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Final Report for LDRD Project 06-ERD-063

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Auspices Statement

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FY07 LDRD Final Report
LDRD Project Tracking Code: 06-ERD-063
Harry R. Beller, Principal Investigator

PROJECT DESCRIPTION

Using the widespread chemolithoautotrophic bacterium *Thiobacillus denitrificans* as a model organism, this project integrated genome-enabled techniques to explore novel metabolic (oxidation-reduction) pathways and bacteria-mineral interactions that could mediate the behavior of uranium in aquifers. This is particularly pertinent to a groundwater remediation process that is of great interest to the U.S Department of Energy: *in situ*, reductive immobilization of metals, including radionuclides. In previous studies, we performed a detailed analysis of the genome of *T. denitrificans* (including manual curation and comparative genomics) and conducted physiological studies of the anaerobic, nitrate-dependent oxidation of U(IV)-containing minerals by *T. denitrificans*. This 1.5-year project built on those previous studies and entailed whole-genome transcriptional analysis with high-density, oligonucleotide microarrays as well as development of a genetic system for *T. denitrificans*, with which targeted genes could be disrupted (“knocked out”) and complemented *in trans*.

RESEARCH ACTIVITIES AND TECHNICAL OUTCOME

OVERVIEW

The activities and technical outcomes of studies undertaken for this 1.5-year LDRD project are described in the two associated journal articles included in this final report:

- Beller, H. R., T. E. Letain, A. Chakicherla, S. R. Kane, T. C. Legler, and M. A. Coleman. 2006. Whole-genome transcriptional analysis of chemolithoautotrophic thiosulfate oxidation by *Thiobacillus denitrificans* under aerobic vs. denitrifying conditions. *Journal of Bacteriology* **188**:7005-7015. [UCRL-JRNL-220798]
- Letain, T. E., S. R. Kane, T. C. Legler, E. P. Salazar, P. G. Agron, and H. R. Beller. 2007. Development of a genetic system for the chemolithoautotrophic bacterium *Thiobacillus denitrificans*. *Applied and Environmental Microbiology* **73**:3265-3271. [UCRL-JRNL-226908]

MAJOR ACCOMPLISHMENTS

- We completed and analyzed whole-genome cDNA microarray (transcriptome profiling) experiments with *T. denitrificans* cells induced under aerobic versus denitrifying conditions (the latter conditions are relevant to nitrate-dependent U(IV) oxidation); these studies revealed many novel aspects of how key metabolic genes in this facultative bacterium are modulated in response to changes in the terminal electron acceptor (Beller et al. 2006).
- We successfully developed a genetic system for *T. denitrificans* and demonstrated its effectiveness by targeting a gene encoding a large subunit of a [NiFe]hydrogenase (Letain et al. 2007); hydrogenases are environmentally relevant in *T. denitrificans*, as we previously showed that nitrate-dependent U(IV) oxidation in this bacterium is dependent on H₂ oxidation. We demonstrated not only that we could successfully disrupt a targeted gene, but also that we could complement the mutant with an expression vector that we engineered and could also generate double mutants. This genetic system will be an invaluable tool for future studies of nitrate-dependent U(IV) oxidation and other metabolic processes in *T. denitrificans*.
- The PI (Beller) was invited to present some of this work at the 106th General Meeting of the American Society for Microbiology (Orlando, FL) in a presentation entitled “Genomic and transcriptomic insights into the metabolism of the obligate chemolithoautotroph *Thiobacillus denitrificans*.” [UCRL-PRES-219902].
- We presented a poster at the 107th General Meeting of the American Society for Microbiology (Toronto, Canada) entitled “Development of a genetic system for the U(IV)-oxidizing bacterium *Thiobacillus denitrificans*.” [UCRL-ABS-229989].



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Whole-Genome Transcriptional Analysis of Chemolithoautotrophic Thiosulfate Oxidation by *Thiobacillus denitrificans* Under Aerobic vs. Denitrifying Conditions

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Whole-Genome Transcriptional Analysis of Chemolithoautotrophic Thiosulfate Oxidation by *Thiobacillus denitrificans* under Aerobic versus Denitrifying Conditions†

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Thiobacillus denitrificans is one of the few known obligate chemolithoautotrophic bacteria capable of energetically coupling thiosulfate oxidation to denitrification as well as aerobic respiration. As very little is known about the differential expression of genes associated with key chemolithoautotrophic functions (such as sulfur compound oxidation and CO₂ fixation) under aerobic versus denitrifying conditions, we conducted whole-genome, cDNA microarray studies to explore this topic systematically. The microarrays identified 277 genes (approximately 10% of the genome) as differentially expressed using RMA (robust multiarray average) statistical analysis and a twofold cutoff. Genes upregulated (ca. 6- to 150-fold) under aerobic conditions included a cluster of genes associated with iron acquisition (e.g., siderophore-related genes), a cluster of cytochrome *cbb*₃ oxidase genes, *cbbL* and *cbbS* (encoding the large and small subunits of form I ribulose 1,5-bisphosphate carboxylase/oxygenase, or RubisCO), and multiple molecular chaperone genes. Genes upregulated (ca. 4- to 95-fold) under denitrifying conditions included *nar*, *nir*, and *nor* genes (associated, respectively, with nitrate reductase, nitrite reductase, and nitric oxide reductase, which catalyze successive steps of denitrification), *cbbM* (encoding form II RubisCO), and genes involved with sulfur compound oxidation (including two physically separated but highly similar copies of sulfide:quinone oxidoreductase and of *dsrC*, associated with dissimilatory sulfite reductase). Among genes associated with denitrification, relative expression levels (i.e., degree of upregulation with nitrate) tended to decrease in the order *nar* > *nir* > *nor* > *nos*. Reverse transcription-quantitative PCR analysis was used to validate these trends.

Thiobacillus denitrificans is an obligately chemolithoautotrophic bacterium characterized by its ability to conserve energy from the oxidation of inorganic sulfur compounds under either aerobic or denitrifying conditions (5). As a facultative anaerobe, *T. denitrificans* may benefit from modulating key components of its energy metabolism, such as sulfur compound oxidation or carbon dioxide fixation, according to whether oxygen or nitrate is the terminal electron acceptor. For example, *T. denitrificans* can express both form I and form II ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), which have different relative affinities for CO₂ and the competing substrate O₂ and therefore may differ in CO₂ fixation efficiency under aerobic versus denitrifying conditions. Also, among its large complement of genes associated with sulfur compound oxidation, *T. denitrificans* shares some genes with aerobic, chemolithotrophic sulfur-oxidizing bacteria and some with anaerobic, phototrophic sulfur bacteria (5). There is very little information on how (or whether) *T. denitrificans* modulates the expression of these sulfur-oxidizing genes as a function of the prevailing terminal electron acceptor. The recent availability of the complete genome sequence of *T. denitrificans* (5) and of high-density oligonucleotide microarrays provided us an opportunity to address

these and other questions by systematically investigating differential expression across the entire *T. denitrificans* genome under aerobic versus denitrifying conditions.

MATERIALS AND METHODS

Cell growth and exposure conditions. To represent gene expression under denitrifying conditions, *T. denitrificans* (ATCC strain 25259, obtained from the American Type Culture Collection) was cultivated at 30°C under strictly anaerobic conditions as described previously (4) with growth medium that contained 20 mM thiosulfate, 20 mM nitrate, and 30 mM bicarbonate (pH ~7). For exposure immediately before harvesting of RNA, 1,200 ml of cells in late exponential phase (1×10^8 to 2×10^8 cells/ml) was harvested anaerobically by centrifugation (13,400 × g, 15°C, 10 min) and resuspended in modified growth medium (phosphate concentration reduced to 1.5 mM), and three 10-ml replicates (ca. 7.3 mg protein each) in sealed vials (90% N₂–10% CO₂ headspace) were incubated for ca. 35 min. Cell growth, resuspension, and incubation were performed in an anaerobic glove box (4).

To represent gene expression under aerobic conditions, *T. denitrificans* was cultivated (two successive transfers) with growth medium that differed from the denitrifying medium in several noteworthy respects: it contained no nitrate, it was equilibrated with atmospheric oxygen (rotating in a shake flask at 200 rpm), and it contained 70 mM phosphate, 0.7 μM copper (compared to 1.2 μM in denitrifying medium), and 10 μM iron (compared to 7.5 μM). The reason for using a higher phosphate buffer concentration in the aerobic medium was that, when lower phosphate concentrations were tested, the pH of aerobic growth medium dropped from ~7 to ~5 as *T. denitrificans* oxidized thiosulfate. This follows from the stoichiometry of thiosulfate oxidation, which yields fivefold more protons per mole of thiosulfate under aerobic than denitrifying conditions. For exposure immediately before harvesting of RNA, 1,200 ml of cells in late exponential phase was harvested by centrifugation and resuspended in aerobic growth medium, and three 10-ml replicates (ca. 3.9 mg protein each) were incubated at 30°C in 125-ml Erlenmeyer flasks rotating at 200 rpm for 60 min. The pH of the cell suspensions remained in the circumneutral range throughout the incubation period.

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† Supplemental material for this article may be found at <http://jb.asm.org/>.

Metabolic activity (thiosulfate oxidation to sulfate, nitrate consumption in anaerobic cultures) was assessed in all anaerobic and aerobic suspensions by sampling each culture twice: immediately upon resuspension and immediately before harvesting for RNA. Ion chromatography was used to determine thiosulfate, sulfate, and nitrate concentrations (4). Previous experiments indicated that metabolic rates during suspensions were sufficiently linear throughout the incubation period that initial and final concentrations could be used to calculate representative specific rates. These analyses demonstrated that specific thiosulfate oxidation rates were comparable under denitrifying and aerobic conditions (0.43 ± 0.005 and $0.56 \pm 0.006 \mu\text{mol thiosulfate} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, respectively).

RNA extraction. Immediately after exposures, two volumes of RNeasy Protect (QIAGEN) were added to each culture. Samples were incubated at room temperature for 12 min, split in half, and centrifuged at 4,000 rpm for 10 min. The supernatant was decanted, and the pellet was stored at -20°C until extraction. RNA extraction was carried out with a MasterPure Complete (MPC) DNA and RNA purification kit (EpiCentre) using a modified protocol. Briefly, 300 μl of lysis solution containing 112 μg proteinase K was added to the cell pellet and the sample was incubated at 65°C for 20 to 25 min. The sample was placed on ice for 3 to 5 min, and 200 μl of MPC solution was added to precipitate protein. The supernatant was recovered after centrifugation at $>10,000 \times g$ at 4°C for 10 min. Nucleic acid was subsequently precipitated from the supernatant after addition of 500 μl 99% isopropanol and centrifugation at $>10,000 \times g$ at 4°C for 10 min. The pellet was treated with DNase I for 20 min at 37°C . To this sample was added 200 μl each of $2\times$ T&C lysis solution and MPC solution with vortexing after each addition. The samples were placed on ice for 3 to 5 min and centrifuged at $>10,000 \times g$ at 4°C for 10 min. RNA in the supernatant was recovered by isopropanol precipitation as described above. The RNA pellet was washed twice with 75% ethanol, dried briefly, suspended in water, and stored at -80°C until cDNA synthesis. Aliquots were analyzed with a Bioanalyzer (Agilent), which indicated minimal degradation and concentrations ranging from 310 to 2,000 ng/ μl . A_{260}/A_{280} ratios ranged from 1.7 to 2.1.

Preparation of labeled cDNA. cDNA production and labeling were performed by NimbleGen Systems, Inc. RNA samples were thawed on ice, and 10 μg total RNA was used to perform cDNA synthesis with SuperScript II reverse transcriptase and random hexamers. After this reaction, RNases A and H were used to digest the RNA. Single-stranded cDNA was subsequently purified by phenol extraction. Glycogen (10 μg) was added as a carrier prior to precipitation with a 1/10 volume ammonium acetate and 2.5 volumes of absolute ethanol. The resulting pellet was suspended in 30 μl water. The cDNA yield was determined by UV/visible spectrophotometry at 260 nm. The cDNA was partially digested with DNase I (0.2 U) at 37°C for 13 min or until 50- to 200-base fragments were observed with the Bioanalyzer. The fragmented cDNA was end labeled using biotin- N_6 -ddATP and terminal deoxynucleotidyl transferase (51 U) with incubation at 37°C for 2 h. The labeled product was concentrated to 20 μl using a Microcon YM-10 10,000 MWCO filter device (Millipore) and frozen at -20°C prior to hybridization.

Array design. The genome sequence from *T. denitrificans* ATCC 25259 (5) (GenBank accession no. CP000116) was submitted to NimbleGen Systems Inc. for microarray design and manufacture using maskless, digital micromirror technology. High-density (approximately 400,000-spot) microarrays employed a randomized design and a four-in-nine pattern to enhance sensitivity. Three replicates of the genome were included per chip. An average of 10 different 60-base oligonucleotides (60-mer probes) represented each open reading frame (ORF) in the genome. 60-mer probes were selected such that each probe had at least three mismatches compared to all other 60-mers in the target genome. A total of 28,320 probes were designed for the genome, which was annotated to have 2,832 ORFs at the time of microarray design (the finished genome is annotated to have 2,827 ORFs [5]). A quality control check (hybridization) was performed for each array, which contained on-chip control oligonucleotides.

