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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 UO_2 (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 H_2 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 H_2 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 H_2 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 H_2 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 H_2 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		
Escherichia coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7967 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Thiobacillus denitrificans		
ATCC 25259	Wild type	ATCC[a]
TL001	*hynL*::kan	This work
TL002	*hynL*::kan *hydA*::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 *mob*$^+$	15
pTn*Mod*-OCm	pMB1, Cam^r *mob*$^+$ Tn*5 tnp*	11
pTn*Mod*-OGm	pMB1, Gent^r *mob*$^+$ Tn*5 tnp*	11
pTn*Mod*-OKm′	pMB1, Kan^r *mob*$^+$ Tn*5 tnp*	11
pTn*Mod*-SmO	pMB1, Spcm^r Str^r *mob*$^+$ Tn*5 tnp*	11
pTn*Mod*-OTc	pMB1, Tet^r *mob*$^+$ Tn*5 tnp*	11
pRR10	IncP, Amp^r *mob*$^+$ *lacZ*α	19
pTL1	IncP, Amp^r, pRR10 with P_{Kan} inserted at MCS	This work
pTL2	IncP, Gent^r Amp^s, pTL1 with *bla*::gent; *T. denitrificans* expression vector	This work
pTL3	IncP, Gent^r, pTL2 with *hynL-hupE* 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 SacI fragment from pTn*Mod*-OGm containing *aacC1* inserted at MCS of pMOD-2	This work
pUC19-*hynL*	pMB1, Amp^r, pUC19 with *hynL* inserted at MCS	This work
pUC19-*hynL*::kan	pMB1, Amp^r Kan^r, pUC19 with *hynL*::kan inserted at MCS	This work
pUC19-*hydA*	pMB1, Amp^r, pUC19 with *hydA* inserted at MCS	This work
pUC19-*hydA*::gent	pMB1, Amp^r Gent^r, pUC19 with *hydA*::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 Tn*5* 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 Tn*5* 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 Na_2HPO_4, 3.0 g of KH_2PO_4, 1.0 g of NH_4Cl, 2.52 g of $NaHCO_3$, 4.96 g of $Na_2S_2O_3 \cdot 5H_2O$, 2.02 g of KNO_3, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.03 g of $CaCl_2 \cdot 2H_2O$, 2.7 mg of $FeCl_3 \cdot 6H_2O$, 0.18 mg of $CuSO_4 \cdot 5H_2O$, 50 μg of vitamin B_{12}, 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 performed 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	GAATTCTAGATGGCAACAACACAGCGCGTC
hynL-1-r	GATAGCATGCTTATTTGACCTTCACCTGCACG
pUC19-f	GCCAGGGTTTTCCCAGTCACGA
pUC19-r	GAGCGGATAACAATTTCACACAGG
kanP-f	AAAGCCACGTTGTGTCTC
kanP-r	GAATGTTAACACCCCTTGTATTACTG
hynL-hupE-f	phos-ATGGCAACAACACAGCGC
hynL-hupE-r	phos-TCACAGCCCGAGGATGAGG
EZ-Tn5-f[b]	ACCTACAACAAAGCTCTCATCAACC
EZ-Tn5-r[b]	GCAATGTAACATCAGAGATTTTGAG
pMOD2-PCR-f[b]	ATTCAGGCTGCGCAACTGGT
pMOD2-PCR-r[b]	GTCAGTGAGCGAGGAAGCGGAAG
pMOD2-Seq-f[b]	GCCAACGACTACGCACTAGCCAAC
pMOD2-Seq-r[b]	GAGCCAATATGCGAGAACACCCGAGAA
hynL-2-f	TCATGGCGGAGCATCTGGAG
hynL-2-r	CGTATTTCGGAACATGGGTGG
hydA-f	GAATGGTACCCAGTTCAACATGCTCTACCT
hydA-r	GATAGGTACCTTGAACGGCGCGTCGAACTG

[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 pTn*Mod* minitransposon plasmids were prepared to 0.1 to 0.2 μg μl^{-1} in 10 mM Tris buffer (pH 8.0), and 0.1 to 0.3 μg μl^{-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 μg ml^{-1} kanamycin; 50 or 100 μg ml^{-1} gentamicin; 50 μg ml^{-1} streptomycin and 100 μg ml^{-1} spectinomycin; 5, 10, or 20 μg ml^{-1} tetracycline; or 20 or 25 μg 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 pTn*Mod*-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 pTn*Mod*-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 hydrogenase, *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 (optical density 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 H_2O and resuspended in 500 μl ultrapure H_2O. 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 μg ml^{-1} kanamycin for the *hynL* mutant, 50 μg 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

