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Hydrogen Metabolism in *Shewanella oneidensis* MR-1▽

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***Shewanella oneidensis* MR-1 is a facultative sediment microorganism which uses diverse compounds, such as oxygen and fumarate, as well as insoluble Fe(III) and Mn(IV) as electron acceptors. The electron donor spectrum is more limited and includes metabolic end products of primary fermenting bacteria, such as lactate, formate, and hydrogen. While the utilization of hydrogen as an electron donor has been described previously, we report here the formation of hydrogen from pyruvate under anaerobic, stationary-phase conditions in the absence of an external electron acceptor. Genes for the two *S. oneidensis* MR-1 hydrogenases, *hydA*, encoding a periplasmic [Fe-Fe] hydrogenase, and *hyaB*, encoding a periplasmic [Ni-Fe] hydrogenase, were found to be expressed only under anaerobic conditions during early exponential growth and into stationary-phase growth. Analyses of Δ*hydA*, Δ*hyaB*, and Δ*hydA* Δ*hyaB* in-frame-deletion mutants indicated that HydA functions primarily as a hydrogen-forming hydrogenase while HyaB has a bifunctional role and represents the dominant hydrogenase activity under the experimental conditions tested. Based on results from physiological and genetic experiments, we propose that hydrogen is formed from pyruvate by multiple parallel pathways, one pathway involving formate as an intermediate, pyruvate-formate lyase, and formate-hydrogen lyase, comprised of HydA hydrogenase and formate dehydrogenase, and a formate-independent pathway involving pyruvate dehydrogenase. A reverse electron transport chain is potentially involved in a formate-hydrogen lyase-independent pathway. While pyruvate does not support a fermentative mode of growth in this microorganism, pyruvate, in the absence of an electron acceptor, increased cell viability in anaerobic, stationary-phase cultures, suggesting a role in the survival of *S. oneidensis* MR-1 under stationary-phase conditions.**

Shewanella oneidensis MR-1 is a facultative, anaerobic γ-proteobacterium frequently found in suboxic sediment and soil environments (5, 31, 47, 50). The microorganism utilizes a wide range of compounds as terminal electron acceptors for anaerobic respiration and growth. These include compounds such as fumarate; dimethyl sulfoxide and trimethylamine *N*-oxide; elemental sulfur; S_2O_3; NO_3^-; NO_2^-; metal ions like Fe(III), Mn(IV), and Cr(VI); radionuclides such as U(VI); and others (20, 26, 27). The spectrum of electron donor usage is more limited and includes metabolic end products of primary fermenting bacteria such as lactate and formate (24, 39). The use of hydrogen as an electron donor by *Shewanella* species was described in earlier reports. Hydrogen served as the electron donor for Fe(III), Mn(IV), NO_3^-, Co(III), U(VI), and Cr(VI) reduction by *S. oneidensis* MR-1 and *S. putrefaciens* (21). In addition, sulfite reduction (11) and the transformation of tetrachloromethane into trichloromethane in *S. putrefaciens* under Fe(III)-respiring conditions have been reported previously (33). A recent report described hydrogen as an electron donor for the reduction of Pd(II) by *S. oneidensis* MR-1 (12).

Hydrogenases catalyze the reversible reduction of protons into molecular hydrogen and have a central role in the energy metabolisms in many anaerobic microorganisms. Hydrogenases can be categorized into two phylogenetically distinct classes according to the metal contents of their active sites (48): [Ni-Fe] hydrogenases and [Fe-Fe] hydrogenases. [Ni-Fe] hydrogenases are found in a variety of anaerobic and facultative heterotrophic bacteria, cyanobacteria, and archaea and typically form heterodimers. An [Fe-Fe] hydrogenase may exist as a distinct monomer or heteromer (34) and is usually found in strict anaerobic bacteria such as *Clostridium* and *Desulfovibrio* spp., as well as in some green algae and several eukaryotic protists such as *Trichomonas.* [Ni-Fe] hydrogenases and [Fe-Fe] hydrogenases contain cyanide and carbon monoxide ligands coordinated with iron atoms at the active site (4, 15). Hydrogenases can also be classified according to their role in the uptake or formation of hydrogen. [Fe-Fe] hydrogenases are typically H_2-forming hydrogenases, while [Ni-Fe] hydrogenases can function either in uptake or release. [Fe-Fe] hydrogenases have an approximately 100-fold-higher turnover number than [Ni-Fe] hydrogenases (15, 46).

Analysis of the *S. oneidensis* MR-1 genome revealed two putative hydrogenase gene clusters, *hydA* (SO3920 to SO3926) and *hyaB* (SO2089 to SO2099) (16). Based on structural features, HydAB is predicted to be a periplasmic [Fe-Fe] hydrogenase and HyaB a periplasmic [Ni-Fe] hydrogenase. The assembly of the [Fe-Fe] hydrogenase and its H-cluster is complex and involves helper proteins (19, 32). The *S. oneidensis* MR-1 *hydA* gene cluster also contains the *hydE*, *hydF*, and *hydG* genes, encoding accessory proteins predicted to be involved in the maturation of the [Fe-Fe] hydrogenase based on sequence homology to proteins in *Chlamydomonas reinhardtii* and several other prokaryotes (34). Interestingly, a gene designated *fdh* encoding a putative formate dehydrogenase (FDH) is located in the *S. oneidensis* MR-1 *hydA* operon between *hydB* and the *hydGEF* genes. This FDH, in conjunction with HydAB, could function as a formate-hydrogen lyase (FHL) (see below).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
Bacterial strains		
E. coli		
DH5α-λpir	ϕ80d*lacZ*Δ*M15* Δ(*lacYZA-argF*)*U196 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* λpir	23
S17-1 λpir	*thi pro recA hsdR* [RP4-2Tc::Mu-Km::Tn*7*]λpir Tp^r Sm^r	41
S. oneidensis		
MR-1 (AS84)	Wild type	44
AS92	Wild type; Gm^r	44
AS50	In-frame deletion of *hydA* (SO3920) in AS84	This study
AS51	In-frame deletion of *hyaB* (SO2098) in AS92; Gm^r	This study
AS52	In-frame deletions of *hydA* (SO3920) and *hyaB* (SO2098) in AS50	This study
AS372	AS50 with chromosomal insertion of *hydA* (SO3920) wild-type allele	This study
AS373	AS51 with chromosomal insertion of *hyaB* (SO2098) wild-type allele; Gm^r	This study
AS374	AS52 with chromosomal insertion of *hydA* (SO3920) and *hyaB* (SO2098) wild-type alleles	This study
Plasmids		
pGP704-Sac28-Km	mobRP4$^+$ oriR6K *sacB*; suicide plasmid for in-frame deletions and insertions; Km^r	45
pGP704-Sac28-Km::F_{hydA}	In-frame-deletion fragment of *hydA* in pGP704-Sac28-Km	This study
pGP704-Sac28-Km::F_{hyaB}	In-frame-deletion fragment of *hyaB* in pGP704-Sac28-Km	This study
pGP704-Sac28-Km-hydA	*hydA* in pGP704-Sac28-Km	This study
pGP704-Sac28-Km-hyaB	*hyaB* in pGP704-Sac28-Km	This study

Here we report experiments investigating the physiological and genetic basis for hydrogen formation in *S. oneidensis* MR-1. Considering the importance of hydrogen in anoxic environments, hydrogen formation by *S. oneidensis* MR-1 may add a new function for this microorganism in mixed-species communities in such environments.

MATERIALS AND METHODS

Growth conditions and media. *Escherichia coli* strains (Table 1) were grown in Luria-Bertani (LB) medium at 37°C. LB medium was solidified with 2% (wt/vol) agar and supplemented with 10 μg/ml gentamicin and/or 25 μg/ml kanamycin, if required. *S. oneidensis* MR-1 strains were grown at 30°C in LB medium or in mineral medium [MM; 5.7 mM K_2HPO_4, 3.3 mM KH_2PO_4, 125 mM NaCl, 485 μM $CaCl_2$, 9 mM $(NH_4)_2SO_4$, 5 μM $CoCl_2$, 0.2 μM $CuSO_4$, 57 μM H_3BO_3, 5.4 μM $FeCl_2$, 1.0 mM $MgSO_4$, 1.3 μM $MnSO_4$, 67.2 μM Na_2EDTA, 3.9 μM Na_2MoO_4, 1.5 μM Na_2SeO_4, 2 mM $NaHCO_3$, 5 μM $NiCl_2$, and 1 μM $ZnSO_4$, pH 7.4 (modified as described in reference 27)] supplemented with 20 to 40 mM lactate as indicated. Anaerobic cultures were grown in 50 ml of MM with either lactate or pyruvate (10 to 20 mM) as the electron donor and fumarate as the electron acceptor (10 to 40 mM) in 120-ml serum bottles sealed with butyl-rubber stoppers (49). Oxygen was removed from the medium by repeatedly flushing the headspace of each bottle with argon gas (99.9% purity; Praxair, Santa Clara, CA) followed by the application of a vacuum. Argon gas and vacuum cycles were repeated 10 times before bottles were inoculated. Anaerobic cultures were grown by using the following procedure. A liquid inoculum from an aerobic overnight culture grown in MM with 20 mM lactate was transferred (1% [vol/vol] culture) into anaerobic 60-ml serum bottles containing 10 ml of anoxic MM supplemented with 10 mM pyruvate and 10 mM fumarate. Cultures were incubated for 24 h at 30°C and at 250 rpm in an S500 orbital shaker (VWR, Brisbane, CA). These anaerobic precultures were used as the inoculum (1%, vol/vol) for anaerobic growth experiments with 120-ml serum bottles containing 50 ml of anoxic MM supplemented with the experiment-specific electron donor and acceptor.

