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Novel Physiological Features of *Carboxydothermus hydrogenoformans* and *Thermoterrabacterium ferrireducens*

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Carboxydothermus hydrogenoformans **is able to grow by conversion of CO to H_2 and CO_2. Besides CO, only pyruvate was described as serving as an energy source. Based on 16S rRNA gene sequence similarity, *C. hydrogenoformans* is closely related to *Thermoterrabacterium ferrireducens*. *T. ferrireducens* is like *C. hydrogenoformans* a gram-positive, thermophilic, strict anaerobic bacterium. However, it is capable of using various electron donors and acceptors for growth. Growth of *C. hydrogenoformans* with multiple electron donors and acceptors was tested. *C. hydrogenoformans* oxidized formate, lactate, glycerol, CO, and H_2 with 9,10-anthraquinone-2,6-disulfonate as an electron acceptor. Sulfite, thiosulfate, sulfur, nitrate, and fumarate were reduced with lactate as an electron donor. *T. ferrireducens* oxidized CO with 9,10-anthraquinone-2,6-disulfonate as an electron acceptor but did not produce H_2 from CO. In contrast to what was published before, *T. ferrireducens* was able to grow on lactate with sulfite, sulfur, and nitrate as electron acceptors.**

Carboxydothermus hydrogenoformans was isolated from hydrothermal freshwater springs on Kunashir Island (Russian Kurils). The bacterium grows by the conversion of CO plus H_2O to H_2 plus CO_2 (2, 13). An obligate dependence on CO for its growth was argued, since no other substrates were shown to support growth. Volcanic exhalations, decomposition of plant material, and interspecies CO transfer are possible sources of CO (13). Growth on CO is fast. Doubling times of 120 min are attained, despite the low standard Gibbs free energy of this reaction (−20 kJ/mol of CO).

Several studies have addressed biochemical aspects of *C. hydrogenoformans*. Two carbon monoxide dehydrogenases (CODHs) and a CO-oxidizing/H_2-evolving enzyme complex have been purified and characterized (10, 16). The first crystal structure of a nickel-CODH was solved for CODH II obtained from *C. hydrogenoformans* (1). In addition, whole-genome sequencing of *C. hydrogenoformans* is in progress (The Institute for Genomic Research, Rockville, Md.; Center of Marine Biotechnology, Baltimore, Md.).

A limitation in further studies on *C. hydrogenoformans* is its restricted substrate use; only pyruvate and CO were reported to support growth of this bacterium (14). However, indications for a wider substrate range exist. *C. hydrogenoformans* and *Thermoterrabacterium ferrireducens* share a close phylogenetic relationship. An identity of more than 98% between 16S rRNA gene sequences of *T. ferrireducens* (U76363) and *C. hydrogenoformans* can be deduced from the published phylogenetic tree (11). *T. ferrireducens* was isolated from Yellowstone National Park and is described as growing by fermentation of pyruvate, lactate, glycerate, glycerol, and 1,2-propanediol. Glycerol and H_2 also support growth with fumarate, 9,10-anthraquinone-2,6-disulfonate (AQDS), ferric iron, or thiosulfate as a terminal electron acceptor (9). These substrate combinations have not been tested for *C. hydrogenoformans*.

Here we demonstrate that *C. hydrogenoformans* is capable of anaerobic respiration in a similar fashion to *T. ferrireducens*. In addition, we show that *T. ferrireducens* is able to use CO as an electron donor for AQDS and fumarate reduction.

MATERIALS AND METHODS

Organisms. *C. hydrogenoformans* (DSM 6008) and *T. ferrireducens* (DSM 11255) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany).

