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Regulation of 5-Aminolevulinic Acid Synthesis in *Rhodobacter sphaeroides* 2.4.1: the Genetic Basis of Mutant H-5 Auxotrophy†

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Rhodobacter sphaeroides **H-5 was isolated as a 5-aminolevulinic acid (ALA) auxotroph following treatment of wild-type cells with *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (J. Lascelles and T. Altshuler, J. Bacteriol. 98:721–727, 1969). The existence in *R. sphaeroides* 2.4.1 of the genes *hemA* and *hemT*, each encoding the enzyme 5-aminolevulinic acid synthase (EC 2.3.1.37), raised questions as to the genetic basis for the ALA auxotrophy in mutant H-5. We therefore cloned both the *hemA* and *hemT* genes from mutant H-5. The *hemA* gene has been sequenced in its entirety and bears four base pair substitutions which encode three amino acid changes relative to the sequence of wild-type strain 2.4.1. Complementation analysis of an *Escherichia coli* ALA auxotroph has revealed that the loss of ALA synthase activity in the HemA mutant enzyme could be localized to two of the amino acid substitutions. On the other hand, the *hemT* gene from mutant H-5 was able to complement an *E. coli* mutant requiring ALA for growth. Complementation analyses were also carried out by introducing the cloned *hemA* or *hemT* gene of mutant H-5 or wild-type 2.4.1 in *trans* into H-5 and, in parallel, into our previously described HemA-HemT double mutant strain AT1 (E. L. Neidle and S. Kaplan, J. Bacteriol. 175:2304–2313, 1993). This analysis revealed that while the complementation pattern of mutant AT1 parallels that for the *E. coli* ALA auxotroph, mutant H-5 could only be complemented by the wild-type *hemA* gene. The ability of the *hemT* gene of either mutant H-5 or wild-type 2.4.1 to complement the ALA auxotrophy of mutant AT1 but not mutant H-5 was consistent with β-galactosidase activities obtained with *hemT-lacZ* transcriptional fusions. We conclude that the ALA auxotrophy of mutant H-5 arises from (i) a nonfunctional HemA protein containing multiple missense substitutions and (ii) an inability of the normal *hemT* gene to be expressed in the mutant H-5 genetic background, i.e., an additional mutation of unknown origin is required for *hemT* expression. These studies bear directly on the regulation of the expression of the *hemA* and *hemT* genes of *R. sphaeroides* 2.4.1.**

The various growth modes of the facultative photosynthetic bacterium *Rhodobacter sphaeroides* require different tetrapyrroles. For example, hemes, as part of cytochromes, are essential for aerobic and anaerobic respiration, whereas photosynthetic growth requires both heme-bound cytochromes and bacteriochlorophylls that are bound to proteins of the photosynthetic machinery. Vitamin B_{12}, yet another tetrapyrrole, is required under all growth conditions. The synthesis of 5-aminolevulinic acid (ALA), the first committed precursor in the biosynthesis of all tetrapyrroles in *R. sphaeroides*, is catalyzed by ALA synthase, encoded in this organism by two genes, *hemA* and *hemT* (15, 16, 23). Whereas most bacteria examined and all plants synthesize ALA from glutamic acid in a series of steps involving a glutamyl-tRNA intermediate through the C5 pathway, *R. sphaeroides* and related bacteria synthesize ALA by the same reaction mechanism that occurs in yeast and animal cell mitochondria. This reaction, also called the Shemin or C4 pathway, involves the condensation of glycine and succinyl coenzyme A with pyridoxal phosphate as a cofactor (succinyl-coenzyme A:glycine *C*-succinyl transferase [decarboxylating]; EC 2.3.1.37) to produce ALA and carbon dioxide. With the only exception identified to date being *Euglena gracilis*, organisms appear to have only one pathway by which ALA is formed (for a review, see reference 1).

The DNA sequences of *hemA* and *hemT* and their locations on the *R. sphaeroides* physical map have been determined (16). The *hemA* gene is located on the large chromosome, whereas *hemT* is found on the small chromosome. Furthermore, the flanking sequences of *hemT* and *hemA* are completely different, and as might be predicted, the expression patterns for each gene are different. For example, Northern (RNA) analyses of the *hemT* and *hemA* messages have determined that the *hemT* message is undetectable in wild-type cells. The *hemA* message is present under both aerobic and photosynthetic growth conditions, but message levels fluctuate according to the growth mode, being two- to threefold higher under photosynthetic growth conditions (10 W/m^2) relative to aerobic conditions (16). Despite the lack of detectable levels of the *hemT* message, however, there is evidence to suggest that the *hemT* product does contribute to the overall cellular levels of ALA. This evidence comes from an analysis of the relative levels of various components of the photosynthetic machinery of a *hemT* deletion strain. Compared with those for the wild type, deletion strain levels are 45% for bacteriochlorophylls, 64% for carotenoids, 50% for B875 complex, and 44% for B800-850 (15). Even more convincing proof that *hemT* does have a function is the observation that although the levels of these spectral complexes are reduced in a mutant containing a *hemA* disruption, levels are actually lower in the *hemT* deletion strain. On the other hand, ALA synthase activities determined for strains capable of synthesizing either HemA or HemT are

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† This work is dedicated to the memory of M. Snel-Zeilstra.

identical to activities determined for the wild type, both aerobically as well as under photosynthetic conditions (15).

In addition to the regulation of gene expression, differences in the biochemical properties of the two isozymes could affect the overall cellular levels of ALA. Indeed, a variety of workers have reported that the ALA synthase activity of *R. sphaeroides* comprises two different populations of enzyme not only differing biochemically (6, 24–26) but also in their cellular localization (5); one activity fractionates cytoplasmically, whereas the other appears to be membrane associated. It is thus evident that a thorough analysis of both the expression and the biochemistry of *hemA* and *hemT* and their gene products is required in order to define the role of these genes and their products in *R. sphaeroides*.

Mutants provide a valuable tool with which to address both of these questions in that altered expression and/or defective gene products can confer phenotypes. In pursuit of our goal of understanding the interactions between and the unique regulatory patterns for both *hemA* and *hemT* of *R. sphaeroides* 2.4.1, we have examined the original ALA auxotrophic strain, *R. sphaeroides* H-5 isolated by Lascelles and Altshuler (11). This mutant was the first ALA auxotroph isolated for any bacterium, and because of our current knowledge of the existence of two genes encoding ALA synthase, the molecular basis for this auxotrophy raised certain questions. This analysis consisted of cloning and sequencing the *hemA* and *hemT* genes from mutant H-5 and, by means of complementation analyses with both the cloned genes and the corresponding genes from wild-type 2.4.1, determining the genetic basis of the ALA auxotrophy of mutant H-5.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. (Additional details about some of the plasmids are provided in Fig. 1 and 4 to 6.) *R. sphaeroides* wild-type strain 2.4.1 was grown in Silstrom's succinic acid minimal medium A (20) as previously described. Mutant strains H-5 and AT1 were grown in media supplemented with 0.2 mM ALA under all conditions described here. *R. sphaeroides* cultures assayed for β-galactosidase activities were grown chemoheterotrophically by sparging them with gas mixtures as previously described (3). *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium (13). When appropriate, the media were supplemented with antibiotics to maintain selection for the presence of plasmids. For *R. sphaeroides*, the final concentrations were 0.8 μg/ml for tetracycline (Tc), 25 μg/ml for kanamycin (Kn), and 50 μg/ml for spectinomycin (Sp) and streptomycin (St); for *E. coli*, the final concentrations were 15 μg/ml for Tc, 25 μg/ml for Kn, 50 μg/ml for Sp and St, and 100 μg/ml for ampicillin. The *E. coli* ALA auxotroph, SASP19, was supplemented with 50 μg of ALA per ml of culture medium. 5-Bromo-4-chloro-3-indolyl β-D-galactoside (at 40 μg/ml for *E. coli* and 15 μg/ml for *R. sphaeroides*) was used to monitor β-galactosidase activity in the construction of plasmids and bacterial strains.