Cell suspension experiments. *S. oneidensis* MR-1 and mutant strains were grown anaerobically for 48 h at 30°C as described above in MM containing 20 mM pyruvate and 10 mM fumarate. Upon growth to an optical density at 600 nm (OD_{600}) of about 0.15 and the detection of hydrogen, cells were harvested anaerobically (10 min at 5,000 × *g*), washed twice with anoxic MM supplemented with 2 mM 1, 4-dithiothreitol, and resuspended in 25-ml serum bottles containing anoxic MM, adjusting the OD_{600} to 30. Additional serum bottles were filled with 6 ml of anoxic MM and supplemented with electron donors or acceptors from anaerobic stock solutions to a final concentration of 10 mM. When needed, the electron uncoupler carbonyl cyanide 3-chloro-phenylhydrazone (CCCP) was added from a 10 mM anoxic stock solution in ethanol to a final concentration of 5 nmol CCCP per 1 mg of protein. These procedures were conducted in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) operated under an atmosphere with an H_2/N_2 ratio of 10:90. All bottles were sealed with rubber stoppers, removed from the anaerobic chamber, and flushed with argon until no hydrogen was detectable in the headspace. Concentrated cells were then transferred into the electron donor-/acceptor-containing bottles with an argon-flushed syringe and diluted to a final OD_{600} of 0.3. The bottles were shaken at 250 rpm at 30°C for 4 h, and hydrogen measurements were taken periodically. Organic acids were quantified at the beginning and end of each experiment by using the procedure described below.

Analytical methods. All samples were taken anaerobically with argon-flushed syringes. The growth of anaerobic cultures was monitored by using OD_{600} measurements (Ultrospec 10; Amersham BioSciences, Piscataway, NJ). Organic acids were identified and quantified by high-performance liquid chromatography (1100 series high-performance liquid chromatography system; Agilent, Palo Alto, CA). Liquid samples were removed from cultures, filtered with 0.2-μm-syringe filters (Nalgene, Rochester, NY), and frozen immediately. Organic acids were separated on an Aminex HPX-87H column (Bio-Rad, Hercules, CA) using 5 mM H_2SO_4 as the running buffer at a flow rate of 0.4 ml/min. The injection volume was 20 μl per sample. Lactate and succinate were analyzed at 55°C and acetate, fumarate, pyruvate, and formate at 20°C. Compounds were identified by comparison to known standards for the retention time, UV absorbance (210 nm), and refractive index signal. Calibration with standards was routinely performed. Samples for hydrogen analysis were removed from the headspace with gas-tight syringes (Hamilton, Reno, NV) and injected into a reduction gas hydrogen analyzer (Peak Performer I; Peak Laboratories, Mountain View, CA) operated at room temperature with 99.998% N_2 as the carrier gas. Hydrogen was quantified according to a standard calibration curve (analytical H_2 standards; Matheson Tri-Gas, Twinsburg, OH). Headspace and solution concentrations were calculated using Henry's law constants (22).

Survival experiments. For viability experiments with anaerobically grown, stationary-phase cells, *S. oneidensis* MR-1 and the Δ*hydA* Δ*hyaB* double-mutant strains were grown anaerobically as described above for 48 h at 30°C in MM containing 20 mM pyruvate and 20 mM fumarate. Cells were then harvested anaerobically as described above and diluted to a final OD_{600} of 0.25 in 120-ml serum bottles containing MM supplemented with 10 mM pyruvate. Serum bottles with only MM served as a control. After the removal of hydrogen by flushing of the headspace with argon gas, bottles were incubated at 30°C at 250 rpm for 1 week. Survival under anaerobic conditions was quantified daily by counting CFU on LB agar plates. The hydrogen concentration, the OD_{600}, and the organic acids profile were quantified daily as described above.

RNA extraction and reverse transcription PCR (RT-PCR). *S. oneidensis* MR-1 cells were grown anaerobically in serum bottles containing 100 ml of MM sup-

TABLE 2. Primers used in this study

Primer name	Sequence (5′→3′)
Primers for *hydA* deletion	
hydA_824SacI_F	GGATGAGCTCGCATTATCAATTCACCAT
hydA_1342EcoRI_RC	TCCGGAATTCATTAATCTTGATCAGCCC
hydA_2309EcoRI_F	GCCGGAATTCGTGAAATCAGCCTCTGTC
hydA_2725NcoI_RC	CATCCCATGGTTTTGCTAGGCTGTCGTC
hydA_600F	CCTATGGATGATGGATGAAGT
hydA_2675RC	TACTCATTACGGCGTTCAAG
Primers for *hyaB* deletion	
hyaB_SacI_802F	GGATGAGCTCGTGGCCGTTTTGATGCAG
hyaB_EcoRI_1284RC	CCGGAATTCTTTCAGTATGACTTCAAT
hyaB_EcoRI_2355F	CCGGAATTCGATGCTGTCAATGCCCTG
hyaB_XbaI_2891RC	ATGCTCTAGAATGCGGGTTTCAGAATGG
hyaB_551F	GATTATCTCTGTTGGCACTTGTG
hyaB_2981_RC	GCAATATAGAATCCGGTGATC
Primers for RT assays	
hydA_22F	ATGACAACGACAACTTATCAAC
hydA_1223RC	CCCAGCCATGAAGAGCCTTT
hyaB_1169F	GCGCGTTGTTATCGACCCT
hyaB_2824RC	CGGAACTCGCTCAATGCTT
cymA_EcoRI_R (reverse)	CCACGAATTCAAGCCAATGCTTTGTCGGCTTGC
cymA_Pst (forward)	GCGCCTGCAGTTCCATCCTAGCGCTACTGG
hydA_hydB_1002F	CGTGAAATCAGCCTCTGTC
hydA_hydB_1489RC	TCCTAATGGCTCGCCACC
hydB_fdh_1386F	GTACATTAAGGCCAGAAGC
hydB_fdh_1744RC	ACAATCCCCGCAATGATG
fdh_hydG_2095F	CTTCTTGCCCGTGCATTT
fdh_hydG_2603RC	CAGCGGTGCAAACATCAC
hydG_hyp_3540F	CTGGCGATCATTTTATGG
hydG_hyp_4126RC	TTGCAGTGGTAACGCTTGG
hyp_hydE_4217F	CTTTGGCTGGAGTTTGAT
hyp_hydE_4696RC	CACTGCGTTAAGGATTTC
hydE_hydF_5226F	GAATATTCCCGCCACCAG
hydE_hydF_5970RC	GCTGTTTACCCGCAAGAC
pflB_3F	GACCGACAAAACTGAACTGT
pflB_1206RC	GGACGCATTAAGTCATCG
pdh_3F	GTCTGAAGATATGCTACAAGA
pdh_1057RC	GCGCCAAATGTCATCGTCC
prdA_156F (SO0698)	TAACGACTCCCTCTGTGAA
prdA_913RC (SO0698)	CTAATGCTTCTTCGGTGAG

plemented with 20 mM pyruvate and 10 mM fumarate for 7, 15, 24, 32, 39, 48, 52, and 71 h. At every time point, 100 ml of culture was withdrawn and centrifuged for 5 min at 4°C and 5,000 × *g*. Cell pellets were washed in ice-cold AE buffer (20 mM sodium acetate, 1 mM EDTA), immediately frozen in liquid nitrogen, and stored at −80°C. RNA was extracted from cell pellets according to the method described in reference 29. Following extraction, the RNA was subjected to DNA digestion with 5 μl of RNase-free DNaseI (10 U/μl; Ambion, Austin, TX) according to the manufacturer's instructions until no residual DNA was detected. RNA from aerobic cultures was extracted by following the above-described protocol with late-log-phase cultures (20 h of growth at 30°C) grown in shake flasks on MM with 20 mM pyruvate. cDNA was synthesized from 0.5 to 3 μg of RNA by using the SuperScript III RT kit (Invitrogen, Carlsbad, CA) and 50 ng of hexanucleotides per reaction in a final volume of 20 μl. PCR amplifications were performed according to the manufacturer's instructions with 25 to 100 ng of cDNA by using the Siegen PCR kit reagents (QIAGEN GmbH, Germany). Amplification parameters included denaturation at 94°C for 5 min and 29 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min, followed by a final extension step at 72°C for 8 min.