Medium composition and cultivation. Both microorganisms were cultivated under strictly anaerobic conditions in a basal carbonate-buffered medium composed as described by Stams et al. (12). However, 1.5 g of $NaHCO_3$ liter^{-1}, 0.2 g of $Na_2S \cdot 9H_2O$ liter^{-1}, 0.1 g of yeast extract liter^{-1}, and 2 μmol of $NiCl_2$ liter^{-1} were used instead of the previously reported concentrations; Na_2S concentrations were lowered to 0.04 g liter^{-1} when AQDS was present in the growth medium. One hundred-twenty-milliliter butyl rubber-stoppered bottles contained 50 ml of medium. AQDS (8.25 g l^{-1}) or sulfur (2 g l^{-1}) was added to bottles as solids prior to sterilization. Bottles were autoclaved for 25 min at 121°C. Sulfur-containing bottles were autoclaved at 110°C for 35 min. Gas phases were pressurized to 170 kPa and were composed of 20% CO_2 and 80% N_2, H_2, or CO. The following compounds were added to the bottles from separately autoclaved stock solutions. Formate, methanol, acetate, DL-lactate, glycerol, and sulfate were used in concentrations of 20 mM unless stated otherwise. Sulfite, thiosulfate, nitrate, and fumarate were used in concentrations of 10 mM, unless stated otherwise. Cultures with an H_2/CO_2 or CO/CO_2 gas phase were incubated at 60°C and shaken at 100 rpm. Cultures with a N_2/CO_2 gas phase were incubated, not shaken, at 65°C.

Analytical methods. H_2, CO, N_2O, and CO_2 were analyzed by gas chromatography on a Chrompack CP9001 gas chromatograph fitted with a TCD detector. The injector and detector temperatures were 60 and 130°C, respectively. CO_2 was analyzed with a Poraplot Q column (Chrompack; 25 m length, 0.53 mm internal diameter; film thickness, 20 μm). Helium was the carrier gas at a flow rate of 18 ml min^{-1}, and the oven temperature was 45°C. H_2 and CO were analyzed with a Molsieve column (Chrompack; 30 m length, 0.53 mm internal diameter; film thickness, 15 μm); argon at a flow rate of 10 ml min^{-1} was the carrier gas. The oven temperature was 50°C.

Organic acids, glycerol, and methanol were analyzed by high-performance liquid chromatography with a Polyspher OA HY column (300 to 6.5 mm; Merck, Darmstadt, Germany) and an RI SE-61 refractive index detector (Shodex, Tokyo Japan) as described previously (8). Sulfate, thiosulfate, nitrate, and nitrite were analyzed by high-performance liquid chromatography with an Ionpac AS9-SC column (Dionex, Sunnyvale, Calif.) and ED 40 electrochemical detector (Di-

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onex) as described previously (8). Sulfide was analyzed according to the modified colorimetric method of Pachmeyer (18). Ammonium was determined by a variation on the Berthelot reaction (7). Reduction of AQDS was indicated by visual color change of the growth medium.

Selective media and DGGE. Purity of the cultures was examined by phase-contrast microscopy, use of selective media, and denaturing gradient gel electrophoresis (DGGE). *C. hydrogenoformans* and *T. ferrireducens* were transferred five times on the medium with lactate and an N_2/CO_2 gas phase. Subsequently the strains were transferred to media supplemented with glucose, sucrose, lactate, or lactate plus nitrate; media with a CO/CO_2 gas phase, either with or without fumarate; and media with a H_2/CO_2 gas phase. Both strains were also tested with the control medium of Svetlichny et al. (13) containing 20 mM glucose, 5 g of yeast extract $liter^{-1}$, 2 g of peptone $liter^{-1}$, and an N_2/CO_2 atmosphere.

DGGE analysis of 16S ribosomal DNA (rDNA) was performed by amplification of the V6-to-V8 region by PCR of DNA isolated from cultures of *T. ferrireducens* and *C. hydrogenoformans* grown on different substrates. DNA was isolated from 5 to 10 ml of culture suspension as described by Zoetendal et al. (19), using bead-beat cell disruption, phenol-chloroform extraction, and ethanol precipitation. Isolated cells from the cultures grown with CO alone were treated with lysozyme for 1 h at 37°C before cell disruption. PCR and DGGE were performed as described by Heilig et al. (3) with primers F0968GC and R1401 (6). A 40 to 60% denaturing gradient gel was used for DGGE.

