Positive and Negative Transcriptional Regulators of Glutathione-Dependent Formaldehyde Metabolism

Jason W. Hickman,^{1†} Vernon C. Witthuhn, Jr.,¹ Miguel Dominguez,²
and Timothy J. Donohue^{1*}

Departments of Bacteriology¹ and Genetics,² University of Wisconsin—Madison,
Madison, Wisconsin

Received 23 April 2004/Accepted 1 September 2004

A glutathione (GSH)-dependent pathway is used for formaldehyde metabolism by a wide variety of prokaryotes and eukaryotes. In this pathway, *S*-hydroxymethylglutathione, produced by the reaction of formaldehyde with the thiolate moiety of glutathione, is the substrate for a GSH-dependent formaldehyde dehydrogenase (GSH-FDH). While expression of GSH-FDH often increases in the presence of metabolic or exogenous sources of formaldehyde, little is known about the factors that regulate this response. Here, we identify two signal transduction pathways that regulate expression of *adhI*, the gene encoding GSH-FDH, in *Rhodobacter sphaeroides*. The loss of the histidine kinase response regulator pair RfdRS or the histidine kinase RfdS increases *adhI* transcription in the absence of metabolic sources of formaldehyde. Cells lacking RfdRS further increase *adhI* expression in the presence of metabolic sources of formaldehyde (methanol), suggesting that this negative regulator of GSH-FDH expression does not respond to this compound. In contrast, mutants lacking the histidine kinase response regulator pair AfdRS or the histidine kinase AfdS cannot induce *adhI* expression in the presence of either formaldehyde or metabolic sources of this compound. AfdR stimulates activity of the *adhI* promoter in vitro, indicating that this protein is a direct activator of GSH-FDH expression. Activation by AfdR is detectable only after incubation of the protein with acetyl phosphate, suggesting that phosphorylation is necessary for transcription activation. Activation of *adhI* transcription by acetyl-phosphate-treated AfdR in vitro is inhibited by a truncated RfdR protein, suggesting that this protein is a direct repressor of GSH-FDH expression. Together, the data indicate that AfdRS and RfdRS positively and negatively regulate *adhI* transcription in response to different signals.

Formaldehyde is a cytogenic compound that is produced by environmental, industrial, and metabolic processes including the oxidative demethylation of amino acids and osmoprotectants or oxidation of one-carbon compounds like methanol, methyl halides, and methane (10, 11, 16, 20–22, 24, 35, 37, 38). The toxicity of formaldehyde stems from its reactivity with amino and sulfhydryl groups of biological molecules, causing alkylation, mutations, and cross-links that destroy the function of membranes, proteins, and nucleic acids (18, 25). When the sources of formaldehyde, its toxicity, and the carbon skeletons and reducing power derived from its oxidation are considered, it is clear that cells benefit from metabolism of this compound.

We are studying formaldehyde metabolism by the facultative bacterium *Rhodobacter sphaeroides*. This α -proteobacterium uses a glutathione (GSH)-dependent pathway for formaldehyde metabolism that is similar to those present in several prokaryotic and eukaryotic cells (5, 7). In this pathway, formaldehyde reacts with the thiolate moiety of GSH to form *S*-hydroxymethylglutathione, a substrate for a GSH-dependent formaldehyde dehydrogenase (GSH-FDH). GSH-FDH oxidizes *S*-hydroxymethylglutathione to *S*-formylglutathione with concomitant reduction of NAD. *S*-Formylglutathione is hydro-

lyzed to glutathione and formate before oxidation of formate to CO₂ by a NAD-dependent formate dehydrogenase. Evidence supporting the role of GSH-FDH in this pathway includes the inability of GSH-FDH mutants to grow in the presence of metabolic sources of formaldehyde and the defect in formaldehyde oxidation seen in cells that lack this enzyme (5, 19).

Given this role of GSH-FDH, it makes sense that expression of this enzyme is regulated. In *Saccharomyces cerevisiae*, GSH-FDH levels are increased by the presence of methylated compounds like methyl methanesulphonate or formaldehyde (36). In many eubacteria, including *R. sphaeroides*, expression of the GSH-FDH gene (*adhI*) is increased when either formaldehyde or metabolic sources of this compound, such as methanol or choline, are present (6, 15, 17). These and other results led to the proposal that a pathway intermediate, possibly formaldehyde itself, increases *adhI* transcription. Additional support for the regulated expression of *R. sphaeroides* GSH-FDH expression includes the existence of mutations, such as the *spd-7* allele, that increase *adhI* transcription (5). Unfortunately, little molecular information is available on the proteins or signals that regulate expression of GSH-FDH homologs.

To increase our understanding of GSH-FDH expression, we have identified genes that control *adhI* transcription. In one set of experiments, we utilized a previously characterized mutant containing the *spd-7* allele, which increases expression of the *adhI-cycI* operon (30). Increased *adhI-cycI* expression by the *spd-7* allele restores photosynthetic growth to a mutant that lacks cytochrome *c*₂ by increasing levels of the cytochrome *c*₂

* Corresponding author. Mailing address: Department of Bacteriology, University of Wisconsin—Madison, Room 390, 420 Henry Mall, Madison, WI 53706. Phone: (608) 262-4663. Fax: (608) 262-9865. E-mail: tdonohue@bact.wisc.edu.

† Present address: Department of Microbiology, University of Iowa, Iowa City, IA 52242.

isoform, isocytochrome c_2 , encoded by *cycl* (31). By screening for wild-type DNA sequences that abolished photosynthetic growth of cells containing the *spd-7* allele, we identified a putative two-component signal transduction system that negatively regulates *adhI-cycl* transcription, which we termed RfdRS (repressor of formaldehyde dehydrogenase). Mutants lacking RfdRS still increase *adhI* transcription in response to metabolic sources of formaldehyde, suggesting the presence of additional regulators of *adhI* transcription. By searching the *R. sphaeroides* genome sequence, we identified a second related two-component regulatory system, AfdRS (activator of formaldehyde dehydrogenase), that stimulates *adhI* transcription in vitro and is necessary for formaldehyde-dependent induction of *adhI* expression in vivo. Both AfdRS and RfdRS display similarity with proteins in the methanol-oxidizing bacterium *Paracoccus denitrificans* (FlhRS) that have been implicated in controlling the expression of GSH-FDH in that organism. Based on these findings, we propose that *R. sphaeroides* GSH-FDH expression is positively and negatively regulated. Our data indicate that AfdRS is necessary for formaldehyde-dependent increases in *adhI* transcription, while negative regulation of *adhI* transcription by RfdRS responds to a different and unknown signal.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Table 1 lists strains and plasmids. *R. sphaeroides* was grown in Siström's minimal medium A containing 35 mM succinate at 30°C (33). For photosynthetic growth on solid medium, plates were placed in GasPak anaerobic jars (Becton Dickinson Microbiology Systems, Cockeysville, Md.) with H₂-CO₂ generators. *Escherichia coli* strain DH5 α was used as a plasmid host, S17-1 was used for conjugation of plasmids into *R. sphaeroides* (11), and ER2566 was used for expression of intein-chitin-binding domain fusions. *E. coli* strains were typically grown in Luria-Bertani medium at 37°C. When necessary, the medium was supplemented with spectinomycin (25 μ g/ml), kanamycin (25 μ g/ml), trimethoprim (30 μ g/ml), or tetracycline (1 μ g/ml) for *R. sphaeroides* and ampicillin (100 μ g/ml), tetracycline (10 μ g/ml), spectinomycin (25 μ g/ml), or kanamycin (25 μ g/ml) for *E. coli*. All primer sequences used are available from the authors upon request.

Isolation of sequences that reduce *adhI-cycl* expression. To identify DNA sequences that reduce expression of the *adhI-cycl* operon, a cosmid bank of wild-type *R. sphaeroides* DNA (34) was screened for isolates that abolish photosynthetic growth of a strain, CYCA65R7, that contains the *spd-7* allele (30). *E. coli* donors were mated with cells containing the *spd-7* allele to identify a cosmid, pUI8017, that abolished photosynthetic growth of this strain.

Cloning and inactivation of *rfdRTS* or *afdRTS*. To create a strain lacking RfdRTS, an Ω Sp^r cartridge was inserted into codon 23 of the *rfdR* gene (*rfd-1* allele). To do this, a ~2.0-kb HindIII fragment containing the Ω Sp^r cartridge was purified from pHP45, and the ends of this fragment were filled in by incubation with the Klenow fragment of DNA polymerase. This fragment was ligated into a filled-in XhoI site of pVW17-4, a plasmid which contains a BamHI fragment encoding *rfdR* from pUI8017. The resultant plasmid, p Δ RfdR1, was digested with BamHI, and a 3.4-kb fragment carrying the interrupted *rfdR* gene was purified and its end was filled in. This fragment was ligated into a filled-in EcoRI site of pSUP202. This Tc^r Sp^r plasmid, p Δ RfdR2, was mobilized into wild-type *R. sphaeroides*, and colonies exhibiting an Sp^r Tc^s phenotype were analyzed with genomic Southern blots to ensure that the *rfd-1* allele was incorporated by an even number of crossovers (data not shown).

