviations (σ_n) provided, are the averages of at least two independent determinations. β-Galactosidase activity of the vector alone in wild-type 2.4.1 is 4 U.

quences flanking the transposon insertion site, we performed DNA sequence analysis with cosmid pUI8180 and the transposon-bearing derivative as the templates and oligonucleotide primers whose sequences we derived from DNA sequence analysis of the restriction fragments. By comparing the DNA sequences flanking the transposon insertion site to sequences in GenBank, we found this region to be homologous to the *ccoNOQP* region of *R. capsulatus* (33). Although this region has not been shown at the mRNA level to comprise an operon, we consider these genes to be cotranscribed on the basis of high similarity of the DNA sequences and spacing of the open reading frames to the homologous *fixNOQP* operons of *Rhizobium meliloti* and *Bradyrhizobium japonicum*. Alignment of the predicted amino acid sequences revealed that the position of the transposon insertion site corresponded to DNA sequences downstream of the *ccoN* gene and 10 bp upstream of the start codon for the CcoO protein of *R. sphaeroides* 2.4.1, as shown in Fig. 3B. This was also consistent with the predicted location of the transposon deduced from the previously established map of the region (see reference 36).

Analysis of *hemA* expression in JZ722 using *hemA*::*lacZ*. To quantitate the effect of the *har-1* mutation on *hemA* transcription, we used plasmids bearing transcriptional fusions between the *hemA* upstream sequences and the *lacZ* gene of *E. coli*. These results are presented in Table 3. For pUI1063, which does not include ORFA2 (Table 1) but does include the FNR-binding consensus sequence and extends 167 bp upstream of the most 5′ transcription initiation site defined for the *hemA* message, the enzymatic activity in extracts of JZ722 cultures was approximately fourfold higher than that in extracts of wild-type 2.4.1 cells. For the reporter plasmid that also contains ORFA2 sequences, pUI1088 (Table 1), the difference in β-galactosidase activity found in extracts of JZ722 and 2.4.1 was approximately 60% higher for JZ722, although the absolute levels of β-galactosidase activity were higher when ORFA2 was present. These values were obtained for cultures grown aerobically. Regardless of the presence or absence of ORFA2 sequences, expression from the *hemA* upstream sequences was higher in JZ722 than in wild-type 2.4.1. As also described elsewhere (36), the presence or absence of ORFA2 shows a substantial effect on *hemA* expression, regardless of the presence or absence of FnrL, the *R. sphaeroides* Fnr homolog. Further, as described above, we did see a greater relative range in *hemA* expression when ORFA2 was not present (4-fold) than when it was present (1.6-fold). Therefore, our initial choice in excluding the ORFA2 sequence from our selection vector appears to have been validated.

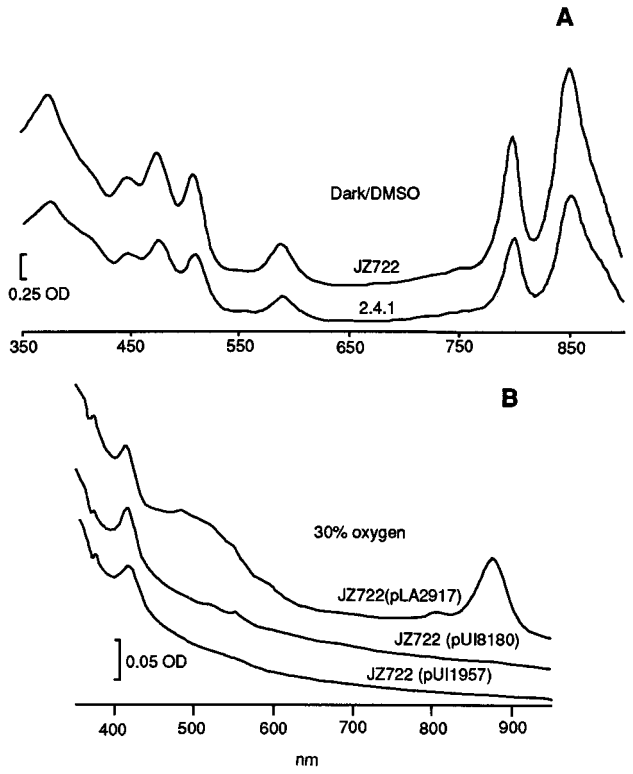

FIG. 4. Spectral analysis of *R. sphaeroides* strains. (A) Spectra of JZ722 and 2.4.1 from cells grown in the dark with DMSO. (B) Spectra of JZ722 exconjugant cell extracts grown in 30% oxygen. OD, optical density.