DNA manipulations, amplification of DNA sequences, and sequencing. Plasmid DNA was isolated according to standard protocols or manufacturers' instructions, as were restriction endonucleases and other enzymatic treatments of plasmids and DNA fragments. Standard electrophoretic techniques were used in DNA analysis.

For template DNA used in amplification reactions, genomic DNA was obtained by performing the standard protocol used in small-scale isolations of plasmid DNA, i.e., alkaline lysis, phenol extraction, and ethanol precipitation of liquid cultures of bacteria. This procedure yielded ample amounts of chromosomal DNA on which to perform multiple amplification reactions, and the samples were stable for several months when stored at −20°C.

The *hemA*- and *hemT*-bearing DNA fragments of mutant H-5 and NCIB8253 (H-5) were amplified from chromosomal DNA of these strains with the following primers. Amplification of *hemA* sequences was executed with primers HEMA-UP, 5′-AGGGACCAATGAACGGTTT-3′, and HEMA-ALL, 5′-GTCCCGAA AGAAGTAGCACA-3′, to amplify 1,404 bp of *hemA* DNA. For *hemT* amplification, primers RDXA, 5′-GAAAGATGGGTGTTCGC-3′, and HEMT-ALL, 5′-GGATCCGATGGCGTGTCTTC-3′, were used to amplify 1,783 bp of *hemT* DNA. The thermocycling program consisted of (i) denaturation at 95°C for 2 min and (ii) annealing and extension at 55°C for 2 min and was repeated 35 times. Reaction mixes were prepared with Ampli-Taq (Perkin-Elmer) according to the manufacturer's instructions, and amplification was carried out in a MJ PTC-100 Thermal Cycler (MJ Research, Inc., Watertown, Mass.). Amplified DNA was purified from agarose gels with the GeneClean Kit (Bio 101). The purified DNA was then either directly sequenced or cloned after treatment with the Klenow fragment of *E. coli* DNA polymerase I. The upstream sequences of *hemT* were similarly amplified from genomic DNA isolated from strain A1 and mutant H-5 with the RDXA primer together with primer HEMT-REVERSE, 5′-CGT CAATGAGCTTCTGGAAG-3′, and the same thermocycling program described above was used.

DNA sequences were determined with appropriately designed oligonucleotides to prime standard sequencing reactions spanning the regions of interest. The sequencing primers used in determining *hemA* sequences were as follows: HEMA-UP, HEMA-ALL, HEMA-DOWN (5′-GAAGCGTGGTTCAACTTG TC-3′), HEMA-2 (5′-CGCTGGATTCGACCGGCG-3′), HEMA-3 (5′-GACG AGGTCCATGCCGTC-3′), HEMA-4 (5′-GAGGTCGAGAAGATGAAG-3′), PBST3 (5′-CCTCACTAAAGGGAACAAAAGC-3′), and EXT7-1 (5′-TAATA CGACTCACTATAG-3′). Upstream sequences of *hemT* were determined with RDXA and HEMT-REVERSE. The positions of all the primers described here, and elsewhere, are shown schematically in Fig. 1. DNA sequence analyses were performed with an ABI 373A automatic DNA sequencer (Applied Biosystems Inc., Foster City, Calif.) at the DNA Core Facility of the Department of Microbiology and Molecular Genetics, University of Texas Health Science Center, Houston, Tex.

Construction of pUI1087. pUI1087 is a derivative of pBSIISK$^+$ in which the polylinker has been modified in two steps. (i) The *Eco*RI site has been eliminated by treatment with the Klenow fragment of *E. coli* DNA polymerase I. (ii) Oligonucleotides MUT-1, 5′-CTAGAATTCCATGGCCAG-3′, and MUT-2, 5′-CTAGCTGGCCATGGAATT-3′, were first annealed to each other and then ligated into the *Spe*I site of the pBS polylinker (Fig. 1). These two modifications, confirmed by DNA sequence analysis, made possible the direct cloning of a variety of DNA molecules used in this study. Also, restriction fragments could subsequently be generated through treatment with endonucleases having recognition sites flanking the original cloning site to accommodate direct cloning into other vectors. For example, this two-step cloning procedure was employed in the construction of the *hemT-lacZ* and *hemT*-lacZ* transcriptional fusion plasmids derived from pCF1010 (Table 1).

Conjugation techniques. Plasmids were mobilized into *R. sphaeroides* by previously described procedures (3). For the ALA auxotroph mutants H-5 and AT1, matings were performed on media containing 0.2 mM ALA, and exconjugants were then placed on media again supplemented with ALA. Alternatively, when appropriate, exconjugants were scored for growth on media with and without ALA.

Cell extract preparation and β-galactosidase activity assays. Crude cell extract preparations and assays of β-galactosidase activity were performed as described elsewhere (22). Reported values are the averages of at least two assays performed on individually prepared cell extracts, with ranges of less than 20%.

Pulsed-field agarose gel electrophoresis. Genomic DNA was prepared and analyzed by pulsed-field agarose gel electrophoresis according to previously described protocols (21). Pulsed-field electrophoresis was performed with a transverse alternating field electrophoresis gel apparatus (Beckman Instruments, Inc., Fullerton, Calif.).

Construction of Ω cartridge insertion-*hemA* gene disruption and deletion strains. *R. sphaeroides* 2.4.1 was used as the recipient for plasmid pUI1007 or pUI1903. Matings were conducted on Luria-Bertani solid medium containing 0.2 mM ALA, and exconjugants were then plated on selective media containing Kn for recipients of plasmid pUI1007 or Sp and St for recipients of pUI1903, both with or without additional supplementation with ALA. Recombinant strains containing even-numbered crossovers were screened for by scoring individual exconjugants for Tc sensitivity, which indicates the absence of vector sequences, and ALA auxotrophy, which indicates displacement of the *hemA* gene by the disrupted gene. Confirmation of the correct DNA structure of the derived strains was obtained through amplification of appropriate DNA segments and pulsed-field agarose gel electrophoresis of genomic DNA isolated from positively identified candidates. Even-numbered crossover events resulting in the insertion of the mutated gene in single copy into the chromosome were identified by the following amplification reactions: (i) amplified products of the expected size from PCR of DNA from presumed even-numbered crossover candidates with either the HEMA-UP and Ω (5′-TGATCCGGTGGATGACCTTT-3′ [Fig. 1]) primers or the HEMA-DOWN and Ω primers being used and the absence of product with DNA from wild-type cells, and (ii) the absence of amplified product with HEMA-UP and HEMA-DOWN primers being used in a PCR with DNA isolated from even-numbered crossover candidates and the presence of product of the expected size with DNA from wild-type cells. Product is not obtained in this reaction with DNA from even-numbered candidates because the Ω cartridge prevents *Taq* polymerase extension through the transcription terminator sequences at the ends of the Ω cartridge. Note, however, that extension is clearly successful through one terminator of the Ω cartridge, since the first reaction described above yields product when appropriate template DNA is provided.