Microarray hybridization and analysis. NimbleGen Systems, Inc. performed array hybridization using their Hybriwheel technology. The arrays were prehybridized at 45°C in a 50 mM 4-morpholineethanesulfonic acid buffer containing 500 mM NaCl, 10 mM EDTA, and 0.005% Tween 20 with herring sperm DNA (0.1 mg/ml) to prevent nonspecific binding to the array. After 15 min, 4 μg of labeled cDNA in hybridization buffer was added and arrays were incubated at 45°C for 16 to 20 h. Several wash steps (initially nonstringent and later stringent conditions) removed free probe, followed by detection of bound probe with Cy3-labeled streptavidin. To amplify the signal, biotinylated anti-streptavidin goat antibody was hybridized to the array. The arrays were analyzed using an Axon GenePix 4000B scanner with associated software (Molecular Devices Corp., Sunnyvale, CA).

Microarray data analysis. Investigation of reproducible differences between treatments was performed using the Bioconductor R software package. Data were processed using quantile normalization (7), and background correction was performed using the RMA (robust multiarray average) method. Data were visualized with box-and-whisker plots and scatter plots (volcano plots). Intensities were adjusted to have the same interquartile range. A linear model fit was determined for each gene using the LIMMA package (Linear Models for Microarray Data; Gordon K. Smyth), and lists of genes with the most evidence of differential expression were obtained.

Reverse transcription-quantitative PCR analysis. Confirmation of transcript levels for modulated genes was performed by reverse transcription-quantitative PCR (RT-qPCR) analysis of RNA samples representing each of the two experimental conditions. Total RNA from samples used for microarray analysis was reverse transcribed and amplified using a QuantiTect SYBR Green RT-PCR kit (QIAGEN) with gene-specific primers. Each gene-specific PCR was performed in triplicate using 25- μl reaction mixtures containing ~ 20 ng of template on a Prism 7000 cyclor (ABI). Calibration curves were performed with genomic DNA serially diluted over a range of 4 to 5 orders of magnitude. The PCR conditions were optimized to be performed as follows for all transcripts: 50°C for 30 min; 95°C for 15 min; 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s; 30 to 35 cycles. The primers are listed in Table S1 of the supplemental material.

RT-PCR analysis of *sqr* and *dsrC* transcripts. Qualitative and quantitative RT-PCR studies were performed to investigate whether a gene associated with sulfur compound oxidation, *dsrC* (Tbd1408), was cotranscribed with upstream genes associated with nitrate reduction (*nar* genes) and sulfide:quinone oxidoreductase (*sqr*; Tbd1407). Forward PCR primers were designed for Tbd1406 (*narI*) and Tbd1407 (Tbd1406F and Tbd1407F, respectively) (see Table S1 in the supplemental material), and reverse primers were designed for Tbd1408 (Tbd1408R and Tbd1408R2) (see Table S1). Control primers for the large transcript (targeting 1406 and 1407) and the Tbd1408 transcript were also designed and tested. The PCR conditions were optimized using *T. denitrificans* genomic DNA. cDNA was produced from RNA samples used in microarray experiments (aerobic and denitrifying conditions) with 150 to 250 ng RNA (pretreated with DNase), 100 units Retroscript reverse transcriptase (Ambion), random decamers, and incubation at 43°C for 75 min. PCR products were visualized by gel electrophoresis on a 1% agarose $1\times$ Tris-acetate-EDTA gel with ethidium bromide staining and UV illumination. RT-qPCR analysis was performed with a forward primer from Tbd1407, the Tbd1407-1408 intergenic region, or Tbd1408 with a reverse primer for Tbd1408 (see Table S1). The primers and template were added to SYBR Green Master Mix (Bio-Rad), and reactions were run on a Cepheid SmartCycler using the following program: 98°C for 15 s; 60°C for 60 s; 40 cycles. Controls for both RT-PCR and RT-qPCR analyses included trials without reverse transcriptase and trials without template.

Microarray data accession number. Microarray data have been deposited in the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE5256.

RESULTS AND DISCUSSION

Genome-wide observations. Microarray analysis identified 277 genes in *T. denitrificans* as differentially expressed under aerobic versus denitrifying conditions using a twofold cutoff ($P < 0.0001$). The top 50 upregulated genes under denitrifying conditions are listed in Table 1, and the top 50 upregulated genes under aerobic conditions are listed in Table 2. A large percentage of the most upregulated genes under either denitrifying or aerobic conditions occur as gene clusters and can be classified within a small number of functional categories. To illustrate, under denitrifying conditions, upregulated genes include those associated with nitrate reductase (Tbd1401 to -1406 [Tbd1401-1406]; *nar* cluster), nitrite reductase (Tbd0070-0077; *nir* cluster), nitric oxide reductase (Tbd0554-0562; *nor* cluster), and sulfur compound oxidation (including Tbd1407-1408, adjacent to the *nar* cluster). Other gene clusters with less obvious functional associations are also included among the most upregulated genes (e.g., Tbd1499-1501 and Tbd1835-1838, which includes divergently transcribed genes) and certain functions are repre-

TABLE 1. Top 50 ORFs upregulated under denitrifying conditions

Tbd ORF	Fold upregulation	p value ^a	Category ^b	Annotation ^c
1402	95	7.5E-37	DN NO3	NarK ₂ protein, nitrate/proton antiporter
1404	94	2.4E-34	DN NO3	NarH protein, nitrate reductase beta subunit
1403	89	1.8E-33	DN NO3	NarG protein, nitrate reductase alpha subunit
1401	58	5.4E-56	DN NO3	NarK protein, nitrate/proton symporter
1406	57	3.8E-52	DN NO3	NarI protein, nitrate reductase gamma subunit
1407	55	3.4E-64	S	Sulfide:quinone oxidoreductase
1405	54	9.5E-52	DN NO3	NarJ protein, nitrate reductase chaperonin
2200	26	6.5E-36		Fimbrial protein PilA
2774	25	2.0E-46	Null	Null
0077	21	2.9E-40	DN NO2	NirS (cytochrome <i>cd</i> ₁), nitrite reductase
0073	20	1.4E-31	DN NO2	NirG protein
2012	18	1.4E-37	S	Sulfate thiol esterase (tentative)
0075	14	1.5E-39	DN NO2	NirF protein
0072	14	1.4E-38	DN NO2	Nitrite reductase heme biosynthesis protein NirH
1408	14	4.0E-32	S	Putative DsrC protein
0076	13	2.2E-33	DN NO2	Probable NirC protein
1835	13	1.4E-37		Blr3518 protein
1649	13	9.9E-33	S?	Null
0071	12	1.6E-37	DN NO2	Heme D1 biosynthesis protein NirJ
0070	12	2.2E-37	DN NO2	Probable NirN protein
2226	11	7.6E-32	S?	Null
2175	11	3.5E-35		Hypothetical protein
0074	11	8.9E-40	DN NO2	NirD protein
2688	10	2.4E-51		TonB-dependent receptor protein
0561	9.5	4.8E-22	DN NO	Nitric oxide reductase subunit B (NorB)
1836	8.9	1.4E-28		Putative RNA polymerase sigma factor
0576	8.9	2.6E-38		Putative long-chain-fatty-acid transport protein
0559	8.9	1.9E-35	DN NO	Null
0562	8.7	1.3E-37	DN NO	Nitric oxide reductase subunit C (NorC)
1650	8.7	5.3E-27	S	Rhodanese-like domain protein
0558	8.3	1.2E-32	DN NO	Probable NorQ protein
1837	8.2	2.5E-40		Hypothetical signal peptide protein
0557	7.1	5.8E-40	DN NO	Hypothetical protein
0554	7.0	3.8E-34	DN NO?	Null
2225	6.5	4.7E-31	S	Sulfide:quinone oxidoreductase
2638	6.0	1.3E-24	CO2	RubisCO form II protein, CbbM
1501	5.9	1.1E-10	Null	Null
2327	5.7	2.0E-20	S	Putative DsrC protein
2317	5.6	3.6E-35		Rubryerythrin/nigerythrin-like protein
0555	5.5	7.7E-25	DN NO	Possible NorD protein
1742	5.4	5.4E-21		F ₁ -ATP synthase, epsilon subunit
1367	4.6	5.4E-26		Predicted periplasmic or secreted lipoprotein
0556	4.6	4.0E-27	DN NO	Iron-sulfur cluster-binding protein
1809	4.4	3.8E-37		Plasmid-related protein
1500	4.4	7.7E-19		V-type H ⁺ -translocating pyrophosphatase
1388	4.3	3.2E-12		Cystathionine beta-synthase domain protein
1739	4.3	6.8E-26	TT	Tetrathionate reductase subunit B
1741	4.3	6.3E-27	TT	Tetrathionate reductase subunit A
1499	4.3	8.3E-25		Hypothetical protein
0907	4.0	3.7E-27		Bacterial neuraminidase repeat

^a The p values were adjusted by procedures to control the FDR (false discovery rate) criterion defined by Benjamini and Hochberg (6).

^b Category definitions: DN NO3, denitrification, nitrate reductase; DN NO2, denitrification, nitrite reductase; DN NO, denitrification, nitric oxide reductase; S, sulfur compound oxidation; TT, tetrathionate reductase; CO2, CO₂ fixation via RubisCO. "Null" indicates no good hits and no clues from context; "?" indicates no good hits but location and expression suggest association with adjacent genes of known function.

^c Best attempt at annotation based on examination of best BLASTP matches and genomic context.

sented by single genes (e.g., *cbbM*, which encodes form II RubisCO). Under aerobic conditions, upregulated genes include a large cluster putatively encoding proteins associated with iron acquisition (Tbd0705-0725, which account for more than 40% of the top 50 upregulated genes), a cytochrome *cbb*₃ oxidase (Tbd0638-0643), multiple chaperones (including Tbd1537-1539), and form I RubisCO (*cbbS* and *cbbL*; Tbd2623-2624). Under aerobic conditions, as under denitrifying conditions, gene clusters with less obvious functional associations are also included among the most up-

regulated genes (e.g., Tbd2355-2358, Tbd2592-2594, and Tbd2777-2778) (Table 2), and certain functions are represented by single genes (e.g., Tbd1365, a putative *dsrC* presumably associated with sulfur compound oxidation).

The observation that a relatively small number of functional categories account for the majority of the most differentially expressed genes is apparent in Fig. 1, which plots log₂ probability of differential expression versus log₂ fold differential expression for all ORFs identified in the genome. The color coding in Fig. 1 corresponds to the major categories listed in

TABLE 2. Top 50 ORFs upregulated under aerobic conditions

Tbd ORF	Fold upregulation	p value ^a	Category ^b	Annotation ^c
0725	159	2.3E-78	Fe	Putative hemin uptake protein HemP
0706	117	2.0E-63	Fe	Anion-specific porin
0722	111	7.9E-59	Fe	Possible PvuA protein, polyhydroxycarboxylate siderophore uptake
0705	73	4.2E-51	Fe	Bacterioferritin-associated ferredoxin
0721	61	3.4E-54	Fe	Possible PvsA protein, ferritin biosynthesis protein
0710	57	2.1E-57	Fe	Putative lipoprotein
0715	46	7.5E-67	Fe	Possible PsuA protein, TonB-dependent receptor
0718	43	1.2E-59	Fe	Possible PvsD protein, ferritin biosynthesis protein
0712	40	8.5E-59	Fe	MotA/TolQ/ExbB proton channel family
0724	37	3.4E-43	Fe?	Null
0720	31	7.2E-46	Fe	Possible PvsB protein, ferritin biosynthesis protein
0709	27	2.0E-48	Fe?	Putative signal peptide protein
0723	27	7.3E-50	Fe	Possible high-affinity Fe ²⁺ /Pb ²⁺ permease
0711	25	5.7E-55	Fe	ExbD/TolR proton channel family
0716	24	6.2E-64	Fe	Putative aldolase, ferritin biosynthesis protein
0717	23	8.6E-41	Fe	Possible PvsE protein, ferritin biosynthesis protein
1078	18	3.4E-50	Chap	Heat shock family protein HtpG
0719	14	3.8E-52	Fe	Possible PvsC protein, siderophore efflux protein
0641	14	3.1E-34	CytOx	<i>cbb</i> ₃ -type cytochrome oxidase subunit IV (CcoQ)
2777	13	3.3E-35		Hypothetical protein
2778	12	4.7E-33		Predicted outer membrane lipoprotein
1252	11	8.1E-44	Chap	Probable ATP-dependent Lon protease
0815	9.6	1.1E-36	Chap	ClpB ATPase-dependent protease
0708	9.6	1.6E-39	Fe?	Possible ApbE protein
0643	9.6	1.7E-41	CytOx	<i>cbb</i> ₃ -type cytochrome oxidase subunit I (CcoN)
0713	9.4	1.6E-35	Fe	Possible TonB-like energy transducer
1538	9.2	1.3E-17	Chap	Chaperone protein DnaK
2593	9.2	1.0E-31		Putative transcriptional regulator
0091	9.1	2.7E-30	Chap	Chaperonin GroEL (HSP60 family)
0640	8.8	7.1E-39	CytOx	<i>cbb</i> ₃ -type cytochrome oxidase subunit III (CcoP)
1053	7.8	2.9E-35		Hypothetical protein
2624	7.4	9.2E-29	CO2	RubisCO form I protein, CbbL (large subunit)
0638	7.4	1.6E-24	CytOx	<i>cbb</i> ₃ cytochrome oxidase maturation protein CcoH
1420	7.4	1.1E-40		ABC transporter phosphate-binding protein
0714	7.1	5.1E-37	Fe	Uncharacterized iron-regulated membrane protein
1585	7.0	7.8E-39		Radical SAM enzyme of unknown function
1365	6.9	2.8E-29	S	Putative DsrC protein
2357	6.8	3.2E-26		Hypothetical protein
2356	6.6	6.6E-25		Hypothetical protein
2623	6.5	1.0E-27	CO2	RubisCO form I protein, CbbS (small subunit)
2398	6.4	7.4E-36		Hypothetical protein
0194	6.4	7.3E-31	Null	Null
2401	6.4	4.9E-15		Thioredoxin domain-containing protein
1370	6.3	3.1E-16	Chap	Chaperone protein IbpA (small heat shock protein)
2270	6.1	4.6E-36		Porcine attaching-effacing-associated protein variant 1
2355	6.1	5.9E-28	Null	Null
0707	6.1	2.9E-40	Fe?	Probable transmembrane protein
0043	5.9	5.5E-32		Hypothetical protein
1539	5.7	3.1E-34	Chap	Chaperone protein DnaJ
2592	5.7	5.9E-29		Putative membrane protein

^a The p values were adjusted by procedures to control the FDR criterion defined by Benjamini and Hochberg (6).