To create a strain lacking RfdS, a ~2.2-kb EcoRI fragment containing the last 250 codons of *rfdT* and the first 459 codons of *rfdS* was cloned into EcoRI-digested pUC19, generating plasmid pVW17-35. This plasmid was digested with ClaI and StuI and filled in with a Klenow fragment of DNA polymerase, deleting codons 6 to 290 of *rfdS*. A 2.0-kb SmaI fragment containing the Ω Sp^r cartridge was purified from pHP45 and inserted into this mutant *rfdS* gene by ligation into digested pVW17-35 (*rfd-2* allele). This plasmid, p Δ RfdS1, was digested with EcoRI, and the ~4.2-kb fragment containing the *R. sphaeroides* DNA with the Ω Sp^r cartridge was isolated and ligated into pSUP202 that had been digested

with EcoRI. This suicide plasmid, p Δ RfdS2, was introduced into wild-type *R. sphaeroides*, and mutants were verified as described above.

A 600-bp in-frame deletion in *rfdT* (codons 71 to 271) was generated by a two-step process. In the first step, PCRs were performed to create products surrounding the region of *rfdT* to be deleted, each of which contained a 10-bp complementary overlap at the 3' end of the upstream product and the 5' end of the downstream product. These products were mixed in a ~1:1 ratio, and a second reaction was performed by using only the outside primers from the first two reactions, resulting in the formation of a ~4-kb fragment containing the *rfd-3* allele and XbaI sites to facilitate cloning. The product from this second reaction was digested with XbaI, cloned into XbaI-digested pUC19 to generate plasmid p Δ rfdT4, and sequenced to confirm the desired in-frame deletion in *rfdT*. This ~4-kb XbaI fragment was cloned into XbaI-digested pCM62. The resultant plasmid, p Δ RfdT1, was mobilized into *R. sphaeroides* RfdR1 to generate a strain lacking RfdT.

For complementation of the *rfdRTS* mutants, a ~4.6-kb fragment from pUI8017 that contains wild-type *rfdRTS* was generated by PCR and cloned into XbaI-digested pUC19, generating plasmid pRfd3. This 4.6-kb XbaI fragment was then cloned into XbaI-digested pCM62, generating a plasmid, pRfd4, that was used for complementation analysis.

To clone the *afdRTS* locus, a ~5-kb fragment containing the *R. sphaeroides* open reading frames RSP2591 (*afdR*), RSP2593 (*afdS*), and RSP2592 (*afdT*) was amplified from genomic DNA, and the PCR product was digested with KpnI, phosphorylated with T4 polynucleotide kinase, and ligated into KpnI-HincII-digested pUC19, resulting in pAfd1.

To generate a polar insertion in *afdR* (*afd-1* allele), a ~1.4-kb region containing this gene was amplified from genomic DNA, placing a KpnI site in the upstream primer. The product was purified, digested with KpnI, phosphorylated, and ligated into HincII-KpnI-digested pUC19, creating pAfdR1. A gene encoding trimethoprim resistance (Tp^r) was inserted into codon 84 of *afdR* by ligation into BtrI-digested pAfdR1 to generate p Δ ElAfdR1. A ~2-kb PCR product was generated from p Δ ElAfdR1 by using vector-specific primers (1224 and 1233) and cloned into ScaI-digested pSup202 to create p Δ ElAfdR3. p Δ ElAfdR3 was conjugated into *R. sphaeroides*, and strains in which *afd-1* had replaced the wild-type gene were verified as described above (strain AfdR7).

To generate a polar insertion in *afdS* (*afd-2* allele), a ~2.5-kb region was amplified from genomic DNA, and the product was phosphorylated and ligated into SmaI-digested pUC19 to generate pAfdS1. A BamHI fragment containing a gene encoding Tp^r was inserted into codon 142 of *afdS* by ligation into pAfdS1 that was digested by BglII, forming p Δ ElAfdS1. Plasmid p Δ ElAfdS1 was used to generate a ~3.5-kb PCR product containing the *afd-2* allele by using vector-specific primers (1224 and 1233). The product was phosphorylated and ligated into ScaI-digested pSUP202, generating p Δ ElAfdS2. p Δ ElAfdS2 was conjugated into *R. sphaeroides*, and strains in which *afd-2* replaced the wild-type gene were verified as described above (strain AfdS1).

To generate an *afdRTS*-containing plasmid for complementation, a ~5-kb PvuII fragment from pAfd1 was ligated into ScaI-digested pSUP202, resulting in pAfdRTS5. For single-copy complementation, pAfdRTS5 was conjugated into *R. sphaeroides*, and merodiploids were identified by screening for resistance to both trimethoprim (from the *afd-1* or *afd-2* allele) and tetracycline (from pSUP202) to generate strains AfdR7-5 and AfdS1-5. The presence of the wild-type *afdRTS* locus or the *afd-1* or *afd-2* allele was confirmed by PCR.

Enzyme assays. The levels of β -galactosidase activity in aerobic cells containing an *adhI::lacZ* reporter gene were assayed (31) while cultures were at cell densities of $\sim 3.0 \times 10^8$ to 7.0×10^8 CFU/ml. Values reported are averages of data from at least three separate isolates grown in separate cultures. The error reported is the standard deviation between individual isolates (31). Values for GSH-dependent formaldehyde dehydrogenase activity (7) are the averages of three independent assays. Protein concentration was determined by the Bradford assay (8a).

Western blots. Western blot analysis with antiserum to isocytochrome c_2 (29) was performed on crude cell extracts prepared by sonication of cells from 500-ml cultures grown to a cell density of approximately 10^9 cells per ml by aeration with a gas mixture of 69% N₂, 30% O₂, and 1% CO₂. Equal amounts of crude extract protein (~12.5 μ g) were loaded on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (4 to 12% gradient) (Invitrogen, Carlsbad, Calif.). Proteins were transferred to nitrocellulose membranes and probed for isocytochrome c_2 with a 1:500 dilution of rabbit antiserum (29). Secondary anti-rabbit immunoglobulin G conjugated to horseradish peroxidase was used for detection.

Purification of AfdR and RfdR. AfdR or a truncated version of RfdR lacking its presumed N-terminal receiver and oligomerization domains (RfdR-CTD) was purified by using an intein-chitin-binding domain fusion system (New England