Strain constructions with *S. oneidensis* MR-1: deletion and complementation of *hydA* and *hyaB*. All genetic work was carried out according to standard protocols (36). Kits for the purification and isolation of plasmids and PCR fragments were obtained from QIAGEN. Enzymes were purchased from New England Biolabs (Beverly, MA). Markerless-in-frame-deletion mutants were constructed with the *S. oneidensis* MR-1 AS84 or AS92 strain (Table 1) by leaving only short 5′ and 3′ sections of the target genes as described previously (45). Briefly, 400- to 500-bp fragments upstream and downstream of *hydA* and *hyaB* were amplified by PCR with the corresponding primer pairs given in Table 2, ligated, and cloned into the suicide vector pGP704-Sac28-Km. The resulting plasmids, harboring truncated genes (pGP704-Sac28-Km::F_{hydA} and pGP704-Sac28-Km::F_{hyaB}), were introduced into the strains listed in Table 1 by conjugation with *E. coli* S-17 λpir. Double recombinants were selected on LB plates containing 8% sucrose and screened for the gene deletion by colony PCR using primers flanking the deleted region (hydA_600F and hydA_2675RC, and hyaB_551F and hyaB_2981_RC). The resulting deletion mutants lacked amino acids 19 to 333 (out of 410 amino acids) in HydA and 48 to 397 (out of 567 amino acids) in HyaB, respectively. To complement the mutations, the corresponding wild-type genes were amplified from *S. oneidensis* MR-1 chromosomal DNA by using the gene-flanking primer pairs for *hydA* and *hyaB* (hydA_824SacI_F and hydA_2725NcoI_RC, and hyaB_SacI_802F and hyaB_XbaI_2891RC) and cloned into the suicide vector pGP704-Sac28-Km. The *hydA* and *hyaB* wild-type alleles were integrated at the chromosomal locus by homologous recombination, as described above, thereby replacing the deleted allele with a wild-type copy ("knock-in" replacement).

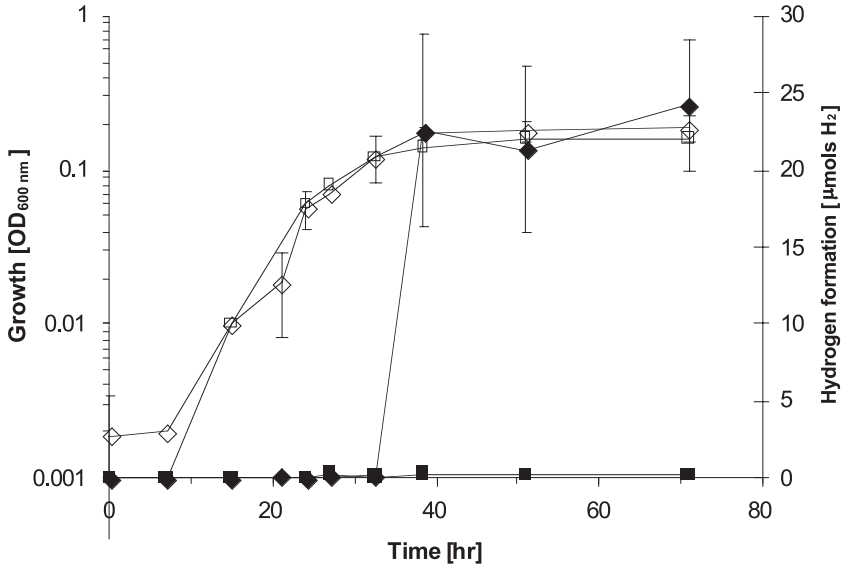
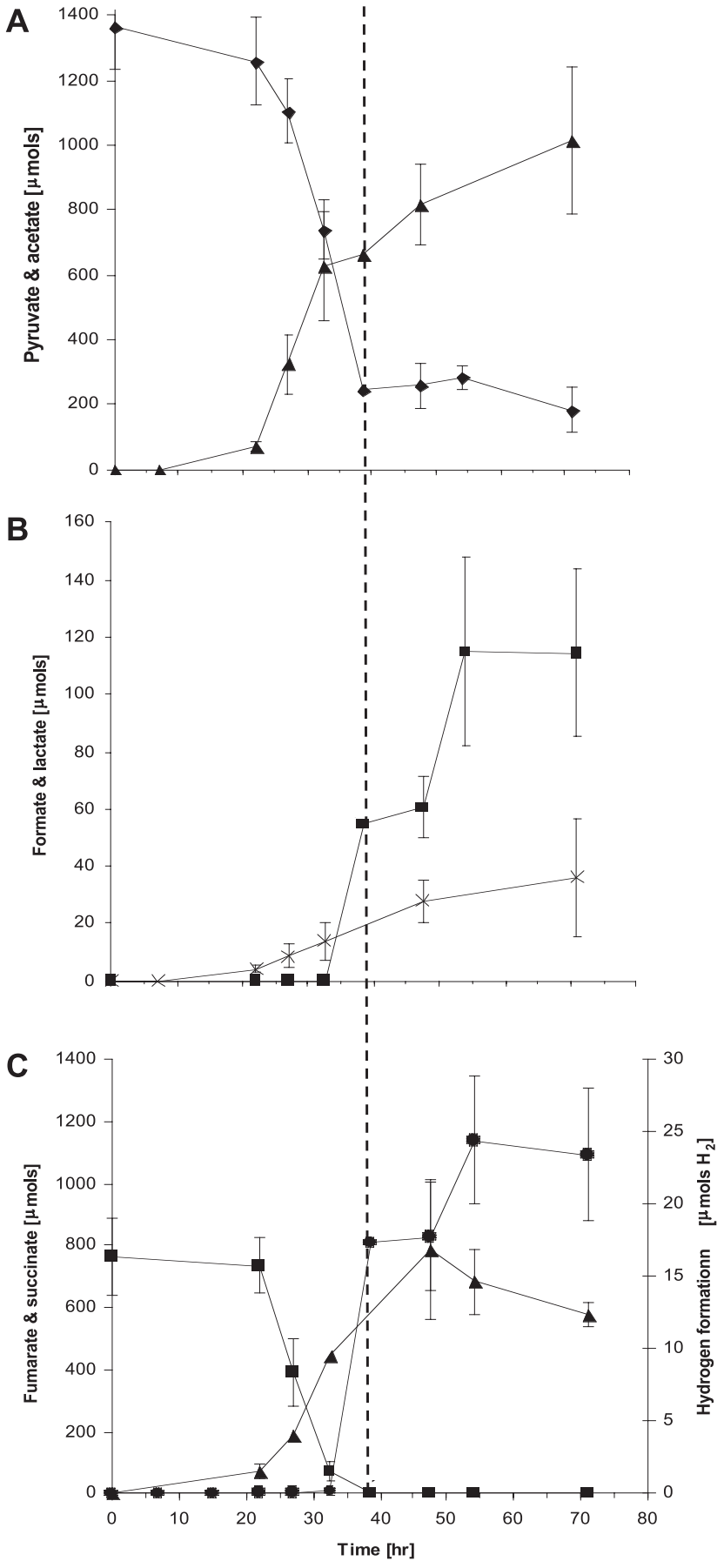


FIG. 1. Hydrogen formation in *S. oneidensis* MR-1. Hydrogen formation by wild-type strain AS84 cells grown on MM amended with either 15 mM pyruvate–15 mM fumarate (squares) or 20 mM pyruvate–10 mM fumarate (diamonds). Open symbols, optical density (600 nm); closed symbols, H_2 formation (μmol). Error bars represent the standard deviations for results for at least three replicate cultures in all experiments.


A
Pyruvate & acetate [µmols]
1400
1200
1000
800
600
400
200
0
B
Formate & lactate [µmols]
160
140
120
100
80
60
40
20
0
C
Fumarate & succinate [µmols]
1400
1200
1000
800
600
400
200
0
Hydrogen formationn [µmols H2]
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
Time [hr]

RESULTS

Hydrogen formation by *S. oneidensis* MR-1 under anaerobic conditions. When *S. oneidensis* MR-1 cells were grown anaerobically in MM supplemented with either lactate or pyruvate as the electron donor and fumarate as the electron acceptor, we discovered the formation of molecular hydrogen (Fig. 1). Hydrogen formation was observed only when the electron donor, pyruvate or lactate (data not shown), was still present after the depletion of the electron acceptor (Fig. 1). The quantification of organic acids in the medium during growth revealed that the onset of hydrogen production correlated with the depletion of the electron acceptor fumarate (Fig. 2C) and the subsequent entrance of cells into the stationary phase (Fig. 1). Hydrogen formation correlated also with the appearance of formate (Fig. 2B). These observations suggested that hydrogen formation occurs under anoxic conditions in the absence of the catabolic electron acceptor fumarate and that hydrogen may be derived directly from pyruvate or indirectly from formate as the intermediate.

To test whether hydrogen can be derived from formate, we grew two sets of cultures anaerobically with an excess of the electron donor and supplemented one set with 10 mM formate after 8 h of growth. In the formate-amended cultures, hydrogen was detected already in logarithmically growing cells about 10 h after formate addition even in the presence of fumarate (Fig. 3). In the control cultures without formate, hydrogen was found only when the cultures had been depleted of the provided electron acceptor, fumarate, and when cells entered stationary phase. Interestingly, the final cell density (OD_{600}) of the formate-supplemented cultures was fourfold lower than that of the nonamended culture (Fig. 3A).