Phenotype of strain JZ722. It was not immediately obvious how an insertion into the intercistronic region of the *ccoNOQP* operon could lead to higher levels of *hemA* transcription under fully aerobic conditions. To both address this question and more fully describe the phenotype of *har-1*, we analyzed the ability of JZ722 to grow under aerobic, photosynthetic, and dark-dimethyl sulfoxide (DMSO) conditions, as well as under nitrogen-fixing conditions. Under all of these culture conditions, growth of JZ722 was similar to that of the wild type (results not shown). However, aerobic cultures were consistently observed to contain higher levels of pigmentation than those of wild-type 2.4.1. Aerobic pigmentation has also been observed for PrrB mutants that aberrantly produce photosynthetic complexes under aerobic conditions (8), as well as in mutants of the repressor molecule PpsR (13). To determine if this was also the case for JZ722, we prepared cell extracts for spectral analysis from cultures grown in the dark in the presence of DMSO and from cultures grown aerobically in 30% oxygen. Analysis of the extracts from anaerobic dark-DMSO cultures revealed that the level of photosynthetic complexes was approximately twofold higher in JZ722 than in wild-type 2.4.1 (Fig. 4A). Spectral analysis of membranes prepared from cultures grown aerobically in 30% oxygen revealed that mutant strain JZ722 expressed the photosynthetic apparatus under aerobic conditions, and importantly, the cellular levels of spectral complexes were highly sensitive to the cell density under these growth conditions. Under these identical growth conditions and at equivalent cell densities, spectral complexes were below detectable levels in wild-type 2.4.1. The relative levels of the pigment-protein complexes are presented in Table 4. We tested the abilities of the cosmid pUI8180 and a subclone that

TABLE 4. Levels of spectral complexes in membrane preparations of *R. sphaeroides* 2.4.1 strains[a]

Strain	Growth conditions	Cell density[b]	Level[c] of spectral complex		[B875/B800-850] ratio
			B800-850	B875	
2.4.1	Dark, DMSO	3.6	32.9	12.8	0.4
JZ722	Dark, DMSO	4.6	60.7	22.0	0.4
2.4.1	30% oxygen	1.6	BD[d]	BD	BD
JZ722	30% oxygen	1.7	0.4	2.5	6.3
2.4.1	30% oxygen	3.1	BD	BD	BD
JZ722	30% oxygen	3.2	3.2	12.8	4.0
JZ722(pLA2917)	30% oxygen	0.8	0.4	2.8	7.0
JZ722(pUI8180)	30% oxygen	0.9	BD	BD	BD
JZ722(pUI1957)	30% oxygen	1.5	BD	BD	BD

[a] Cells were cultured either anaerobically in the dark with DMSO or aerobically by sparging with a mixture of 30% O_2, 1% CO_2, and 69% N_2.
[b] Cell densities are expressed in 10^8 cells per milliliter.
[c] The levels of the spectral complex components were determined as previously described (25) and are expressed as nanomoles of spectral complex per milligram of crude membrane protein.
[d] BD, below detection.

contains *ccoNOQP* (pUI1957 [Table 1]) to complement the oxygen-insensitive expression of the photosynthetic apparatus by spectral analysis of extracts prepared from JZ722 exconjugants grown aerobically. The results of this analysis, shown in Fig. 4B, revealed that when the *ccoNOQP* operon was present in *trans* in mutant JZ722, photosynthetic complexes were below the detection limit, while JZ722 bearing pLA2917, the cosmid vector (Table 1), contained photosynthetic membranes. The relative levels of the pigment-protein complexes present in all of these extracts were determined, and the values are presented in Table 4.

The *trans* complementation of strain JZ722 by both the cosmid and a subclone containing the *ccoNOQP* operon suggests that the only mutation in JZ722 is the one introduced by the insertion of the transposon, which itself is present only in single copy in the mutant strain. Thus, the transposon insertion in JZ722 represents a second class of mutation, in addition to that represented by PRRB78 (8), that leads to expression of the photosynthetic apparatus in the presence of oxygen. A clear distinction between the two classes is that, while PRRB78 is highly unstable, which is reflected in the variegated pigmentation of colonies incubated aerobically, this was not observed for JZ722, which has a uniform colony pigmentation, irrespective of whether Tp is present.