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>R. sphaeroides</i>		
Ga	<i>crtI</i>	Laboratory strain
CYCA65	Ga derivative, <i>cycA-1</i> allele	13
CYCA65R7	Ga derivative, <i>spd-7</i> allele in CYCA65	28
RfdR1	Ga derivative, Sp ^r insertion at codon 23 of <i>rfdR</i> (<i>rfd-1</i>)	This study
RfdS1	Ga derivative, 850-bp deletion and Sp ^r insertion at codon 6 of <i>rfdS</i> (<i>rfd-2</i>)	This study
AfdR7	Ga derivative, Tp ^r insertion at codon 84 of <i>afdR</i> (<i>afd-1</i>)	This study
AfdS1	Ga derivative, Tp ^r insertion at codon 142 of <i>afdS</i> (<i>afd-2</i>)	This study
AfdR7-5	Ga derivative, merodiploid containing <i>afd-1</i> allele and <i>afdRTS</i> from plasmid pAfdRTS5 integrated into the chromosome	This study
AfdS1-5	Ga derivative, merodiploid containing <i>afd-2</i> allele and <i>afdRTS</i> from plasmid pAfdRTS5 integrated into the chromosome	This study
JWH1	Ga derivative, Sp ^r insertion at codon 23 of <i>rfdR</i> (<i>rfd-1</i>) and Tp ^r insertion at codon 84 of <i>afdR</i> (<i>afd-1</i>)	This study
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR178</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	8
S17-1	C600::RP-4 2-(Tc::Mu)(Kn::Tn7) <i>thi pro hsdR hsdM⁺ recA</i>	32
ER2566	F ⁻ λ <i>fhuA2</i> [<i>lon</i>] <i>ompT</i> <i>lacZ::T7 gene1</i> <i>gal</i> <i>sulA11</i> Δ (<i>mcrC-mrr</i>)114::IS10R(<i>mcr-73::miniTn10-TetS</i>)2 <i>R(zgb-210::Tn10)</i> (<i>TetS</i>) <i>endA1</i> [<i>dcm</i>]	New England Biolabs
Plasmids		
pUC19	Ap ^r	27
pSUP202	Ap ^r Tc ^r Cm ^r Mob ⁺ ; pBR325 derivative	32
pCM62	Tc ^r Mob ⁺	26
pTYB2	C-terminal intein-chitin-binding expression vector, Ap ^r	New England Biolabs
pRKK96	Vector for in vitro transcription; filled-in SpeI fragment containing Sp ^r cloned into filled-in HindIII site of pUC19 Δ <i>spI</i>	23
pEPS296	316-bp HindIII-BsaI fragment containing <i>adhI</i> promoter in HindIII-AccI sites of pUC21	6
pLA2917	Kn ^r Tc ^r ; RK2 derivative; <i>cos</i>	1
pUI8017	Tc ^r ; pLA2917 derivative, cosmid containing <i>rfdRTS</i>	This study
pVW17-4	1.7-kb BamHI fragment from pUI8017 containing <i>rfdR</i> cloned into pUC19	This study
pAfdS1	2.5-kb PCR product containing <i>afdS</i> cloned into SmaI-digested pUC19	This study
pDelAfdS1	0.7-kb Tp cassette cloned into BglII site in <i>afdS</i> at codon 142 (<i>afd-2</i>)	This study
pDelAfdS2	3.5-kb PCR product from pDelAfdS1 cloned into ScaI-digested pSUP202	This study
pAfd1	5-kb PCR product containing <i>afdRTS</i> cloned into KpnI-HincII-digested pUC19	This study
pAfdRTS5	5-kb PvuII fragment from pAfd1 cloned into ScaI-digested pSUP202	This study
pAfdR-Int1	750-bp PCR product containing <i>afdR</i> cloned into HincII-digested pUC19	This study
pAfdR-Int2	750-bp NdeI-SmaI fragment from pAfdR-Int1 cloned into pTYB2	This study
pMAD1	~250-bp PCR product containing last ~230 bp of <i>rfdR</i> cloned into SmaI pUC19	This study
pMAD47	~230-bp NdeI-SmaI fragment from pMAD1 cloned into pTYB2	This study
pJWH20	300-bp KpnI-HincII fragment from pEPS296 cloned into KpnI-StuI-digested pRKK96	This study
pTY296	<i>adhI</i> promoter sequences from pEPS296 cloned into pRKK200	6
pARfdR1	2.0-kb Sp ^r cassette cloned into XhoI site in <i>rfdR</i> at codon 23 (<i>rfd-1</i>)	This study
pARfdR2	3.7-kb BamHI fragment from pARfdR1 cloned into filled-in EcoRI-digested pSUP202	This study
pVW17-35	2.2-kb PCR EcoRI fragment from pUI8017 containing part of <i>rfdT</i> and <i>rfdS</i> ligated into EcoRI digested pUC19	This study
pARfdS1	2.0-kb Sp ^r cassette cloned into ClaI-StuI-digested pVW17-35 interrupting <i>rfdS</i> at codon 6 (<i>rfd-2</i>)	This study
pARfdR2	4.2-kb EcoRI fragment from pARfdS1 cloned into EcoRI-digested pSUP202	This study
pDelT4	4.0-kb PCR product containing <i>rfdRTS</i> with a 600-bp deletion of <i>rfdT</i> (<i>rfd-3</i>) cloned into XbaI-digested pUC19	This study
pARfdT4	4.0-kb XbaI fragment from pDelT4 cloned into XbaI-digested pCM62	This study
pRfd3	4.6-kb PCR product containing wild-type <i>rfdRTS</i> cloned into XbaI-digested pUC19	This study
pRfd4	4.6-kb XbaI fragment from pRfd3 containing wild-type <i>rfdRTS</i> cloned into pCM62	This study
pAfdR1	1.4-kb PCR fragment containing <i>afdR</i> cloned into KpnI-HincII-digested pUC19	This study
pDelAfdR1	0.7-kb Tp cassette cloned into BtrI site in <i>afdR</i> at codon 84 (<i>afd-1</i>)	This study
pDelAfdR3	2.1-kb PCR product from pDelAfdR1 cloned into ScaI-digested pSUP202	This study

Biolabs, Beverly, Mass.) that had been previously used to obtain active *R. sphaeroides* response regulators (9).

To generate an intein fusion to AfdR, the *afdR* gene was amplified and cloned into HincII-digested pUC19, generating pAfdR-Int1. After pAfdR-Int1 was digested with NdeI and SmaI, a ~750-bp fragment was ligated into pTYB2 that was digested with NdeI and SmaI, forming pAfd-Int2. Before use, pAfd-Int2 was

sequenced to ensure that *afdR* was fused in frame with the intein-chitin-binding domain. To purify AfdR, 1 liter of ER2566 (pAfdR-Int2) cells were grown at 37°C to an optical density at 600 nm of 0.4 to 0.5. IPTG (isopropyl- β -D-thiogalactopyranoside) was added to 0.3 mM, and the culture was incubated at 30°C for 3 h. Cells were harvested by centrifugation, resuspended in 5 ml of column buffer (20 mM HEPES [pH 8.0], 250 mM KCl, 0.1 mM EDTA, 0.1% Triton X-100), and

lysed by sonication. The extract was centrifuged at $15,000 \times g$ for 30 min, and the supernatant was loaded onto a 6-ml chitin column equilibrated with 10 volumes of column buffer. The column was washed with 10 volumes of column buffer, flushed with column buffer containing 60 mM dithiothreitol, and stored for 36 h at 4°C to allow intein cleavage. Released protein was eluted in column buffer and collected in 2-ml fractions before analysis by SDS-PAGE. Fractions containing AfdR were pooled and dialyzed against storage buffer (20 mM HEPES, 250 mM KCl, 0.1 mM EDTA, 50% glycerol, pH 8.0). AfdR used for in vitro transcription experiments was estimated to be >95% pure by SDS-PAGE analysis of pooled fractions. Phosphorylated AfdR was prepared by mixing the protein with 25 mM acetyl phosphate for 1 h at 30°C (9).

To generate an intein fusion to RfdR-CTD, DNA encoding the C-terminal domain (amino acids 150 to 226) was amplified and cloned into SmaI-digested pUC19, generating pMAD1. After pMAD1 was digested with NdeI and SmaI, a ~230-bp fragment was ligated into pTYB2 that was digested with NdeI and SmaI, forming pMAD47. Before use, pMAD47 was sequenced to ensure that the C-terminal region of *rfdR* was fused in frame with the intein-chitin-binding domain. To purify RfdR-CTD, 800 ml of ER2566 (pMAD47) cells were grown at 37°C to an optical density at 600 nm of 0.4 to 0.5. IPTG was added to 0.3 mM, and the culture was incubated at 30°C for 3 h. Cells were harvested by centrifugation, resuspended in 5 ml of column buffer (20 mM Tris-HCl [pH 7.9], 5 mM MgCl₂, 500 mM KCl, 0.1 mM EDTA, 0.1% Triton X-100), and lysed by sonication. The cell extract was centrifuged at $15,000 \times g$ for 30 min, and the supernatant was loaded onto a 10-ml chitin column equilibrated with 10 column volumes of column buffer. The column was washed with 10 column volumes of column buffer, flushed with column buffer containing 50 mM dithiothreitol, and stored overnight at 4°C to allow intein cleavage. Released protein was eluted in column buffer and collected in 1-ml fractions before analysis by SDS-PAGE. Fractions containing RfdR-CTD were pooled and dialyzed against storage buffer (40 mM Tris-HCl [pH 7.9], 5 mM MgCl₂, 50 mM KCl, 1 mM dithiothreitol, 20% glycerol).

Gel shift assays. A 476-bp DNA fragment containing the *adhI* promoter that extends from -296 to +180 bp relative to the known transcription initiation site (5) was end labeled with [α -³²P]ATP. The indicated concentrations of pure recombinant RfdR-CTD were incubated with 2 ng of the end-labeled *adhI* promoter DNA in a reaction mix containing 10 mM KPO₄ (pH 7.5), 100 mM potassium glutamate, 1 mM EDTA, 0.05 mM dithiothreitol, 0.5 mg of bovine serum albumin/ml, and 30 μ g of salmon sperm DNA/ml. Binding reactions were incubated at 30°C for 40 min. After incubation, protein-DNA complexes were resolved on a 6% polyacrylamide gel (Invitrogen) by using 0.5 $\times$ Tris-borate-EDTA as a running buffer. The gel was run at 4°C for 1.5 h at 100 V. The gel was dried and exposed to a PhosphorImager screen overnight and visualized by using a PhosphorImager and ImageQuant software (Molecular Dynamics).