RESULTS AND DISCUSSION

The close phylogenetic relationship between *C. hydrogenoformans* and *T. ferrireducens* indicates a wider spectrum of substrates used for growth by *C. hydrogenoformans* than CO and pyruvate alone. Especially anaerobic respiration is of interest as *T. ferrireducens* uses various electron donors and acceptors as energy sources while no data on anaerobic respiration by *C. hydrogenoformans* are documented.

The utilization of different electron donors and acceptors for growth by *C. hydrogenoformans* was tested in batch cultures in a direct comparison with *T. ferrireducens*. Formate, methanol, acetate, glycerol, H_2, and CO were tested as electron donors with AQDS as the terminal electron acceptor. Sulfate, sulfite, thiosulfate, sulfur, nitrate, and fumarate were tested as electron acceptors with lactate as the electron donor. Use of substrates was qualified by multiple approaches. AQDS reduction results in a bright orange color of the cultures and can be used to score conversion. Other substrates (except sulfur) and their products were analyzed after 3 and 14 days of cultivation. Furthermore, microscopic observations were used to indicate increases in the numbers of cells. Table 1 lists the results for the use of the different substrates in anaerobic respiration by *C. hydrogenoformans* and *T. ferrireducens*. The expected results for *T. ferrireducens* based on published data (9) are included as well. The range of substrates used by *C. hydrogenoformans* was identical to that of *T. ferrireducens*. All tested substrates were used by *C. hydrogenoformans* and *T. ferrireducens*, except methanol, acetate, and sulfate (Table 1). Differences from reported data and specific observations during experimentation prompted us to perform more detailed studies. These are discussed below.

CO had a different fate in the metabolism of *C. hydrogenoformans* and *T. ferrireducens*. Both microorganisms reduced AQDS with CO as the electron donor. However, H_2 was detected in cultures of *C. hydrogenoformans* as well, while H_2 was not detected in cultures of *T. ferrireducens*. Additional experiments with CO as the sole substrate for carbon and energy were performed with *T. ferrireducens* to check for the presence of the carboxydotrophic hydrogenogenic activity. No growth or

TABLE 1. Anaerobic respiration of selected electron donors and acceptors by *C. hydrogenoformans* and *T. ferrireducens*

Condition	Result for:		
	C. hydrogenoformans	*T. ferrireducens*	
		Actual	Expected[a]
AQDS as acceptor and with donor:			
Formate	+	+	−
Methanol	−	−	−
Acetate	−	−	−
Lactate	+	+	+
Glycerol	+	+	+
H_2	+	+	+
CO	+	+	NR[b]
Lactate as donor and with acceptor:			
Sulfate	−	−	−
Sulfite	+	+	−
Thiosulfate	+	+	+
Sulfur	+	+	−
Nitrate	+	+	−
Fumarate	+	+	+

[a] Expected results based on the data reported by Slobodkin et al. (9).
[b] NR, not reported.

H_2 formation was observed in incubations of *T. ferrireducens* with gas phases that contained CO in the range of 5 to 80%. These results indicate that the capacity to perform the characteristic metabolism of *C. hydrogenoformans*, which accumulates H_2 with CO as a substrate, is absent in *T. ferrireducens*.

Growth of *C. hydrogenoformans* and *T. ferrireducens* with CO as the electron donor was studied in more detail in 200-ml cultures with fumarate (50 mM) as the electron acceptor. Bottles with 200 ml of medium and 385 ml of CO/CO_2 gas phase were inoculated with *C. hydrogenoformans* or *T. ferrireducens*. Reduction of fumarate and formation of H_2 occurred simultaneously in cultures of *C. hydrogenoformans* (Fig. 1A and C). No H_2 was formed in cultures of *T. ferrireducens* (Fig. 1B). Simultaneous fumarate reduction and hydrogen production resulted in a higher consumption rate of CO for *C. hydrogenoformans* (Fig. 1A) than for *T. ferrireducens* (Fig. 1B). Intermediate accumulation of malate was observed in cultures of *C. hydrogenoformans* (Fig. 1C) and *T. ferrireducens* (Fig. 1D). Changes in fumarate, succinate, and malate concentrations were similar for *C. hydrogenoformans* (Fig. 1C) and *T. ferrireducens* (Fig. 1D). Succinate was formed at constant rates of similar magnitude (0.3 mmol h^{-1}) by cultures of both microorganisms. Rates of biomass development (Fig. 1A and B) were also similar. The mass balance of CO oxidation (114.3 mmol/liter of medium) for *C. hydrogenoformans* cultures was 92% complete with 56.6 mM fumarate reduced and 48.7 mmol of H_2 formed per liter of medium. In *T. ferrireducens* cultures, somewhat more fumarate was reduced (56.2 mM) than can be accounted for by CO oxidation (51.6 mM).