These amplification reactions were carried out in parallel, with wild-type 2.4.1 genomic DNA and pUI1903 plasmid DNA being used as controls. One confirmed Ω insertion of the *hemA* deletion strain, constructed with pUI1903, was designated A2. The creation of a new *Ase*I restriction endonuclease site at the site of the insertion of the mutated gene because of the presence of the Ω cartridge was further demonstration of the correct structure of the recombinant

TABLE 1. Bacterial strains and plasmids

Organism and strain or plasmid	Relevant characteristic(s)	Reference or source
E. coli		
DH5α	(ϕ80d*lacZ*ΔM15) Δ*lacU169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*	8
DH5αphe	DH5α *phe*::Tn*10d*Cm	4
HB101	*lacY1 galK2 supE44 ara14 proA2 rpsL20 recA13 xyl-5 mtl-1 hsdS20 mcrB mrr*	2
SASP19	*hemA8*	18
R. sphaeroides		
2.4.1	Wild type	27
H-5	ALA^- derivative of NCIB8253 (H-5)	11
HemA1	2.4.1 derivative, *hemA*::ΩKn^r	15
HemA2	2.4.1 derivative, Δ*hemA*::ΩSt^r-Sp^r	This study (Fig. 5)
HemAT1	2.4.1 derivative, *hemA*::ΩKn^r and *hemT*::ΩSt^r-Sp^r	15
NCIB8253	Wild type, identical in *Ase*I macrorestriction pattern to 2.4.1	NCIMB[a]
NCIB8253 (H-5)	Wild-type parent of mutant H-5, identical in *Ase*I macrorestriction pattern to mutant H-5	R. Niederman
Plasmids		
pBS	Ap^r, with *lac* promoter	Stratagene
pCF1010	RSF1010 derivative featuring a polylinker positioned upstream of *lacZYA′* and an ΩSt^r-Sp^r cartridge positioned at the 5′ end of the polylinker	12
pHP45Ω	Source of ΩSt^r-Sp^r cassette	17
pRK415	Tc^r	9
pSUP202	pBR325 derivative, Mob$^+$ Ap^r Cm^r Tc^r	19
pUI1004	pUC19 derivative with 1.8-kb *hemT* region from *R. sphaeroides* 2.4.1	15
pUI1014	pUC18 derivative with 2-kb *hemA* region from *R. sphaeroides* 2.4.1	15
pUI1023	pRK415 derivative with 2.5-kb *hemT-rdxA′* fragment (*Pst*I-*Sal*I) from *R. sphaeroides* 2.4.1	14
pUI1071	pUI1023 derivative with Kn^r gene fragment of Tn*903* (*Xho*I-*Pst*I) replacing the *hemT* (*Xho*I-*Pst*I) sequences	This study (Fig. 6)
pUI1087	pBSIISK$^+$ derivative with an altered polylinker	This study (Fig. 1)
pUI1090	pUI1087 derivative with 0.5-kb *rdxA′-hemT′* fragment (*Bam*HI-*Nco*I)	This study (Fig. 6)
pUI1091	pUI1087 derivative with 0.5-kb *rdxA′-hemT′** fragment (*Bam*HI-*Nco*I) (*hemT** has a C → T substitution at −13 from the *hemT* transcription start site)	This study (Fig. 6)
pUI1092	pUI1087 derivative with 0.5-kb *rdxA′-hemT** fragment (*Bam*HI-*Nco*I) (*hemT** has a T → C substitution at −31 from the *hemT* transcription start site)	This study (Fig. 6)
pUI1098	pCF1010 derivative with 0.5-kb *rdxA′-hemT* fragment (*Pst*I-*Xba*I) of pUI1090	This study (Fig. 6)
pUI1099	pCF1010 derivative with 0.5-kb *rdxA′-hemT′** fragment (*Pst*I-*Xba*I) of pUI1091	This study (Fig. 6)
pUI1900	pCF1010 derivative with 0.5-kb *rdxA′-hemT′** fragment (*Pst*I-*Xba*I) of pUI1092	This study (Fig. 6)
pUI1903	pSUP202 derivative with Δ*hemA*::ΩSt^r-Sp^r	This study (Fig. 5)
pUI1905A and B	pUI1087 derivative with PCR *hemA* product from *R. sphaeroides* H-5, cloned in both orientations	This study (Fig. 1)
pUI1909	pUI1905 derivative, with the *Sma*I fragment deleted	This study (Fig. 1)
pUI1915A and B	pUI1087 derivative with PCR *hemT* product from *R. sphaeroides* H-5, cloned in both orientations	This study (Fig. 1)
pUI1917	pUI1905 derivative with substitution of the *Bgl*II (in *hemA*)-*Hin*dIII (polylinker-derived) fragment from pUI1014	This study (Fig. 4)
pUI1918	pUI1014 derivative with substitution of the *Bgl*II (in *hemA*)-*Hin*dIII (polylinker-derived) fragment from pUI1905	This study (Fig. 4)
pUI1919	pRK415 derivative with *hemA* fragment (*Xba*I-*Hin*dIII) from pUI1905	This study (Fig. 4)
pUI1920	pRK415 derivative with *hemA* fragment (*Xba*I-*Hin*dIII) from pUI1917	This study (Fig. 4)
pUI1921	pRK415 derivative with *hemA* fragment (*Eco*RI) from pUI1918	This study (Fig. 4)
pUI1922	pRK415 derivative with *hemA* fragment (*Eco*RI-*Hin*dIII) from pUI1014	This study (Fig. 4)
pUI1923A and B	pRK415 derivative with *hemT* fragment (*Bam*HI-*Bam*HI) from pUI1004 in both orientations	This study (Fig. 4)
pUI1924	pRK415 derivative with *hemT* fragment (*Bam*HI-*Bam*HI) from pUI1915A	This study (Fig. 4)

[a] NCIMB, National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland.

clone. Finally, the mutated strains showed a requirement for exogenous ALA, distinguishing them from single crossover recombinants, which were present in large numbers following conjugation.

Chemicals and materials. Restriction endonucleases and other nucleic acid-modifying enzymes were 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.). 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside was obtained from Boehringer Mannheim Biochemicals. Proteinase K, antibiotics, ALA, *o*-nitrophenyl β-D-galactopyranoside, and other reagents were of reagent grade and were purchased from Sigma Chemical Co. (St. Louis, Mo.). Synthetic deoxyoligonucleotide primers were synthesized at the Core Facility of the Department of Microbiology and Molecular Genetics, University of Texas Health Science Center, Houston, Tex.

RESULTS

Lineage of H-5. A first step in the genetic analysis of this mutant strain was to define its lineage. We subjected *Ase*I-restricted genomic DNA preparations to pulsed-field gel electrophoresis and compared their overall band patterns to those of the wild-type strain 2.4.1 and to two other strains of different origins but identified as NCIB8253, the reported parent of mutant H-5 (11). The results, as shown in Fig. 2, demonstrate that H-5 is most similar to an NCIB8253 strain obtained from R. Niederman, while 2.4.1 and the NCIB8253 now on deposit at the National Collection of Industrial and Marine Bacteria

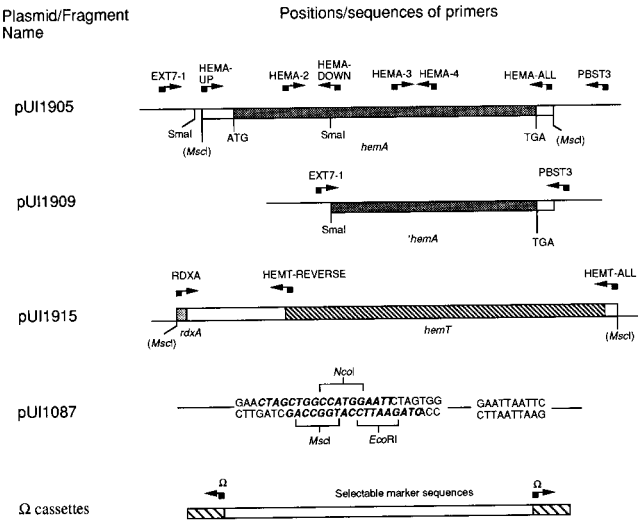

FIG. 1. Schematic diagram of the positions of the various oligodeoxynucleotide primers used in this study. Plasmid and DNA fragment names refer to DNA molecules associated with the use of primers diagrammed above the relevant regions of the plasmid or DNA fragment. Arrows refer to the relative orientations, indicating 5′ to 3′, of the primers. Primer sequences are provided in the Materials and Methods section, as are further details regarding primer usage.