Analyses of Crt and Bchl levels in JZ722. As has been demonstrated previously, the presence of Bchl stabilizes the apoproteins of the reaction center (34), and it has been proposed that the assembly and stabilization of the B800-850 spectral complexes are contingent on the presence of not only Bchl but also the Crt spheroidene (8). To determine whether the levels of Crt and Bchl in JZ722 were different than in wild-type 2.4.1, Crt and Bchl concentrations were compared in extracts from wild-type and mutant cells cultured under highly aerobic conditions. At a cell density of 1.6×10^8 cells per ml, the total levels of Crt and Bchl in extracts of wild-type 2.4.1 were 250 and 310 ng/10^8 cells, respectively. At a cell density of 1.7×10^8 cells per ml for mutant JZ722, the Crt level was 760 and the Bchl level was 1,060 ng/10^8 cells. Thus, both Crt and Bchl levels were approximately threefold higher in mutant JZ722 extracts than in wild-type 2.4.1 extracts.

Analyses of *puc* and *puf* expression in JZ722 using *puc*::*lacZ* and *puf*::*lacZ*. The presence of photosynthetic membrane complexes under aerobic conditions may indicate a change in the level of transcription of genes encoding structural polypeptides of the photosynthetic apparatus. To determine if such was the case for JZ722, we assessed transcription levels of the *puc* and *puf* operons, using plasmids bearing transcriptional fusions to *lacZ*. The results are shown in Table 5 and show that at most there are a twofold-higher β-galactosidase activity in extracts of JZ722 bearing the *puc*::*lacZ* plasmid pCF200 than in extracts of wild-type 2.4.1 bearing pCF200 and a threefold-higher activity in extracts of JZ722 bearing the *puf*::*lacZ* plasmid pUI1830 than in extracts of wild-type 2.4.1 bearing pUI1830. When the same strains were grown anaerobically in the dark with DMSO, increases in the β-galactosidase activity in both JZ722 and wild-type extracts were found for both the *puc* and *puf* transcriptional fusions compared with that in cells grown aerobically. Thus, while the transposon insertion has resulted in the development of photosynthetic membranes under aerobic conditions, the expression of these operons still remains responsive to changes in oxygen tension. However, the levels of β-galactosidase activity in extracts of JZ722 bearing these transcriptional fusion plasmids were similar to or lower than the activity in extracts of wild-type exconjugants cultured anaerobically in the dark. One reasonable explanation is that the limiting reactant leading to increased spectral complex formation in JZ722 under anaerobic conditions is not the cellular abundance of transcripts encoding the apoproteins corresponding to each spectral complex. Eraso and Kaplan (8) have shown for mutant PRRB78 that both *puf* and *puc* operon transcription under aerobic conditions are increased approximately 8- and 20-fold from wild type, respectively. Since the

TABLE 5. β-Galactosidase activity from cell extracts of *R. sphaeroides* 2.4.1 strains a bearing *puf*::*lacZ* or *puc*::*lacZ* transcriptional fusion plasmid grown in 30% oxygen[a]

Strain	β-Galactosidase activity[b]			
	With 30% O_2		In the Dark with DMSO	
	$\bar{x}$	σ_n	$\bar{x}$	σ_n
2.4.1(pCF200)	275	72	1329	103
JZ722(pCF200)	481	91	849	103
2.4.1(pUI1830)	83	16	384	42
JZ722(pUI1830)	240	51	479	115

[a] The strains were grown in Sistrom's minimal medium, supplemented with antibiotics as appropriate.
[b] Equal to micromoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein extract. Average values ($\bar{x}$), with the standard deviations (σ_n) provided, are the averages of at least three independent determinations. β-Galactosidase activity of the vector alone in wild-type 2.4.1 is 4 U.

amounts of the spectral complexes under both aerobic and anaerobic conditions are the same for JZ722 and PRRB78 (8), the mechanisms leading to this common phenotype are different for the two strains.

DISCUSSION

The genetic screen described here has led to the isolation of mutant strains showing increased expression of *hemA* in the presence of oxygen. The locations of the transposon and therefore presumably the loci affecting *hemA* expression were placed on the physical map of *R. sphaeroides* 2.4.1 by mapping the positions of new restriction sites introduced by insertion of the transposon. Each insertion maps to a different location on chromosome I. One of these insertions is closely linked to the *rdxB* gene (see reference 36), and its exact position, determined at the nucleotide level, is immediately upstream of the start codon for translation of the second gene in the *R. sphaeroides* 2.4.1 *ccoNOQP* operon, which is also immediately downstream of *ccoN*. Thus, each structural gene of the operon remains intact.

