Differential expression of NiFe uptake-type hydrogenase genes in *Salmonella enterica* serovar Typhimurium

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Salmonella enterica serovar Typhimurium possesses three similar NiFe hydrogenases important to its virulence. Here we show that the three hydrogenase operons *hyb*, *hya* and *hyd* are expressed under different environmental conditions and are subject to control by different regulatory proteins. Hydrogenase promoter-*lacZ* fusion plasmids were transferred into the wild-type strain or into *arcA*, *fnr*, *iscR*, *narL* and *narP* deletion mutants, or into a *fnr/arcA* double mutant. The *hyb* promoter had highest β -galactosidase activity under growth conditions promoting anaerobic respiration (glycerol plus fumarate) and may be subject to glucose repression, since cells grown with glucose had about half the transcriptional activity of cells grown with mannose. Based on the phenotype of regulatory mutant strains, *IscR* represses *hyb* aerobically, and *ArcA* plays a role in both *hyb* and *hyd* regulation. The *hyd* promoter had about five times more activity in cells grown under aerobic conditions compared to anaerobic levels, and its activity tripled in an *arcA* mutant grown anaerobically. The *hya* promoter had the highest activity when cells were grown anaerobically with glucose, and the growth yield of the *hya* mutant was about 25 % lower than for wild-type cells grown fermentatively, suggesting that *Hya* may be utilized during fermentation. The *hya* promoter is repressed by nitrate and this repression was abolished when the *NarL*-binding site was mutated, or in a *narL* mutant background. *FNR* is involved in *hyb* and *hya* regulation, since β -galactosidase activity decreased significantly in a *fnr* mutant. These findings suggest that the three hydrogenases are used under different conditions, likely enhancing the pathogen's capacity to survive in a variety of environments.

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INTRODUCTION

Enteric pathogens are responsible for about two million deaths annually and the diarrhoeal illness they cause results in billions of dollars of treatment costs (de Bruyn, 2000). *Salmonella* species are some of the most common pathogens associated with sporadic diarrhoea in adults living in developed countries (de Bruyn, 2000). According to the World Health Organization, the cost associated with *Salmonella* infections alone is estimated at three billion dollars annually in the United States.

It was recently shown that hydrogen gas (H_2) is an important energy source for the survival of pathogens *in vivo* (Mehta *et al.*, 2005; Olson & Maier, 2002). H_2 is produced in the host by colonic bacterial fermentations and diffuses throughout the animal's body (Maier, 2003). Bacterial NiFe uptake-type hydrogenases catalyse the H_2 -oxidizing reaction $H_2 \rightarrow 2e^- + 2H^+$, yielding protons that can contribute to energy production via generation of a

proton gradient across the cytoplasmic membrane. In addition, electrons produced in the H_2 -splitting reaction can be passed to other membrane-bound electron carriers, eventually contributing to ATP production (Maier *et al.*, 2004).

Physiological studies indicated that *Salmonella enterica* serovar Typhimurium has at least two Ni-containing hydrogenases that are responsible for hydrogen uptake (Jamieson *et al.*, 1986; Sawers *et al.*, 1986). The genomic sequence (TIGR; <http://www.tigr.org>) indicates that three NiFe membrane-bound hydrogenases exist in the *S. enterica* strain SGSC1412 (McClelland *et al.*, 2001). The results from mouse infection studies suggest that these hydrogenases are individually important for virulence (Maier *et al.*, 2004), and the removal of all three hydrogenases (creating a triple mutant) resulted in a strain that was avirulent and unable to colonize the mouse liver or spleen. However, the presence of any one hydrogenase on a low-copy-number plasmid restored some virulence characteristics to the triple mutant and each double-mutant strain with a deletion in two hydrogenases was less virulent than the parent strain (Maier *et al.*, 2004).

Abbreviation: CRP, cyclic AMP receptor protein.

The reason that *S. enterica* serovar Typhimurium has three very similar NiFe hydrogenases is unknown, but it may be expected that they are utilized under different environmental conditions. If so, the mechanism of gene expression of these operons is likely to be different. Park *et al.* (1999) studied a reporter gene fusion to one of the *S. enterica* serovar Typhimurium NiFe hydrogenase promoters that is homologous to *hya* of a similar bacterium, *Escherichia coli*. They determined that the hydrogenase operon was acid-inducible, repressed by nitrate at acidic pH, but not at neutral or alkaline pH, and induced by anaerobiosis. Cyclic AMP receptor protein (CRP) and tyrosine were required for expression (Park *et al.*, 1999). Several studies that measured hydrogenase protein levels in *S. enterica* serovar Typhimurium under different growth conditions suggested that hydrogenase protein levels are affected by anaerobiosis and catabolite repression (Jamieson *et al.*, 1986; Sawers *et al.*, 1986).