^b Category definitions: Fe, iron acquisition; Chap, chaperones and stress proteins; CytOx, cytochrome oxidase; CO2, CO₂ fixation via RubisCO; S, sulfur compound oxidation. "Null" indicates no good hits and no clues from context; "?" indicates no good hits but location and expression suggest association with adjacent genes of known function.

^c Best attempt at annotation based on examination of best BLASTP matches and genomic context.

Tables 1 and 2, namely, denitrification, sulfur compound oxidation, CO₂ fixation via RubisCO (forms I and II), iron acquisition, cytochrome *cbb*₃ oxidase, and chaperones and stress proteins; all genes not falling within these categories in Tables 1 and 2, and all genes not included in Tables 1 and 2, are gray in Fig. 1.

Denitrification. Although it is not surprising that genes associated with denitrification (*nar*, *nir*, and *nor* genes) were among the most upregulated genes under denitrifying conditions, subtler trends in expression of these genes were more

novel. Most notably, relative expression levels (i.e., degree of upregulation under denitrifying conditions) tended to decrease in the order *nar* > *nir* > *nor* > *nos* (Fig. 2). With the exception of a few genes (primarily associated with transcriptional regulators, such as *narXL* and Tbd0078-0079), fold upregulation for denitrification genes fell in the following ranges: *nar*, 54- to 95-fold; *nir*, 10- to 21-fold; *nor*, 4- to 10-fold; *nos*, 0.5- to 0.9-fold. This trend was both a function of generally decreasing absolute expression levels under denitrifying conditions (except for the structural genes *nirS*, *norCB*, and *nosZ*) and in-

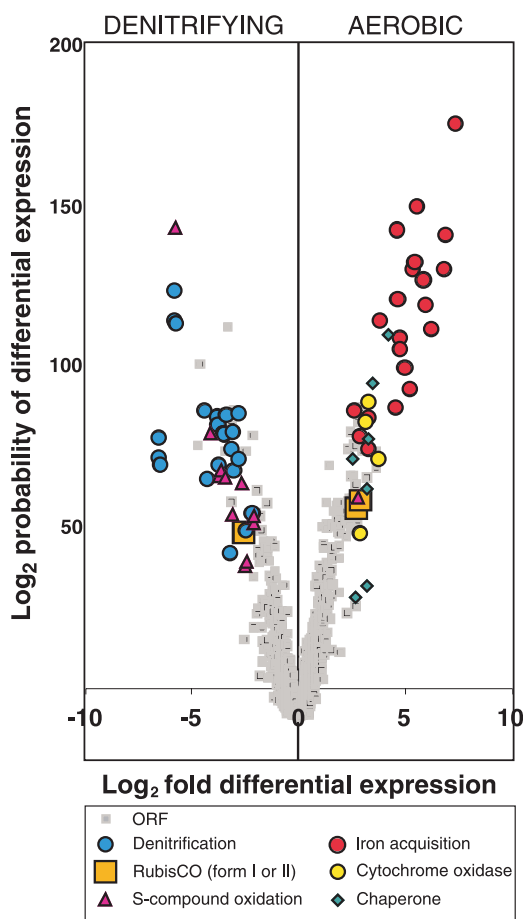


FIG. 1. Plot of log₂ probability of differential expression versus log₂ fold differential expression for all genes identified in the *T. denitrificans* genome. The color coding corresponds to the major categories listed in Tables 1 and 2, namely, denitrification, sulfur compound oxidation, CO₂ fixation via RubisCO (forms I and II), iron acquisition, cytochrome *cbb*₃ oxidase, and chaperones and stress proteins; all genes not falling within these categories in Tables 1 and 2, as well as all genes not included in Tables 1 and 2, are colored gray.

creased expression of *nos* genes (especially *nosZ*) under aerobic conditions (Fig. 2).

To our knowledge, this is the most complete data set for differential aerobic/denitrifying expression across the complement of denitrification genes; previous transcriptional studies have focused primarily on structural genes or on gene clusters associated with only one of the four denitrification enzymes. In a general sense, the microarray results for *T. denitrificans* are consistent with the well-documented transcriptional activation of denitrification genes as a function of low O₂ tension and the presence of a nitrogen oxide (NO₃⁻, NO₂⁻, NO, or N₂O) (reviewed in reference 36). With respect to *T. denitrificans* specifically, the microarray results are generally consistent with greatly increased NAR and NIR enzyme activities (in crude extracts) that were observed to accompany the transition from aerobic to denitrifying conditions in continuous culture (18). Furthermore, the decreasing trend in upregulation shown in Fig. 2 could be consistent with induction of each reductase component by its cognate substrate, as one might expect the

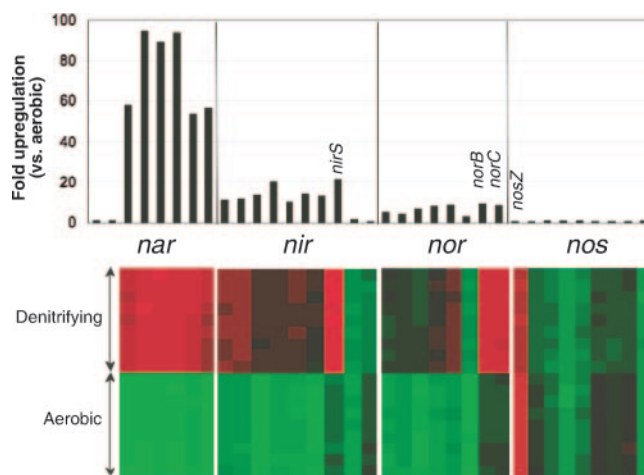


FIG. 2. Histogram displaying fold upregulation (denitrifying versus aerobic conditions) for genes associated with denitrification, including *nar* cluster genes (Tbd1399-1406), *nir* cluster genes (Tbd0070-0079), *nor* cluster genes (Tbd0555-0562), and *nos* cluster genes (Tbd1389-1397). Structural *nir*, *nor*, and *nos* genes are labeled. Absolute expression levels for these genes are shown below the histogram, with green representing the lowest levels of expression, black representing intermediate levels, and red representing the highest levels. The plot of absolute expression levels shows all nine replicates for each condition.

concentration pattern of [NO₃⁻] > [NO₂⁻] > [NO] > [N₂O] in a denitrifying cell (although this concept clearly oversimplifies the regulation of denitrification).

However, for *nos* genes in particular, the results for *T. denitrificans* appear to deviate from findings for other denitrifying species for which data are available, namely, *Pseudomonas stutzeri*, *Paracoccus denitrificans*, and *Paracoccus pantotrophus* (formerly *Thiosphaera pantotropha*). For example, whereas expression of *nos* genes (including *nosD*) in *T. denitrificans* was comparable under aerobic and denitrifying conditions, the amount of *nosD* transcripts in *P. stutzeri* (revealed by Northern blot analysis) increased steadily and dramatically during the first hour following a shift from aerobic to denitrifying conditions in continuous culture (15). In another continuous culture study of *P. stutzeri* (20), *NosZ* levels were at least 10-fold greater for cells under denitrifying conditions than for cells under fully aerobic conditions (in the presence of nitrate). A continuous culture study of *Paracoccus denitrificans* revealed more than a 10-fold increase in the amount of *nosZ* transcripts during the first hour following transition from aerobic to denitrifying conditions (3). This temporal trend was qualitatively similar to those of other denitrification genes; however, *narH* and *nirS* transcript copy numbers increased more (approximately 30- to 45-fold) (3). In continuous culture and batch culture studies of *P. pantotrophus* (23), *NosZ* expression was 2- to ca. 20-fold greater under denitrifying conditions than under aerobic conditions (in the presence or absence of nitrate) and clearly decreased as a function of increasing oxygen concentration in continuous culture. Differences in experimental approach preclude a direct comparison of the results of the present study with those just cited for *P. stutzeri* and *P. denitrificans*; such differences in experimental approach include the use of continuous cultures versus batch cultures and measurement after aerobic/anaerobic transitions versus comparisons of

cultures grown exclusively under aerobic or denitrifying conditions. Acknowledging this caveat, the available data suggest inconsistent trends for differential aerobic/denitrifying *nos* gene expression in *T. denitrificans* compared to other species studied. In *P. stutzeri*, *P. denitrificans*, and *P. pantotrophus*, there appears to be considerable upregulation of *nos* genes (at least *nosZ* and *nosD*) under denitrifying conditions; this is clearly not the case for *T. denitrificans* (indeed, there is slight upregulation of these genes under aerobic conditions) (Fig. 2).

In the absence of additional experimental evidence, we cannot explain the anomalous lack of differential transcription of *nosZ* and other *nos* genes in *T. denitrificans* under aerobic versus denitrifying conditions. Nonetheless, examination of promoter regions for some key genes associated with denitrification did reveal possible clues. Specifically, these promoter regions in *T. denitrificans* were examined with respect to potential FNR boxes (i.e., DNA-binding motifs for FNR-like transcription factors). When compared to the canonical FNR box 5'-TTGAT-N₄-ATCAA-3' described for *Escherichia coli* (36), slightly degenerate sequences were found upstream of *narK* (aTGAc...ATCtt, located 778 nucleotides [nt] from the translational start site of Tbd1401), *nirS* (TTGAc...ATCAA, located 76 nt from the translational start site of Tbd0077), *norC* (aTGAc...ATCAA, located 163 nt from the translational start site of Tbd0562), and *nosZ* (TTGAg...gTCAg, located 1,310 nt from the translational start site of Tbd1389). Two features shared by the *narK*, *nirS*, and *norC* versions and not in the *nosZ* version are the c and A shown in boldface type above. Also, upstream of *nosZ*, there were five additional sequences with 5' ends that matched either the canonical FNR box or the *narK*, *nirS*, or *norC* FNR boxes cited above, but these were more degenerate on the 3' end (with only 0 to 2 bases agreeing with the canonical sequence). Degeneracy at the 3' end of FNR boxes upstream of *nosZ* has been observed for some denitrifying species (8, 36) but not for others (2). Considering that FNR boxes for positive regulation of denitrification genes are typically centered at a distance of -41.5 nt from the transcription start site (36), most of the putative FNR boxes just described for *T. denitrificans* seem to be very far upstream. It is not clear whether any of these characteristics of putative FNR boxes in *T. denitrificans* could explain the lack of *nosZ* upregulation under denitrifying conditions.

Sulfur compound oxidation. Although a diverse complement of more than 50 genes associated with sulfur compound oxidation has been described in *T. denitrificans* ATCC 25259 (5), those genes associated with activity under aerobic versus denitrifying conditions have not been elucidated to date. Many of the *T. denitrificans* genes associated with sulfur compound oxidation (5) were not found to be differentially expressed in this study (Tables 1 and 2). Among the genes not appearing in Tables 1 and 2 were clusters of sulfur compound oxidation genes that were very highly expressed under both aerobic and denitrifying conditions. These include *soxXYZA* (Tbd0567-0564), *dsrABEFHCKMLJOP* (Tbd2485-2474), and the genes encoding ATP sulfurylase and APS reductase (Tbd0874-0872). The expression levels of these genes were typically at or above the 95th percentile expression level observed across the genome. Indeed, many of these genes are likely to be constitutively expressed in *T. denitrificans* as, in most cases, their expression levels were similarly high under Fe(II)-oxidizing,

denitrifying conditions when no sulfur-containing electron donor was present (H. Beller et al., unpublished microarray data).

Differential expression was observed for certain genes associated with sulfur compound oxidation; in some cases, the absolute expression levels of these genes when upregulated were also in the range of the 95th percentile expression level observed across the genome. Among the most differentially regulated genes putatively associated with sulfur compound oxidation, all but one were upregulated under denitrifying rather than aerobic conditions (Tables 1 and 2; Fig. 1). These included two copies of sulfide:quinone oxidoreductase (*sqr*) that share 43% amino acid identity (Tbd1407 and Tbd2225; 55- and 6.5-fold upregulated under denitrifying conditions), a rhodanese-like domain protein (Tbd1650; 8.7-fold upregulated), and two putative copies of *dsrC* that share 88% amino acid identity (Tbd1408 and Tbd2327; 14- and 5.7-fold upregulated). Another putative copy of *dsrC* (Tbd1365) was upregulated 6.9-fold aerobically. Another rhodanese copy (Tbd2399) was less upregulated aerobically (3.8-fold) but was included in a gene cluster that exhibited some stronger aerobic upregulation (Tbd2398-2401) (Table 2).