In vitro transcription reactions. A typical 20- μ l reaction included *R. sphaeroides* RNA polymerase (9), 20 nM plasmid pJWH20 (see below), and AfdR or RfdR-CTD in transcription buffer (40 mM Tris-Cl [pH 7.9], 50 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 0.1 mM dithiothreitol, 0.1 mg of bovine serum albumin/ml). This mixture was incubated for 20 min at 30°C prior to initiating the assay. After transcription was initiated by the addition of nucleoside triphosphates (0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, and 0.05 mM UTP plus 10 μ Ci of [α -³²P]UTP), the reaction mixture was incubated at 30°C for 20 min. Assays were stopped by adding 10 μ l of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). Samples were heated to 95°C for 5 min, and the products were separated on a 6% polyacrylamide-urea gel. The gel was dried and exposed to a PhosphorImager screen. Transcript levels were quantified by using ImageQuant software (Molecular Dynamics). Data shown for induction of *adhI* transcription were calculated relative to the background level and amount of the control RNA1 product that was present in each reaction.

Potential *adhI* promoter sequences (-296 to +14 relative to known transcriptional initiation site) were obtained by digestion of pEPS296 (6) with KpnI and HincII. This 310-bp fragment was cloned into pRKK96 digested with KpnI and StuI to generate pJWH20. The sequencing of pJWH20 with plasmid-specific primers was used to ensure the desired orientation of *adhI* promoter sequences relative to the SpoT40 transcription terminator on pRKK96.

RESULTS

A negative regulator of GSH-FDH expression. To search for negative regulators of GSH-FDH expression, we utilized an *R. sphaeroides* mutant, CYCA65R7, which contains the *spd-7* allele (28). The *spd-7* allele restores photosynthetic growth to

FIG. 1. Cosmid pUI8017 reduces *adhI-cycI* expression. (Top) A Western blot monitoring the relative abundance of isocytochrome *c*₂ in extracts of aerobically grown cells is shown. The strains used in this analysis are indicated above the lanes. (Bottom) GSH-FDH specific activities (in nanomoles per minute per milligram of protein) measured in extracts of these strains. Numbers in parentheses indicate the standard deviations in GSH-FDH specific activity.

cells lacking cytochrome *c*₂ by increasing transcription of the *adhI-cycI* operon, which encodes GSH-FDH (*adhI*) and isocytochrome *c*₂ (*cycI*) (7). Increased levels of isocytochrome *c*₂ are required for photosynthetic growth in the absence of cytochrome *c*₂, so we reasoned that wild-type DNA sequences reducing *adhI-cycI* expression would abolish photosynthetic growth of cells containing the *spd-7* allele.

To search for DNA that reduced *adhI-cycI* expression, a wild-type cosmid library was mobilized into cells containing an *spd-7* allele (CYCA65R7), and exconjugants were scored for photosynthetic growth. One cosmid that blocked photosynthetic growth, pUI8017, was identified. To test that the loss of photosynthetic growth of cells containing an *spd-7* allele was due to reduced expression of the *adhI-cycI* operon, we measured the levels of these gene products. To measure levels of the *cycI* gene product, a Western blot with CycI-specific antibody was used to determine the relative abundance of isocytochrome *c*₂ in aerobically grown cells (Fig. 1). This analysis showed that there were low, but detectable, amounts of isocytochrome *c*₂ in both wild-type cells and cells lacking cytochrome *c*₂ (CYCA65). There is increased abundance of isocytochrome *c*₂ in cells containing the *spd-7* allele, similar to results reported previously (29). The presence of pUI8017 in the *spd-7* mutant decreased the abundance of isocytochrome *c*₂, providing an explanation for why the presence of this cosmid blocked photosynthetic growth of cells containing the *spd-7* allele.

To provide a more accurate measure of the effects of this plasmid on *adhI-cycI* expression, we assayed the specific activity of GSH-FDH in crude cell extracts from these same strains. As expected from the above results, the ~4-fold increase in GSH-FDH-specific activities caused by the *spd-7* mutation is abolished by pUI8017 (Fig. 1). Control experiments indicate that these decreases in GSH-FDH and isocytochrome *c*₂ are

FIG. 2. Physical map of the region around *rfdRTS* and *afdRTS* in *R. sphaeroides*. Hypothetical genes are indicated by their gene numbers in the annotated *R. sphaeroides* genome (<http://genome.ornl.gov/microbial/rsph/>). The sizes of the genes (in base pairs) are indicated above the map, and the direction of transcription is indicated by the arrows below. (A) Open reading frames corresponding to *rfdRTS* are indicated in the boxes (RSP2882 to RSP2880). The positions of mutations in *rfdR*, *rfdT*, and *rfdS* used in this study are indicated by the triangles. (B) The top shows the region surrounding *afdRTS*, including RSP2575 to RSP2596 on *R. sphaeroides* chromosome I. The bottom shows the region surrounding *afdRTS*. The places where *Tp^r* genes were inserted to generate the *afd-1* and *afd-2* alleles are indicated by the triangles.

accompanied by a reduction in the abundance of *adhI-cycI* mRNA (data not shown), suggesting that sequences on this plasmid are lowering transcription from the *adhI* promoter (6).

To identify genes responsible for this decrease in *adhI-cycI* expression, we tested a series of plasmids derived from this cosmid for their ability to prevent photosynthetic growth of cells containing the *spd-7* allele. Sequence analysis of a smaller region that prevented photosynthetic growth of cells containing an *spd-7* allele revealed the presence of a potential operon (*rfdRTS*) predicted to encode a two-component regulatory system (RfdRS) and an additional protein (RfdT) with no predicted function (Fig. 2a); these correspond to open reading frames named RSP2880 to RSP2882 in the *R. sphaeroides* genome sequence (<http://genome.ornl.gov/microbial/rsph/>).

We named these genes *rfdRTS* (repressor of GSH-dependent formaldehyde dehydrogenase) because it appears that these gene products are negative regulators of *adhI* expression (see below). The predicted amino acid sequence of RfdR has significant similarity to the NarL family of response regulators, while RfdS is a member of the sensor histidine kinases. The other predicted open reading frame, RfdT, displays significant amino acid similarity to a group of hypothetical conserved proteins often found to be encoded by genes between RfdR and RfdS homologs.

RfdRTS negatively regulates *adhI* expression. The reduced expression of *adhI-cycI* when RfdRTS are present in cells containing the *spd-7* allele predicts that these proteins are negative

regulators of GSH-FDH expression. If RfdRTS negatively regulated *adhI* transcription, then the loss of RfdRS should increase *adhI* expression. To test this hypothesis, we constructed strains that lack RfdRTS (RfdR1, *rfd-1* allele) or RfdS (RfdS1, *rfd-2* allele). Expression of *adhI* in mutant and wild-type strains was monitored by using a previously described transcriptional fusion of *adhI* promoter sequences (−296 to +14 relative to the transcriptional start site) to a promoterless *lacZ* gene on a low-copy plasmid (pTY296) (6).

Aerobically grown wild-type cells display levels of β-galactosidase activity that are well above background (Table 2), as reported previously (6). Aerobic cells lacking RfdRTS have three- to fourfold-higher levels of β-galactosidase activity than wild-type cells grown in a succinate-based minimal medium. This result supports the hypothesis that RfdRTS negatively regulate *adhI* transcription. Cells lacking only RfdS also have ~3-fold-higher levels of activity from the *adhI* reporter gene (Table 2), suggesting that the presumed kinase activity of RfdS is necessary for negative regulation of *adhI* transcription. The amount of *adhI* transcription in each of these mutants is restored to wild-type levels by introducing a plasmid (pRfd4) containing wild-type *rfdRTS*, confirming that the loss of RfdRTS is responsible for the increase in cells lacking either RfdRTS or RfdS (Table 2). The presence of *rfdRTS* on this ~6- to 10-copy plasmid does not decrease *adhI* expression compared to the uninduced levels seen in the wild type (Table 2). This result is likely due to the fact that *adhI* expression is

TABLE 2. β -Galactosidase activities from an *adhI-lacZ* reporter gene in wild type cells and those lacking combinations of *rfdRTS* or *afdRTS*