The nomenclature of *S. enterica* serovar Typhimurium hydrogenase genes is somewhat confusing. Therefore, the *Salmonella* hydrogenases were renamed in this paper, according to nomenclature used for similar uptake-type hydrogenases in *E. coli*. The *E. coli* hydrogenase genes are located in the *hya* operon (containing the hydrogenase 1 genes) and *hyb* operon (containing the hydrogenase 2 genes). Amino acid sequence alignments using the BLAST program from the NCBI website have shown that the *S. enterica* serovar Typhimurium hydrogenase Hyb large subunit has about 94 % sequence similarity to the Hyb large subunit of *E. coli*. The other two *S. enterica* serovar Typhimurium uptake-type hydrogenases (both annotated as Hyd) are similar to the single *E. coli* Hya (www.tigr.org). The *S. enterica* serovar Typhimurium *hyd* STM1786–STM1787 was called *hya* in this study because the hydrogenase large subunit has a high percentage sequence similarity to *E. coli* Hya large subunit (91 %). The *S. enterica* serovar Typhimurium *hyd* genes STM1538–1539 were not renamed, since the hydrogenase large subunit has a much lower percentage sequence similarity to the *E. coli* Hya large subunit (66 %). In a recently published study on decreased virulence in strains lacking the hydrogenases, the *hyb* genes STM3147–STM3150 were called Group I hydrogenase, *hyd* genes STM1538–STM1539 were Group II hydrogenase, and *hya* genes STM1786–STM1787 were called Group III hydrogenase (Maier *et al.*, 2004). Here, the nomenclature was modified for ease of comparison with previous work done in *E. coli* and *Salmonella*.

Studies of reporter genes fused to *E. coli* *hya* and *hyb* promoters have shown that their expression is regulated by anaerobiosis, nitrate, catabolite repression and pH. Several proteins are involved in this regulation, including fumarate nitrate respiration (FNR) protein and aerobic respiration control (Arc) protein. The *hyb* operon in *E. coli* is affected by catabolite repression, mediated by CRP (Richard *et al.*, 1999). Both *hyb* and *hya* operons are regulated by the NarP and NarL proteins, which repress the operons in the presence of nitrate (Richard *et al.*, 1999). Since *E. coli* and

S. enterica are closely related, these studies led us to test whether the hydrogenases in *S. enterica* serovar Typhimurium were regulated in a similar fashion.

Here we examine the hydrogenase gene expression level in *S. enterica* serovar Typhimurium using reporter gene fusions. We show that the hydrogenases are each expressed under different growth conditions, and are differentially regulated by FNR, ArcA and NarL. The genes *hyb* and *hya* had the highest expression under anaerobic conditions and were indirectly regulated by FNR, and *hyb* may be glucose repressed. Our study shows that *hya* was nitrate repressed, and the repression was mediated by NarL, but NarP was not involved. ArcA activated *hyb* expression. *S. enterica* serovar Typhimurium *hya* was not under the control of ArcA. Unlike what has been shown for either *hya* or *hyb* in *E. coli*, the *hyd* operon was expressed highest aerobically and was repressed by ArcA.

METHODS

Strains, growth conditions and reagents. The *S. enterica* serovar Typhimurium and *E. coli* strains and plasmids used in this study are listed in Table 1. Strains were maintained in Luria–Bertani (LB) broth or on agar plates with appropriate antibiotics: 50 µg ampicillin ml⁻¹, 34 µg chloramphenicol ml⁻¹, 25 µg kanamycin ml⁻¹. CR-HYD medium was used where indicated (Sawers *et al.*, 1986). The medium was supplemented with glucose (0.4 %), mannose (0.4 %), glycerol (0.4 %), sodium formate (20 mM), sodium fumarate (20 mM) or sodium nitrate (20 mM), where indicated. Cells were grown at 37 °C either anaerobically or aerobically. Anaerobic conditions were established by sparging sealed 165 ml bottles with N₂ for 15 min, then anaerobic mix (10 % H₂, 5 % CO₂ and 85 % N₂) for 20 min. ONPG was purchased from Pierce. Primers were purchased from Integrated DNA Technologies.

Construction of reporter gene fusions. The entire intergenic region between the gene upstream of each hydrogenase and the start codon of the hydrogenase was amplified by PCR using primer pairs HybHindIII and HybNotI, HydHindIII and HydNotI, or HyaHindIII and HyaNot I (Table 2) to generate 300–500 bp fragments. Primers were engineered to create a HindIII site on one side of the PCR product and a NotI site on the other end. PCR fragments and a low-copy-number vector pNN387, which contains a promoterless *lacZ* gene and the *lacY* gene, were sequentially digested with HindIII and NotI. Each PCR fragment was ligated into the digested pNN387 and transformed into *E. coli* TOP10 cells, yielding plasmids pZBL1, pZBL2 and pZBL3, respectively (Table 1). To facilitate transformation of plasmids into strain JSG210, plasmids were first introduced into the restriction-deficient strain E218 before being transformed into JSG210. Fusions were confirmed by PCR using a *lacZ*-specific primer, Lac, and HybNotI, HydNotI, or HyaNotI to generate a 2.7 kb fragment. The resulting PCR products were sequenced, using the promoter-specific primer (Integrated Biotech Labs, University of Georgia).