Transcriptional analysis, organization, and expression of *hydA* and *hyaB* genes. We examined the expression patterns of the two *S. oneidensis* hydrogenase genes, *hydA* (SO3920) and *hyaB* (SO2098), under anoxic growth conditions and performed RT-PCR analyses of cDNA derived at different time points from anaerobically grown cultures. As Fig. 4 shows, both *hydA* and *hyaB* transcripts were detected during exponential growth phase (15 to 24 h) (Fig. 4C and D) while fumarate was still present (Fig. 4A and B). Elevated transcription (inferred from the comparison of gel band intensities) was found after 32 h for *hydA* (Fig. 4C) and after 39 h for *hyaB* (Fig. 4D), while cultures were entering stationary phase. However, hydrogen formation was detected only after 32 h. The tetraheme cytochrome gene *cymA* (SO4591) that is constitutively expressed under anoxic conditions and involved in mediating electron transfer from menaquinone to periplasmic electron carriers (10) was used as a positive control (Fig. 4E). *hydA* and *hyaB* expression was also detected in cultures that were grown without electron donor excess and where no hydrogen was detected (data not shown). Amplification products of *hydA* and *hyaB* were not obtained from the cDNA derived from aerobically grown cultures (Fig. 5A).

We investigated the transcriptional organization of the *hydA* gene cluster by using a series of RT-PCR experiments. PCR amplification with primer pairs designed to amplify intergenic regions between *hydA* and *hydG*, *hydG* and *hydX*, *hydX* and *hydE*, and *hydE* and *hydF* was performed with cDNA isolated from anaerobic cultures of strain AS84 grown for 32 h. The amplification yielded products of the expected sizes (2, 0.5, 0.5, and 0.65 kb, respectively), as shown in Fig. 5B. This result demonstrates that *hydA*, *hydB*, *fdh*, *hydG*, *hydX*, *hydE*, and *hydF* are expressed as a polycistronic unit during growth under the experimental conditions. *hydG*, *hydE*, and *hydF* have been reported to be involved in the correct assembly and folding of the [Fe-Fe] hydrogenase (4, 19, 32, 34, 35). Collectively, these data show that *hydA* and *hyaB* are expressed only under anaerobic growth conditions during logarithmic growth through early stationary phase.

Role of HydA and HyaB in hydrogen formation and consumption. To examine the role of the two hydrogenases in hydrogen metabolism in *S. oneidensis* MR-1, we constructed and analyzed markerless-in-frame-deletion mutants with the deletion of *hydA* (strain AS50) and *hyaB* (strain AS51) and the double deletion Δ*hydA* Δ*hyaB* (strain AS52). All strains showed similar anaerobic growth rates ($0.08 \pm 0.02\ h^{-1}$), indicating that the introduced deletions did not affect growth (Fig. 6A). Anaerobic growth experiments were performed with batch cultures in MM with 20 mM pyruvate and 10 mM fumarate. Hydrogen formation from the Δ*hydA* strain AS50 (282 ± 31 μmol H_2 OD_{600} $unit^{-1}$) was similar to that from the wild type AS84 (300 ± 44 μmol H_2 OD_{600} $unit^{-1}$) after 60 h of growth. Significantly smaller amounts of hydrogen were detected from the Δ*hyaB* strain AS51 (70 ± 19 μmol H_2 OD_{600} $unit^{-1}$), and no hydrogen was detected in experiments using the Δ*hyaB* Δ*hydA* mutant (AS52), indicating that HydA and HyaB are the only hydrogenases in *S. oneidensis* MR-1 (Fig. 6B). Hydrogen formation in the Δ*hydA* and Δ*hyaB* mutants containing knock-in replacements with the respective wild-type alleles was restored to the wild-type level (Fig. 6C).

In cell suspension experiments with pyruvate as the electron donor, the Δ*hydA* strain (AS50) showed only a slight decrease in hydrogen formation compared to the wild type (AS84) (Fig. 7). However, the Δ*hyaB* mutant (AS51) was severely deficient in hydrogen formation. The same observation was made when formate was added (Fig. 7B). Overall, six- to eightfold less hydrogen was produced from cell suspensions with formate than from cell suspensions with pyruvate. We also investigated the consumption of hydrogen in cell suspensions that were amended with hydrogen and fumarate. Wild-type cells and the Δ*hydA* mutant consumed hydrogen in nearly equal amounts (Fig. 7C). No hydrogen consumption in the Δ*hyaB* mutant (Fig. 7C) or in control experiments without fumarate (data not shown) was detected. The Δ*hydA* Δ*hyaB* strain was deficient in hydrogen formation and consumption as well (data not shown). Collectively, these experiments showed that HydA and

FIG. 2. Hydrogen and formate formation from pyruvate after depletion of fumarate. *S. oneidensis* wild type AS84 was grown in 120-ml serum bottles with 50 ml of MM supplemented with 20 mM pyruvate and 10 mM fumarate. Hydrogen was quantified in headspace samples. (A) Pyruvate (◆) oxidation to acetate (▲). (B) Lactate (×) and formate (■) formation. (C) Fumarate (■) reduction to succinate (▲) and hydrogen (●) formation. The first time point of formate and hydrogen detection as well as fumarate depletion at 38.5 h is indicated by a dashed vertical line.

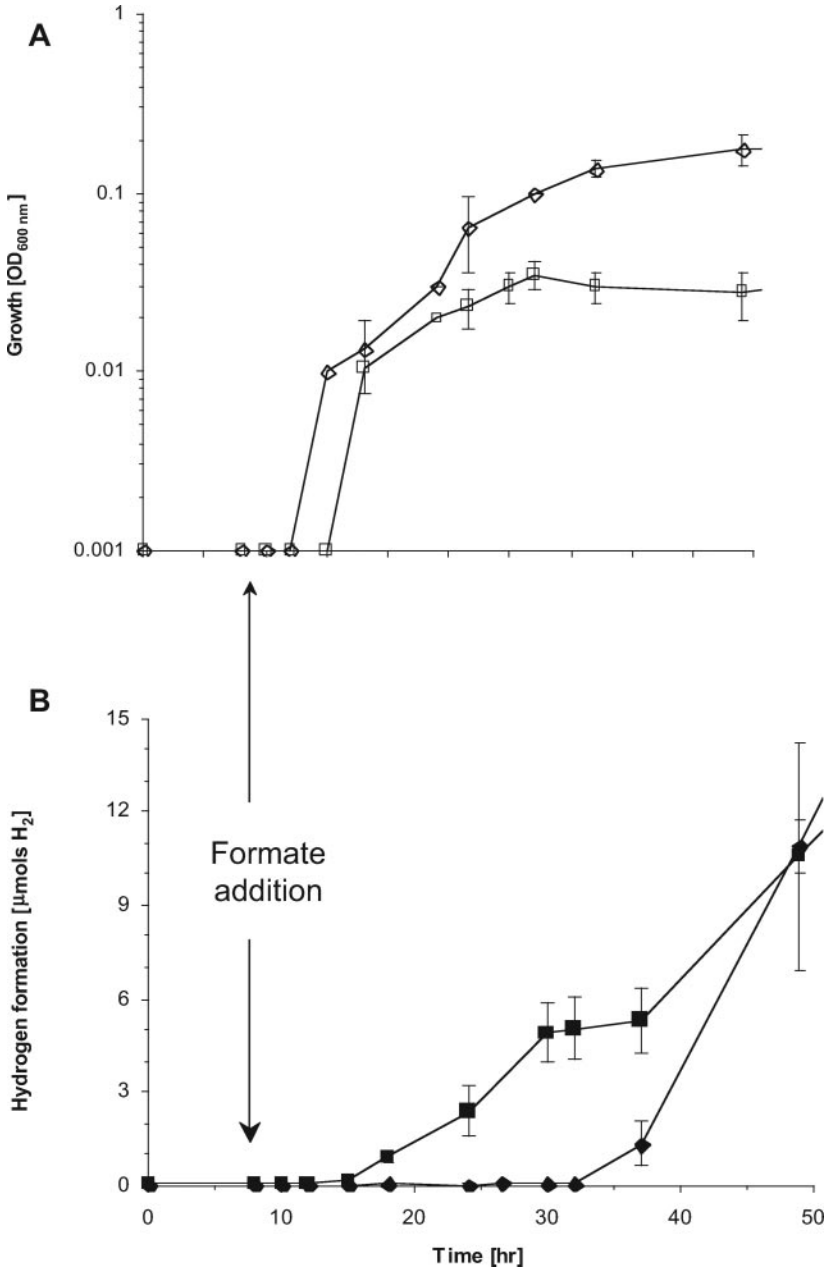


FIG. 3. Hydrogen formation from formate in *S. oneidensis* MR-1. Wild-type AS84 cells were grown on MM with 20 mM pyruvate–10 mM fumarate (diamonds) and additionally with 10 mM formate (squares). (A) Optical density at 600 nm (open symbols). (B) Hydrogen formation (closed symbols). Error bars represent the standard deviations for results for at least three replicate cultures in all experiments.

HyaB are the only two hydrogenases in *S. oneidensis* MR-1. HydA functions as a hydrogen-forming hydrogenase, while HyaB functions as bidirectional hydrogenase.