In contrast to the results of Slobodkin et al. (9), sulfite was reduced by *T. ferrireducens* in our experiments. In that study, potentially toxic concentrations of sulfite (20 mM) were used. *C. hydrogenoformans* and *T. ferrireducens* were inoculated in medium with 2, 5, 10, and 20 mM sulfite and lactate (20 mM) as electron donors to test this hypothesis. Formation of sulfide

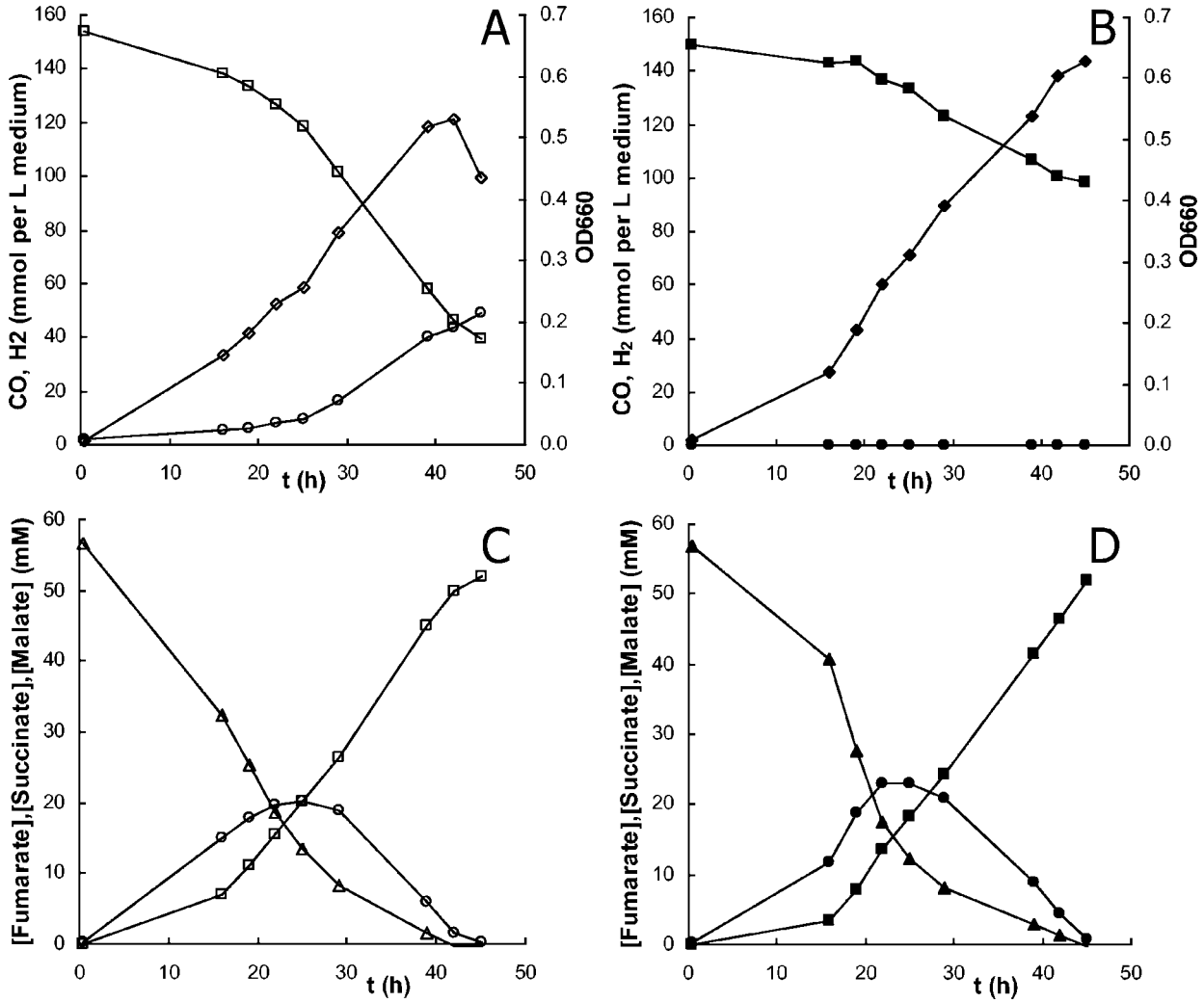

FIG. 1. Reduction of fumarate to succinate with carbon monoxide as the electron donor. *C. hydrogenoformans* (open symbols) and *T. ferrireducens* (closed symbols) were cultivated in 585-ml bottles with 200 ml of medium. Plotted are the average data of duplicate incubations. t, time. (A and B) Development of biomass represented by optical density at 660 nm (OD_{660}; diamonds) and quantities of CO (squares) and H_2 (circles) present per liter of medium during incubation of *C. hydrogenoformans* (A) and *T. ferrireducens* (B). (C and D) Millimolar concentrations of fumarate (triangles), succinate (squares), and malate (circles) during incubation of *C. hydrogenoformans* (C) and *T. ferrireducens* (D).

was detected in cultures of *C. hydrogenoformans* and *T. ferrireducens* started with 2, 5, and 10 mM sulfite, but not in cultures started with 20 mM sulfite. *C. hydrogenoformans* cultures with 10 mM sulfite produced 6.9 mM sulfide in 76 h of cultivation (Fig. 2). A total of 12.6 mM lactate was consumed, and 14.1 mM acetate was produced. The formation of 6.9 mM sulfide requires oxidation of 10.35 mM lactate. If homoacetogenesis is considered, then the remaining consumed lactate (2.25 mM) balances with 