Strain	Plasmid	Relevant genotype	Inducer ^b	β -Galactosidase activity Miller units (SD) ^a
Wild type	None	<i>afdRTS</i> ⁺ / <i>rfdRTS</i> ⁺	None	26 (7)
Wild type	None	<i>afdRTS</i> ⁺ / <i>rfdRTS</i> ⁺	MeOH	381 (125)
Wild type	None	<i>afdRTS</i> ⁺ / <i>rfdRTS</i> ⁺	HCHO	295 (100)
RfdR1	None	<i>rfdRTS</i> deficient	None	74 (2)
RfdR1	pRfd4	<i>rfdRTS</i> ⁺	None	31 (7)
RfdR1	pRfdT4	<i>rfdT</i> deficient	None	23 (1)
RfdR1	None	<i>rfdRTS</i> deficient	MeOH	755 (145)
RfdS1	None	<i>rfdS</i> deficient	None	66 (9)
RfdS1	pRfd4	<i>rfdRTS</i> ⁺	None	33 (2)
RfdS1	None	<i>rfdS</i> deficient	MeOH	651 (77)
AfdR7	None	<i>afdRTS</i> deficient	None	17 (5)
AfdR7	None	<i>afdRTS</i> deficient	MeOH	17 (5)
AfdR7	None	<i>afdRTS</i> deficient	HCHO	16 (5)
AfdR7-5	None	<i>afdRTS</i> ⁺	None	31 (4)
AfdR7-5	None	<i>afdRTS</i> ⁺	MeOH	482 (113)
AfdR7-5	None	<i>afdRTS</i> ⁺	HCHO	317 (59)
AfdS1	None	<i>afdS</i> deficient	None	28 (13)
AfdS1	None	<i>afdS</i> deficient	MeOH	42 (14)
AfdS1	None	<i>afdS</i> deficient	HCHO	42 (21)
AfdS1-5	None	<i>afdRTS</i> ⁺	None	37 (15)
AfdS1-5	None	<i>afdRTS</i> ⁺	MeOH	410 (176)
AfdS1-5	None	<i>afdRTS</i> ⁺	HCHO	371 (37)
JWH1	None	<i>afdRTS</i> and <i>rfdRTS</i> deficient	None	19 (1)
JWH1	None	<i>afdRTS</i> and <i>rfdRTS</i> deficient	MeOH	19 (1)
JWH1	None	<i>afdRTS</i> and <i>rfdRTS</i> deficient	HCHO	20 (2)

^a Standard deviations are in parentheses. Averages are data from activities of at least three independent isolates.

^b MeOH, 100 mM methanol was added to culture for 3 h prior to analysis; HCHO, 50 μ M formaldehyde was added to culture for 1.5 h prior to analysis.

repressed in uninduced wild-type cells, so the presence of 6 to 10 copies of the genes encoding this potential negative regulator does not cause an additional decrease in *adhI* expression.

The fact that *rfdT* appears to be part of an *rfdRTS* operon prompted us to test if RfdT has a role in regulating *adhI* transcription. To test this hypothesis, we monitored the activity of the *adhI* promoter in a strain containing an in-frame deletion in RfdT. We constructed this strain by introducing a plasmid with an in-frame deletion within *rfdT* (p Δ RfdT4) into cells lacking RfdRTS (RfdR1). Analysis of this strain revealed that *adhI* expression was similar in aerobic cells lacking RfdT to that found in wild-type cells or an RfdR mutant that contained a plasmid-encoded *rfdRTS* region (Table 2). This observation indicates that RfdT is not necessary for negative regulation of *adhI* transcription by RfdRS.

***adhI* expression in cells lacking RfdRTS or RfdS is increased by metabolic sources of formaldehyde.** We know that metabolic sources of formaldehyde, like methanol, increase *adhI* transcription (6). To determine if *rfdRTS* was responsible for this increase, we measured β -galactosidase activity from the *adhI* reporter in aerobically grown wild-type cells and those lacking either RfdRTS or RfdS in the presence or absence of this metabolic source of formaldehyde. We found that *adhI* expression is induced $\sim$ 15-fold by adding methanol to aerobic cells grown in a succinate-based minimal medium (Table 2), similar to the increase reported previously (6). In addition, we found that *adhI* expression in aerobic cells lacking either RfdRTS or RfdS is increased $\sim$ 10-fold by the addition of methanol (Table 2). This finding indicates that RfdRTS are not essential for increased *adhI* transcription in the presence of metabolic sources of formaldehyde. The similar level of *adhI*

transcription in either wild-type or RfdRTS mutant cells in the presence of metabolic sources of formaldehyde could reflect the maximal activity of the *adhI* promoter in vivo. In summary, these results show that RfdRS are negative regulators of *adhI* expression but that these proteins are not required for increased *adhI* transcription in response to metabolic sources of formaldehyde.

Genes encoding possible signal transduction proteins are within 8 kb of *adhI*. The ability of formaldehyde to increase *adhI* transcription in cells lacking RfdRS suggests that cells contain an additional pathway that increases activity of this promoter in the presence of formaldehyde. To search for such an additional regulator of *adhI* expression, we queried the *R. sphaeroides* genome sequence (<http://genome.ornl.gov/microbial/rsph/>) for gene products related to proteins that are predicted to control similar pathways in other proteobacteria.

This analysis identified additional potential regulators of *adhI* expression near a putative formaldehyde metabolism gene cluster (Fig. 2b). In the region surrounding *adhI-cycI* are genes that could encode enzymes to oxidize methanol to formaldehyde (XoxF), periplasmic *c*-type cytochromes (CycI and CycB), a protein to facilitate the formation of *S*-hydroxymethylglutathione from formaldehyde and GSH (GfaA), and open reading frames which encode homologs of proteins of unknown function that are derived from genes typically linked to those encoding GSH-FDH family members in other eubacteria (14, 15, 17). This region also contains genes (RSP2591 to RSP2593) predicted to encode a sensor histidine kinase, a response regulator, and a predicted integral membrane protein of unknown function (Fig. 2b). We called these latter genes

afdRTS, for activator of glutathione-dependent formaldehyde dehydrogenase, based on the results of the following analyses.

The predicted sensor histidine kinase, AfdS, has significant amino acid sequence similarity to RfdS (39% identity, 52% similarity) and to a *P. denitrificans* protein, FlhS (51% identity, 64% similarity), that is required to increase GSH-FDH expression (see Fig. 6) (17). The potential response regulator, AfdR, is predicted to contain a C-terminal DNA-binding domain in the NarL response regulator family with significant amino acid sequence similarity to RfdR (44% identity, 59% similarity) and *P. denitrificans* FlhR (61% identity, 76% similarity). The predicted integral membrane protein has significant amino acid sequence similarity to RfdT and the uncharacterized gene product encoded by *P. denitrificans* *orf2*.

***afdRS* encodes a formaldehyde-responsive two-component regulator of *adhI* transcription.** The similarity of AfdRS to *P. denitrificans* FlhRS, and the observation that *flhRS* mutants of this bacterium fail to activate GSH-FDH expression (17), led us to test if these *R. sphaeroides* proteins regulated *adhI* expression. To do this, we monitored *adhI* expression in wild-type cells and in mutants that lack either AfdRTS (AfdR7) or AfdS (AfdS1) in the absence and presence of methanol. Expression of *adhI* was detectable when each of these strains was grown aerobically in a succinate-based minimal medium (Table 2). However, the addition of 100 mM methanol failed to stimulate transcription from the *adhI::lacZ* fusion in cells lacking either AfdRTS or AfdS, suggesting that these proteins are required for increased *adhI* transcription under these conditions (Table 2). Consistent with this notion, these defects in *adhI* expression are complemented by placing a wild-type copy of *afdRTS* in single copy in cells lacking either AfdRTS or AfdS (AfdR7-5 and AfdS1-5 [Table 2]). From this result, we conclude that AfdRTS are necessary for increased *adhI* expression when methanol is present under aerobic conditions.

The results of previous experiments led us to propose that formaldehyde, or possibly the glutathione adduct S-hydroxymethylglutathione, is an inducer of *adhI* transcription (6). Thus, the failure of methanol to stimulate *adhI* expression in cells lacking AfdRTS could reflect an inability of these mutants to generate formaldehyde from methanol or a defect in transcriptional control by formaldehyde. To distinguish between these possibilities, we monitored the ability of sublethal concentrations of formaldehyde to increase *adhI* expression. If the defect in cells lacking AfdRTS reflected an inability in generating this inducer from methanol, *adhI* expression should be increased by formaldehyde in these mutants. However, if formaldehyde were a potential signal molecule for AfdRTS, then cells lacking these proteins would be defective in stimulating *adhI* expression when formaldehyde was added.

In wild-type cells, *adhI* transcription is stimulated ~10-fold within 1.5 h after the addition of 50 μ M formaldehyde (Table 2). However, there was no significant increase in *adhI* expression when 50 μ M formaldehyde was added to cells lacking either AfdRTS or AfdS (Table 2). Placing a single copy of *afdRTS* in either mutant restored induction of *adhI* expression by formaldehyde (Table 2), suggesting that AfdRS responds to this compound.