(Torry Research Station, Aberdeen, Scotland) appear to be one and the same. Therefore, the genetic backgrounds of mutant H-5 and 2.4.1 are not identical. At the present time, this is as close as we can come to designating the parent of mutant H-5, since that strain appears to be no longer available. However, the sequencing results obtained for *hemA* and *hemT* from both strains demonstrated that these two strains are likely to be very similar, if not identical, for these loci. For the sake of clarity here, we will refer to the strain which by the above criterion is considered to be the parent of H-5 as NCIB8253 (H-5).

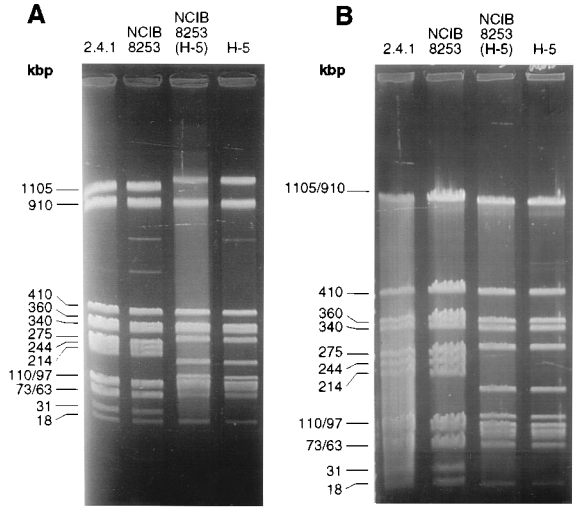

FIG. 2. Comparative pulsed-field agarose gel electrophoresis of genomic DNA digested with *Ase*I of various strains of *R. sphaeroides*. Separation conditions are as previously described by Suwanto and Kaplan (21). For further details regarding restriction fragment sizes and positions on the physical map of wild-type 2.4.1, see Suwanto and Kaplan (21). Each panel shows one gel. (A) Optimum separation of the largest restriction fragments. (B) Optimum separation of the medium-sized restriction fragments.

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 1 MDYNLALDTALNRLHTEGRYRTFIDIERRKGAFPKAMWRKPDGSEKEITV 50

51 WCGNDYLGMGQHPVVLGAMHEALDSTGAGSGGTRNISGTTLYHKRLEAEL 100

          (GCG->ACG)
             Thr
             |
101 ADLHGKEAALVFSSAYIANDATLSTLPQLIPGLVIVSDKLNHASMIEGIR 150

                    (CGG->CGA)
                       Arg
                       |
151 RSGTEKHIFKHNDLDDLRRILTSIGKDRPILVAFESVYSMDGDFGRIEEI 200

                (GTC->ATC)  (GGC->GAC)
                     Ile    Asp
                     |      |                  *
                     |      |                  *********|**
201 CDIADEFGALKYIDEVHAVGMYGPRGGGVAERDGLMDRIDIINGTLGKAY 250
    ***
251 GVFGGYIAASSKMCDAVRSYAPGFIFSTSLPPVVAAGAAASVRHLKGDVE 300

301 LREKHQTQARILKMRLKGLGLPIIDHGSHIVPVHVGDPVHCKMISDMLLE 350

351 HFGIYVQPINFPTVPRGTERLRFTPSPVHDSGMIDHLVKAMDVLWQHCAL 400

401 NRAEVVA* 408
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FIG. 3. Amino acid sequence of the HemA protein, indicating the substitutions encoded by the *hemA* gene of mutant H-5. Highly conserved amino acid residues (16) are in boldface type. The pyridoxal phosphate-binding region identified by Ferriera et al. (7) is indicated by asterisks, with a line drawn to the lysine residue identified as binding to pyridoxal phosphate. Provided above the amino acid substitutions encoded by the *hemA* gene of mutant H-5 are the base pair substitutions determined from the DNA sequence analysis.

PCR amplification of *hemA* and *hemT* from mutant strain H-5. By means of the primers appropriate for each gene (see Materials and Methods), *hemA* and *hemT* were amplified by PCR as described in Materials and Methods. For *hemA*, the product was 1,404 bp in length, and for *hemT*, the product was 1,783 bp in length. The products were then treated with the Klenow fragment of *E. coli* DNA polymerase I and cloned into the *Msc*I site of pUI1087 (see Materials and Methods), a derivative of pBlueScriptII(SK$^+$) with a modified polylinker sequence. Candidate clones were confirmed by restriction analysis. PCR was also used to amplify the *hemA* gene from strain NCIB8253 (H-5).

Sequence analysis of the *hemA* and *hemT* genes cloned from H-5. One clone of *hemA* from mutant H-5 was sequenced in its entirety. All base pair changes relative to the *hemA* sequence of wild-type strain 2.4.1 were confirmed by sequencing both strands of the cloned *hemA* gene at these positions and by sequencing the PCR product directly. Three of the four base pair differences identified by the DNA sequence analysis result in amino acid substitutions, i.e., missense alterations. As indicated in Fig. 3, these three substitutions are at highly conserved positions in the HemA protein, but none are within the pyridoxal phosphate-binding region identified by Ferreira et al. (7). DNA sequence analysis was also performed with the PCR product obtained from the presumed wild-type parental strain of mutant H-5, strain NCIB8253 (H-5), being used as template. This analysis revealed that NCIB8253 (H-5) and 2.4.1 have identical *hemA* sequences, and thus all four of the base pair differences of the H-5 *hemA* gene are considered to be true mutations in the *hemA* gene of mutant H-5. Thus, given the unavailability of the true parent of mutant H-5, we believe that these results provide an accurate description of the mutations present in the *hemA* gene of mutant H-5 and, importantly, help to define the lineage of H-5.