Inasmuch as three *dsrC* copies were among the most differentially regulated genes, it is noteworthy that the *T. denitrificans* genome includes eight putative *dsrC* copies overall (5); the phylogenetic relationships and genomic organization of these homologs have been presented elsewhere (5). Only one copy, Tbd2480, is located in the large gene cluster *dsrABEFHCKMLJOPNR* (Tbd2485-2472) and is constitutively expressed at a high level (5). Although the exact function of DsrC is not known, it is almost certainly involved with sulfur compound oxidation; the associated *dsrAB* genes encode a siroheme-containing sulfite reductase that has been proposed to catalyze the oxidation of certain inorganic sulfur species (e.g., hydrogen sulfide or sulfane-sulfur derived from thiosulfate) to sulfite (27, 33).

In light of the strong upregulation of Tbd1407 (*sqr*) and Tbd1408 (putative *dsrC*) under denitrifying conditions (Table 1), the genomic location of these genes is noteworthy: they are immediately downstream of the *narKK₂GHJI* cluster (Tbd1401-1406) (5), which encodes a membrane-bound, dissimilatory nitrate reductase (and associated nitrate/nitrite transporters) (Fig. 3A). As there is not even a single intergenic base separating Tbd1406 and Tbd1407, it follows that Tbd1407 is part of a polycistronic transcript including *nar* genes (probably *narKK₂GHJI*). However, the intergenic region between Tbd1407 and Tbd1408 includes a putative ribosomal-binding site and FNR box (Fig. 3B). Thus, coregulation rather than cotranscription of Tbd1407 and Tbd1408 is plausible and, indeed, is suggested by the anomalously high expression of Tbd1408 relative to Tbd1407 under aerobic conditions (Fig. 3A). To further investigate whether Tbd1408 was transcribed independently of Tbd1407 and upstream *nar* genes, RT-qPCR studies were conducted. These studies confirmed that, for the most part, Tbd1408 was transcribed separately from Tbd1407 and upstream *nar* genes: under denitrifying or aerobic conditions, the copy number of transcripts of Tbd1408 (*dsrC*) was at least 10-fold greater than the copy number of transcripts including Tbd1407 and Tbd1408 (*sqr* and *dsrC*) (Fig. 3C). Although the RT-qPCR studies were constrained by amplicon length and did

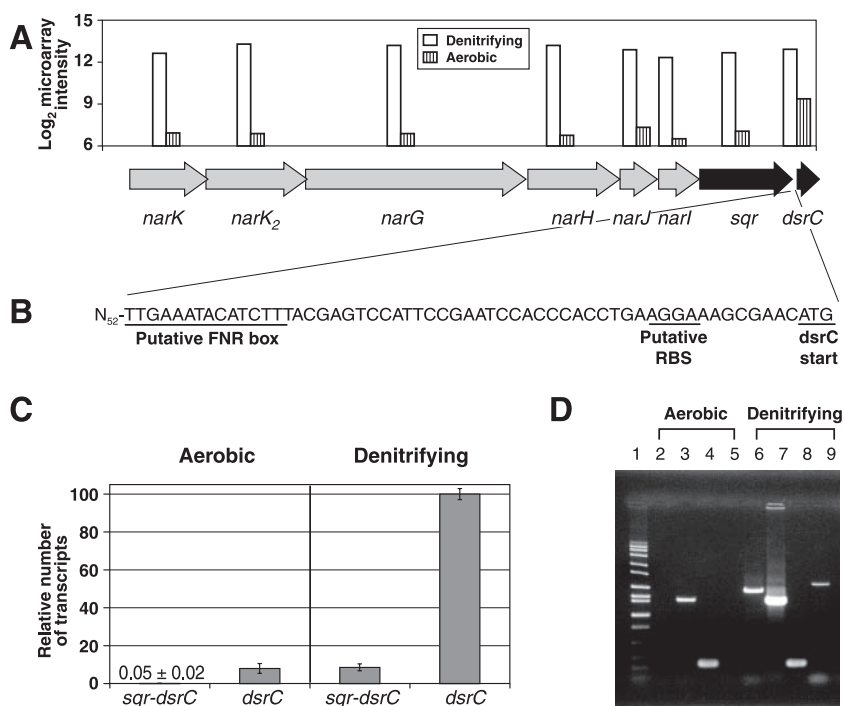


FIG. 3. (A) Histogram of absolute expression levels of *sqr* (Tbd1407), *dsrC* (Tbd1408), and adjacent *nar* genes under aerobic and denitrifying conditions. (B) Partial sequence of the intergenic region between *sqr* and *dsrC*; the putative ribosomal-binding site (RBS) and FNR box are indicated by underlining. (C) RT-qPCR results for cells exposed to aerobic and denitrifying conditions showing the relative number of transcripts that include *dsrC* (primers Tbd1408F and Tbd1408R2) or *dsrC* plus the intergenic region between *sqr* and *dsrC* (primers Tbd1407-1408IG_F and Tbd1408R). Numbers of transcripts are normalized to the largest value (*dsrC*; denitrifying conditions). Error bars represent 1 standard deviation based upon triplicate analyses. (D) Electropherogram of RT-PCR products from RNA extracts for cells exposed to aerobic (lanes 2 to 5) and denitrifying (lanes 6 to 9) conditions. Lane 1, Hi-Lo DNA marker, 50 bp to 10 kbp (Bionexus Inc., Oakland, CA); lanes 2 and 6, Tbd1407F/Tbd1408R2; lanes 3 and 7, Tbd1407F/Tbd1407R; lanes 4 and 8, Tbd1408F/Tbd1408R2; lanes 5 and 9, Tbd1406F/Tbd1408R2. All bands represent cDNA amplicons of the expected length and are consistent with PCRs using genomic DNA as the template (not shown). No bands were visible for negative controls lacking reverse transcriptase (not shown).

not address transcripts extending upstream beyond Tbd1407, semiquantitative RT-PCR analyses (Fig. 3D) suggested that, at least under denitrifying conditions, the Tbd1407-1408 transcripts actually extended at least from Tbd1406 (*narI*) to Tbd1408 (lane 9). Overall, the microarray and RT-qPCR results suggest that the promoter(s) controlling the expression of *dsrC* (Tbd1408), while clearly effecting stronger activation under denitrifying than aerobic conditions, may be further enhanced by the presence of sulfur compounds.

Carbon dioxide fixation. The genome of *T. denitrificans* encodes both form I and form II RubisCO enzymes for CO₂ fixation (11, 14). The microarray results show clearly that the structural genes encoding form I and II RubisCO were differentially expressed: *cbbM* (Tbd2638, which encodes form II) was upregulated 6-fold under denitrifying conditions (Table 1), whereas *cbbL* and *cbbS* (Tbd2624-2623, which encode the large and small subunits of form I) were upregulated 7.4- and 6.5-fold, respectively, under aerobic conditions (Table 2). The other *cbb* genes included in the form I and form II RubisCO gene clusters were also differentially expressed, albeit to a lesser extent than the structural genes. Thus, *cbbQ* and *cbbO* (Tbd2637 and Tbd2636) in the form II cluster were upregulated 2.7- and 3.5-fold under denitrifying conditions, whereas their homologs in the form I cluster (Tbd2622 and Tbd2621) were upregulated 5.5- and 2.6-fold under aerobic conditions.

These results are consistent with the biochemical characterization of form I and II RubisCO in *T. denitrificans* with respect to their relative affinities to CO₂ and O₂. Molecular oxygen competes with CO₂ for the active site of RubisCO and thereby decreases its efficiency for carbon fixation. The relative specificity of RubisCO enzymes for CO₂ and O₂ (the CO₂/O₂ specificity factor, or τ) was determined in *T. denitrificans* (14); form I was shown to have considerably higher CO₂/O₂ specificity ($\tau = 46$) than form II ($\tau = 14$). Thus, expressing form I under aerobic conditions would tend to maximize the efficiency of CO₂ fixation.

We are not aware of any previous studies of differential expression of form I and II RubisCO under aerobic versus denitrifying conditions. The most relevant studies are those that investigated expression of form I and II RubisCO under a variety of chemoautotrophic, chemoheterotrophic, photoautotrophic, and photoheterotrophic conditions in *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* (recently reviewed in reference 10). Differential transcription of forms I and II was observed in some of these studies. In the absence of more experimental data for *T. denitrificans*, these existing studies allow us only to speculate about regulatory systems that might be involved in differential transcription of form I and II RubisCO in *T. denitrificans*.

RegB/RegA is a global, two-component, redox-responsive

regulatory system that appears to have a role in differential expression of form I and II RubisCO in *Rhodobacter* species (reference 10 and references therein). For example, in work with *regA* mutants of *R. sphaeroides* grown under aerobic, chemolithotrophic conditions, Gibson et al. (12) indicated that RegA (PrrA) functioned as a strong activator of form II RubisCO genes but had no effect on, or acted as a mild repressor of, the form I genes. However, rocket electroimmunoassay studies of *R. sphaeroides* strain HR-CAC showed that approximately 2.5-fold more form I than form II RubisCO protein was expressed under aerobic chemolithoautotrophic conditions (25). RegA also influences the differential, redox-responsive transcription of other genes, including those associated with photosynthesis, cytochrome *cbb*₃ oxidase, and Cu-containing nitrite reductase (*nirK*).

Several lines of evidence suggest that the RegB/RegA system is present in *T. denitrificans* and may contribute to transcriptional regulation of RubisCO genes: (i) genes putatively encoding RegA and RegB have been identified in *T. denitrificans* (Tbd2690 and Tbd2689, respectively), (ii) possible RegA-binding sites are present upstream of *cbbM* and *cbbL*, and (iii) at least one putative RegA-binding site is present in the intergenic region upstream of the aerobically upregulated *ccoN* gene (Tbd0643), which encodes a subunit of cytochrome *cbb*₃ oxidase and has been associated with RegB/RegA regulation in *R. capsulatus* (29, 30). BLASTP searches for RegA in the *T. denitrificans* genome using the RegA (PrrA) sequence from *R. sphaeroides* (GenBank YP_351562) revealed that Tbd2690 was the best match; the deduced amino acid sequence of Tbd2690 shares 51% identity with the RegA sequence of *R. sphaeroides*. Alignment of these (and other) RegA sequences showed that the *T. denitrificans* homolog also includes the highly conserved helix-turn-helix DNA-binding motif described for a range of RegA homologs (10). RegB is putatively encoded by Tbd2689 in *T. denitrificans* (26% sequence identity with the RegB sequence of *R. sphaeroides*; GenBank YP_351564). Alignment of the deduced amino acid sequence of Tbd2689 with known RegB sequences revealed that the *T. denitrificans* homolog contains a highly conserved, redox-active cysteine residue that has been shown to exert control over the activity of the sensor kinase in *R. sphaeroides* (31). Searches for RegA-binding sites upstream of *cbbM* and *cbbL* in *T. denitrificans* revealed possible degenerate sequences. Laguri et al. (22) described the following main features of RegA-binding sites derived from studies of *R. sphaeroides* and *R. capsulatus*: (i) a palindromic 5'-GCGNC...GNCGC-3' consensus, (ii) a central AT-rich section, and (iii) a variable number of bases between the 5' and 3' palindromic regions (with an apparent total of 9 to 15 bases in the binding site motif). Sequences conforming to these characteristics were found upstream of *cbbM* (5'-GCGACAGCCGC-3') and *cbbL* (5'-GCGCCTCTT GTCGC-3'). Notably, both of these putative RegA-binding sites were located at least 950 nt upstream of the translational start sites of *cbbM* and *cbbL* and occurred in a complementary *cbbR* coding region (i.e., in a *cbbR* coding region on the opposite strand from *cbbM* and *cbbL*). Since the RegA-binding consensus features were based on only two bacterial species, it is possible that other RegA-binding sites occur upstream of *cbbM* or *cbbL* but could not be detected because they diverge from *Rhodobacter* motifs.

Transcriptional regulation of RubisCO genes is characteristically complex and is controlled by more than just the RegB/RegA system. For example, there is undoubtedly also some positive control of form I and II RubisCO expression by the LysR-type transcriptional regulator CbbR (21). Both the form I and form II operons in *T. denitrificans* are adjacent to divergently transcribed *cbbR* genes (5), and multiple putative CbbR-binding sites were found in upstream regions of both *cbbM* and *cbbL*. To illustrate, in the intergenic region between *cbbL* and the upstream *cbbR* gene, there were putative, often overlapping CbbR-binding sites located from 4 to 17 nt and 89 to 152 nt upstream from the translational start site. In the intergenic region between *cbbM* and the upstream *cbbR* gene, there were putative CbbR-binding sites located from 4 to 24 nt and 64 to 127 nt upstream from the translational start site. The motif used to identify putative CbbR-binding sites was T-N₁₂-A, which deviates from the T-N₁₁-A motif characteristic of LysR-type transcriptional regulators but may be more applicable to CbbR-binding sites in autotrophic bacteria (21). There is currently no evidence suggesting that CbbR influences differential expression of RubisCO genes under aerobic versus denitrifying conditions, and it is very possible that as-yet-identified transcriptional regulators may influence expression of RubisCO genes (9, 12).