A two-step PCR approach was used to make a *hya* NarL-binding-site mutant promoter fusion. In two separate reactions, primers UpSTHyaNarL and HyaHindIII or RevSTHyaNarL and HyaNotI were used to generate two PCR products that were about 250 bp each. Both PCR products were mixed together and the final PCR product of about 500 bp was obtained using the primers HyaHindIII and HyaNotI. The resulting PCR product had a modified putative NarL-binding site, as confirmed by sequencing (DNA Sequencing Core,

Table 1. Strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
Strains		
<i>S. enterica</i> serovar Typhimurium		
JSG210	14028s (wild-type)	ATCC
ALZ7	JSG210 with $\Delta hyb::FRT^*$	This work
ALZ8	JSG210 with $\Delta hyd::FRT$	This work
ALZ9	JSG210 with $\Delta hya::FRT$	This work
ALZ10	JSG210 with $\Delta arcA::FRT$	This work
ALZ11	JSG210 with $\Delta fnr::FRT$	This work
ALZ12	JSG210 with $\Delta narL::FRT$	This work
ALZ13	JSG210 with $\Delta narP::FRT$	This work
ALZ14	JSG210 with $\Delta arcA::FRT \Delta fnr::FRT$	This work
ALZ25	JSG210 with $\Delta iscR::FRT$	This work
E218	<i>hsdL6</i> (r^-m^+) <i>hsdSA29</i> (r^-m^+) <i>hsdSB121</i> (r^-m^+) <i>galE856 xyl-404 leu-3121 metA22 metE551 trpC2 ilv-452 rpsL120</i>	T. Hoover, University of Georgia
<i>E. coli</i>		
TOP10	Cloning strain	Invitrogen
Plasmids		
pCP20	Amp ^r ; contains flippase gene for λ Red mutagenesis	Datsenko & Wanner (2000)
pKD46	Amp ^r ; contains Red genes γ , β and <i>exo</i>	Datsenko & Wanner (2000)
pKD4	Kan ^r ; contains <i>kan</i> cassette	Datsenko & Wanner (2000)
pNN387	Cm ^r ; vector containing promoterless <i>lacZ</i>	Elledge & Davis (1989)
pZBL1	Cm ^r ; pNN387 with <i>hyb-lacZ</i> fusion	This work
pZBL2	Cm ^r ; pNN387 with <i>hyd-lacZ</i> fusion	This work
pZBL3	Cm ^r ; pNN387 with <i>hya-lacZ</i> fusion	This work
pZBL4	Cm ^r ; pNN387 with mutant <i>hya-lacZ</i> fusion	This work

*FRT, FLP recognition target.

University of Michigan). The PCR product and the pNN387 plasmid were restriction-digested and ligated into pNN387 as described above to make pZBL4. *E. coli* and *S. enterica* serovar Typhimurium cells were then transformed with pZBL4.

Mutant strain construction. Deletion mutants of the *arcA*, *fnr*, *narL*, *narP* and *iscR* genes were constructed using the lambda Red system, as described by Datsenko & Wanner (2000). In this system, the target gene is replaced by an antibiotic-resistance cassette, which is then removed by site-specific recombination at the FRT [flippase (FLP) recognition target] sites that flank the resistance gene. Gene deletions were confirmed by PCR using primers homologous to regions flanking the deleted gene (Table 2) and by sequencing across the deletion (DNA Sequencing Core, University of Michigan). The mutant strains are listed in Table 1.

The double mutant was constructed using the *fnr* deletion mutant and an *arcA* mutant in which the kanamycin cassette replaced the *arcA* gene. The phage P22Htint (J. Gunn, The Ohio State University, Columbus) was used to transduce the antibiotic marker from the $\Delta arcA::kan$ strain into the *fnr* mutant, making an *arcA/fnr* double mutant. The kanamycin cassette was removed as described above. The double mutant was confirmed by PCR.

Hydrogenase deletion mutants were constructed using the lambda Red system as described previously (Maier *et al.*, 2004). Here, deletion of the Group I genes (STM3147–3150) resulted in the *hyb* mutant, deletion of the Group II genes (STM1538–1539) resulted in the *hyd* mutant, and deletion of the Group III genes (STM1786–1787) resulted in the *hya* mutant (Table 1).

Enzyme assay. Gene expression was determined using β -galactosidase assays according to Miller (1992). *S. enterica* serovar Typhimurium fusion strains were grown overnight anaerobically or aerobically in sealed bottles containing LB broth or CR-HYD medium. Cultures were assayed for β -galactosidase activity at 37 °C as described previously (Miller, 1992).

Growth studies. Wild-type *S. enterica* serovar Typhimurium JSG210, or *hyd*, *hyb* or *hya* deletion mutant strains were grown in bottles containing CR-HYD medium supplemented with glucose, or glycerol and sodium fumarate, as indicated. Cells were grown aerobically or anaerobically overnight at 37 °C. Growth yield was determined by measuring OD₆₀₀.

RESULTS

Effect of growth conditions on hydrogenase gene expression

In order to study the expression of hydrogenase genes, transcriptional reporter fusions were made. A sequence including 300–500 bases upstream of the first gene of each hydrogenase operon was PCR amplified from strain JSG210. Each PCR product was then cloned into the vector pNN387 (Elledge & Davis, 1989) directly upstream of a promoterless *lacZ* gene. The resulting plasmids are

Table 2. Primers

Restriction enzyme sites are in bold, and the mutated putative NarL-binding site is in lower case.