Pyruvate metabolism and hydrogen formation. Genome analysis revealed the presence of several genes involved in pyruvate metabolism: genes for the pyruvate-formate lyase (PFL) and its activator protein (*pflAB*; SO2912 to SO2913), the pyruvate dehydrogenase (PDH) complex gene (*pdh*; SO0424 to SO0426), and the gene SO0968 that has recently been shown to encode a pyruvate reductase (*prdA*) (Grigoriy Pinchuk, personal communication). In order to evaluate whether these genes are transcribed during anaerobic growth on pyruvate plus fumarate, we performed a series of RT-PCR experiments. All three genes were found to be expressed from early log to stationary phase (Fig. 8). The expression of *pflB* paralleled the expression of *hydA* and *hyaB*. The transcription of *pdh* was detected at all time points. The transcription of *prdA* was detected from the beginning of growth (15 h) through early stationary phase (39 h).

The observation of the expression of *pflB*, together with the stimulation of hydrogen formation upon the addition of formate to anaerobic, exponentially growing cells (Fig. 3), suggested that

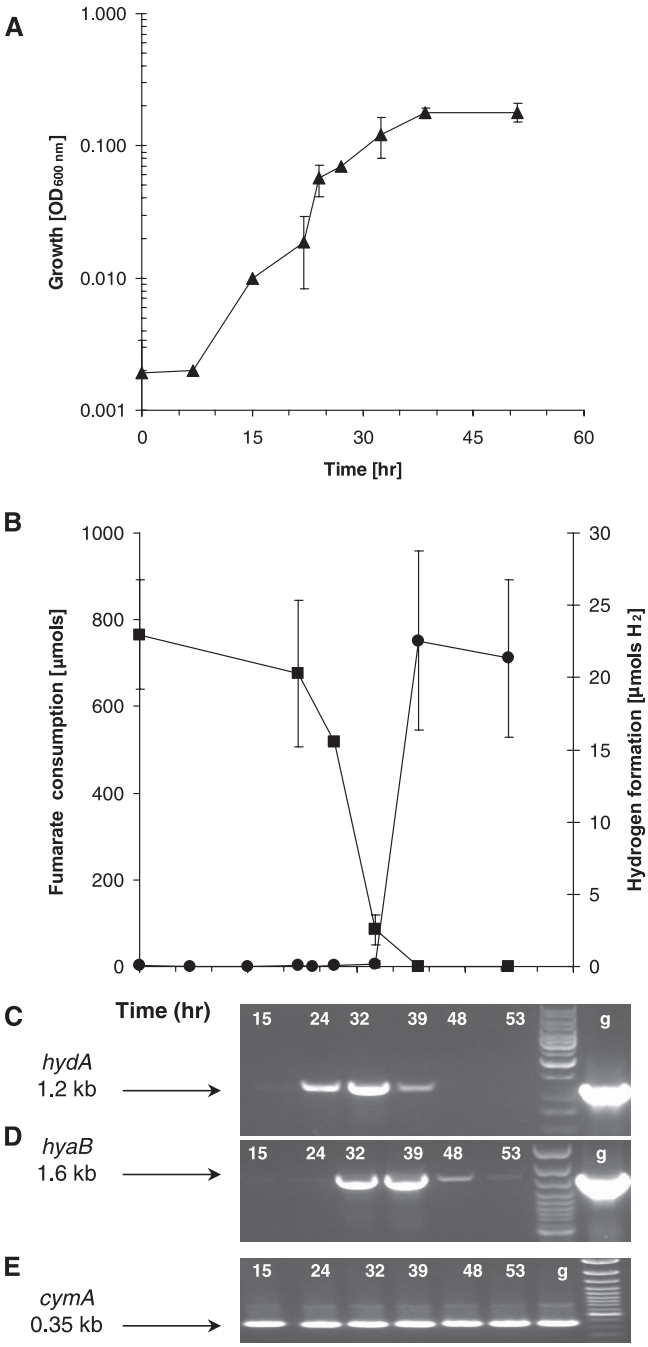


FIG. 4. Expression of *hydA* and *hyaB* during growth of *S. oneidensis* MR-1. Correlation between growth phase (A); fumarate consumption and hydrogen formation (B); and expression of *hydA* (C), *hyaB* (D), and *cymA* (E). All RT assays were performed with 25 ng of cDNA. g, genomic DNA; ▲, *S. oneidensis* wild type anaerobic growth with 20 mM pyruvate and 10 mM fumarate; ■, fumarate concentrations during growth; ●, hydrogen formation during growth. Error bars represent the standard deviations for results for at least three replicate cultures in all experiments.

pyruvate-derived formate could be a source of reducing equivalents for hydrogen via a putative formate-hydrogen lyase encoded by the *hydA* operon. Alternatively, hydrogen could be formed from formate via one of the three formate dehydrogenases encoded by the *S. oneidensis* MR-1 genome or from pyruvate by PDH, both in conjunction with a reverse electron transport. To test whether a reverse electron transport may be involved in hydrogen formation, we conducted cell suspension experiments using CCCP, a protonophore that collapses the electrochemical membrane potential, thus inhibiting reverse electron transport (30). The addition of CCCP to anaerobic cell suspensions metabolizing pyruvate in the absence of an electron acceptor reduced hydrogen production, while pyruvate was converted into acetate and lactate (data not shown). The level of hydrogen formation from formate was generally very low (Fig. 7B), and the addition of CCCP did not further reduce hydrogen formation in cell suspensions with formate.

Effect of pyruvate on anaerobic survival of *S. oneidensis* MR-1 in stationary phase. Despite the presence of PFL, *S. oneidensis* MR-1 did not grow fermentatively with pyruvate as the sole catabolic substrate (data not shown). To test whether pyruvate supplementation affected the viability of stationary-phase cells, we conducted anaerobic viability experiments with the wild type (AS84) and the Δ*hydA* Δ*hyaB* mutant (AS52). When stationary-phase wild-type cells were incubated in the presence of 10 mM pyruvate, cells remained viable for 3 days before a 65-fold decrease in the number of CFU was detected on day 4 (Fig. 9). In contrast, wild-type cells incubated without pyruvate showed an immediate decrease in the counts of viable cells after day 1. By day 4, the numbers of viable cells had decreased by a factor of 2,000 compared to those in cultures amended with pyruvate. Similar results were obtained with the Δ*hydA* Δ*hyaB* double mutant.

These data suggest that the presence of pyruvate enhances the survival of *S. oneidensis* MR-1 in stationary phase under anaerobic conditions. No significant difference was observed between the wild type and the Δ*hydA* Δ*hyaB* double mutant, suggesting that hydrogen production is not required for stationary-phase viability.

DISCUSSION

S. oneidensis MR-1 has been shown previously to utilize molecular hydrogen as an electron donor (11, 21, 33). We report here hydrogen formation from pyruvate or lactate under anoxic conditions in the absence of the electron acceptor fumarate. Since *S. oneidensis* MR-1 cannot grow fermentatively with pyruvate, such electron acceptor-limiting conditions lead to the cessation of growth and to the entrance of cells into stationary phase.

In the *S. oneidensis* MR-1 genome, two putative hydrogenase gene clusters were identified (16). The *hydA* cluster (SO3920 to SO3926) encoding the catalytic subunit HydA and the small subunit HydB is predicted to form a periplasmic [Fe-Fe] hydrogenase (16). The presence of an [Fe-Fe] hydrogenase in a facultative microorganism is unique (16). Sequence analysis of the genome of *Geobacter sulfurreducens*, a metal-reducing microorganism that shares many physiological features with *S. oneidensis* MR-1 (14), revealed two periplasmic [Ni-Fe] hydrogenases but no [Fe-Fe] hydrogenase (9). HydA in *S. oneidensis* MR-1 is highly similar to [Fe-Fe] hydrogenases from other *Shewanella* species (*S. decolorationis*, *Shewanella* sp. strain MR-4, and *Shewanella* sp. strain ANA-3) and other strictly anaerobic microbes, such as *Syntrophomonas wolfei*, *Desulfo-*