Cytochrome *cbb*₃ oxidase. The gene cluster Tbd0643-0637, which includes genes putatively encoding one of two cytochrome *cbb*₃ oxidases in *T. denitrificans* (5), was upregulated under aerobic conditions (Table 2; Fig. 1). The first four genes in this cluster (Tbd0643-0640) appear to be *ccoNOQP*, and the entire cluster is highly similar in terms of gene sequence and organization to a cluster in the related β -proteobacterium *Azoarcus* sp. strain EbN1. Throughout this cluster in *T. denitrificans*, genes were upregulated 3.9- to 13.5-fold relative to denitrifying conditions. The highest upregulation was for *ccoN* (9.6-fold) and *ccoQ* (13.5-fold).

The results for *T. denitrificans* are generally consistent with those from *ccoN::lacZ* transcriptional fusion studies of *R. capsulatus* and *R. sphaeroides*, which showed greater expression of *ccoN* under aerobic, and particularly microaerophilic, conditions compared to anaerobic conditions (24, 29, 30). Studies with *regA* mutants of *R. capsulatus* suggest that RegA activates cytochrome *cbb*₃ oxidase expression semiaerobically or aerobically but represses expression anaerobically (29, 30). In contrast, FnrL apparently activates cytochrome *cbb*₃ oxidase expression semiaerobically or anaerobically in these two *Rhodobacter* species (24, 29).

The promoter region upstream of *ccoN* was examined for potential RegA- and FNR-binding sites, as these transcription factors have been implicated in the regulation of cytochrome *cbb*₃ oxidases in *Rhodobacter* species. We focused on the promoter region of *ccoN* because it is the first gene in this cluster and its upstream intergenic region is nearly 500 nt long, whereas the intergenic regions upstream of *ccoO*, *-Q*, and *-P* only range from 0 to 11 nt. A probable RegA-binding site (5'-GCGACACGTTGGCGC-3') was identified upstream of *ccoN*; this putative binding site was located much closer to the translational start site (ca. 280 nt upstream) than those we have identified in promoter regions of *cbbL* and *cbbM* (discussed previously). The most likely FNR-binding site identified in the *ccoN* promoter region was TTGAT...cTCgc, which was no-

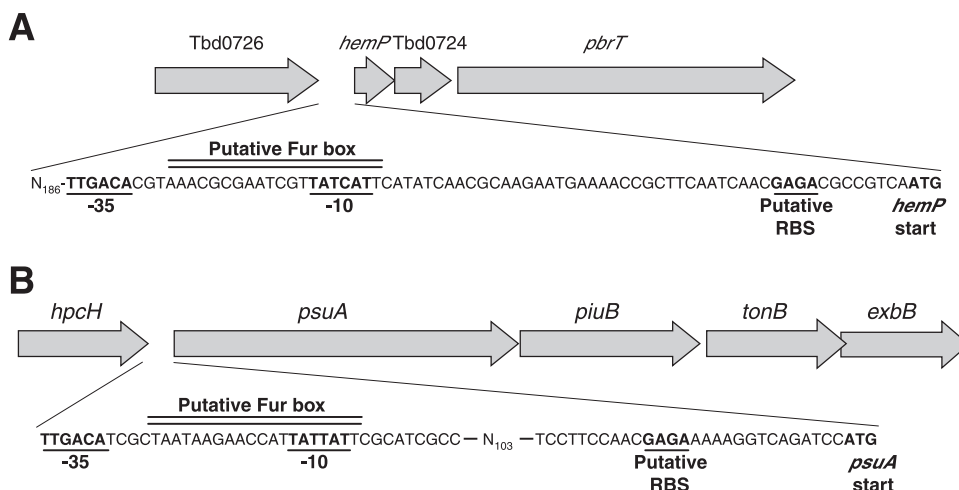


FIG. 4. Nucleotide sequences in the promoter regions of the *hemP* (A) and *psuA* (B) genes. The putative -35 and -10 promoter sequences as well as the putative Fur box sequences for both genes are indicated.

tably degenerate at the 3' end and was located 374 nt upstream of the translational start site.

Chaperones and stress proteins. A number of genes associated with protein folding and turnover were upregulated under aerobic conditions (Table 2; Fig. 1). These include the genes encoding the molecular chaperones ClpB (Tbd0815; 9.6-fold upregulated), GroEL and GroES (Tbd0091-0092; 9.1- and 5.1-fold upregulated, respectively), GrpE, DnaK, and DnaJ (Tbd1537-1539; 5.1- to 9.2-fold upregulated), and IbpA (Tbd1370; 6.3-fold upregulated). Several genes occurring in a cluster with GroEL and GroES were also aerobically upregulated, albeit to a lesser extent (Tbd0094-0096; 2.1- to 2.6-fold). Other aerobically upregulated genes encoding proteins that are putatively associated with protein folding and turnover include genes for HtpG (Tbd1078; 18-fold upregulated) and Lon protease (Tbd1252; 11-fold upregulated) (Table 2). Several of these genes have been found to be regulated in *Escherichia coli* by sigma 32, the heat shock/stress alternative sigma factor (Tbd0345). Sigma 32-regulated genes include *clpB*, *grpE*, *dnaJ/dnaK*, *ibpA*, *htpG*, and *lon* (35). In turn, several of the proteins encoded by these genes regulate intracellular levels of sigma 32, as do GroEL and GroES (13).

GroEL, an essential chaperone, and DnaK have been shown to play a significant role in the viability of *E. coli* (16). In *E. coli*, it has been demonstrated that about 250 proteins interact with GroEL, of which several could also utilize DnaK for proper folding (19). In the current study, both DnaK and GroEL were found to be significantly upregulated under aerobic conditions along with form I RubisCO, which has been shown to be a substrate of GroEL (17).

Iron acquisition. A cluster of 21 genes (Tbd0705 to Tbd0725), many of which are associated with Fe³⁺ uptake (5), includes the 16 most aerobically upregulated genes observed in this study (Table 2). In fact, all 21 genes in the cluster are among the top 50 aerobically upregulated genes (Table 2). The level of upregulation within the cluster varies widely, ranging from 6.1-fold upregulation for Tbd0707 to 159-fold upregulation for Tbd0725. Aerobic upregulation of iron transport genes in bacteria occurs in response to limited iron availability due to

the lower solubility of Fe(III) species compared to Fe(II) species (1). To illustrate for the conditions used in this study, although the amounts of iron added to the aerobic and denitrifying cultures were similar (10 and 7.5 μM, respectively), equilibrium geochemical modeling (26) indicated that the amounts of dissolved iron under these two conditions differed dramatically. Whereas all of the 7.5 μM iron would be present in solution under denitrifying conditions (~75% as FeHCO₃⁺), less than 0.7 μM would be soluble under aerobic conditions [>93% of the Fe would be present as Fe(OH)₃ precipitate].

Genes found in the cluster include those that encode proteins involved in siderophore biosynthesis and export (Tbd0716-0721), Fe³⁺-siderophore uptake across the outer membrane (Tbd0711-0713, Tbd0715, and Tbd0722), iron storage and mobility (Tbd0705), and heme uptake (Tbd0725). Systems involved in iron acquisition have been found to be regulated by the ferric uptake regulator protein, Fur (Tbd1123), which acts as a repressor in the presence of Fe²⁺ and a derepressor in the absence of Fe²⁺ (1). Putative DNA-binding sites allowing for Fur-dependent regulation (1, 34) were identified upstream of two genes in the cluster (Tbd0725 and Tbd0715) and overlapped with an *E. coli*-type sigma 70 promoter sequence for both genes (Fig. 4). The most highly upregulated gene, Tbd0725, encodes a putative homolog of HemP, a Fur-regulated protein associated with heme uptake in *Yersinia en-*

TABLE 3. *E. coli*-like sigma 70 consensus sequences identified in promoter regions of selected genes that were upregulated aerobically in *T. denitrificans*

Tbd ORF	Gene	-35 sequence	-10 sequence
0705	<i>bfd</i>	TTGACA	TAGAAT
0715	<i>psuA</i>	TTGACA	TATTAT
0725	<i>hemP</i>	TTGACA	TATCAT
0092	<i>groES</i>	TTGAAA	TATTAT
1252	<i>lon</i>	TTGAAA	GATACT
1538	<i>dnaK</i>	TTGAAA	CATATT
0643	<i>ccoN</i>	TTGACA	TATATT
1389	<i>nosZ</i>	TAGACA	TACATG

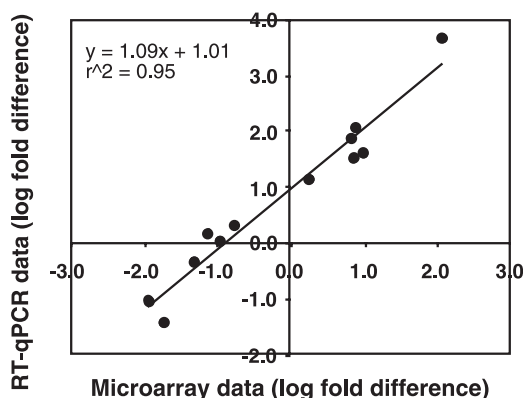


FIG. 5. Correlation between aerobic fold upregulation as determined by RT-qPCR versus microarray analysis for 12 genes: *narG* (Tbd1403), *nirS* (Tbd0077), *norB* (Tbd0561), *nosZ* (Tbd1389), *cbbS* (Tbd2623), *cbbL* (Tbd2624), *cbbM* (Tbd2638), *sqr* (Tbd1407), *ccoN* (Tbd0643), *dsrC* (Tbd1408), *dsrC* (Tbd1365), and *pvuA* (Tbd0722).

terocolitica (28). Although other genes associated with heme uptake are not found in this gene cluster, they are found scattered throughout the *T. denitrificans* genome. The second gene, Tbd0715, encodes a homolog of PsuA, a Fur-regulated Fe^{3+} -siderophore outer membrane receptor in *Vibrio parahaemolyticus* found associated with siderophore biosynthesis genes similar to those occurring in this *T. denitrificans* cluster (32).

Sequences closely matching the *E. coli* sigma 70 "consensus" sequences were also found in promoter regions of several other aerobically upregulated genes (Table 3), including *bfd* (Tbd0705), *groES* (Tbd0092), *lon* (Tbd1252), *dnaK* (Tbd1538), and *ccoN* (Tbd0643). As discussed previously, these genes encode proteins involved in a variety of functions, including iron uptake and storage, protein folding and turnover, and aerobic respiration via cytochrome *cbb*₃ oxidase. In contrast, promoter regions for anaerobically upregulated genes, including those involved in denitrification (*narK*, *nirS*, and *norC*) and carbon fixation (*cbbM*), did not have sequences that closely matched the *E. coli* consensus sequence. Interestingly, the promoter region for *nosZ*, which was slightly upregulated aerobically despite being associated with denitrification (Fig. 2), also contains sequences similar to the *E. coli* sigma 70 consensus sequences (Table 3).

RT-qPCR validation of microarray trends. Twelve genes were selected for analysis by RT-qPCR to confirm that differential expression indicated by the microarray data was supported by an independent method. The selected genes (listed in the legend for Fig. 5) cover a wide range of expression and include genes that were most upregulated under aerobic conditions and under denitrifying conditions. Overall, the RT-qPCR data and microarray data were very consistent (Fig. 5); the data were highly correlated ($r^2 = 0.95$) and had a slope that approached unity (1.085).

Concluding remarks. As one of the first whole-genome transcriptional studies of a chemolithotrophic bacterium, and one of the few studies addressing transcriptional analysis of genes associated with chemolithotrophic sulfur compound oxidation, this study provides a number of novel findings, including the

following: (i) strong upregulation under denitrifying conditions of two copies of *sqr* (which is explained by genomic location adjacent to the *nar* gene cluster for only one *sqr* copy), (ii) a variety of expression behaviors for the eight *dsrC* copies (ranging from aerobic upregulation to anaerobic upregulation to constitutive expression at a high level), (iii) consistently high-level expression under aerobic and denitrifying conditions of several important gene clusters associated with sulfur compound oxidation (including *soxXYZA*, *dsrABEFHCKMLJOP*, and the genes encoding ATP sulfurylase and APS reductase), (iv) differential expression of genes putatively encoding rhodanese (an enzyme function previously lacking direct evidence for its involvement in thiosulfate oxidation), and (v) differential expression of form I and II RubisCO under aerobic versus denitrifying conditions. Whereas this study provides some insight into the unusual ability of *T. denitrificans* to oxidize sulfur compounds under aerobic and denitrifying conditions, additional whole-genome transcriptional studies by our group will provide information on other unusual abilities of this bacterium, namely, catalysis of anaerobic, nitrate-dependent Fe(II) and U(IV) oxidation. Combining these microarray results with the use of a newly developed genetic system in *T. denitrificans* (T. Letain, S. Kane, T. Legler, H. Beller, E. Salazar, and P. Agron, unpublished data) will facilitate better understanding of the biochemical and genetic basis of the oxidative metabolism of this widespread but unusual bacterium.