Primer	Sequence (5'–3')	Application
HybHindIII	GCACA AAGCTT CTGGCTTTTATTTTGCAC	<i>hyb</i> fusion plasmid
HybNotI	ACTGCAGT GCGGCCG CTCCAACAGGGGTATTATC	<i>hyb</i> fusion plasmid
HydHindIII	GCACA AAGCTT AGTTAGTGTGGGCATC	<i>hyd</i> fusion plasmid
HydNotI	ACTGCAGT GCGGCCG CTCTCCTTGTTTTGTGA	<i>hyd</i> fusion plasmid
HyaHindIII	GCACA AAGCTT GCACCACGCTCCGC	<i>hya</i> fusion plasmid
HyaNotI	ACTGCAGT GCGGCCG CCCTCTTTCAAACAGTC	<i>hya</i> fusion plasmid
Lac	GACCATTTTCAATCCGA	Confirming fusions
UpSTHyaNarL	AAGCTagcaaaagtcACTTCACATTGTTTCTTA	NarL mutant plasmid
RevSTHyaNarL	AAGTgacttttctAGCTTATAGCGGTATATT	NarL mutant plasmid
UpSTArcADel	ATTTAGTTGGCAATTTAGGTAGCAAACATGTGTGTAGGCTGGAGCTGCTTC	<i>arcA</i> deletion
RevSTArcADel	GTTTTTACAGTTTGGCGCCTGGGCCGAATTACATATGAATATCCTCCTTA	<i>arcA</i> deletion
UpSTArcACheck	ACATGCATCAGGCAGGTCAG	Confirming <i>arcA</i> deletion
RevSTArcACheck	TAGCGGAAGGGGGACATAAGAAAC	Confirming <i>arcA</i> deletion
UpSTFNRDel	GCTCAGACTTACGCGCTCACCAAAAAGATGTGTGTAGGCTGGAGCTGCTTC	<i>fnr</i> deletion
RevSTFNRDel	GGCAGAAGATAACATCAATGGTTTAGCTGACGTTTACATATGAATATCCTCCTTA	<i>fnr</i> deletion
UpSTFNRCheck	GTGCCTTATCGGTCACACTTTTATG	Confirming <i>fnr</i> deletion
RevSTFNRCheck	GAAACAGAGGAAGCGCTTCTGCCAGATC	Confirming <i>fnr</i> deletion
UpSTNarLDel	ACTTCACAGAAACCCAGGGAGATACCCATGTGTGTAGGCTGGAGCTGCTTC	<i>narL</i> deletion
RevSTNarLDel	CATTGGCAACCGTTCCAGGAGCAATAATTACATATGAATATCCTCCTTA	<i>narL</i> deletion
UpSTNarLCheck	CAAAGTTTGC CGCGGCGATTGCCAGG	Confirming <i>narL</i> deletion
RevSTNarLCheck	CTGACTGAACCGTTTATCACGATGC	Confirming <i>narL</i> deletion
UpSTNarPDel	CATTCTAATAAACTCAGGAGATACAGCATGTGTGTAAGCTGGAGCTGCCTC	<i>narP</i> deletion
RevSTNarPDel	TGGCGCGGCGCCACCGGGCCTGGCTTGTTACATATGAATATCCTCCTTA	<i>narP</i> deletion
UpSTNarPCheck	GCTTGACAATGTGGCAAACTCTGGAC	Confirming <i>narP</i> deletion
RevSTNarPCheck	GGTGATAGAGCGCAGTATCCGGCTG	Confirming <i>narP</i> deletion
UpSTIscRDel	GGCAGGGGCGAGTTTGAGGTTAAGTAAGACTGTGTAGGCTGGAGCTGCTTC	<i>iscR</i> deletion
RevSTIscRDel	GCCGCCAGAAGAGATGGCGTAATATTTTAACATATGAATATCCTCCTTA	<i>iscR</i> deletion
UpSTIscrCheck	TTGTCCCTGCTATGCAATACCCC	Confirming <i>iscR</i> deletion
RevSTIscrCheck	ATGTACGACCGTGTGACGGAC	Confirming <i>iscR</i> deletion

listed in Table 1. Strain JSG210 was transformed with the plasmids.

To examine the possible roles of these hydrogenases, promoter activity was determined after cells were grown under different environmental conditions in CR-HYD medium. The *hyb* promoter expression was about 13-fold higher when cells were grown under anaerobic respiration conditions (glycerol plus fumarate), compared to fermentation (anaerobic glucose) conditions (Table 3), suggesting that the Hyb proteins are used during anaerobic respiration. The *hyb* mutant had a 16 % lower growth yield compared to wild-type when grown anaerobically with glycerol and fumarate; however, this reduced growth yield was not significant. The *hyd* promoter had more than seven times more activity under aerobic conditions, compared to anaerobic glucose levels. The *hya* promoter had the highest activity when grown under fermentative conditions (anaerobic glucose or mannose), and the *hya* mutant had about 25 % lower growth yield than wild-type when grown fermentatively with glucose, indicating that this hydrogenase is used during fermentation. The presence of nitrate

resulted in a twofold reduction in β -galactosidase activity for the *hya* promoter (when grown with glucose), suggesting that *hya* is repressed by nitrate.