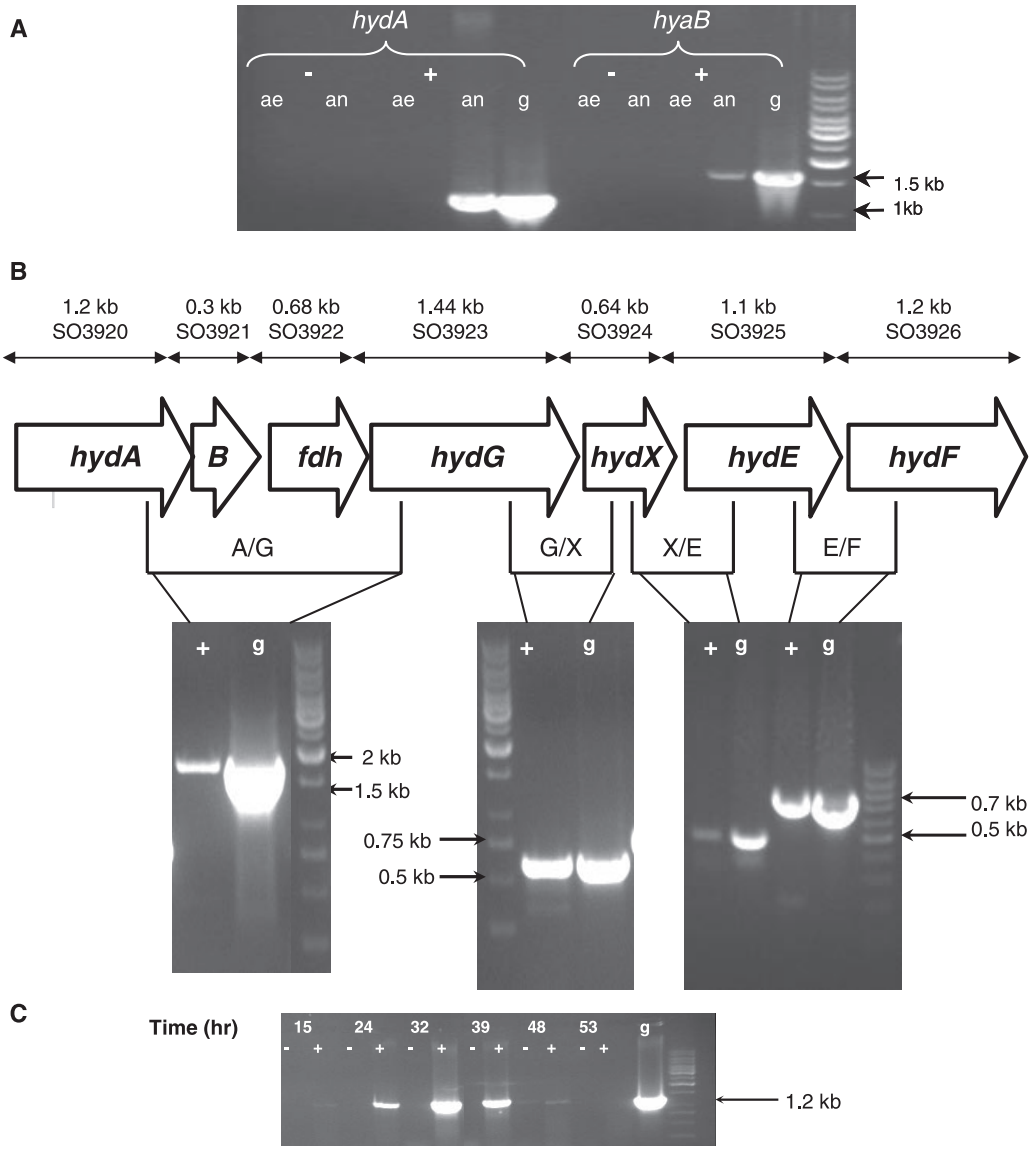


FIG. 5. Control of expression and transcriptional organization of the *hydA* and *hyaB* genes in *S. oneidensis* MR-1. (A). Transcription of *hydA* and *hyaB* under aerobic and anaerobic conditions. g, genomic DNA; +, cDNA synthesis conducted with reverse transcriptase; −, cDNA synthesis conducted without reverse transcriptase; ae, cDNA library obtained from aerobic cultures; an, cDNA library obtained from anaerobic cultures. (B) Physical map and transcriptional organization of the *hydA* gene locus in *S. oneidensis* MR-1. RT-PCR products were obtained with primers flanking the intergenic regions between *hydA* (SO3920) and downstream genes of the *hydA* operon (*hydB*, SO3921; *fdh*, SO3922; *hydG*, SO3923; *hydX*, SO3924; *hydE*, SO3925; and *hydF*, SO3926). All RT assays were performed with 25 ng of cDNA. (C) Control reactions for cDNA purity. *hydA* was amplified from genomic DNA. +, cDNA synthesis conducted with reverse transcriptase; −, cDNA synthesis conducted without reverse transcriptase.

vibrio vulgaris, and some other *Desulfovibrio* spp. (17). Interestingly, a gene designated *fdh* (SO3922) encoding a putative FDH is located in the *S. oneidensis* MR-1 *hydA* operon between *hydB* and the *hydGEF* genes. The latter genes are predicted to be involved in HydA folding and maturation. This FDH, in conjunction with HydAB, could function as a formate-hydrogen lyase (see below).

The second hydrogenase gene cluster in the *S. oneidensis* MR-1 genome (SO2098 to SO2099) encodes a putative quinone-reactive, periplasmic [Ni-Fe] hydrogenase (HyaB) with high sequence similarity to the [Ni-Fe] hydrogenases from *Thiomicrospira* sp., *Helicobacter* sp., *Wolinella succinogenes*, and *Geobacter metallireducens* and the *E. coli* hydrogenase-2. Our physiological and genetic analyses demonstrated that these two hydrogenases are the only hydrogenases present in *S. oneidensis* MR-1. HyaB functions as a bidirectional hydrogenase, whereas HydA is a hydrogen-forming hydrogenase only under the anoxic conditions tested. HyaB appears to provide the dominant hydrogenase activity for hydrogen formation in *S. oneidensis* MR-1. *hydA*, *hydB*, and *fdh* genes were previously reported to be up-regulated four- to sixfold under thiosulfate-reducing conditions in comparison to fumarate-reducing conditions, while *hyaB* had the same expression level under the different conditions (2). Thus, while HyaB provided the dom-

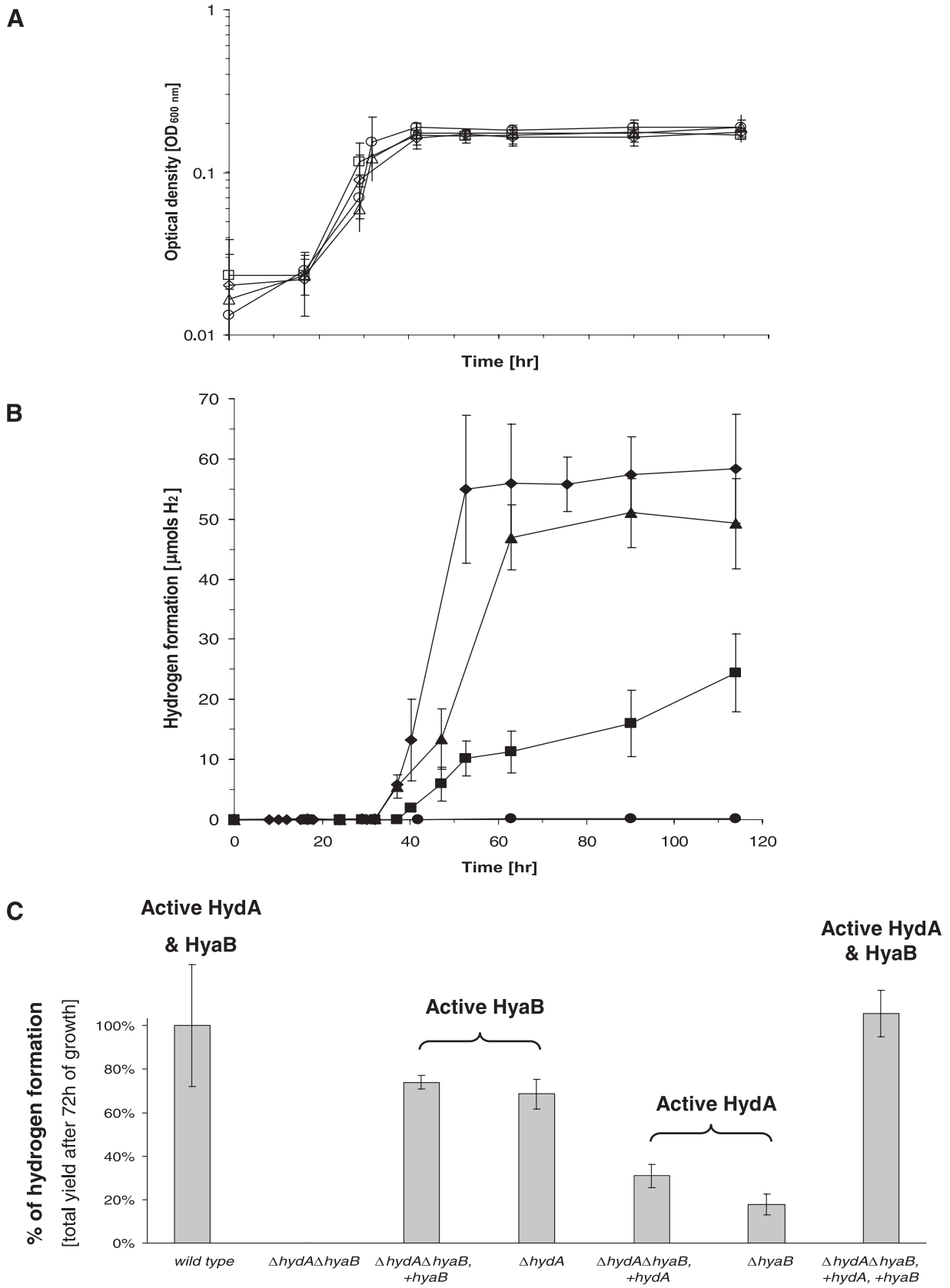


FIG. 6. Mutant phenotypes and complementation of Δ*hydA*, Δ*hyaB*, and Δ*hydA* Δ*hyaB* mutations. (A and B) Growth, measured by optical density increase over time (open symbols) (A), and hydrogen production (closed symbols) (B) for the *S. oneidensis* wild type (diamonds), the Δ*hydA* mutant (triangles), the Δ*hyaB* mutant (squares), and the Δ*hydA* Δ*hyaB* double mutant (circles). (C) Rescue of the defect in hydrogen formation in Δ*hydA*, Δ*hyaB*, and Δ*hydA* Δ*hyaB* mutants after knock-in complementation (see Materials and Methods). Error bars represent the standard deviations for results from three independent experiments.