The Cco protein complexes have been purified from both *R. sphaeroides* (11) and *R. capsulatus* (14) and characterized biochemically. They encode *cb*-type cytochrome *c* oxidases, in which *ccoN* is thought to encode the oxidase active site, while *ccoO* and *ccoP* are thought to encode components of the electron donor chain, and the function of *ccoQ* is unknown (10, 33). In *R. capsulatus*, the entire operon has been disabled through the insertion of an omega cassette in *ccoN*, the first gene of the operon (33). This mutant was reported to be unaltered with respect to its ability to grow under a variety of conditions, including nitrogen-fixing conditions (33). While in some organisms, such as *R. meliloti*, these genes are essential for symbiotic nitrogen fixation, they have recently been identified in bacteria that are not believed to be capable of fixing nitrogen, such as *Agrobacterium tumefaciens* (30, 33) and *Pseudomonas aeruginosa* (33). Thus, it seems reasonable to conclude that this operon and encoded function(s) are probably not directly involved in the metabolic process of fixing nitrogen. The observed phenotype in *R. meliloti* could be more complex and explained by the participation of these gene products, or more likely the redox state or redox intermediates which they help to maintain, in the mechanism(s) that regulates expression of at least the nitrogen fixation genes.

A second operon, *rdxBHIS* of *R. sphaeroides* 2.4.1, which bears homology to the *fixGHIS* operon of *R. meliloti* and *B. japonicum* (36), may also be involved in the same redox process. In all of these bacteria, this operon is located downstream of the *fixNOQP* operon (or the analogous *ccoNOQP* in *R. sphaeroides* 2.4.1). The only known phenotype for the *fixGHIS* operon is the Fix phenotype first identified in *R. meliloti* (17). In *R. sphaeroides* 2.4.1, *rdxBHIS* and *rdxA*, a homolog of *rdxB*, are both present, but these genes are not essential for nitrogen fixation, since a mutant strain with an omega cassette insertion in both *rdxA* and *rdxB*, presumably polar to the genes downstream of *rdxB*, is able to grow diazotrophically (28). Inspection of the predicted protein sequences of FixG and RdxA reveals the presence of two conserved [4Fe-4S] clusters, suggestive of a redox function for these proteins.

Since the transposon in JZ722 is inserted between the *ccoN* and *ccoO* genes, it is likely, although not proven, that the levels of the downstream gene products, including CcoO, CcoP, and CcoQ, in JZ722 are different from those in the wild-type 2.4.1, while the levels of CcoN may remain unaffected. Whether the transposon insertion has resulted in increased or decreased expression of the genes downstream of the insertion site has not been established. In other organisms, expression of the analogous *fixNOQP* operon is reported to be activated by the *fixK* gene product under anaerobic conditions. The role of FixK in *R. meliloti* is similar to Fnr in *E. coli* and presumably to FnrL in *R. sphaeroides* 2.4.1, in that it binds to a DNA sequence identical to the FNR-binding consensus sequence and regulates the anaerobic expression of a number of genes. In *R. sphaeroides* 2.4.1, an FNR-binding sequence has been identified upstream of *ccoN* (36), suggesting that this operon is regulated in wild-type cells by anaerobiosis mediated through FnrL. This seems a reasonable assumption in light of the fact that we have determined that expression of the *hemA* gene, whose upstream sequence also contains a consensus FNR-binding sequence, is regulated by FnrL (36). A consensus FNR-binding sequence is also present upstream of the *fixGHIS* operons in *R. meliloti* and *B. japonicum*, and a sequence with two mismatches to the consensus has been identified upstream of *rdxBHIS* in *R. sphaeroides* 2.4.1 (36). The presence of these FNR consensus sequences establishes a tentative connection between the *cco* operon and the *hemA* gene and possibly the *rdxB* operon as well.

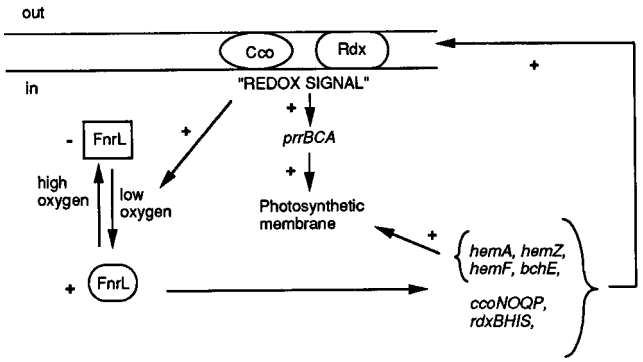

FIG. 5. A model of the anaerobic regulation of the photosynthesis genes. A box around FnrL indicates inactive protein, while an oval around FnrL represents active protein; these hypothetical features are based on the activities described for the *E. coli* homolog of FnrL, Fnr (see references 19 and 21). For further details, see Discussion.