Since some of the *E. coli* hydrogenases are catabolite repressed when cells are grown with glucose, glucose repression of *S. enterica* serovar Typhimurium hydrogenase genes was investigated. Cells were grown in CR-HYD with glucose or mannose as a carbon source. The *hyb* promoter had about twice as much activity when cells were grown anaerobically on mannose, compared to glucose, suggesting that these genes may be repressed in the presence of glucose (Table 3). Such an effect was not observed for the *hyd* and *hya* genes, indicating that they are not glucose repressed.

Anoxic regulation of hydrogenase promoters

A mutant analysis approach was used to further examine the regulation of *S. enterica* serovar Typhimurium hydrogenase operons in response to oxygen availability. Upon sequence analysis, several putative ArcA-binding sites were

Table 3. β -Galactosidase activities of wild-type *S. typhimurium* containing hydrogenase promoter-*lacZ* fusions

Condition	β -Galactosidase activity (Miller units)*			
	Vector-only†	<i>hyb</i> fusion	<i>hyd</i> fusion	<i>hya</i> fusion
Aerobic glucose	2.5 ± 0.8	3.9 ± 1.1	63 ± 8.7 ⁽²⁾	3.3 ± 1.0
Anaerobic glucose	2.8 ± 0.7	16.5 ± 1.7 ^(1,4)	8.2 ± 1.2 ⁽²⁾	45.7 ± 3.2 ⁽³⁾
Anaerobic mannose	3.4 ± 1.3	30.5 ± 1.3 ⁽⁴⁾	9.3 ± 0.6	57.3 ± 6.7
Anaerobic glucose + formate	3.7 ± 0.3	34.8 ± 2.0	20.0 ± 3.3	9.9 ± 1.6
Anaerobic glycerol + fumarate	3.9 ± 0.6	215.1 ± 24.8 ⁽¹⁾	15.4 ± 1.1	3.9 ± 1.4
Anaerobic glucose + nitrate	4.0 ± 0.7	15.4 ± 1.1	9.9 ± 1.1	23.9 ± 1.0 ⁽³⁾

*Cells were grown overnight in bottles containing CR-HYD medium with various growth substrates added as indicated. Results are means of six replicates from three or more independent experiments. Like superscript numbers after the standard deviation values indicate a significant difference in mean between two values, based on Student's *t*-test ($P < 0.01$).

†Vector-only cells are wild-type cells containing the vector pNN387 with a promoterless *lacZ*.

found in all three hydrogenase promoters. Although no putative FNR-binding sites were found in the *S. enterica* serovar Typhimurium hydrogenase promoters, FNR was previously shown to regulate *E. coli* hydrogenase genes (Richard *et al.*, 1999). Therefore, non-polar deletions were made in the *arcA* and *fnr* genes using the lambda Red system (Datsenko & Wanner, 2000). Mutant strains that contained the hydrogenase promoter fusion plasmids were grown overnight in LB medium and β -galactosidase activity was measured. LB broth was used in this experiment because specific growth conditions were not required, and since overall hydrogen uptake activity is higher when cells are grown in rich media (Maier *et al.*, 2004). β -Galactosidase activity was also assayed using wild-type and mutant strains grown anaerobically in CR-HYD medium supplemented with glucose.

β -Galactosidase activity from anaerobically grown cells decreased at least threefold for the *hyb* promoter in the

arcA mutant background as compared to the wild-type (Table 4), suggesting that *hyb* is upregulated by ArcA. Interestingly, an opposite effect was seen for *hyd*. β -Galactosidase activity tripled for the *hyd* fusion in the *arcA* mutant background. This result indicates that ArcA represses the *hyd* genes. ArcA did not appear to regulate *hya* gene expression when cells were grown in LB, as β -galactosidase activity remained unchanged in the mutant as compared to the wild-type.

The *fnr* mutant strain carrying the *hyb* fusion plasmid showed a more than threefold decrease in β -galactosidase activity when compared to wild-type (Table 4). Likewise, β -galactosidase activity decreased fourfold for the *hya* fusion in the *fnr* mutant background, indicating that both *hyb* and *hya* are regulated by FNR.