The upstream region of the PCR-amplified *hemT* gene of mutant H-5 was also sequenced, from positions −346 to +84 relative to the start site of transcription, and no differences relative to the wild-type sequence of strain 2.4.1 were found. No further sequence analysis was done on the *hemT* clones, because complementation analysis in both *E. coli* and *R. sphaeroides* (AT1) demonstrated that the *hemT* gene of mutant H-5 encodes a functional ALA synthase. These findings further

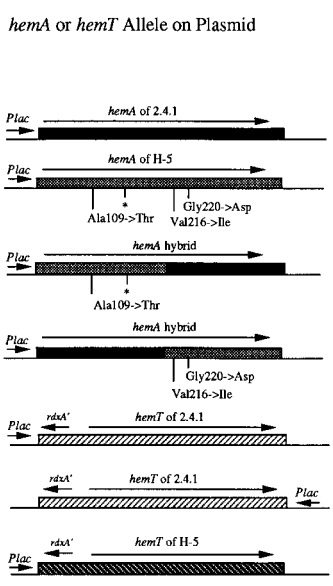

FIG. 4. Schematic diagram of the results of complementation analysis of the *E. coli* ALA auxotrophic strain SASP19 and of the *R. sphaeroides* ALA auxotrophic mutants H-5 and AT1. Further details regarding bacterial strains and the structures of the various plasmids are provided in Table 1 and the text. The derivation of each *hemA* or *hemT* gene is indicated, as are their orientations with respect to the *lac* promoter sequences on the plasmids used for analysis of *E. coli*. The orientations are the same relative to the *tet* promoter sequences present on the plasmids used for analysis of *R. sphaeroides*. Amino acid substitutions encoded by the plasmid-borne genes are as indicated. For complementation of *E. coli* SASP19, a + symbol indicates bacterial growth equivalent to that of the pUI1014 transformant of SASP19 in the absence of exogenous ALA, which is also equivalent to the growth of SASP19 transformed with the vector alone in the presence of ALA. A − symbol indicates no growth observed in the absence of exogenous ALA. For complementation of *R. sphaeroides* H-5 and AT1, a + symbol indicates bacterial growth equivalent to that of the H-5(pUI1922) and AT1(pUI1922) exconjugants in the absence of exogenous ALA, which is also equivalent to the growth of exconjugants of the vector alone in the presence of 0.2 mM ALA. A − indicates no growth observed in the absence of exogenous ALA.

support the premise that the *hemA* and *hemT* genes of NCIB8253 (H-5) and 2.4.1 are identical.

Complementation of an ALA auxotroph of *E. coli*. Previous work has shown that both the *hemA* and the *hemT* genes of *R. sphaeroides* 2.4.1 can complement ALA auxotrophs of *E. coli*, strains SASX77 (here referred to as SASP19) and SASX41B (15, 23). In addition to complementation analysis of the cloned PCR products, the four mutations within the *hemA* gene were further subdivided by substitution of the wild-type *hemA* sequences for either the proximal two changes or the distal two changes, as shown in Fig. 4. The ability of the derived chimeric genes, present in multicopy, to complement the ALA requirement of *E. coli* SASP19 (18) is also indicated. We found that the presence of the two distal mutations abolished the ability of the chimeric gene to complement SASP19. However, the two proximal mutations in an otherwise wild-type gene did not result in a loss of ALA synthase activity, as judged by the ability of such a chimeric protein to complement the ALA requirement.

The *hemT* clone from *R. sphaeroides* H-5 complemented SASP19 as effectively as the wild-type *hemA* or *hemT* gene of *R. sphaeroides* 2.4.1. This result unequivocally demonstrated that the *hemT* gene cloned from H-5 encodes an ALA synthase that is functional in *E. coli*.

Complementation of H-5 and AT1 of *R. sphaeroides*. All of the *hemA* and *hemT* alleles used in the complementation analysis of *E. coli* were subsequently cloned into pRK415, which can replicate at four to six copies per cell in both *E. coli* and *R. sphaeroides* (3). The plasmids were then introduced by conjugation into both strains H-5 and AT1. Strain AT1 is a derivative of wild-type strain 2.4.1 and has a truncated *hemA* gene through the insertion of an ΩKn^r cartridge and a complete *hemT* deletion into which an ΩSp^r-St^r cartridge has been inserted (15). Figure 4 schematically shows the structure of the pRK415 derivatives and presents the results of the complementation analysis.

We found that the complementation pattern of the various *hemA* alleles in *trans* was different for mutant H-5 than for AT1. While both wild-type *hemA* (pUI1922) and the plasmid encoding the protein for which threonine substitutes for alanine at position 109 (Ala-109 → Thr) (pUI1921), which are the same *hemA* alleles that complemented the *E. coli* ALA auxotroph, also complemented the ALA requirement of strain AT1, only the wild-type *hemA*-bearing plasmid complemented the ALA requirement for strain H-5. Since both plasmids are likely to possess the same copy number and both genes are under identical regulatory controls, the presence or absence of complementation in mutant H-5 must be attributable to some structural difference between the proteins produced. One possible explanation for the difference in the complementation patterns of AT1 and H-5 by pUI1921 is the presence of a dominant negative phenotype due to the presence of the chromosomal *hemA* gene of mutant H-5. Implicit in this explanation is that while the Ala-109 → Thr mutation must be mild, the ALA synthase activity of this mutant protein is very likely reduced relative to that of the wild-type protein, since the plasmid bearing the wild-type *hemA* gene could complement the ALA auxotrophy of mutant H-5 and the plasmid-borne *hemA* mutant allele could not. Thus, it might well be that the formation of heterodimers (the native form of *hemA*-encoded ALA synthase is a dimer) consisting of one triply substituted subunit encoded by the chromosomal, mutated *hemA* allele and the other subunit, substituted at residue 109, encoded by the plasmid-borne *hemA* allele reduces the overall ALA synthase activity to a level incapable of meeting the total ALA requirement of the cell. Conversely, a heterodimer made up of a fully wild-type subunit and a triply altered H-5-derived subunit could possess activity.

We did not determine further which one of these distal mutations present in the *hemA* gene from mutant H-5 was effective or if both were effective in disrupting ALA synthase activity. However, to extend our analysis of the *hemA* allele cloned from mutant H-5, we isolated spontaneous ALA^+ colonies arising from AT1 containing in *trans* various defective *hemA* alleles (either Val-216 → Ile and Gly-220 → Asp or with all three amino acid changes; see pUI1919 and pUI1920 in Fig. 4) derived completely or in part from mutant H-5 and which were initially not complemented. Sequence analysis of plasmid DNA from one ALA^+ isolate derived from exconjugants of pUI1919 and one isolate derived from exconjugants of pUI1920 revealed that both of the base pair substitutions encoding Val-216 → Ile and Gly-220 → Asp substitutions in each parental plasmid had reverted to the wild type. It is therefore likely that either the Val-216 → Ile or the Gly-220 → Asp substitution alone abolishes ALA synthase activity. Since the chromosomal *hemA* sequences are disrupted but not deleted in AT1, the restoration of wild-type sequences most likely resulted from either recombination, reversion, or both when multiple (four to six for these plasmids) copies of the *hemA* gene exist in *trans*. The presence of these two mutations in mutant H-5, either of which alone apparently abolishes ALA synthase activity, explains the observation that reversion of the mutated *hemA* gene to a functional *hemA* gene in H-5, when present in single copy, is not routinely observed.

The results of our complementation analysis of the *hemT* gene of mutant H-5 demonstrated that while the H-5 *hemT* gene encoded a functional ALA synthase, as had already been demonstrated by its ability to complement the *E. coli* ALA auxotroph, the ability of this gene to complement *R. sphaeroides* was strain specific in that it did complement AT1 but not H-5. This apparent paradox must be due to differences in the genetic backgrounds of H-5 and AT1. The *hemT* gene from strain 2.4.1 complemented only AT1, irrespective of its orientation in pRK415 relative to the *tet* promoter. Thus, expression of *hemT* was from its own upstream sequences. Yet, it has been shown that the *hemT* message is undetectable in wild-type cells (16). These results suggested that the inability of *hemT* to complement the ALA^- phenotype of mutant H-5 was due to a missing component required for functional *hemT* expression which, although normally absent from wild-type cells, can be induced and is present in strain AT1. Results described in the next sections demonstrate that activation of *hemT* mediated by *trans*-acting elements can occur.