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Development of a Genetic System for the Chemolithoautotrophic Bacterium *Thiobacillus denitrificans*[∇]

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Thiobacillus denitrificans is a widespread, chemolithoautotrophic bacterium with an unusual and environmentally relevant metabolic repertoire, which includes its ability to couple denitrification to sulfur compound oxidation; to catalyze anaerobic, nitrate-dependent oxidation of Fe(II) and U(IV); and to oxidize mineral electron donors. Recent analysis of its genome sequence also revealed the presence of genes encoding two [NiFe]hydrogenases, whose role in metabolism is unclear, as the sequenced strain does not appear to be able to grow on hydrogen as a sole electron donor under denitrifying conditions. In this study, we report the development of a genetic system for *T. denitrificans*, with which insertion mutations can be introduced by homologous recombination and complemented in *trans*. The antibiotic sensitivity of *T. denitrificans* was characterized, and a procedure for transformation with foreign DNA by electroporation was established. Insertion mutations were generated by *in vitro* transposition, the mutated genes were amplified by the PCR, and the amplicons were introduced into *T. denitrificans* by electroporation. The IncP plasmid pRR10 was found to be a useful vector for complementation. The effectiveness of the genetic system was demonstrated with the *hynL* gene, which encodes the large subunit of a [NiFe]hydrogenase. Interruption of *hynL* in a *hynL::kan* mutant resulted in a 75% decrease in specific hydrogenase activity relative to the wild type, whereas complementation of the *hynL* mutation resulted in activity that was 50% greater than that of the wild type. The availability of a genetic system in *T. denitrificans* will facilitate our understanding of the genetics and biochemistry underlying its unusual metabolism.

Thiobacillus denitrificans is a widespread, obligate chemolithoautotrophic bacterium with an unusual and environmentally relevant metabolic repertoire, which includes its ability to couple denitrification to sulfur compound oxidation; to catalyze anaerobic, nitrate-dependent oxidation of Fe(II) and U(IV); and to oxidize mineral electron donors such as FeS and UO₂ (6 and references therein). More information about the metabolism of *T. denitrificans* emerged from recent analysis of its genome sequence, which revealed the presence of genes encoding two [NiFe]hydrogenases (6). Hydrogenases are metalloenzymes that catalyze the reversible oxidation of H₂ to protons and are vital components of the energy metabolism of many microbes. Hydrogenases had not previously been reported in *T. denitrificans* even though this species was first isolated over a century ago (4). The role of these hydrogenases in *T. denitrificans* is unclear, as the sequenced strain does not appear to be able to grow on hydrogen as a sole electron donor under denitrifying conditions (6). Notably, hydrogen oxidation appears to be required for nitrate-dependent U(IV) oxidation by *T. denitrificans* (5), although the biochemical linkage between H₂ and U(IV) oxidation has not been elucidated.

Based upon genome annotation, the two [NiFe]hydrogenases in *T. denitrificans* have been putatively characterized as follows (6): (i) a periplasmic group 1 [NiFe]hydrogenase (following the classification system described by Vignais et al. [22])

presumed to catalyze H₂ oxidation *in vivo* and (ii) a cytoplasmic, heterotetrameric, group 3b [NiFe]hydrogenase (following the classification system described by Vignais et al. [22]) that is typically associated with H₂ evolution as a means of disposing of excess reducing equivalents under fermentative conditions. A noteworthy feature of the group 1 hydrogenase is that it is encoded by an unusual gene cluster (*hynS-isp1-isp2-hynL*; Tbd_1378-1375) that has only been observed in four other microbes to date, none of which is a mesophilic, chemolithoautotrophic bacterium like *T. denitrificans* (6).

In this article, we describe the development of a genetic system in *T. denitrificans* that focuses on genetic disruption (and complementation in *trans*) of the group 1 hydrogenase. We chose to target a gene associated with the group 1 hydrogenase for several reasons: (i) presumably, this is the hydrogenase that catalyzes anaerobic H₂ oxidation in *T. denitrificans*; (ii) accordingly, this is probably the hydrogenase that drives nitrate reduction during nitrate-dependent U(IV) oxidation (5); and (iii) the gene cluster encoding this hydrogenase is unusual, as noted previously. Specifically, we targeted the *hynL* gene, as this gene encodes the hydrogenase's large subunit, which harbors the active site (22). As described in this article, the somewhat unexpected results for the hydrogenase activity of the *hynL* knockout mutant led us to generate a double knockout containing mutations in the genes for the large subunits of both [NiFe]hydrogenases.

Relatively few genetic systems have been described for chemolithoautotrophic bacteria such as *T. denitrificans*. These include *Halothiobacillus neapolitanus*, which represents the earliest published example of the successful introduction of a

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TABLE 1. Strains and plasmids used in this study

Strain, plasmid, or transposon	Genotype or markers; characteristics and uses	Source or reference
Strains		
<i>Escherichia coli</i> TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7967 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
<i>Thiobacillus denitrificans</i> ATCC 25259	Wild type	ATCC ^a
TL001	<i>hynL</i> ::kan	This work
TL002	<i>hynL</i> ::kan <i>hydA</i> ::gent	This work
Plasmids		
pUC19	pMB1, Amp ^r ; cloning vector	24
pPA20	pMB1, IncP Amp ^r Kan ^r ; shuttle vector	1
pANT4	IncQ, Amp ^r Kan ^r <i>mob</i> ⁺	15
pTnMod-OCm	pMB1, Cam ^r <i>mob</i> ⁺ Tn5 <i>tnp</i>	11
pTnMod-OGm	pMB1, Gent ^r <i>mob</i> ⁺ Tn5 <i>tnp</i>	11
pTnMod-OKm'	pMB1, Kan ^r <i>mob</i> ⁺ Tn5 <i>tnp</i>	11
pTnMod-SmO	pMB1, Spcm ^r Str ^r <i>mob</i> ⁺ Tn5 <i>tnp</i>	11
pTnMod-OTc	pMB1, Tet ^r <i>mob</i> ⁺ Tn5 <i>tnp</i>	11
pRR10	IncP, Amp ^r <i>mob</i> ⁺ <i>lacZ</i> α	19
pTL1	IncP, Amp ^r , pRR10 with P _{Kan} inserted at MCS	This work
pTL2	IncP, Gent ^r Amp ^r , pTL1 with <i>bla</i> ::gent; <i>T. denitrificans</i> expression vector	This work
pTL3	IncP, Gent ^r , pTL2 with <i>hynL-hupE</i> inserted next to P _{Kan} allowing for expression	This work
EZ-Tn5 pMOD-2<MCS>	ColE1, Amp ^r ; transposon construction vector	EPICENTRE Biotechnologies
pMOD-2<gent>	ColE1, Amp ^r Gent ^r with <i>SacI</i> fragment from pTnMod-OGm containing <i>aacC1</i> inserted at MCS of pMOD-2	This work
pUC19- <i>hynL</i>	pMB1, Amp ^r , pUC19 with <i>hynL</i> inserted at MCS	This work
pUC19- <i>hynL</i> ::kan	pMB1, Amp ^r Kan ^r , pUC19 with <i>hynL</i> ::kan inserted at MCS	This work
pUC19- <i>hydA</i>	pMB1, Amp ^r , pUC19 with <i>hydA</i> inserted at MCS	This work
pUC19- <i>hydA</i> ::gent	pMB1, Amp ^r Gent ^r , pUC19 with <i>hydA</i> ::gent inserted at MCS	This work
Transposons		
Tn-kan	Kan ^r , EZ-Tn5<KAN-2> DNA fragment with kanamycin resistance selection marker located between Mosaic End Tn5 transposase recognition sequences	EPICENTRE Biotechnologies
Tn-gent	Gent ^r , EZ-Tn5 pMOD-2<gent> DNA fragment with gentamicin resistance selection marker located between Mosaic End Tn5 transposase recognition sequences	This work

^a ATCC, American Type Culture Collection, Manassas, VA.

wide-host-range plasmid into a chemolithoautotrophic bacterium (3, 14), and *Acidithiobacillus ferrooxidans* (16, 18), a bacterium widely used in mineral leaching and often associated with acid mine drainage. Other sulfur compound-metabolizing bacteria for which genetic systems have been described include the photolithoautotrophic bacterium *Chlorobium tepidum* (12) and the heterotrophic bacterium *Geobacter sulfurreducens* (9), both environmentally relevant organisms.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids and *T. denitrificans* (ATCC 25259) and *Escherichia coli* strains that were used in this study are described in Table 1.

Culturing conditions and growth media. *E. coli* was propagated according to established methods (20).

T. denitrificans cultures for electroporation and plasmid preparations were grown in modified M9 minimal medium (20) with additional constituents and vitamins prepared as described by Beller (5) and Widdel and Bak (23). The medium composition, per liter of water, was as follows: 6.8 g of Na₂HPO₄, 3.0 g of KH₂PO₄, 1.0 g of NH₄Cl, 2.52 g of NaHCO₃, 4.96 g of Na₂S₂O₃ · 5H₂O, 2.02 g of KNO₃, 0.2 g of MgSO₄ · 7H₂O, 0.03 g of CaCl₂ · 2H₂O, 2.7 mg of FeCl₃ · 6H₂O, 0.18 mg of CuSO₄ · 5H₂O, 50 μg of vitamin B₁₂, 100 μg of thiamine, and 1 ml of stock solution 6 (23). Solid medium also contained 15 g purified agar/liter (Oxoid, Hampshire, United Kingdom). Liquid cultures were grown on the bench top without aeration in capped flasks and tubes. Incubation of *T. denitrificans* on solid medium was per-

formed inside an anaerobic glove box at 30°C, as colony growth of *T. denitrificans* was found to be limited under aerobic conditions. Ultrapurified water (18-MΩ resistance) obtained from a Milli-Q UV Plus system (Millipore, Bedford, MA) was used to prepare the growth medium and all other aqueous solutions.

Antibiotics were used where appropriate in agar plates and liquid cultures at the following concentrations (unless indicated otherwise): ampicillin, 100 μg ml⁻¹; chloramphenicol, 50 μg ml⁻¹; gentamicin, 50 μg ml⁻¹; kanamycin, 50 μg ml⁻¹; streptomycin, 50 μg ml⁻¹; spectinomycin, 100 μg ml⁻¹; tetracycline, 20 μg ml⁻¹.

Determination of plating efficiency. The cell density of suspended *T. denitrificans* cultures was determined by measuring the absorbance at 600 nm of washed cell suspensions and was correlated with microscopic cell counts made with a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA). The plating efficiency was determined by serially plating 10-fold dilutions of the cell suspensions in triplicate and counting the CFU obtained.

DNA manipulations. Genomic DNA was isolated from *T. denitrificans* using a cetyltrimethylammonium bromide precipitation method (2). Plasmid DNA was isolated from *E. coli* and *T. denitrificans* using midi- or mini-plasmid purification kits (QIAGEN, Valencia, CA). PCR products and gel-excised plasmid fragments were purified with a QIAquick gel extraction kit (QIAGEN). Primers for amplification of *T. denitrificans* genomic DNA were designed from the whole genome sequence available through the Joint Genome Institute website at <http://genome.ornl.gov/microbial/tbden/> and in the GenBank/EMBL database under accession no. CP000116. PCR amplification of DNA fragments containing *T. denitrificans* sequence was typically performed using Advantage-GC 2 polymerase (Clontech, Mountain View, CA), except where noted, to improve yields for polymerization of the high-GC (66%) *T. denitrificans* DNA. Manufacturer's

TABLE 2. Sequences of primers used in this study

Primer	Sequence ^a (5' → 3')
hynL-1-f.....	GAATTCTAGATGGAACAACACAGCGGTC
hynL-1-r.....	GATAGCATGCTTATTTGACCTTACCTGCACG
pUC19-f.....	GCCAGGGTTTTCCAGTCACGA
pUC19-r.....	GAGCGGATAACAATTCACACAGG
kanP-f.....	AAAGCCACGTTGTGTCTC
kanP-r.....	GAATGTTAACACCCCTTGATTACTG
hynL-hupE-f.....	phos-ATGGCAACAACACAGCGC
hynL-hupE-r.....	phos-TCACAGCCCGAGGATGAGG
EZ-Tn5-f ^b	ACCTACAACAAAGCTCTCATCAACC
EZ-Tn5-r ^b	GCAATGTAACATCAGAGATTTTGAG
pMOD2-PCR-f ^b	ATTGAGGCTGCGCAACTGGT
pMOD2-PCR-r ^b	GTCAGGTGAGCGAGGAAAGCGGAAG
pMOD2-Seq-f ^b	GCCAACGACTACGCACTAGCCAAC
pMOD2-Seq-r ^b	GAGCCAAATATGCGAGAACACCCGAGAA
hynL-2-f.....	TCATGGCGGAGCATCTGGAG
hynL-2-r.....	CGTATTTGCGAACATGGGTGG
hydA-f.....	GAATGGTACCCAGTTCAACATGCTCTACCT
hydA-r.....	GATAGGTACCTTGAACGGCGCGTCAACTG

^a phos denotes phosphorylated ends. Relevant restriction sites are underlined.

^b EPICENTRE Biotechnologies, Madison, WI.

protocols were used when working with the EZ-Tn5<KAN-2> insertion kit and the EZ-Tn5 pMOD-2<MCS> vector (EPICENTRE Biotechnologies, Madison, WI). DNA sequencing was performed by Davis Sequencing (Davis, CA).

Determination of antibiotic resistance. Various pTnMod minitransposon plasmids were prepared to 0.1 to 0.2 $\mu\text{g } \mu\text{l}^{-1}$ in 10 mM Tris buffer (pH 8.0), and 0.1 to 0.3 $\mu\text{g } \mu\text{l}^{-1}$ was electroporated into *T. denitrificans* as described below. Recipient cells were plated on modified M9 solid medium containing one of the following: 20, 25, or 50 $\mu\text{g } \text{ml}^{-1}$ kanamycin; 50 or 100 $\mu\text{g } \text{ml}^{-1}$ gentamicin; 50 $\mu\text{g } \text{ml}^{-1}$ streptomycin and 100 $\mu\text{g } \text{ml}^{-1}$ spectinomycin; 5, 10, or 20 $\mu\text{g } \text{ml}^{-1}$ tetracycline; or 20 or 25 $\mu\text{g } \text{ml}^{-1}$ chloramphenicol.