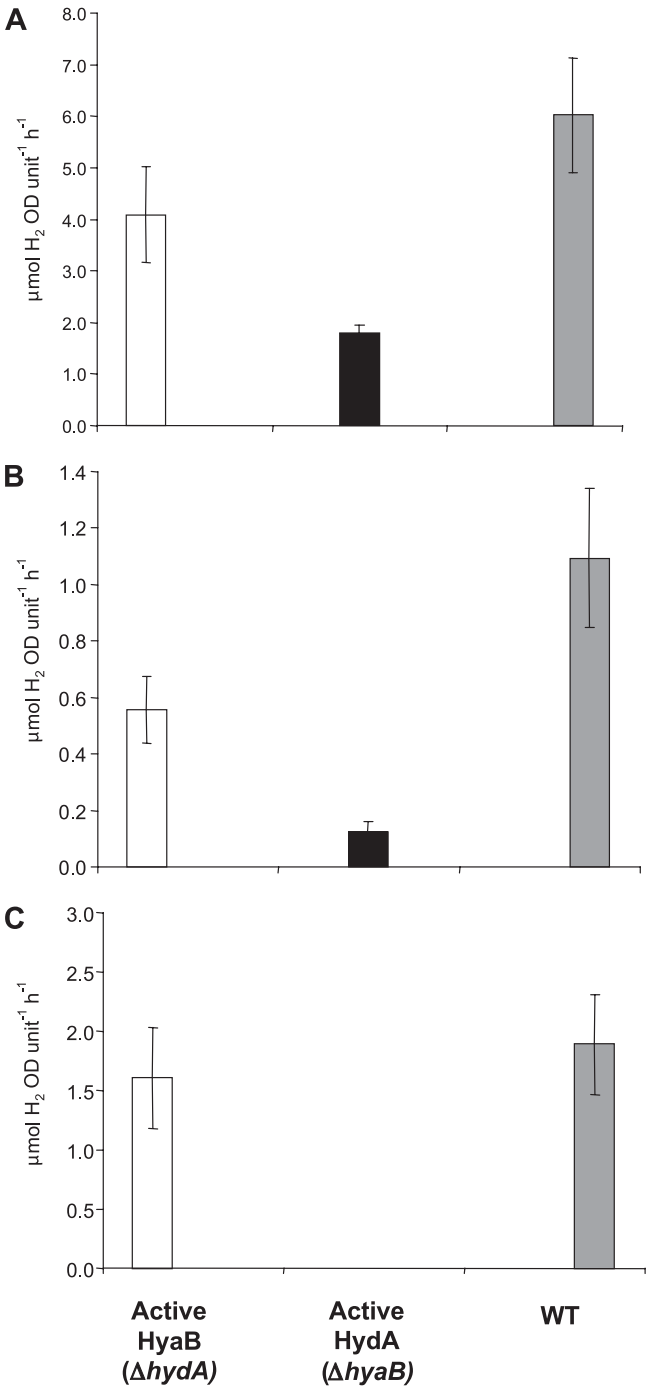


FIG. 7. Formation and consumption of hydrogen in cell suspension experiments with *S. oneidensis* MR-1 strains. (A) Hydrogen formation with 10 mM pyruvate as the electron donor. (B) Hydrogen formation with 10 mM formate as the electron donor. (C) Hydrogen consumption with 10 mM fumarate as the electron acceptor. Gray bars, wild-type (WT) cells; black bars, Δ*hyaB* mutant; white bars, Δ*hydA* mutant. Error bars represent the standard deviations for results from three independent experiments.

inant hydrogenase activity under our conditions, HydA might be responsible for most of the hydrogenase activity under thiosulfate-reducing conditions.

A more detailed molecular and physiological analysis of the involvement of HydA and HyaB in hydrogen formation revealed several interesting features. While hydrogen was detected only in cultures or cell suspensions entering stationary phase, the expression of *hydA* and *hyaB* was observed already at the beginning of the exponential growth phase (Fig. 5). Since at these time points no hydrogen formation was observed, this finding suggests the involvement of posttranscriptional/-translational mechanisms in the activation of hydrogenase activity in *S. oneidensis* MR-1. These RT-PCR results for *hydA* and *hyaB* expression are consistent with previous findings from whole-genome microarray studies (6, 28); the *hydA* and *hyaB* genes were induced after a switch from aerobic growth to growth under fumarate-, Fe(III)-, or nitrate-reducing conditions (3).

While we identified the hydrogenases and their specific roles in hydrogen formation in *S. oneidensis* MR-1, the analysis of the flow of electrons from the electron donor pyruvate to protons suggests the involvement of several parallel pathways (Fig. 10). Two pyruvate-metabolizing enzyme genes, *pfl* and *pdh*, were simultaneously expressed under the anoxic growth conditions (Fig. 8). This pattern is similar to the regulation of these genes in *E. coli* (7, 18, 37). In addition to these RT-PCR data, the observations that formate is detected in pyruvate-metabolizing cultures (Fig. 2) and that formate addition induces hydrogen formation in the presence of the electron acceptor fumarate (Fig. 3) suggested that one route of electron flow to protons proceeds via formate. Formate could be formed by the activity of an expressed PFL and consumed by an FHL. Our transcriptional analysis revealed that *hydA* is cotranscribed with *fdh* (SO3922) (Fig. 5), which is consistent with the two encoded proteins' forming an FHL complex in vivo. FHL in *E. coli* is also a membrane-associated complex, but FDH is linked to the hydrogenase-3-type [Ni-Fe] hydrogenase (1). Formate dehydrogenases that form complexes with [Fe-Fe] periplasmic hydrogenases were found in *Desulfovibrio* spp. periplasm, but these were suggested to interact with a *c*-type cytochrome network instead of directly with the hydrogenases (17). Formate conversion to hydrogen via an FHL in *S. oneidensis* MR-1 would thermodynamically favor hydrogen formation since the redox potentials of formate and hydrogen are both −420 mV under standard-state conditions (43).

Although our data suggest that formate is an intermediate in hydrogen formation, they also indicate that FHL-mediated hydrogen formation does not represent the dominant electron flow to protons under our experimental conditions. This conclusion is based on the finding that a Δ*hydA* mutant exhibited only minor reduction in hydrogen formation (Fig. 6). Furthermore, we detected formate consumption, which was independent of hydrogen production, in the Δ*hydA* Δ*hyaB* double mutant AS52 (data not shown). FHL-independent formate oxidation could proceed via one or more of the annotated formate dehydrogenase genes in clusters SO0101 to SO0103, SO4509 to SO4511, and SO4513 to SO4515. Those FDHs are predicted to be similar to three-subunit FDH enzymes that participate in respiration, using either oxygen or nitrate as the electron acceptor (40). Hydrogen formation via FDH that uses NAD^+ as an electron acceptor would be thermodynamically less favorable because of the more-positive redox potential of $NADH/NAD^+$ ($E_0 = -320$ mV) (43). Moreover, such a mode of hydrogen formation would significantly reduce the amount

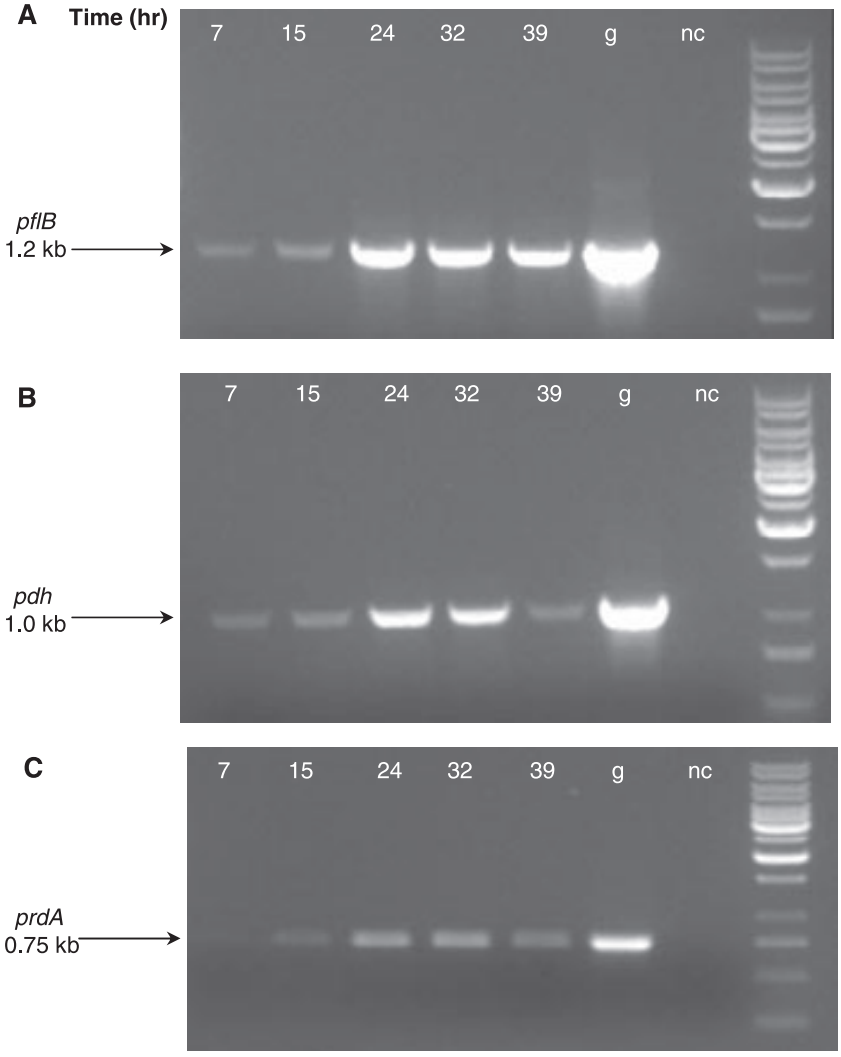


FIG. 8. RT-PCR analysis of selected genes involved in pyruvate metabolism in *S. oneidensis* MR-1. Transcriptional analysis of *pflB* (SO2913) (A), *pdh* (SO0424) (B), and *prdA* (SO0968) (C) at different time points (indicated by numbers) during the growth of *S. oneidensis* MR-1 with 20 mM pyruvate and 10 mM fumarate. All RT assays were performed with 100 ng of cDNA. g, genomic DNA; nc, negative control (no template added).