Construction of a complete *hemA* deletion in *R. sphaeroides* 2.4.1. If *hemT* expression is absent from wild-type cells, why is strain A1, bearing a *hemA* disruption (15, 16), an ALA prototroph? In light of our complementation results, we reevaluated the status of *hemT* expression in strains lacking *hemA*-encoded ALA synthase activity by construction of a deletion of the entire *hemA* gene. By conjugation in independent experiments, we introduced either pUI1903, which via gene replacement would create a complete deletion of the *hemA* gene accompanied by the insertion of an Ω cassette, or pUI1007 (Fig. 5), which was used to construct the *hemA* disruption in A1 and was isolated by Neidle and Kaplan (15). We found that the only exconjugants retaining Sp-St resistance and Tc sensitivity, in the case of plasmid pUI1903, or Kn resistance and Tc sensitivity, in the case of plasmid pUI1007, were those that also required ALA. Thus, deletion or disruption of the *hemA* gene alone in an otherwise wild-type strain conferred ALA auxotrophy.

The difference between the ALA auxotroph we have now identified and the observation of Neidle and Kaplan (15) that in the absence of *hemA*, the cellular ALA requirement can be met by the *hemT*-encoded ALA synthase, is most likely due to the fact that there is a high frequency of reversion to an ALA^+ phenotype among strains of *R. sphaeroides* harboring nonrevertible mutations in the *hemA* gene. The absence of detectable levels of a *hemT* message in wild-type cells and its presence in A1 (16) demonstrate that reversion to ALA prototrophy is coupled to an increase in *hemT* expression at the level of transcription. Similar attempts to isolate revertants to ALA prototrophy of mutant H-5 were unsuccessful.

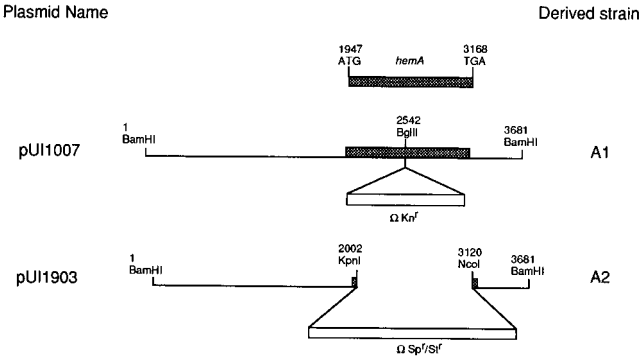

FIG. 5. Construction of *hemA*-disrupted or -deleted *R. sphaeroides* strains. Suicide vector pUI1007 or pUI1903, bearing the *hemA* genes and flanking sequences, was introduced into wild-type 2.4.1. The resulting strains, generated by homologous recombination between the plasmid-borne sequences and the chromosomal sequences, were as indicated. A1 is an isolate previously described by Neidle and Kaplan (15). Further details are provided in Table 1 and the text.

The growth of exconjugants giving rise to the *hemA* deletion or disruption was markedly slower on selective plates containing ALA than on identical plates without ALA. In fact, as has been previously demonstrated (10, 15), we observed that growth of the wild-type 2.4.1 strain was also inhibited in the presence of exogenous ALA. The conclusion drawn from these observations is that ALA, when provided exogenously at concentrations beyond what is required for growth, is deleterious. During the construction of strain AT1, which is absolutely dependent on added ALA for growth, it was not known what minimal concentration of exogenous ALA would select for events whereby the cells become resistant to an inhibitory ALA concentration. It may be that one means by which the cell overcomes ALA sensitivity involves the same mechanism that activates the expression of *hemT*. We have demonstrated that AT1 is activated for *hemT* expression and that its growth in medium containing ALA at concentrations 10-fold above that which inhibits the growth of wild-type cells (15) is similar to the growth of wild-type 2.4.1 in medium without ALA.

Our complementation analyses demonstrated that the *hemT* gene of mutant H-5 encodes a functional ALA synthase, and DNA sequence analysis of the upstream sequences of the *hemT* gene of mutant H-5, described above, ruled out any contribution of *cis*-linked regulatory differences. Once again, the evidence suggested that the basis for the ability of the *hemT* gene of either mutant H-5 or wild-type 2.4.1 to complement AT1 but not mutant H-5 must be due to differences in the genetic backgrounds of the two strains. Furthermore, whatever the event that occurred in strain AT1 and led to *hemT* activation, this event did not or cannot occur in mutant H-5, since unlike the case with the *hemA* disruption or deletion strains, we have not observed mutant H-5 to revert to ALA prototrophy.

DNA sequence analysis of the *hemT* upstream sequences in the ALA^+ A1 strain. We confirmed that *trans*-acting elements can lead to the activation of *hemT* expression at the level of

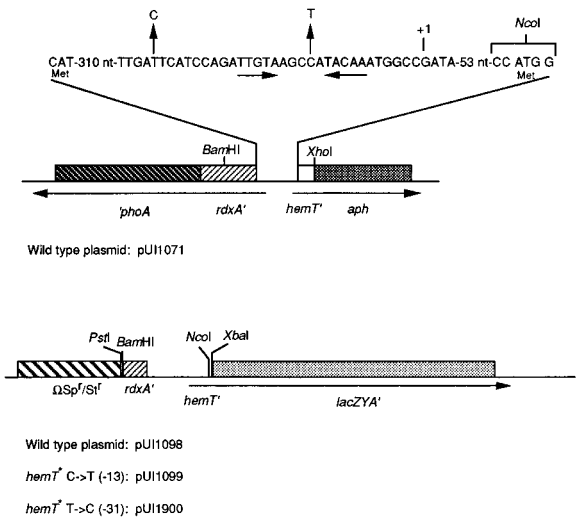

FIG. 6. Schematic representation of plasmids bearing transcriptional fusions of the *hemT* upstream sequences to the *aph* gene of Tn*903* Kn resistance for pUI1071 or *lacZYA'* of *E. coli* for plasmids pUI1098, pUI1099, and pUI1900. Also indicated are the positions of base pair substitutions of *hemT** mutations. For further details about these plasmids, see Table 1 and the text.

transcription, as was strongly suggested by the mRNA studies of Neidle and Kaplan (16). We analyzed the PCR-amplified *hemT* upstream sequences of the ALA prototroph A1 by determining the DNA sequence of residues −346 to +84 relative to the start site of *hemT* transcription. There were no changes found in these sequences relative to the wild-type 2.4.1 sequences. Thus, we confirmed that *hemT* expression can be activated by means of extragenic events.

Isolating and characterizing mutants of a *hemT* Kn^r transcriptional fusion plasmid that have increased levels of transcription. If *hemT* transcription is being down-regulated in wild-type cells, we might expect that appropriate changes in the upstream sequences of *hemT* could bypass this regulation, leading to increased levels of *hemT* transcription. We constructed a plasmid in which the upstream sequences of *hemT* are positioned proximal to the coding sequences for the Kn resistance gene of Tn*903* (Fig. 6). Exconjugants of the wild-type construct, pUI1071, were incapable of growth on media containing Kn at concentrations above 5 μg/ml. However, by plating exconjugants on media containing higher concentrations of Kn, spontaneous mutants were selected, which conferred resistance to a concentration of at least 25 μg/ml. A total of eight independently isolated, mutated plasmids were analyzed at the DNA sequence level, and all were found to contain a C → T base pair substitution at position −13 relative to the start site of *hemT* transcription. Another selection from plates containing 100 μg of Kn per ml yielded a mutant plasmid bearing a T → C base pair substitution at position −31 relative to the start site of *hemT* transcription. Thus, we have demonstrated that up mutations (*hemT** mutations), which increased the levels of *hemT* transcripts, can occur in wild-type cells. However, these mutations were not present in the upstream *hemT* sequences of the ALA$^+$ A1 strain, and these up mutations could not account for the complementation of the ALA auxotroph AT1 by the plasmid-borne *hemT* gene. For these strains, it must be that increased *hemT* expression was brought about by changes in components mediating *hemT* expression, i.e., *trans*-acting mutations.