To test if AfdRS was also required for the methanol-dependent increases in *adhI* expression seen in cells lacking RfdRTS, we compared the ability of this compound to increase *adhI*

FIG. 3. AfdR treated with acetyl phosphate activates *adhI* transcription in vitro. Positions of the *adhI*-specific and RNA1-specific transcripts are indicated by arrows. The *adhI* transcript appears as two discrete products due to RNA polymerase terminating transcription at adjacent bases downstream of the terminator on the plasmid template (2). The amount of AfdR in reactions and treatment with (+) acetyl phosphate (Acetyl-P) is listed at top. Transcript levels were quantified by using ImageQuant software (Molecular Dynamics). Indicated increases in *adhI* transcript levels were calculated relative to background in each lane.

expression in cells lacking both AfdRS and RfdRS (JWH1) to that of wild-type cells or strains lacking one of these pathways. In the absence of methanol, strains lacking both RfdRS and AfdRS have a level of *adhI* expression similar to that of wild-type cells but less than that observed in an RfdRTS mutant (Table 2). When either methanol or formaldehyde was added to induce *adhI* expression, there was no detectable increase in transcription in cells lacking both RfdRTS and AfdRTS (Table 2). Thus, we conclude that AfdRTS is necessary for the increased *adhI* expression in the presence of formaldehyde that is seen in both wild-type cells and strains lacking RfdRTS.

AfdR activates *adhI* transcription. To test if AfdR is a direct regulator of *adhI* expression, we asked if this protein stimulates activity of this promoter in vitro. To accomplish this, recombinant AfdR was added to in vitro transcription reactions containing *R. sphaeroides* RNA polymerase holoenzyme (20 nM) and an *adhI*-specific template. Many response regulators activate transcription only when phosphorylated, so reactions were performed with both AfdR and protein that was incubated with acetyl phosphate prior to the in vitro transcription assays.

No *adhI*-specific transcript was detected in assays lacking AfdR or when up to 5 μ M AfdR that was not incubated with acetyl phosphate was used (Fig. 3). However, the addition of 2 to 5 μ M concentrations of AfdR that was incubated with acetyl phosphate (AfdR-Pi) resulted in the production of an *adhI*-specific transcript of the expected size (184 nucleotides) based on the position of the known transcription initiation site rela-

tive to a transcription terminator on the plasmid template (Fig. 3). Of equal significance, the addition of acetyl phosphate-treated AfdR caused no detectable increase in the abundance of control RNA1 transcript that is contained on the plasmid template, suggesting that this increase in *adhI* expression is not due to a general increase in transcription.

We are unable to quantify the ability of acetyl phosphate-treated AfdR to stimulate *adhI* transcription because there is no detectable transcript in the absence of this protein or when untreated samples of this protein are used. However, there is a ~17-fold increase in *adhI* transcript levels when reactions containing 2 μ M AfdR that had been treated with acetyl phosphate were compared to those where 0.5 μ M AfdR was treated under identical conditions, and there is a ~22-fold increase in transcript levels when 5 μ M AfdR that was treated with acetyl phosphate was used. These results demonstrate that AfdR is a direct activator of *adhI* transcription and they suggest that this protein, like other members of the NarL family of response regulators, must be phosphorylated in order to activate transcription.

RfdR binds the *adhI* promoter. The increased *adhI* promoter activity in cells lacking RfdR suggested that this protein was a repressor of GSH-FDH expression (see above). To provide evidence supporting this hypothesis, we tested the ability of RfdR to interact with the *adhI* promoter by a gel mobility shift assay. To accomplish this, we used a truncated form of RfdR that contains only its putative C-terminal DNA binding domain (RfdR-CTD). Control experiments indicate that that half-life of 32 P-labeled full-length RfdR is less than 1 min (data not shown), suggesting that it would be difficult to maintain phosphorylation of full-length RfdR during the time needed to observe any interactions with *adhI* promoter sequences by a gel mobility shift assay. Thus, the use of RfdR-CTD meant that this protein should not need to be treated with acetyl phosphate in order to observe an interaction with the *adhI* promoter.

A DNA fragment containing *adhI* promoter sequences that extend from -296 to +180 relative to the known *adhI* transcription start site was incubated with purified recombinant RfdR-CTD prior to separating any DNA-protein complexes from promoter DNA on a 6% polyacrylamide gel. A shift in the mobility of the *adhI* promoter fragment can be seen when 0.5 μ M RfdR-CTD is added to the reaction (Fig. 4). The amount of this slower-migrating species increases when 1.0 μ M RfdR-CTD is present. This finding indicates that RfdR-CTD is capable of interacting with the *adhI* promoter at micromolar concentrations. This interaction between RfdR-CTD and the *adhI* promoter appears to be specific due to the fact that a 300-fold excess of a nonspecific competitor was present in these reactions.

RfdR is a direct inhibitor of *adhI* transcription. Since RfdR-CTD binds the *adhI* promoter, we wanted to test if RfdR-CTD could inhibit *adhI* transcription in vitro. It was not feasible to test the ability of RfdR alone to inhibit *adhI* transcription since no product was detectable from this promoter in the absence of acetyl phosphate-treated AfdR (Fig. 3). However, the ability of acetyl phosphate-treated AfdR to stimulate *adhI* transcription in vitro provided a way to test if RfdR acted directly to repress transcription from this promoter. To test this hypothesis, we used RfdR-CTD, which was deleted in the N-terminal

FIG. 4. RfdR-CTD binds to *adhI* promoter sequences. The amounts of RfdR-CTD added to the binding reaction are indicated above the lanes. The arrows indicate the migration position of the *adhI* promoter fragment and migration position of the presumed complex between DNA and RfdR-CTD. In this assay, 2 ng of end-labeled DNA (extending from -296 to +180 relative to the *adhI* transcription initiation site) was present in each reaction (6).

response regulator domain, and its presumed oligomerization region in order to minimize the potential for formation of mixed oligomers between the related AfdR and RfdR proteins in vitro. The use of RfdR-CTD also meant that this protein should not need to be treated with acetyl phosphate in order to observe an effect on *adhI* transcription in vitro.

To test if RfdR was a direct inhibitor of *adhI* transcription, we monitored the abundance of this transcript when increasing amounts of RfdR-CTD (0.5 to 4 μ M) were added to multiple-round transcription reactions containing *R. sphaeroides* RNA polymerase holoenzyme (95 nM), an *adhI*-specific template, and AfdR (2 μ M) that was treated with acetyl phosphate (Fig. 5). As expected, pretreatment of AfdR with acetyl phosphate prior to the assay resulted in the accumulation of an *adhI* transcript. There was a concentration-dependent decrease in the amount of the *adhI* transcript when increasing amounts of RfdR-CTD were present in the reactions. This negative effect of RfdR-CTD did not appear to reflect a general decrease in transcription since the addition of this protein did not reduce the amount of the control RNA1 transcript in a concentration-dependent manner (Fig. 5). If one measures the amount of *adhI* transcript present at different concentrations of RfdR-CTD relative to the level of RNA1 transcript that is present in each of these assays, ~2 μ M RfdR-CTD is sufficient to reduce the amount of this product by 50% (Fig. 5). By comparing the ability of RfdR-CTD to interact with *adhI* promoter DNA (Fig. 4) and its ability to inhibit transcript production from this promoter (Fig. 5), it appears that the apparent affinity of this truncated protein is in the micromolar range. Thus, we conclude that RfdR is a direct inhibitor of *adhI* transcription because it binds to this promoter and can act as a repressor of GSH-FDH expression.

DISCUSSION

The ability to metabolize formaldehyde and regulate the expression of genes required for its oxidation is likely to be

FIG. 5. RfdR is a direct inhibitor of *adhI* transcription in vitro. The top panel indicates positions of the *adhI*-specific and RNA1-specific transcripts with arrows. The presence of AfdR (2 μ M) and the concentration of RfdR-CTD (see the text) present (+) or absent (–) in reactions and pretreatment with acetyl phosphate (Acetyl-P) are indicated above the figure. Transcript levels were quantified by using ImageQuant software (Molecular Dynamics). The graph at the bottom shows the relative decrease in *adhI* transcript levels as the concentration of RfdR-CTD is increased, after correction for background and the relative amount of RNA1 transcript that is produced in each assay.

beneficial for cells, considering the ability of formaldehyde to act as a mutagen, destroy membrane function, inactivate proteins, and serve as a metabolic intermediate (16, 18, 21, 22, 24). GSH-FDH enzymes have been shown to play a key role in formaldehyde oxidation by glutathione-dependent pathways in prokaryotic and eukaryotic cells. However, the question of how GSH-FDH expression is regulated at the molecular level has received less attention. Here, we identify multiple two-component signal transduction pathways that directly control expression of *adhI*, the gene that encodes GSH-FDH in *R. sphaeroides*. Conclusions from our analysis of *adhI* transcription,

new questions, and the relevance to other cells are summarized below.