Sequence analysis did not reveal any FNR-binding sites in either promoter [using the consensus sequence

Table 4. Anaerobic regulation of hydrogenase expression

Strain	β -Galactosidase activity (Miller units)*			
	Vector-only	<i>hyb</i> fusion	<i>hyd</i> fusion	<i>hya</i> fusion
JSG210 (wt) aerobic	7.1 ± 0.8	241.7 ± 33.2	57.8 ± 2.0	10.0 ± 0.7
JSG210 (wt) anaerobic	3.3 ± 3.5	421.5 ± 75.9 ^(1,2,5)	29.7 ± 6.6 ^(4,6)	61.5 ± 12.6 ^(3,7)
ALZ10 (<i>arcA</i>)	4.1 ± 0.9	118.6 ± 11.8 ⁽¹⁾	85.4 ± 3.1 ⁽⁴⁾	67.0 ± 12.3
ALZ11 (<i>fnr</i>)	2.8 ± 2.1	117.9 ± 10.4 ⁽²⁾	36.9 ± 5.6	14.7 ± 2.7 ⁽³⁾
ALZ14 (<i>arcA fnr</i>)	0.1 ± 4.4	28.8 ± 6.3 ⁽⁵⁾	65.8 ± 5.4 ⁽⁶⁾	26.6 ± 8.8 ⁽⁷⁾
ALZ25 (<i>iscR</i>) aerobic	6.9 ± 1.1	512.9 ± 15.8	63.1 ± 1.8	11.0 ± 1.7
ALZ25 (<i>iscR</i>) anaerobic	5.8 ± 0.9	476.6 ± 14.1	40.7 ± 1.5	66.2 ± 4.1

*Wild-type (wt) and *iscR* mutant cells containing hydrogenase promoter fusions were grown aerobically or anaerobically overnight in LB medium and β -galactosidase activity was measured. The *arcA*, *fnr* and *arcA/fnr* mutant strains were grown anaerobically. Results are means of six to eight replicates from three or more independent experiments. Like superscript numbers after the standard deviation values indicate a significant difference in mean between two values, based on Student's *t*-test ($P < 0.01$).

TTGATNNNNATCAA (Zhang & Ebright, 1990)]. It has been shown that FNR can upregulate *arcA* expression in *E. coli* (Compan & Touati, 1994). In order to examine the possibility that the FNR effect is mediated by ArcA in *Salmonella*, an *arcA/fnr* double mutant was constructed using the lambda Red method. If FNR activates ArcA, it would be expected that β -galactosidase activity in the double mutant would be the same as in the FNR or ArcA mutant. In contrast, *hyb* expression decreased about 14-fold in the double mutant, 11-fold lower than in either the *arcA* or *fnr* single mutant. This result suggests that both FNR and ArcA are separately involved in *hyb* expression. Expression of *hyd* was similar between the *arcA/fnr* double mutant and the *arcA* mutant, supporting the conclusion that *fnr* does not regulate *hyd*. Likewise, *hya* expression was similar in the *arcA/fnr* double mutant compared to the *fnr* mutant when cells were grown in LB, indicating that *arcA* does not regulate *hya*.

A recent study reported that *E. coli hya* and *hyb* genes were repressed by IscR (iron-sulphur cluster regulator) under aerobic conditions, but not anaerobically (Giel *et al.*, 2006). A non-polar *iscR* deletion mutant was made in order to determine whether *S. typhimurium hyb*, *hyd* and *hya* genes are regulated similarly. Aerobic *hyb* expression in the *iscR* mutant was about twofold greater than in wild-type cells, and near the anaerobically achieved levels. These results indicate that *hyb* is repressed by IscR aerobically; however, unlike in *E. coli*, *S. typhimurium hya* genes do not seem to be regulated by IscR.

Similar trends were seen for *hyd* and *hya* when *arcA*, *fnr*, *arcA/fnr*, *iscR* mutant and wild-type cells were grown in CR-HYD medium compared to LB, although the actual values varied. This variation is likely due to the fact that LB is a richer medium than CR-HYD. There were a few differences when cells were grown in CR-HYD, namely a significant increase in *hyd* expression in the *fnr* mutant and a significant decrease in *hya* expression in the *arcA* mutant (data not shown). This may indicate a role for FNR in the regulation of *hyd* expression and a role for ArcA in the regulation of *hya* expression under some conditions.

Overall *hyb* expression was low for both wild-type and mutant strains when cells were grown in CR-HYD plus glucose, and only slight differences were observed between the mutant and wild-type cells. It seems that LB is a more appropriate medium than CR-HYD to assay *hyb* expression levels. Clearly, growth conditions play a role in the regulation of these genes, and the ArcA and FNR regulation of these operons may depend on other factors present under certain growth conditions. Gel-shift and DNA-binding studies will be useful to address whether this regulation is direct, or dependent on other factors.

Regulation of *hya* by nitrate

The *hya* promoter in the wild-type background was shown to be downregulated about twofold in the presence of nitrate (Table 3). NarX/NarL and NarQ/NarP are common two-component systems that control the nitrate response in *S. enterica* serovar Typhimurium (Rabin & Stewart, 1993). *narL* and *narP* deletion mutants were constructed using the lambda Red system. Wild-type and mutant strains were grown on CR-HYD with glucose and nitrate, and β -galactosidase assays were then performed. Nitrate repression was abolished in the *narL* strain (Table 5), indicating that NarL mediates the nitrate regulation of *hya*. NarP did not appear to play a role in the regulation of this gene, since β -galactosidase activity in the *narP* strain was not significantly different from wild-type levels (Table 5).