Construction of hynL insertion mutant. In order to perform gene disruption via homologous recombination, a hynL::kan product was created. The hynL gene (Tbd_1375) was amplified from *T. denitrificans* genomic DNA using Vent DNA polymerase (New England BioLabs, Ipswich, MA) and hynL-1 primers containing an XbaI site in the forward primer and an SphI site in the reverse primer (Table 2). The resulting 1.8-kb PCR product was digested with XbaI and SphI and ligated into XbaI/SphI-digested pUC19. The resulting pUC19-hynL plasmid was subjected to in vitro transposition with an EZ-Tn5<KAN-2> cassette system (EPICENTRE Biotechnologies) and transformed into *E. coli*. Resulting kanamycin-resistant colonies were screened for Tn-kan (Table 1) placement within hynL and sequenced for the exact Tn-kan location using EZ-Tn5 primers (Table 2). Linear DNA containing hynL::kan for electroporation into *T. denitrificans* was generated from pUC19-hynL::kan with pUC19 primers (Table 2) and Hi-Fi Taq polymerase (Invitrogen, Carlsbad, CA).

Construction of complementation plasmid. A complementation plasmid (Fig. 1) was developed based on the IncP broad-host-range vector pRR10 (19). The kanamycin promoter, P_{Kan} (10), was amplified from pTnMod-OKm' using kanP primers (Table 2) and Platinum Taq polymerase (Invitrogen). The resulting 110-bp amplicon was ligated into the SmaI site of pRR10, generating pTL1 (Table 1) and creating a unique HpaI site immediately downstream of P_{Kan}. In order to introduce gentamicin resistance into pTL1, a transposon containing the aminoglycoside acetyltransferase gene aacC1 was created. First, an 836-bp fragment containing aacC1 was cut from pTnMod-OGm (Table 1) with SacI. This same fragment was ligated into the SacI site of EZ-Tn5 pMOD-2<MCS>, creating pMOD-2<gent> (Table 1). The gentamicin transposon Tn-gent (Table 1) was created according to the manufacturer's instructions via PCR amplification of pMOD-2<gent> with pMOD2-PCR primers (Table 2) and transposed into pTL1, completing the complementation vector pTL2 (Table 1; Fig. 1). The transposition reaction products were electroporated into TOP10 *E. coli* cells. The resulting gentamicin-resistant colonies were screened for ampicillin sensitivity, and the exact location of aacC1 was confirmed by sequence analysis. To generate the final hynL expression vector, pTL3 (Table 1), a fragment containing hynL and the downstream gene hupE was amplified from *T. denitrificans* genomic DNA using hynL-hupE primers (Table 2) and Phusion DNA polymerase (Finnzymes, Espoo, Finland) and was ligated into the HpaI site of pTL2. The resulting clones were screened by PCR analysis for correct orientation of hynL-hupE relative to P_{Kan}, and the sequence was confirmed.

Construction of hydA insertion mutant. A disruption was created in the *T. denitrificans* gene encoding the large structural subunit of a group 3b hydroge-

nase, hydA (Tbd_1263), as follows. The hydA gene with approximately 1 kb flanking DNA on each end was amplified from *T. denitrificans* genomic DNA using KpnI-modified hydA primers (Table 2). The resulting 3.8-kb PCR product was digested with KpnI and ligated into KpnI-digested pUC19, yielding pUC19-hydA (Table 1). This plasmid was subjected to in vitro transposition with Tn-gent and transformed into *E. coli*. Resulting gentamicin-resistant colonies were screened for Tn-gent placement within hydA and sequenced for the exact Tn-gent location using pMOD2-Seq primers (Table 2). Linear DNA containing hydA::gent for electroporation into *T. denitrificans* strain TL001 (Table 1) was generated from pUC19-hydA::gent with pUC19 primers (Table 2).

Electrocompetent cell preparation and electrotransformation. One Shot TOP10 Electrocomp *E. coli* cells (Table 1; Invitrogen) were purchased and transformed according to the manufacturer's instructions.

Electrocompetent *T. denitrificans* cells were prepared from 48-h (late log phase), 200-ml cultures at 600 nm = 0.1 to 0.25; 1×10^8 to 2.5×10^8 cells ml⁻¹. All manipulations were carried out on ice, and all solutions were sterile and ice cold. Cells were harvested by centrifugation at 4°C for 10 min at $3,220 \times g$. The cells were washed twice in 100 ml ultrapure H₂O and resuspended in 500 μl ultrapure H₂O. Cell integrity of *T. denitrificans* was monitored throughout the wash procedure by phase-contrast microscopy. Cells were freshly prepared immediately before electroporation.

All electrotransformations were performed in 0.2-cm-gap Bio-Rad Gene Pulser cuvettes using a Bio-Rad Gene Pulser II equipped with a Pulse Controller Plus. Electrocompetent *T. denitrificans* cells (50 μl) were pulsed at 12.5 kV cm⁻¹ for ~5 ms (resistance = 200 Ω ; capacitance = 25 μF). Typically, 2 to 5 μl containing ~100 to 500 ng DNA was used for electroporation. Cells were recovered in 1 ml ice-cold, modified M9 medium immediately following electroporation and transferred into sterile 1.7-ml Microfuge tubes. Electroporated cells were allowed to recover for 24 h at room temperature prior to plating on solid medium containing antibiotics. Each *T. denitrificans* electrotransformation experiment included a negative control in which no DNA was added. In all cases, no background growth of *T. denitrificans* on selective medium was observed.

Hydrogenase activity assay. In vivo assays were conducted with wild-type, mutant, and complemented mutant strains of *T. denitrificans* to assess specific hydrogenase activity. Cells (100 to 200 ml) were grown with thiosulfate and nitrate under strictly anaerobic conditions as described elsewhere (5), except that antibiotics were included in the growth medium (e.g., 50 $\mu\text{g } \text{ml}^{-1}$ kanamycin for the hynL mutant, 50 $\mu\text{g } \text{ml}^{-1}$ gentamicin for the complemented mutant). Cultivation and handling of cells for the in vivo assay were conducted in an anaerobic glove box with headspace containing ~3% hydrogen (5). *T. denitrificans* cells in

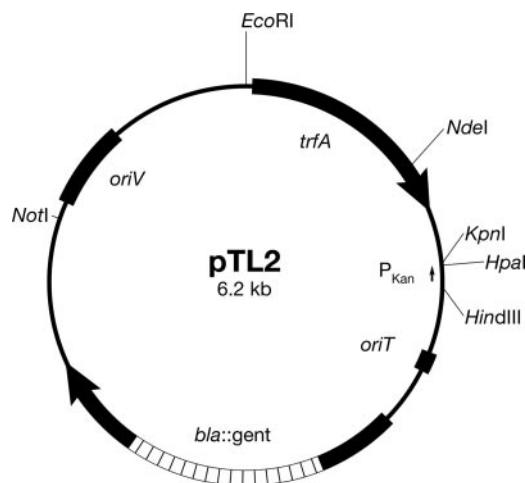


FIG. 1. Schematic diagram of the complementation vector (pTL2; Table 1) used in *T. denitrificans*. Plasmid construction is described in Materials and Methods. The following features are shown: oriV, the RK2 minimal vegetative origin of replication; oriT, the origin of transfer; trfA, encodes the RK2 replication initiation protein; bla, the beta-lactamase gene encoding ampicillin resistance; P_{Kan}, the 110-bp promoter of the kanamycin resistance gene from pTnMod-OKm'; and relevant restriction sites. The HpaI restriction site is unique and allows placement of genes for complementation. KpnI and HindIII bracket the original MCS from pRR10.

late exponential phase ($\sim 2 \times 10^8$ cells ml^{-1}) were harvested anaerobically by centrifugation in sealed polycarbonate bottles and washed once with an anaerobic resuspension buffer described previously (5). Cells were then resuspended in the anaerobic buffer at a final density of 1×10^9 to 2×10^9 cells ml^{-1} . One milliliter of this cell suspension was added to a quartz cuvette with a polytetrafluoroethylene stopper (1-cm path length; Spectrocell, Oreland, PA). Then, 10 μl of an anaerobic 100 mM benzyl viologen solution was added to the cuvette. The cuvette was then immediately sealed with the stopper, gently mixed, and quickly removed from the glove box for analysis. Reduction of benzyl viologen was monitored spectrophotometrically by measuring absorbance at 555 nm every 20 s for 5 min. All materials used in the assays were stored in the anaerobic glove box for at least 1 day prior to use. Negative controls included assays without benzyl viologen or without hydrogen. The hydrogen-free negative controls involved a butyl rubber stopper rather than a polytetrafluoroethylene stopper and were prepared in the same manner as regular samples except that, after sealing the cuvette, the glove box atmosphere was replaced by alternately evacuating the cuvette headspace under vacuum and flushing with an H_2 -free, anaerobic mixture of 90% N_2 -10% CO_2 .

RESULTS AND DISCUSSION

Growth on solid medium and characterization of antibiotic sensitivity. In order to allow the isolation of clonal populations of genetically modified *T. denitrificans*, plating onto solid growth medium was optimized. Medium solidified by the addition of purified agar (Oxoid) yielded the best results (see Materials and Methods), with visible colonies observed after 7 to 10 days of growth under anaerobic conditions at 30°C. When late logarithmic liquid medium cultures containing $(1.3 \pm 0.2) \times 10^8$ cells ml^{-1} (mean \pm standard deviation; $n = 16$)—as determined by direct cell counts in a Petroff-Hausser counting chamber—were plated on this medium, $(1.6 \pm 0.6) \times 10^8$ CFU ml^{-1} ($n = 3$) were recovered, demonstrating a high plating efficiency.

The growth of *T. denitrificans* on solid medium was inhibited by a variety of common antibiotics. Growth of 1×10^9 cells ml^{-1} plated on solid medium was inhibited by chloramphenicol (50 $\mu\text{g ml}^{-1}$), gentamicin (50 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$), streptomycin (50 $\mu\text{g ml}^{-1}$) plus spectinomycin (100 $\mu\text{g ml}^{-1}$), and tetracycline (5 $\mu\text{g ml}^{-1}$). The growth of *T. denitrificans* in liquid medium was similarly inhibited, with one difference: *T. denitrificans* grown in liquid medium also displayed sensitivity to ampicillin (100 $\mu\text{g ml}^{-1}$). As *T. denitrificans* takes much longer to grow on solid medium (10 days, as opposed to 2 days in liquid medium), it is likely that the ampicillin had degraded during the 10 days it took for growth to occur on solid medium. These results indicated that several different antibiotic resistance markers could be used for selection of genetic variants.

Identification of potential expression vectors. Both broad- and narrow-host-range plasmids (13) were tested for the ability to replicate within *T. denitrificans*. The plasmids were introduced by electroporation, and viability after electroporation was demonstrated by both N_2 bubble formation (evidence of denitrification) and growth on solid media.

An IncP plasmid containing the RK2 minireplicon, pPA20 (1), and an IncQ plasmid, pANT4 (15), were evaluated for the ability to replicate in *T. denitrificans*. Electroporation of pPA20 into *T. denitrificans* resulted in multiple kanamycin-resistant isolates (5 to 10 isolates μg^{-1} DNA). To ensure that pPA20 was successfully replicating in *T. denitrificans*, the plasmid was purified from one of the kanamycin-resistant transformants, checked for correct size by gel electrophoresis (data not

shown), and reintroduced into *T. denitrificans* via electroporation. A significant increase in the number of transformants μg^{-1} of plasmid DNA was seen with pPA20 isolated from *T. denitrificans* (1.4×10^6 transformants μg^{-1}) when compared to pPA20 isolated from *E. coli* (5 to 10 transformants μg^{-1}), a 10^5 -fold increase. The low number of transformants resulting from the *E. coli*-purified plasmid is likely a result of plasmid degradation by host restriction/modification systems, of which *T. denitrificans* has several (6). Electroporation of pANT4 into *T. denitrificans* yielded no kanamycin-resistant isolates after several attempts.

Analysis of antibiotic resistance determinants in *T. denitrificans*. A series of minitransposon vectors (Table 1) were used to confer functional antibiotic resistance in *T. denitrificans*, including pTnMod-OKm' (Kan^r), pTnMod-SmO (Str^r Spcm^r), pTnMod-OGm (Gent^r), pTnMod-OCm (Cam^r), and pTnMod-OTc (Tet^r), containing Tn5 inverted repeats, the transposase external to the repeats, and a conditional origin of replication (11). Evaluation of these five different antibiotic resistance determinants contained on pTnMod vectors showed that three constructs resulted in functional antibiotic resistance after transposition into the *T. denitrificans* genome, including kanamycin, gentamicin, and streptomycin-spectinomycin resistance. Electroporation of *T. denitrificans* with pTnMod-OTc and pTnMod-OCm did not result in tetracycline- or chloramphenicol-resistant colonies, respectively, using 5, 10, and 20 μg tetracycline ml^{-1} or 25 and 50 μg chloramphenicol ml^{-1} . Since no colonies were obtained, it is not clear whether the lack of resistance resulted from lack of gene expression or lack of functionality of the antibiotic resistance marker in *T. denitrificans*.