of hydrogen formed unless a reverse electron transport were involved. The involvement of such reverse electron flow could be inferred from our results from the experiments using the protonophore CCCP. In these experiments, we observed a dramatic decrease in hydrogen formation from pyruvate in the presence of CCCP while pyruvate utilization was largely unaffected. Such an observation is consistent with a reverse electron transport's driving formate oxidation, e.g., via NADH, and hydrogen formation.

However, there is also hydrogen-independent formate metabolism under electron acceptor-limiting conditions. In the absence of fumarate, we detected formate consumption without concomitant hydrogen formation in the Δ*hydA* Δ*hyaB* double mutant AS52 (data not shown). In addition, we detected more formate consumption than hydrogen production in wild-type cultures growing with pyruvate, fumarate, and formate (7 ± 1.7 μmol of formate consumed per 1 μmol of H_2 produced).

In addition to these two formate-dependent pathways of hydrogen formation from pyruvate, pyruvate oxidation via the expressed PDH would also involve the reduction of NAD^+ and the necessity for NADH oxidation for hydrogen formation via a similar mechanism. To this point, the *S. oneidensis* MR-1 PDH has not been studied in detail. A third pyruvate-metabolizing enzyme, the pyruvate reductase (SO0968), was also identified (Fig. 8), and lactate formation from pyruvate was observed under those conditions, i.e., growth with pyruvate and fumarate (Fig. 2). In this third electron pathway, one molecule of pyruvate would be oxidized and a second molecule of pyruvate would be reduced to lactate. Regardless of the exact path of electron flow to protons, all operating pathways of pyruvate oxidation result in the generation of acetyl coenzyme A, which can then be converted to acetate, thereby enabling the microorganism to conserve one ATP molecule. Acetate is a known end product under anoxic conditions in *S. oneidensis* (39).

In summary, our data show that under fumarate-limiting

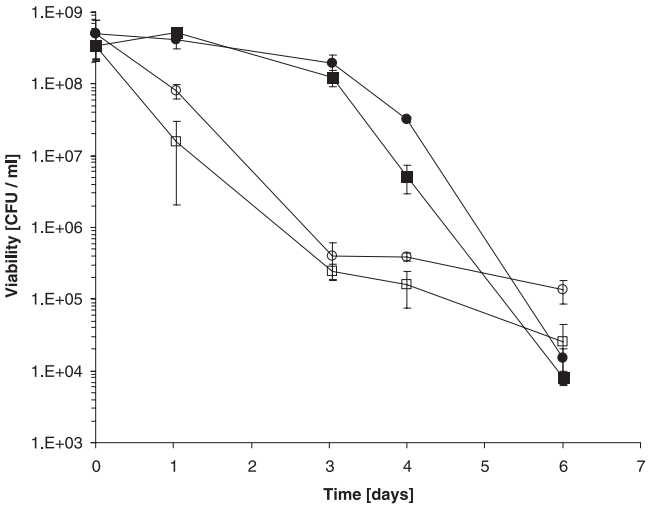


FIG. 9. Effect of pyruvate on the viability of anaerobic, stationary-phase cells of *S. oneidensis* MR-1. Cells were grown with equimolar amounts of pyruvate and fumarate. The *S. oneidensis* wild type (squares) and the Δ*hydA* Δ*hyaB* double mutant (circles) were separated from the growth medium and resuspended in anaerobic MM in rubber stopper-sealed serum bottles with (closed symbols) and without (open symbols) the addition of 10 mM pyruvate. Over a time period of 6 days, aliquots were removed daily and the numbers of viable cells were determined by counting CFU on LB agar plates. Error bars represent the standard deviations for results from three independent experiments.

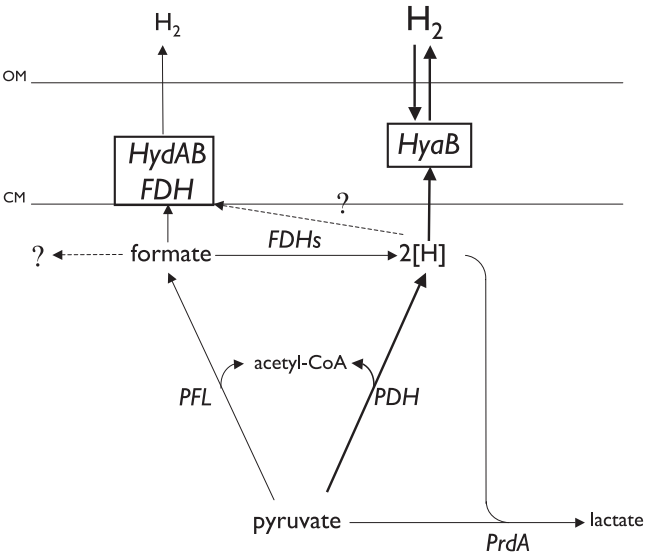


FIG. 10. Working model of hydrogen metabolism in stationary phase of *S. oneidensis* MR-1 cells grown with pyruvate and fumarate after electron acceptor depletion. See the text for details. Emphasis is placed on the roles of the HydA and HyaB hydrogenases and potential patterns of electron flow from pyruvate. The hypothesis is that HydAB and FDH, encoded by SO3920 to SO3922, form a periplasm-facing, membrane-associated complex mediating formate-hydrogen lyase activity. HyaB is a bifunctional hydrogenase under the experimental conditions tested. The thickness of the arrow indicates the predominant electron flow from pyruvate. At this point it is unclear whether or not reducing equivalents that are not associated with formate can be transferred to the HydAB-FDH complex and result in hydrogen formation (dotted line). HydAB-FDH is encoded by SO3920 to SO3922, HyaB by SO2089 to SO2099, PDH by SO0424, PFL by SO2913, PrdA by SO0968, and FDHs by SO0101to SO0103, SO4509 to SO4511, or SO4513 to SO4515. OM, outer membrane; CM, cytoplasmic membrane; aceytl-CoA, acetyl coenzyme A.

conditions, which are frequently encountered by *Shewanella* species in organically rich environments, the processes of the metabolism of pyruvate, hydrogen, and formate are intrinsically linked, presumably by parallel and overlapping electron transport pathways (Fig. 10). A flow of reducing equivalents from formate to protons via an FHL, including HydA, is probably of only minor importance for hydrogen formation under these conditions, while most hydrogen is formed via HyaB. It is interesting to note that the operation of multiple parallel electron-transferring pathways appears to be a general feature of *Shewanella* species metabolism. Anoxic conditions lead to the expression of numerous anaerobic terminal oxidoreductases, regardless of whether the specific electron acceptors are present (2, 3).

Pyruvate metabolism as we describe here appears to play a significant role in the survival of *S. oneidensis* MR-1 under stationary-phase conditions (Fig. 9). The addition of pyruvate to a stationary-phase wild-type culture yielded about 1 μmol of H_2 per 4 μmol of pyruvate (data not shown). Recent reports on the anaerobic survival of the opportunistic pathogen *Pseudomonas aeruginosa* described a similar phenomenon. While pyruvate does not support growth, long-term survival of up to 18 days was enhanced by pyruvate fermentation (13, 38). We were not able to account for all the pyruvate metabolized by stationary-phase cells, suggesting that a so-far-undocumented, hydrogen-independent pyruvate metabolism process is occurring. However, in *S. oneidensis* MR-1, hydrogen formation is not essential for survival during stationary phase (Fig. 9).

The ability of *S. oneidensis* MR-1 to also produce hydrogen under anaerobic, fumarate-limiting conditions is significant in that hydrogen is a key electron donor for other important anaerobic reductive transformations, such as the reductive dehalogenation of chloroethenes and chloroaromatic compounds (8, 25, 42). Therefore, *S. oneidensis* MR-1 and perhaps other *Shewanella* species may, next to their role in heavy-metal transformations, also play a role as fermenting microorganisms in providing hydrogen to mixed microbial communities.

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