To further explore the regulation of *hya* by NarL, a potential NarL-binding site in the *hya* promoter was identified and mutated. The potential binding site (ATACCCACAC) had six out of seven identical bases as compared with the *E. coli* consensus sequence (NTACCCATN) described by Darwin *et al.* (1997). The entire binding site in the *hya* promoter was replaced by a random sequence of nucleotides (AGCAAAAGTC) using a two-step PCR approach. Cells containing *lacZ* driven by the mutant promoter were grown overnight in CR-HYD with glucose and nitrate and β -galactosidase activity was subsequently determined. We found that the strain containing the mutant NarL-binding site was not repressed

Table 5. Nitrate regulation of hydrogenase expression

Strain	β -Galactosidase activity (Miller units)*	
	Glucose	Glucose + nitrate
JSG210 (wild-type) <i>hya</i> fusion	37.2 $\pm$ 8.8 ⁽¹⁾	23.4 $\pm$ 2.4 ^(1,2)
JSG210 pZBL4 (<i>hya</i> mutant promoter fusion)	9.6 $\pm$ 3.8	13.0 $\pm$ 1.4
ALZ12 (<i>narL</i>) <i>hya</i> fusion	36.3 $\pm$ 5.5	38.5 $\pm$ 4.5 ⁽²⁾
ALZ11 (<i>narP</i>) <i>hya</i> fusion	33.4 $\pm$ 13.6	25.7 $\pm$ 2.1

*Mutant and wild-type cells containing hydrogenase promoter fusions were grown anaerobically overnight in CR-HYD medium. For strain JSG210 pZBL4, results are means of six replicates; for the other strains the data are the mean and standard deviation of at least 14 replicates. Like superscript numbers after the standard deviation values indicate a significant difference in mean between the two values, based on Student's *t*-test ($P < 0.01$).

by nitrate (Table 5). Expression was likely reduced in this strain because ten base pairs were replaced in the promoter, which could affect the binding of other regulatory proteins. DNA-binding and gel-shift assays will be useful to confirm whether NarL binds to the potential binding site in the *hya* promoter, or whether the nitrate regulation is indirect.

DISCUSSION

The *S. enterica* serovar Typhimurium NiFe uptake-type hydrogenases are expressed under different growth conditions, and are differentially regulated at the transcriptional level. Whereas the *hyb* genes were expressed the most when the cells were undergoing anaerobic respiration, the *hyd* genes had the highest expression under aerobic conditions, and the *hya* genes were expressed under conditions favouring fermentation (Table 3). The expression of the *hyd* genes was unique in comparison to either *hya* or *hyb* in the similar bacterium, *E. coli*, and it appears that *hyd* is absent from *E. coli*.

The results agree with previous work in which researchers analysed the active hydrogenase enzyme levels of cells grown under different conditions (Jamieson *et al.*, 1986; Sawers *et al.*, 1986). Those studies were based on immunoreaction to antibodies raised against *E. coli* hydrogenases, and it was concluded that there were at least two H₂-oxidizing hydrogenase isoenzymes. Isoenzyme 2 was necessary for anaerobic respiration-dependent hydrogen uptake, since this activity was greatly reduced in a strain lacking that isoenzyme (Sawers *et al.*, 1986). Isoenzyme 2, so named because of its sequence similarity to *E. coli* hydrogenase 2, is called Hyb in this work. Therefore, it is logical that *hyb* would be transcribed maximally when cells are grown with glycerol and fumarate. In light of these results, it seems that *S. enterica* serovar Typhimurium Hyb, like hydrogenase 2 in *E. coli* (Menon *et al.*, 1994), is used for hydrogen oxidation coupled to fumarate respiration. Nevertheless, based on mutant analysis (Maier *et al.*, 2004), this hydrogenase was capable of coupling H₂ oxidation to O₂ as well.

An early report using immunoelectrophoretic methods to study the active hydrogenase activity of wild-type and mutant strains indicated that isoenzyme 2 was catabolite repressed by glucose (Jamieson *et al.*, 1986). This repression was mediated by CRP, since, in a *crp* mutant, levels of isoenzyme 2 could not be increased by the addition of cAMP (Jamieson *et al.*, 1986). Our study took a different approach to examine catabolite repression of the uptake-type hydrogenases. We determined the gene-expression activity of each hydrogenase grown with different sugars and found that the *hyb* genes may be repressed in the presence of glucose (Table 3). Our results agree qualitatively with the early *S. enterica* serovar Typhimurium report, showing that *hyb* is glucose repressed and *hya* is not. In addition, our work shows that *hyd* is not glucose repressed, which was not examined previously.

Sawers *et al.* (1986) reported that *S. enterica* serovar Typhimurium isoenzyme 1 (analogous to both Hya and Hyd) was necessary for hydrogen uptake under fermentative growth in the absence of nitrate. Our results support this conclusion, since *hya* had the highest activity under fermentative conditions, and it was repressed by nitrate (Table 3). These results indicate that the function of Hya may be to oxidize H₂ while the cell is undergoing fermentation. Such hydrogen oxidation would be expected to generate additional energy for metabolism under this condition, and may be necessary for optimal growth, since growth yield was decreased in the *hya* mutant. Previous studies did not distinguish between Hya and Hyd, because it was not known at that time that *S. enterica* serovar Typhimurium had three uptake-type hydrogenases.