Our results predict that one regulator of *adhI* expression, RfdRS, is a repressor of *adhI* expression, while the other, AfdRS, activates *adhI* transcription. Both systems contain a predicted sensor histidine kinase (RfdS and AfdS) and a response regulator (RfdR and AfdR). The *R. sphaeroides* genome sequence predicts that AfdRS is encoded within a putative five-gene operon that includes *afdRTS*, RSP2594, and RSP2595. The function of AfdT and the latter two genes are unknown, but homologs exist in *P. denitrificans* (17). If these

FIG. 6. The putative sensor domain of RfdS is different from those of AfdS and FlhS. Amino acid sequence alignment of the N-terminal regions of *R. sphaeroides* RfdS, *P. denitrificans* FlhS, and *R. sphaeroides* AfdS is shown. Identical residues are shaded in black, and functionally similar residues are shaded in grey. A consensus sequence is shown below the alignment.

five genes are cotranscribed from one promoter, RSP2594 and RSP2595 are not necessary for AfdRS function under the conditions tested since *afdRTS* complements cells containing a polar insertion in *afdR*.

Features of RfdRTS and AfdRTS. The deduced amino acid sequence of AfdS and RfdS predict that they are soluble sensor histidine kinases. Of the histidine kinases that have been described, RfdS and AfdS display the greatest similarity to the presumed sensor in a pathway that activates *P. denitrificans* GSH-FDH expression, FlhS (17). The C termini of RfdS, AfdS, and FlhS are each predicted to also contain an additional receiver domain that is found on the N terminus of response regulators (RfdS residues 618 to 720 and AfdS residues 350 to 470). The predicted presence of a receiver domain in these presumed sensors suggests that these are hybrid histidine kinases that use multiple phosphotransfer reactions as part of their signaling pathway. AfdS and FlhS each have a relatively small N-terminal sensor domain (residues 1 to 83) compared to that of RfdS (residues 1 to 373) (Fig. 6). The possible significance of this larger sensor domain in RfdS is presented below.

The amino acid sequence of RfdR and AfdR predicts that they each contain typical N-terminal receiver domains and C-terminal helix-turn-helix motifs related to the DNA binding domain of NarL. AfdR appears to require phosphorylation to

activate *adhI* transcription. This is consistent with the behavior of the related *E. coli* NarL protein and many other response regulators (3). It is also likely that the related RfdR protein also requires phosphorylation to interact with DNA since it is also a response regulator in the NarL family. It is unknown where either AfdR or RfdR binds within the *adhI* promoter and whether they contain one or multiple binding sites as is the case for NarL at some promoters (12). There is also considerable amino acid sequence identity in the C-terminal domains of AfdR and RfdR, so it may be possible that they have similar or overlapping target sites for DNA binding.

RfdT and AfdT are not related to any characterized proteins, but hydropathy analysis (using the Kyte-Doolittle scale [<http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html>]) indicates that they contain at least one transmembrane domain. An in-frame deletion in *R. sphaeroides* *rfdT* had no detectable effect on *adhI* expression in the absence or presence of known inducers. Therefore, a role for AfdT/RfdT in *R. sphaeroides* and the *P. denitrificans* homolog (Orf2) remains to be determined.

Multiple pathways control *adhI* expression. Transcription of *R. sphaeroides adhI* is induced by formaldehyde or several metabolic sources of this compound (6), suggesting that a metabolic intermediate acts as an inducer of gene expression. It appeared that an early pathway intermediate was responsi-

FIG. 7. Multiple two-component systems regulate *adhI* expression. AfdRS activates *adhI* transcription in response to a signal derived from formaldehyde metabolism, possibly formaldehyde (HCHO) or *S*-hydroxymethylglutathione (HMGS). RfdRS represses *adhI* transcription. The signal that relieves repression by RfdRS is unknown.

ble for this increase since the addition of formate or bicarbonate does not induce *adhI* transcription (4). Additionally, mutants lacking GSH-FDH have elevated *adhI* expression even in the absence of exogenous formaldehyde, indicating that a pathway intermediate upstream of GSH-FDH is stimulating this promoter. Because of this, it was proposed that formaldehyde, or its glutathione adduct *S*-hydroxymethylglutathione, is a potential metabolic signal that induces *adhI* expression. Our results suggest that the AfdRS pathway is responsible for this induction since these proteins were needed to increase *adhI* expression in response to metabolic or exogenous sources of formaldehyde in vivo (Fig. 7). We further propose that phosphorylated AfdR is responsible for this increase since this protein was a direct activator of *adhI* transcription in vitro.

While RfdRS has a negative effect on *adhI* expression, this signaling pathway does not appear to respond to formaldehyde, since transcription of this promoter was activated by metabolic sources of this compound in cells lacking RfdRS. Rather, it appears that repression of *adhI* transcription by RfdR is relieved by another, unknown signal (Fig. 7). In this regard, it is possible that the additional domain in the N terminus of RfdS may allow it to sense a signal different from AfdS (Fig. 6). It may seem puzzling for cells to use the RfdRS pathway to reduce *adhI* transcription even in the presence of formaldehyde. However, our results show that the level of repression by RfdR is relatively small compared to the activation that is observed in the presence of formaldehyde. Of equal or greater importance, this level of repression by RfdRS does not inhibit the ability of cells to generate energy and carbon skeletons from the oxidation of formaldehyde since cells with a wild-type RfdRS pathway are able to perform this conversion.

While we identified *R. sphaeroides* RfdRS for its ability to reduce *adhI-cycI* expression and prevent photosynthetic growth of cells containing the *spd-7* allele, inactivating *rfdRS* does not restore photosynthetic growth to a cytochrome *c*₂ mutant (data not shown). Thus, it appears that the unknown signal which relieves the negative effect of RfdRS on *adhI-cycI* expression would be unable to allow sufficient production of isocytochrome *c*₂ to support photosynthetic growth in the absence of cytochrome *c*₂.

Other homologs of AfdRS and RfdRS. There are important similarities and differences in the regulation of formaldehyde metabolism between *R. sphaeroides* and other proteobacteria. In *R. sphaeroides*, GSH-FDH expression is directly regulated by multiple two-component regulatory circuits (RfdRS and AfdRS) that appear to respond to different signals. To date, only a single positive regulator of GSH-FDH has been described in *P. denitrificans* (FlhRS), and it has not been demonstrated that FlhR directly activates transcription of the GSH-FDH gene in this organism (17).

If FlhRS is a direct activator of GSH-FDH expression in *P. denitrificans*, there appears to be differences in the control of formaldehyde metabolism. For example, the loss of FlhRS in *P. denitrificans* abolishes expression of formyl-GSH hydrolase, cytochrome *c*_{553i}, and methanol dehydrogenase (17). As a result, *P. denitrificans* requires FlhRS for growth on formaldehyde-generating carbon sources like methanol, methylamine, and choline (17). It does not appear that the loss of AfdRS blocks expression of a putative methanol dehydrogenase and the formyl-GSH hydrolase in *R. sphaeroides* since *afdRS* mutants of this bacterium can use methanol as a sole photosynthetic carbon source but at a reduced growth rate compared to that of the wild-type (data not shown). Possibly, the basal levels of GSH-FDH expression in *afdRS* mutants are sufficient to support growth under these conditions. Also supporting this notion is the fact that both *adhI* transcription in vivo and activity of GSH-FDH (data not shown) are still detectable in the AfdRS mutant strain.

There are several predicted homologs of AfdRS and RfdRS in the microbial genome database. In virtually every microbial genome that contains an AfdRS homolog, the genes are adjacent to regions predicted to encode either GSH-FDH enzymes or putative PQQ-dependent alcohol dehydrogenases that could produce formaldehyde from methanol. Among the bacteria for which genomes have been sequenced, *R. sphaeroides* is the only one predicted to contain homologs of both RfdRS and AfdRS. Thus, it will be interesting to see why this facultative α -proteobacterium contains pathways to negatively and positively regulate GSH-FDH expression compared to other or-

ganisms which appear to contain only an AfdRS or RfdRS system.

In conclusion, we have identified multiple two-component systems that directly regulate expression of the *R. sphaeroides* GSH-FDH structural gene *adhI*. One of these systems, AfdRS, is a direct activator of *adhI* transcription that appears to respond to an intermediate in formaldehyde oxidation, while RfdRS is a direct negative regulator of GSH-FDH expression in response to an unknown signal. These observations indicate that regulation of GSH-dependent formaldehyde oxidation in *R. sphaeroides* is an intricate process with multiple two-component systems that could each respond to different physiological signals. The AfdRS system shares similarities with other two-component regulators, but this is the first report of a transcription factor acting directly to regulate transcription of a formaldehyde-inducible gene. We are now in a position to determine how AfdRS senses signals from formaldehyde metabolism and regulates genes needed for metabolism of this compound. Additional work to identify the signal that controls RfdRS function will characterize the role of this two-component system and could help elucidate additional roles for GSH-FDH in cells.