TABLE 2. β-Galactosidase activities from cell extracts of *R. sphaeroides* strains bearing *hemT-lacZ* or *hemT*-lacZ* transcriptional fusion plasmids

Transcriptional fusion plasmid	Relative β-galactosidase activity[a]		
	2.4.1	H-5	AT1
Wild-type plasmid: pUI1098	4.3 (1)	16 (4)	78 (18)
C → T (−13): pUI1099	303 (70)	29 (7)	1,624 (378)
T → C (−31): pUI1900	237 (55)	19 (4)	2,099 (488)

[a] Activities are expressed in units of β-galactosidase activity (micromoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein extract). All values represent the average of at least two independent determinations, for which values differed by less than 20%. The strains were grown in Sistrom's minimal medium, supplemented with ALA and antibiotics as appropriate. Relative β-galactosidase activities normalized to the activity of cell extracts of wild-type 2.4.1 with plasmid pUI1098 are given in parentheses. β-Galactosidase activity of the vector alone in wild-type 2.4.1 is 4 U.

Comparing the levels of *hemT* transcription in 2.4.1, H-5, and AT1. In the above sections, we reached the conclusion that extragenic differences among the three strains, wild-type 2.4.1, mutant AT1, and mutant H-5, resulted in different levels of *hemT* expression. To quantitatively demonstrate the differences in *hemT* expression in these strains, we constructed plasmids bearing transcriptional fusions between the wild-type *hemT* upstream sequences or the *hemT** upstream sequences and the *lacZ* gene of *E. coli* encoding β-galactosidase (Fig. 6). Assays of β-galactosidase activity were then performed on extracts of exconjugants containing these plasmids in the three different genetic backgrounds, i.e., wild-type 2.4.1, mutant H-5, and mutant AT1. The results are summarized in Table 2. We found that the wild-type upstream sequences were expressed at very low levels in wild-type 2.4.1 (4.3 U) and in mutant H-5 (16 U). In contrast, expression of wild-type upstream sequences was 18-fold higher in mutant AT1 (78 U) than in wild-type 2.4.1. This result confirmed that *hemT* expression was indeed turned on in the mutant AT1 background relative to that of either wild-type 2.4.1 or mutant H-5 and strongly supports the existence of one, or more, positively acting transcriptional factor(s).

We also found that while each of the two *hemT** mutations dramatically increased the levels of *lacZ* expression in wild-type 2.4.1 (70-fold for pUI1099 and 55-fold for pUI1900), in the H-5 mutant background, the effects of either of these mutations were marginal at best. Only a twofold increase was observed in mutant H-5 for the plasmid bearing a mutation at the −13 position, and the mutation at −31 showed no increase at all relative to expression from the wild-type upstream sequences in mutant H-5. Therefore, mutations that clearly activated *hemT* expression in other strains did not lead to higher expression when present in the H-5 background. These findings further exclude the possibility that a heterodimer between a multiply defective HemA and a normal HemT in mutant H-5 was responsible for the H-5 requirement for ALA.

These results demonstrate that both *cis* and *trans* mutations were capable of influencing *hemT* expression. Furthermore, these effects were synergistic, since expression from the *hemT** mutations in the *trans*-activated strain AT1 was an additional fivefold higher in the case of pUI1099 and ninefold higher in the case of pUI1900 relative to the activities observed in wild-type 2.4.1.

DISCUSSION

The *hemA* gene of mutant H-5 encodes a nonfunctional ALA synthase. Since earlier studies had demonstrated that in a strain bearing a nonrevertible mutation in *hemA*, the *hemT* gene product is capable of providing ALA to the cell (15), we had expected that we would find the *hemT* gene product of mutant H-5 to have been rendered nonfunctional. However, we have demonstrated, through our complementation analyses, that the *hemT* gene of H-5 does encode a functional HemT protein. Why, then, is mutant H-5 an ALA auxotroph? Formulating an answer to this question required an analysis of the mechanisms by which *hemT* expression can be increased.

First, we demonstrated that disruption or deletion of the *hemA* gene in otherwise wild-type cells actually conferred ALA auxotrophy. But these auxotrophs readily revert to ALA prototrophy, an event which clearly obscured earlier results from which it was concluded that ALA synthase encoded by *hemT* already fulfilled the ALA requirement in HemA$^-$ strains (16).

Second, we showed that reversion to ALA prototrophy was not brought about by changes in the upstream sequences of *hemT*, in that DNA sequence analyses of these sequences in the ALA prototrophic *hemA*-disrupted strain A1 revealed the absence of any changes relative to wild-type sequences. Furthermore, we showed that changes upstream of *hemT* were not the basis for mutant H-5 auxotrophy, since the results of our complementation analysis of the *hemT* gene of H-5 and the wild-type *hemT* gene were the same, i.e., either *hemT* gene could complement AT1, but neither could complement mutant H-5. From this result, it became evident that there were differences between strain AT1 and mutant H-5 which were extragenic to *hemT* and which determined whether the *hemT* gene product could fulfill the ALA requirement of HemA$^-$ strains. Thus, we concluded that AT1 cells were activated for *hemT* expression, while mutant H-5 cells, like wild-type cells, were not. The consequence for mutant H-5 and other strains bearing a nonfunctional *hemA* gene is that unless *hemT* expression is turned on, the cells require ALA for growth.

The identification of mutations in DNA sequences immediately upstream of *hemT* that increased the level of *hemT* expression in wild-type 2.4.1 meant that not only *trans*-acting events, as conclusively demonstrated by our complementation analysis, but *cis*-acting alterations could increase *hemT* expression as well. Yet, mutant H-5 is apparently unable to employ either of these two mechanisms (designated *cis* and *trans* activation) to bring about an increase in transcription of its functional *hemT* gene. A partial explanation of this observation is that the same *cis*-acting mutations that dramatically increased *hemT* expression in wild-type cells, even if they can occur in H-5, leave *hemT* expression virtually unchanged in mutant H-5. Thus, it appears that this mechanism alone is neither effective nor sufficient to increase *hemT* expression in mutant H-5.

With respect to *trans* activation, no single genetic event is sufficient to enable *hemT* expression to physiologic levels in mutant H-5, unlike the case for the wild-type 2.4.1 genetic background of strain AT1. We consider H-5 to be highly mutagenized on the basis of the fact that no less than four base pair substitutions were identified in *hemA* alone, given the qualification relating to the proposed parent of H-5. Further, the ability of H-5 to take up ALA is altered relative to that of wild-type cells (11). We believe that H-5 is so highly mutagenized that *trans* activation is simply not possible.