Although early work examined hydrogenase production under anaerobic conditions, we report here that *hyd* has the highest expression when cells are grown aerobically (Table 3). It has been shown that there is significant respiratory hydrogenase activity coupled to oxygen in *S. enterica* serovar Typhimurium cells grown under low oxygen conditions (O₂ levels were below 0.2% partial pressure) (Maier *et al.*, 2004). Therefore, we suggest that Hyd is used to completely oxidize hydrogen in a low oxygen environment.

Several transcriptional regulators of each operon were identified in this study. Expression of *hyb* was shown to be downregulated in both the *arcA* and *fnr* mutants (Table 4). These results agree with an early study by Jamieson *et al.* (1986), which reports that the presence of isoenzyme 2 was *fnr* dependent, based on immunoreaction to *E. coli* anti-hydrogenase 2 antibodies. In addition, Jamieson *et al.* (1986) showed that protein levels of isoenzyme 1 (analogous to both Hya and Hyd) were dependent on *fnr*. Our work clarifies this result, since we found that *hya* expression was downregulated in an *fnr* mutant, yet *hyd* expression was unaffected by FNR (Table 4). Interestingly, ArcA was shown to repress *hyd* expression under anaerobic conditions (Table 4). This is further evidence that Hyd is used under aerobic conditions, since ArcA usually represses genes used for aerobic growth and induces anaerobic genes (Lynch & Lin, 1996). As in *E. coli*, IscR repressed *hyb* expression aerobically; however, *hya* expression was not regulated by IscR under the conditions tested.

Using sequence analysis, we were able to find several potential binding sites for ArcA in the region directly upstream from the first gene in the *hyb* operon, indicating that ArcA may directly regulate *hyb* transcription. DNA-binding studies with purified ArcA will be needed to confirm this result. We were unable to find a binding site for FNR in either the *hyb* or *hya* promoter, indicating that FNR regulation is indirect. It was shown that FNR indirectly regulates the hydrogenase genes in *E. coli*, and was speculated that this may occur through altering nickel metabolism (Richard *et al.*, 1999). To test this hypothesis in *S. enterica* serovar Typhimurium, we added up to 1 mM

nickel to the growth medium, but such nickel supplementation did not significantly change *hyb*, *hyd* or *hya* promoter activity in *fnr* mutants (data not shown). Therefore, it appears that the FNR effect is not due to nickel availability in *S. enterica* serovar Typhimurium. In order to test the hypothesis that the FNR effect is mediated by ArcA, an *fnr/arcA* double mutant was constructed. Our results demonstrated that both FNR and ArcA are needed for full expression of *hyb* (Table 4).

The *hya* operon was shown to be regulated by nitrate (Table 3). Nitrate repression of *S. enterica* serovar Typhimurium hydrogenase genes is not well understood. Previous knowledge is limited to the study by Sawers *et al.* (1986), which showed that hydrogen uptake was abolished when cells were grown with nitrate. However, the mechanism of nitrate repression of *E. coli* hydrogenases has been studied. Richard *et al.* (1999) demonstrated that both *E. coli* *hya* and *hyb* operons are repressed by nitrate, mediated by both NarL and NarP. In contrast, we found that nitrate repression of *hya* was only mediated by NarL, and not by NarP (Table 5). We found a potential NarL heptamer recognition sequence in the promoter region of the *hya* operon. The heptamer was not in the 7-2-7 arrangement, as described by Darwin *et al.* (1997), which provides further evidence that NarL was involved in the observed repression, but NarP was not. When this site was mutated, gene expression was no longer repressed by nitrate (Table 5).

In summary, the three uptake-type hydrogenases in *S. enterica* serovar Typhimurium are transcribed under different physiological conditions and they are differently regulated. Although some of these results are similar to those described for the homologous enzymes in *E. coli*, other aspects are unique. For example, the regulation by ArcA and the nitrate repression is different in *S. enterica* serovar Typhimurium. In addition, the regulation and expression of *hyd* is unique.

The uptake-type hydrogenases are essential for virulence in *S. enterica* serovar Typhimurium (Maier *et al.*, 2004). Since the cell encounters a variety of environments during infection, it may be beneficial to be able to express a hydrogenase capable of oxidizing H₂ at different stages of colonization. Perhaps Hyd is used to conserve energy by oxidizing hydrogen in the initial stages of infection, when the cell encounters a low oxygen environment. As the bacterium progresses through the digestive system into a highly competitive and putatively anaerobic environment, *hyb* expression may be favoured to exploit H₂ as an energy source. Maier and co-workers reported H₂ levels to be 43 µM in the stomach and 118–239 µM in the small intestine of mice, levels much higher than needed to saturate the hydrogenase enzymes (Maier *et al.*, 2004; Olson & Maier, 2002). When the bacterium reaches the bowel, it probably both ferments available substrates and encounters large amounts of H₂ produced by the colonic flora. Hya may be very important in this environment. Future investigation

examining the transcriptional level of these hydrogenase operons in the animal model and experiments that examine the H₂ affinity of each *S. enterica* serovar Typhimurium enzyme will be useful to test these ideas.

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