ACKNOWLEDGMENTS

This work was supported by grant DE-FG02-97ER20262 from the U.S. Department of Energy to T.J.D. During these studies, J.W.H. was supported by the William H. Peterson memorial fellowship awarded by the Department of Bacteriology, University of Wisconsin—Madison, and M.D. was supported by predoctoral training grant 5 T32 GM07133 from NIGMS.

We thank Federico Rey for critical reading of the manuscript.

REFERENCES

- Allen, L. N., and R. S. Hanson. 1985. Construction of broad-host-range cosmid cloning vectors: identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. *J. Bacteriol.* **161**:955–962.
- Anthony, J., H. Green, and T. J. Donohue. 2003. *Rhodobacter sphaeroides* RNA polymerase and its sigma factors. *Methods Enzymol.* **370**:54–65.
- Baikalov, I., I. Schroder, M. Kaczor-Grzeskowiak, K. Grzeskowiak, R. P. Gunsalus, and R. E. Dickerson. 1996. Structure of the *Escherichia coli* response regulator NarL. *Biochemistry* **35**:11053–11061.
- Barber, R. D. 1997. Formaldehyde sensing and metabolism in *Rhodobacter sphaeroides*. PhD dissertation. University of Wisconsin—Madison, Madison, Wis.
- Barber, R. D., and T. J. Donohue. 1998. Function of a glutathione-dependent formaldehyde dehydrogenase in *Rhodobacter sphaeroides* formaldehyde oxidation and assimilation. *Biochemistry* **37**:530–537.
- Barber, R. D., and T. J. Donohue. 1998. Pathways for transcriptional activation of a glutathione-dependent formaldehyde dehydrogenase gene. *J. Mol. Biol.* **280**:775–884.
- Barber, R. D., M. A. Rott, and T. J. Donohue. 1996. Characterization of a glutathione-dependent formaldehyde dehydrogenase from *Rhodobacter sphaeroides*. *J. Bacteriol.* **178**:1386–1393.
- Bethesda Research Laboratories. 1986. BRL pUC host: *Escherichia coli* DH5 α competent cells. Bethesda Res. Lab. Focus **8**:9–10.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Comolli, J. C., A. J. Carl, C. Hall, and T. Donohue. 2002. Transcriptional activation of the *Rhodobacter sphaeroides* cytochrome *c*₂ gene P2 promoter by the response regulator PrrA. *J. Bacteriol.* **184**:390–399.
- Correia, M. A. 1995. Rat and human liver cytochromes P450: substrate and inhibitor specificities and functional markers, p. 607–630. In P. R. Ortiz de Montellano (ed.), *Cytochrome P450 structure, mechanism, and biochemistry*, 2nd ed. Plenum Press, New York, N.Y.
- Dahl, A. R., and W. M. Hadley. 1983. Formaldehyde production promoted by rat nasal cytochrome P-450-dependent monooxygenases with nasal decongestants, essences, solvents, air pollutants, nicotine, and cocaine as substrates. *Toxicol. Appl. Pharmacol.* **67**:200–205.
- Darwin, A. J., J. Li, and V. Stewart. 1996. Analysis of nitrate regulatory protein NarL-binding sites in the *fdnG* and *narG* operon control regions of *Escherichia coli* K-12. *Mol. Microbiol.* **20**:621–632.
- Donohue, T. J., A. G. McEwan, S. Van Doren, A. R. Crofts, and S. Kaplan. 1988. Phenotypic and genetic characterization of cytochrome *c*₂ deficient mutants of *Rhodobacter sphaeroides*. *Biochemistry* **27**:1918–1925.
- Goenrich, M., S. Bartoschek, C. H. Hagemeyer, C. Griesinger, and J. A. Vorholt. 2002. A glutathione-dependent formaldehyde-activating enzyme (Gfa) from *Paracoccus denitrificans* detected and purified via two-dimensional proton exchange NMR spectroscopy. *J. Biol. Chem.* **277**:3069–3072.
- Gutheil, W. G., E. Kasimoglu, and P. C. Nicholson. 1997. Induction of glutathione-dependent formaldehyde dehydrogenase activity in *Escherichia coli* and *Haemophilus influenza*. *Biochem. Biophys. Res. Commun.* **238**:693–696.
- Handler, P., M. L. C. Bernheim, and J. R. Klein. 1941. The oxidative demethylation of sarcosine to glycine. *J. Biol. Chem.* **138**:211–218.
- Harms, N., W. N. Reijnders, S. Koning, and R. J. van Spanning. 2001. Two-component system that regulates methanol and formaldehyde oxidation in *Paracoccus denitrificans*. *J. Bacteriol.* **183**:664–670.
- Heck, H. D., M. Casanova, and T. B. Starr. 1990. Formaldehyde toxicity—new understanding. *Crit. Rev. Toxicol.* **20**:397–426.
- Hickman, J. W., R. D. Barber, E. P. Skaar, and T. J. Donohue. 2002. Link between the membrane-bound pyridine nucleotide transhydrogenase and glutathione-dependent processes in *Rhodobacter sphaeroides*. *J. Bacteriol.* **184**:400–409.
- Jones, D. P., H. Thor, B. Andersson, and S. Orrenius. 1978. Detoxification reactions in isolated hepatocytes. Role of glutathione peroxidase, catalase, and formaldehyde dehydrogenase in reactions relating to *N*-demethylation by the cytochrome P-450 system. *J. Biol. Chem.* **253**:6031–6037.
- Kalasz, H. 2003. Biological role of formaldehyde, and cycles related to methylation, demethylation, and formaldehyde production. *Mini Rev. Med. Chem.* **3**:175–192.
- Kalasz, H., M. Bathori, and E. Tyihak. 1998. Formaldehyde generation by *N*-demethylation. *Acta Biol. Hung.* **49**:339–344.
- Karls, R. K., J. R. Wolf, and T. J. Donohue. 1999. Activation of the *cycA* P2 promoter for the *Rhodobacter sphaeroides* cytochrome *c*₂ gene by the photosynthesis response regulator. *Mol. Microbiol.* **34**:822–835.
- Ling, K., and T. Tung. 1948. The oxidative demethylation of monomethyl-L-amino acids. *J. Biol. Chem.* **174**:643–645.
- Ma, T. H., and M. M. Harris. 1988. Review of the genotoxicity of formaldehyde. *Mutat. Res.* **196**:37–59.
- Marx, C. J., and M. E. Lidstrom. 2001. Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria. *Microbiology* **147**:2065–2075.
- Messing, J. 1979. A multipurpose cloning system based on single-stranded DNA bacteriophage M13. *Recomb. DNA Tech. Bull.* **2**:43–48.
- Rott, M. A., and T. J. Donohue. 1990. *Rhodobacter sphaeroides* *spd* mutations allow cytochrome *c*₂-independent photosynthetic growth. *J. Bacteriol.* **172**:1954–1961.
- Rott, M. A., J. Fitch, T. E. Meyer, and T. J. Donohue. 1992. Regulation of a cytochrome *c*₂ isoform in wild-type and cytochrome *c*₂ mutant strains of *Rhodobacter sphaeroides*. *Arch. Biochem. Biophys.* **292**:576–582.
- Rott, M. A., V. C. Witthuhn, B. A. Schilke, M. Soranno, A. Ali, and T. J. Donohue. 1993. Genetic evidence for the role of isocytochrome *c*₂ in photosynthetic growth of *Rhodobacter sphaeroides* *spd* mutants. *J. Bacteriol.* **175**:358–366.
- Schilke, B. A., and T. J. Donohue. 1995. ChrR positively regulates transcription of the *Rhodobacter sphaeroides* cytochrome *c*₂ gene. *J. Bacteriol.* **177**:1929–1937.
- Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for *in vitro* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Bio/Technology* **1**:748–791.
- Sistrom, W. R. 1960. A requirement for sodium in the growth of *Rhodospirillum rubrum*. *Gen. Microbiol.* **22**:778–785.
- Suwanto, A., and S. Kaplan. 1989. Physical and genetic mapping of the *Rhodobacter sphaeroides* 2.4.1 genome: presence of two unique circular chromosomes. *J. Bacteriol.* **171**:5850–5859.
- U.S. Environmental Protection Agency. 1993. Motor-vehicle related air toxics study. Office of Air and Radiation, U.S. Environmental Protection Agency, Washington, D.C.
- Wehner, E. P., E. Rao, and M. Brendel. 1993. Molecular structure and genetic regulation of *sfa*, a gene responsible for resistance to formaldehyde in *Saccharomyces cerevisiae*, and characterization of its protein product. *Mol. Gen. Genet.* **237**:351–358.
- World Health Organization. 2002. Concise international chemical assessment document: formaldehyde 40. World Health Organization, Geneva, Switzerland.
- World Health Organization. 1989. Environmental health criteria for formaldehyde 89. World Health Organization, Geneva, Switzerland.