Development of a gene disruption system for *hynL* and *hydA*. In order to disrupt the genes encoding the hydrogenase large structural subunits within *T. denitrificans*, gene replacement of *hynL* by *hynL::kan*, and of *hydA* by *hydA::gent*, was performed by using homologous recombination. Electroporation of wild-type *T. denitrificans* with 250 ng of *hynL::kan* linear DNA resulted in 10 CFU on modified M9 Km₅₀ plates after 7 to 10 days. PCR analysis with *hynL*-2 primers (Table 2), which anneal outside of the *hynL* gene (Fig. 2), was used to confirm homologous recombination within the *T. denitrificans* genome. Production of the expected 3.1-kb band (Fig. 2, lane 3) signified that *hynL::kan* recombined properly within the genome. Sequence analysis of the PCR products confirmed that the Tn-kan insertion was located 801 bases downstream from the *hynL* translation start site in the *T. denitrificans* genome. PCR analysis of pUC19-*hynL::kan* (Table 1; the plasmid used to generate the linear DNA fragment used for electroporation) with *hynL*-2 primers generated no product, as expected (data not shown).

The confirmed *hynL* knockout strain (TL001; Table 1) was used to generate a second knockout in a group 3b hydrogenase, *hydA*. Homologous recombination of wild-type *hydA* with *hydA::gent* was successful, resulting in 60 to 75 colonies μg^{-1} of transformed linear DNA. One strain, TL002 (Table 1), showed the correct-size product for *hydA::gent* amplification (data not shown) and was also confirmed by sequence analysis, which showed that the disruption was located 182 bp from the translation start site.

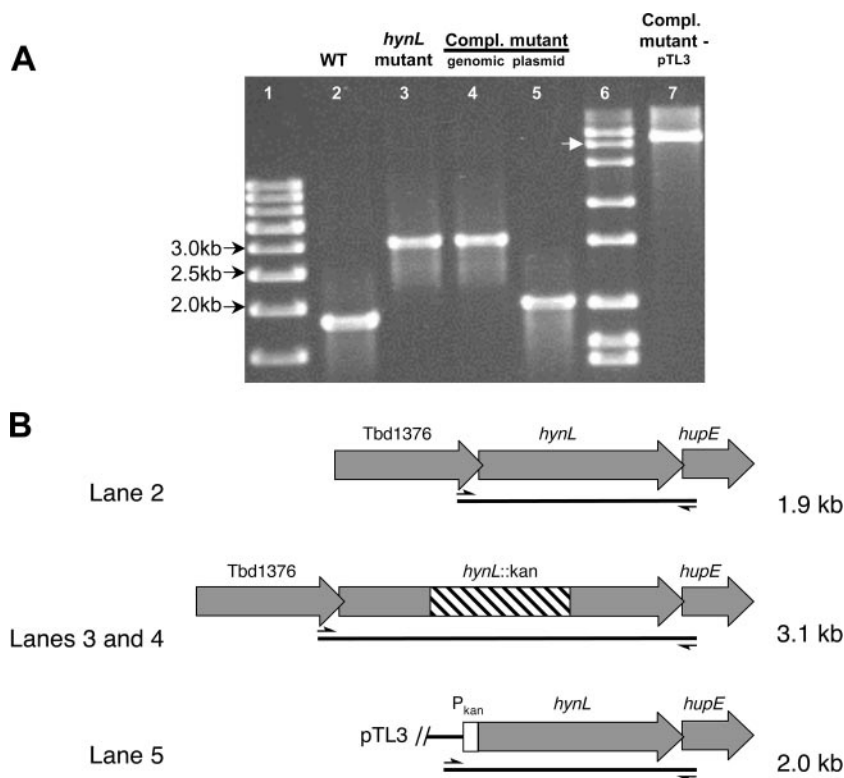


FIG. 2. (A) Electropherogram of PCR products from wild-type (WT) *T. denitrificans*, the *hynL* mutant, and the complemented (Compl.) *hynL* mutant, as well as digested plasmid DNA from the complemented mutant. Lane 1, HyperLadder III, Bionline; lane 2, wild-type DNA, primers *hynL*-2-f and -r; lane 3, *hynL* mutant (strain TL001) DNA, primers *hynL*-2-f and -r; lane 4, complemented-mutant genomic DNA, primers *hynL*-2-f and -r; lane 5, complemented-mutant plasmid DNA, primers *hynL*-2-r and pUC19-r; lane 6, Hi-Lo Marker, Bionexus (the arrow indicates 8 kb); lane 7, complemented-mutant pTL3 plasmid DNA, NdeI digested. (B) Maps of primer positions and amplicon sizes corresponding to lanes 2 to 5 in panel A. Note that the *hynL*-2-f primer anneals with genomic DNA upstream of the *hynL* gene, whereas the pUC19-r primer anneals with pTL3 plasmid DNA, rendering these primers specific to the *T. denitrificans* genome and the complementation plasmid pTL3, respectively.

For both strains TL001 and TL002, no growth defects were apparent during cultivation with thiosulfate and nitrate.

Development of a complementation system to allow for intras expression of the *hynL* gene. To demonstrate that the phenotype of the mutant strain TL001 was due to disruption of the hydrogenase large structural subunit (*hynL*), and not due to a secondary gene mutation, an expression vector containing *hynL* was constructed. While pPA20 was able to replicate in *T. denitrificans*, it is not ideally suited as an expression vector due to its relatively large size (8 kb) and lack of a multiple cloning site (MCS). Since the RK2 minireplicon of plasmid pPA20 was sufficient for replication in *T. denitrificans*, other plasmids containing the same replicon were considered. The broad-host-range plasmid pRR10 (19) was used as the basis for the complementation vector since it contains the same replicon as pPA20, is relatively small, and contains the pUC19 MCS and *lacZ* α fragment (for insert screening). As ampicillin resistance is not an effective selectable marker for *T. denitrificans* on solid medium, a gentamicin-resistant variant of pRR10 was generated. The *aacCI* gene from pTnMod-OGm served as the gentamicin resistance marker in pRR10; after transposition, Tn-gent (containing *aacCI*) (Table 1) was determined by sequence analysis to be located 400 bp into the *bla* gene (Fig. 1).

The gene cluster containing the group 1 hydrogenase gene disruption has five genes between *hynL* and the promoter, with one additional gene, *hupE*, located immediately downstream of *hynL* (6). Microarray expression data (7 and unpublished data), as well as the short intergenic gaps between genes in this cluster, are consistent with the transcription of both *hynL* and *hupE* being dependent on a promoter located at the beginning of this cluster, approximately 4 kb upstream of *hynL*. Since identification of the *hynL* promoter was uncertain, an alternate promoter from the *aph(3')*-I gene encoding kanamycin resistance on pTnMod-OKm' was used instead of the native *hynL* promoter.

Approximately 100 ng of the complementation vector pTL3 was electroporated into TL001, and two to five gentamicin-resistant isolates were obtained. An 8.6-kb plasmid purified from a gentamicin-resistant TL001 isolate (Fig. 2, lane 7) was confirmed to contain *hynL*-*hupE* by both PCR analysis (Fig. 2, lane 5) and sequence analysis of the recovered plasmid. In addition, *hynL*-2 primers were used to confirm the maintained presence of *hynL::kan* within complemented strain TL001 (Fig. 2, lane 4).

Effect of genetic manipulations on hydrogenase activity. Interruption of the *hynL* gene, which encodes the large, active-site-bearing subunit of a periplasmic [NiFe]hydrogenase (6),

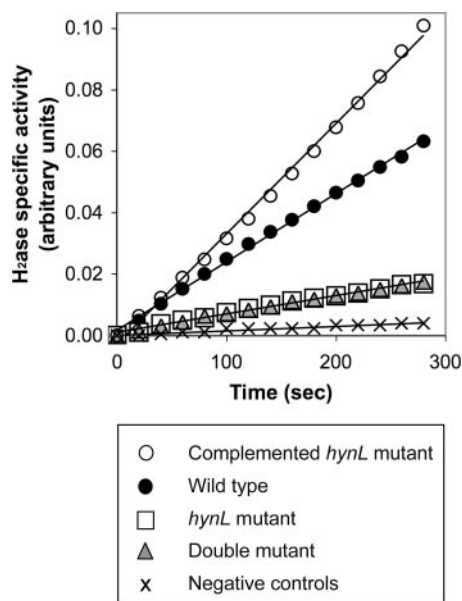


FIG. 3. Hydrogen oxidation (measured as *in vivo* benzyl viologen reduction) versus time for wild-type *T. denitrificans*, strain TL001 (*hynL* mutant), strain TL002 (*hynL hydA* double mutant), and the complemented *hynL* mutant (TL001/pTL3). Negative controls (averaged results for controls with no H_2 and controls with no benzyl viologen) are also shown. Each datum point represents the average of duplicate or triplicate assays. Linear-regression fits are plotted.

resulted in a dramatic decrease in specific hydrogenase activity (Fig. 3). Compared to the rate of specific hydrogen oxidation in wild-type cells, the rate in mutant strain TL001 was, on average, 75% reduced in replicate experiments. The hydrogenase assay proved to be generally robust, as specific rates in replicate experiments with wild-type cells agreed within 1%. Negative control assays that excluded hydrogen or benzyl viologen resulted in rates that were negligible relative to the wild type (Fig. 3). Complementation of the *hynL* mutation (strain TL001/pTL3; Table 1) resulted in specific hydrogenase activity that exceeded that of the wild type (Fig. 3). Overall, the assay results for the *hynL::kan* mutant (strain TL001) and the complemented mutant indicate that the genetic system described here enabled successful manipulation of a targeted gene in *T. denitrificans*.

The observation that interruption of the *hynL* gene did not completely eliminate hydrogenase activity merits further discussion. Contamination with a wild-type copy of *hynL* is largely ruled out because we grew the TL001 culture in medium containing $50 \mu\text{g ml}^{-1}$ kanamycin and could not detect the wild-type *hynL* gene in PCR analyses (including more sensitive real-time PCR analyses [data not shown]) of TL001 cultures that were subjected to the hydrogenase assay (Fig. 2, lane 3). We first hypothesized that the other hydrogenase in *T. denitrificans*, putatively a cytoplasmic, group 3b [NiFe]hydrogenase (6), was responsible for this residual activity in strain TL001. This would have been a noteworthy phenomenon, as homologous group 3b hydrogenases (e.g., in the well-studied hyperthermophile *Pyrococcus furiosus*) are characteristically H_2 -evolving enzymes involved in disposing of excess reductant under fermentative conditions (22) rather than H_2 -oxidizing

enzymes that would function under the denitrifying conditions of this study. However, hydrogenase assays conducted with a double mutant (TL002; with mutations in the large subunits of both [NiFe]hydrogenases) indicated very similar activity to that of the single *hynL* mutant (Fig. 3), suggesting that the group 3b hydrogenase was not catalyzing significant hydrogen oxidation activity. The residual hydrogenase activity in the double mutant leaves open the possibility that an additional hydrogenase is encoded in the *T. denitrificans* genome but has not yet been identified. However, BLASTP searches for other kinds of hydrogenases (e.g., [Fe]hydrogenases, H_2 -forming methylenetetrahydromethanopterin dehydrogenase, *E. coli* hydrogenase 3) against the *T. denitrificans* genome did not reveal convincing evidence of additional hydrogenases.

An alternative explanation for residual hydrogenase activity in the *hynL* mutant is that the translated product of the disrupted gene was still capable of catalyzing hydrogen oxidation, albeit at a greatly reduced level. This seems unlikely for two reasons: (i) the EZ-Tn5<KAN-2> cassette contains stop codons in all three reading frames, which should preclude expression of a large portion (more than half) of the *hynL* gene, and (ii) the assembly and maturation of the active site of the hydrogenase should not proceed properly in the absence of key features at the C-terminal end of HynL, including the two cysteine residues in the very highly conserved C-terminal motif DPCxxCxxH/R and the endolytically formed peptide with a cleavage site located immediately downstream of this motif (8). The two conserved cysteine residues in this motif (along with two conserved cysteine residues in the N-terminal region of HynL) ligate the metal center to the large subunit (17).

The physiological role of hydrogenases in *T. denitrificans* remains unclear. Although the bacterium can oxidize H_2 as a sole electron donor under denitrifying conditions, this metabolism does not support growth (6) and appears to be relatively slow. Under the assay conditions used in this study (i.e., cells grown under denitrifying conditions with thiosulfate in the presence of H_2 and then resuspended with H_2 as the sole electron donor), *in vivo*, specific activity was on the order of $3 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ (based on a molar absorption coefficient for benzyl viologen of $7,780 \text{ M}^{-1} \cdot \text{cm}^{-1}$) (21). The use of hydrogenases in an H_2 -evolving mode to dispose of excess reducing equivalents under fermentative conditions cannot be reconciled with the obligate chemolithotrophic lifestyle of *T. denitrificans*, which has never been shown to function under fermentative conditions. Now that a genetic system is available for *T. denitrificans*, mutants defective in each of the two encoded hydrogenases can be compared under a range of physiological conditions, which should enhance our understanding of the roles of each hydrogenase. In addition, traditional biochemical studies can be conducted with the double mutant (strain TL002) to investigate the possibility that there is a third, as-yet-unidentified hydrogenase in *T. denitrificans*.

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