Therefore, the genetic basis for the ALA auxotrophy of mutant H-5 appears to be a combination of a nonfunctional *hemA* gene product and insufficient transcription of the *hemT* gene, which nevertheless encodes a functional ALA synthase. We have demonstrated that in a wild-type background, both *cis* and *trans* mutations could increase *hemT* expression but that in the mutant H-5 background, these same *cis* mutations did not increase *hemT* expression and the *trans* events have not or cannot occur in mutant H-5. It may be that these two kinds of events, as they pertain to the restoration of ALA synthesis, are related. The *cis* mutations, because of their locations, are likely to represent promoter mutations which, in the presence of an appropriate transcriptional factor(s), are potentiated and thus permit increased transcriptional expression of *hemT*. The H-5 background may contain one or more genetic lesions in the gene encoding this same putative transcription factor(s), which in AT1 is also mutated but is rendered activating for *hemT* expression, leading to enhanced transcription of both *hemT* and *hemT** sequences. In the case of mutant H-5, the original scoring was for ALA auxotrophy, and in the case of AT1, ALA prototrophy was selected. Our working hypothesis is that increased expression of a unique sigma factor in AT1 would fulfill these expectations.

The ALA auxotrophy of H-5 has been observed under both aerobic and photosynthetic growth conditions (11). Thus, it seems that the inability of *hemT* alone in mutant H-5 to fulfill the ALA requirement is not unique to one growth mode. However, the *hemT* gene is able to fulfill the ALA requirement of the *hemA*-truncated strain A1 under both aerobic and photosynthetic growth conditions (15), and the associated increase in *hemT* message levels compared with those of wild-type cells is observed for both of these modes of growth. Note, too, that as with the *hemA* message, *hemT* message levels are three times higher in photosynthetically grown cells compared with those in aerobically grown A1 cells (16). Thus, in addition to turn-on or turn-up of *hemT* gene expression, it appears that *hemT* expression, at the level of transcription, is regulated in a manner paralleling that of the regulation of the *hemA* gene, though it undoubtedly involves a different mechanism.

Despite the manifold insights we have gained into *hemT* expression, there are aspects of *hemT* expression that remain unresolved. For example, if *hemT* is not normally expressed at all, we would not expect any phenotype to be associated with *hemT*-deleted cells, and yet this does not appear to be the case, since bacteriochlorophyll and carotenoid levels and antennae complex levels are significantly reduced in *hemT*-deleted cells relative to those in wild-type cells. The identification and isolation of the component(s) that triggers the conversion of ALA auxotrophy to prototrophy in *hemA* cells will no doubt be of great value in our further analyses of the expression of both the *hemA* and *hemT* genes and their respective contributions to the total levels of ALA synthase activity of the cell.

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REFERENCES

1. **Beale, S. I., and J. D. Weinstein.** 1991. Biosynthesis of 5-aminolevulinic acid in phototrophic organisms, p. 385–406. *In* H. Scheer (ed.), Chlorophylls. CRC Press, Inc., Boca Raton, Fla.
2. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
3. **Davis, J., T. J. Donohue, and S. Kaplan.** 1988. Construction, characterization, and complementation of a *puf* mutant of *Rhodobacter sphaeroides*. J. Bacteriol. **170:**320–329.

4. **Eraso, J. M., and S. Kaplan.** 1994. *prrA*, a putative response regulator involved in oxygen regulation in photosynthesis gene expression in *Rhodobacter sphaeroides*. J. Bacteriol. **176:**32–43.
5. **Fanica-Gaignier, M., and J. Clement-Metral.** 1973. Cellular compartmentation of two species of δ-aminolevulinic acid synthetase in a facultative photohetero-trophic bacterium (*Rps. spheroides* Y). Biochem. Biophys. Res. Commun. **55:**610–615.
6. **Fanica-Gaignier, M., and J. D. Clement-Metral.** 1971. ATP inhibition of aminolevulinate (ALA) synthetase activity in *Rhodopseudomonas spheroides* Y. Biochem. Biophys. Res. Commun. **44:**192–198.
7. **Ferreira, G. C., P. J. Neame, and H. A. Dailey.** 1993. Heme biosynthesis in mammalian systems: evidence of a Schiff base linkage between the pyridoxal 5′ phosphate cofactor and a lysine residue in 5-aminolevulinate synthase. Protein Sci. **2:**1959–1965.
8. **Jessee, J.** 1986. New subcloning efficiency competent cells: $>1 \times 10^6$ transformants/μg. Focus (Bethesda Research Laboratories) **8:**9.
9. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70:**191–197.
10. **Lascelles, J.** 1966. The accumulation of bacteriochlorophyll precursors by mutant and wild-type strains of *Rhodopseudomonas spheroides*. Biochem. J. **100:**175–183.
11. **Lascelles, J., and T. Altshuler.** 1969. Mutant strains of *Rhodopseudomonas spheroides* lacking δ-aminolevulinate synthase: growth, heme, and bacteriochlorophyll synthesis. J. Bacteriol. **98:**721–727.
12. **Lee, J. K., and S. Kaplan.** Transcriptional regulation of *puc* operon expression in *Rhodobacter sphaeroides*: analysis of the *cis*-acting downstream regulatory sequence. Submitted for publication.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
14. **Neidle, E. L., and S. Kaplan.** 1992. *Rhodobacter sphaeroides rdxA*, a homolog of *Rhizobium meliloti fixG*, encodes a membrane protein which may bind cytoplasmic [4Fe-4S] clusters. J. Bacteriol. **174:**6444–6454.
15. **Neidle, E. L., and S. Kaplan.** 1993. 5-Aminolevulinic acid availability and control of spectral complex formation in HemA and HemT mutants of *Rhodobacter sphaeroides*. J. Bacteriol. **175:**2304–2313.
16. **Neidle, E. L., and S. Kaplan.** 1993. Expression of the *Rhodobacter sphaeroides hemA* and *hemT* genes, encoding two 5-aminolevulinic acid synthase isozymes. J. Bacteriol. **175:**2292–2303.
17. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
18. **Săsărman, A., M. Surdeanu, and T. Horodniceanu.** 1968. Locus determining the synthesis of δ-aminolevulinic acid in *Escherichia coli* K-12. J. Bacteriol. **96:**1882–1884.
19. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**37–45.
20. **Sistrom, W. R.** 1960. A requirement for sodium in the growth of *Rhodopseudomonas sphaeroides*. J. Gen. Microbiol. **22:**778–785.
21. **Suwanto, A., and S. Kaplan.** 1989. Physical and genetic mapping of the *Rhodobacter sphaeroides* 2.4.1 genome: genome size, fragment identification, and gene localization. J. Bacteriol. **171:**5840–5849.
22. **Tai, T.-N., W. A. Havelka, and S. Kaplan.** 1988. A broad-host-range vector system for cloning and translational *lacZ* fusion analysis. Plasmid **19:**175–188.
23. **Tai, T.-N., M. D. Moore, and S. Kaplan.** 1988. Cloning and characterization of the 5-aminolevulinate synthase gene(s) from *Rhodobacter sphaeroides*. Gene **70:**139–151.
24. **Tuboi, S., and S. Hayasaka.** 1972. Control of δ-aminolevulinate synthetase activity in *Rhodopseudomonas sphaeroides*. II. Requirements of a disulfide compound for the conversion of the inactive form of fraction I to the active form. Arch. Biochem. Biophys. **150:**690–697.
25. **Tuboi, S., H. J. Kim, and G. Kikuchi.** 1970. Differential induction of form I and form II of δ-aminolevulinate synthetase in *Rhodopseudomonas sphaeroides* under various incubation conditions. Arch. Biochem. Biophys. **138:** 155–159.
26. **Tuboi, S., H. J. Kim, and G. Kikuchi.** 1970. Occurrence and properties of two types of δ-aminolevulinate synthetase in *Rhodopseudomonas sphaeroides*. Arch. Biochem. Biophys. **138:**147–154.
27. **van Neil, C. B.** 1944. The culture, general physiology, morphology, and classification of the non-sulfur purple and brown bacteria. Bacteriol. Rev. **8:**1–118.