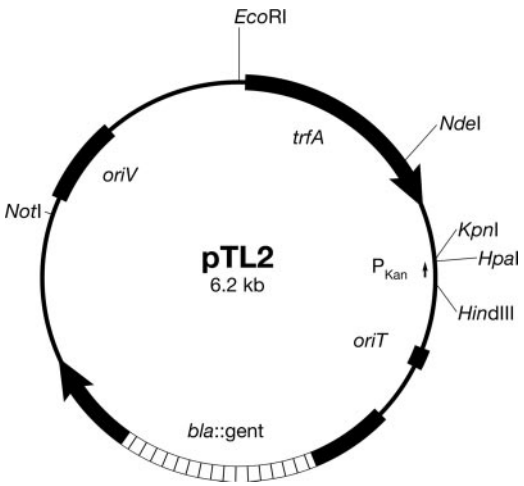

FIG. 1. Schematic diagram of the complementation vector (pTL2; Table 1) used in *T. denitrificans*. Plasmid construction is described in Materials and Methods. The following features are shown: *oriV*, the RK2 minimal vegetative origin of replication; *oriT*, the origin of transfer; *trfA*, encodes the RK2 replication initiation protein; *bla*, the beta-lactamase gene encoding ampicillin resistance; P_{Kan}, the 110-bp promoter of the kanamycin resistance gene from pTn*Mod*-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 (~2×10^8 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 ± 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 μg ml^{-1}), gentamicin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), streptomycin (50 μg ml^{-1}) plus spectinomycin (100 μg ml^{-1}), and tetracycline (5 μg 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 μg 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 pTn*Mod*-OKm′ (Kan^r), pTn*Mod*-SmO (Str^r $Spcm^r$), pTn*Mod*-OGm ($Gent^r$), pTn*Mod*-OCm (Cam^r), and pTn*Mod*-OTc (Tet^r), containing Tn*5* 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 pTn*Mod* 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 pTn*Mod*-OTc and pTn*Mod*-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 Km_{50} 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.

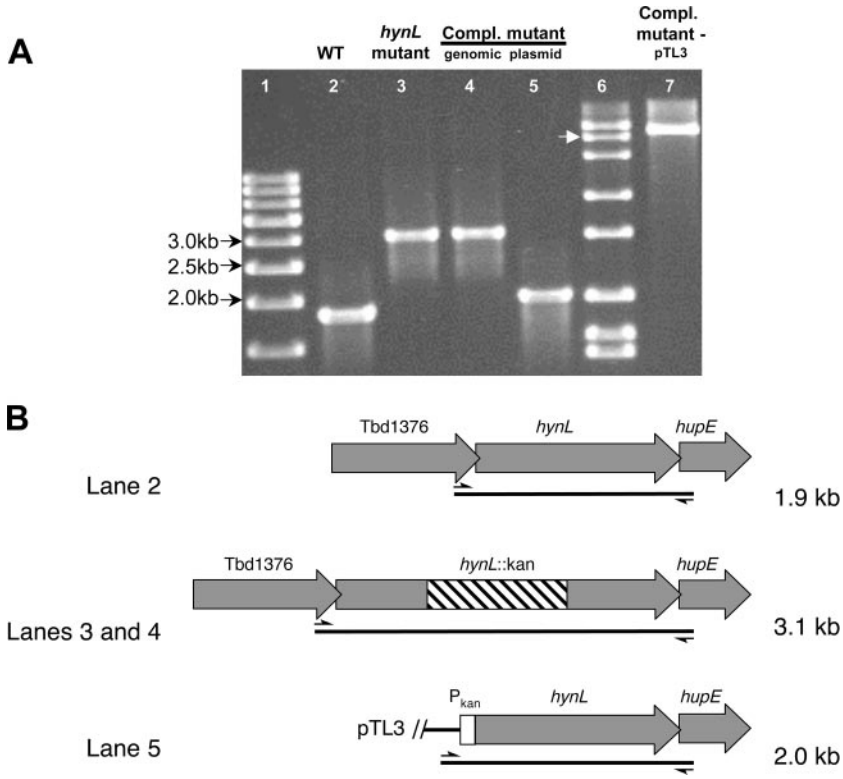

FIG. 2. (A) Electropherogram of PCR products from wild-type (WT) *T. denitrificans*, the *hynL* mutant, and the complemented (Compl.) *hynL* mutant, as well as digested plasmid DNA from the complemented mutant. Lane 1, HyperLadder III, Bioline; lane 2, wild-type DNA, primers hynL-2-f and -r; lane 3, *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 *in-trans* 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 *aacC1* gene from pTn*Mod*-OGm served as the gentamicin resistance marker in pRR10; after transposition, Tn-gent (containing *aacC1*) (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 pTn*Mod*-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),

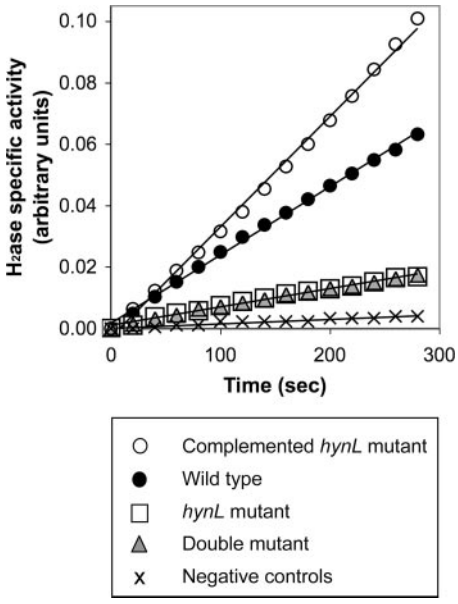

FIG. 3. Hydrogen oxidation (measured as in vivo benzyl viologen reduction) versus time for wild-type *T. denitrificans*, strain TL001 (*hynL* mutant), strain TL002 (*hynL hydA* double mutant), and the complemented *hynL* mutant (TL001/pTL3). Negative controls (averaged results for controls with no H_2 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 $\mu g\ ml^{-1}$ 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 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$ (based on a molar absorption coefficient for benzyl viologen of 7,780 $M^{-1} \cdot cm^{-1}$) (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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