Analysis of the phenotype associated with Har-1, in addition to increased *hemA* expression, revealed that the transposon insertion apparently affected expression of the photosynthetic apparatus. Thus, there are at least two possibilities; either increased expression of *hemA* and the aerobic expression of the photosynthetic apparatus are unrelated events stemming from the transposon insertion, or they are directly dependent on one another. In the former case, increased expression of *hemA* or activity of the *hemA* gene product could lead to increased flow of intermediates through the tetrapyrrole biosynthesis pathway, resulting in the synthesis of Bchl under aerobic conditions with concomitant assembly of the photosynthetic apparatus (34). In the other extreme, each of these seemingly disparate events could be related, and as such we propose a model, shown schematically in Fig. 5, based on the information currently available. If we assume that a redox signal, normally emanating from the activity of the CcoNOQP pathway serves to activate FnrL, then we could imagine that in the mutant strain, a spurious signal may increase the activity of FnrL under aerobic conditions. This increase in FnrL activity could, in turn, amplify the signal by altering the normal expression of the genes under its control, including *hemA*, *rdxBHIS*, and the *cco* operon, all of which have upstream

sequences with homology to the FNR-binding consensus sequence. We have also identified sequences with homology to the FNR-binding consensus sequence in the sequences upstream of *hemZ* (36), *hemF* (36), and *bchE* (12, 38) genes encoding additional steps in the Bchl biosynthesis pathway. With either model, we propose that a redox "signal" is generated through the normal activities of the CcoNOQP pathway under either low-oxygen or anaerobic conditions. In the mutant strain, this "signal" accumulates even in the presence of high oxygen levels. Consistent with this premise is the observation that the level of spectral complexes present in mutant JZ722 increased with increasing cell density under highly aerobic conditions in which there are no detectable complexes present in wild-type 2.4.1. Thus, mutant JZ722 behaves as if its ability to discriminate between oxygen levels has been displaced relative to that of the wild type. This conclusion is based upon the assumption that cell density determines the rate of oxygen utilization and, in turn, the residual level of free oxygen in the medium.

Because there is no impairment of growth in the mutant and because the normal aerobic cytochrome *c* oxidase pathway is intact, we presume that the membrane potential is unaltered in the mutant strain. Therefore, we believe the redox "signal" consists of a redox intermediate of the CcoNOQP pathway or is immediately derived from it. Such a redox intermediate could activate either gene expression (e.g., *fnrL* and *hemA*) or protein function (e.g., PRRBCA, FnrL, or HemA) or any combination of the two. With respect to protein function, several observations have relevance to this conclusion. First, a strong similarity has been noted between the *prrC* gene product and products of *Saccharomyces cerevisiae* genes that are thought to play a role in cytochrome *c* oxidase assembly in the mitochondria of this organism (8), suggesting a possible relationship or interaction at the protein level between the CcoNOQP protein complex and PRRC. Furthermore, Lin and Iuchi (23) have indicated that the two-component ArcA-ArcB system in *E. coli* might respond to an electron carrier in reduced form that accumulates because of oxygen deficiency. In *R. sphaeroides* 2.4.1, the Prr system has been identified as a similar two-component system that responds to changes in oxygen tension (7, 8). Second, there is increasing evidence to suggest that activity of *E. coli* Fnr is correlated with the redox state of the cell, the most recent being a demonstration of the effect of oxygen on the DNA binding activity of a mutant form of Fnr containing a redox-sensitive Fe-S center (19). Finally, other workers have previously noted activation of ALA synthase by disulfides or cystine trisulfide (9, 26), evidence suggesting that a more reduced form of the enzyme has higher activity.

While our results have clearly demonstrated that when ORFA2 is present, transcription of *hemA* is much higher, the mechanism of action of ORFA2 remains to be resolved. A first step must be to determine whether ORFA2 can act in *trans* to increase *hemA* transcription. Studies are in progress to address this issue.

We expect that analysis of the other transposon-generated mutants, *har-2* and *har-3*, showing increased *hemA* expression, will also make a meaningful contribution to our understanding of gene regulation in this organism. However, there is no doubt that even these analyses will not define the entire scope of the regulated expression of the *hemA* gene, since we have not yet addressed other known factors affecting ALA synthase activity, such as incident light intensity and iron concentration, to name a few.

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