90% of the surplus of acetate formed (3.75 mM). A functional acetyl-coenzyme A synthase, essential for homoacetogenesis, is present in *C. hydrogenoformans* (15). Cultures of *T. ferrireducens* formed 7.5 mM sulfide and 22.6 mM acetate (Fig. 2). A total of 18.6 mM lactate was consumed. Ninety-seven percent of surplus acetate formed (11.35 mM) can be accounted for if homoacetogenic oxidation of lactate (7.35 mM) is considered. Cultures with 5 mM sulfite obtained the highest turbidity, whereas cultures with 20 mM did not show visible growth. These results validate that *C. hydrogenoformans* and *T. ferrireducens* do grow with sulfite as an electron acceptor.

In our experiments, thiosulfate was reduced to sulfide by *T. ferrireducens*, whereas sulfur was formed in experiments of Slobodkin et al. (9). Sulfur was not reduced in the latter study. *Thermoanaerobacter* species are reported to produce either sulfide or sulfur from thiosulfate, depending on medium composition and growth stage (4, 5). *Carboxydothermus* and *Thermoterrabacterium* are closely related to the genus *Thermoanaerobacter* (11). Growth conditions may determine the product of thiosulfate reduction by *T. ferrireducens* as well. The difference observed between the sulfur results in our experiments and those of Slobodkin et al. may be explained similarly.

Two pathways are known for dissimilatory nitrate reduction; denitrification, which yields N_2 and nitrate ammonification, which yields ammonium (17). *C. hydrogenoformans* and *T. ferrireducens* both reduced nitrate (10 mM) with lactate (20 mM) as an electron donor to nitrite. Additional experiments were

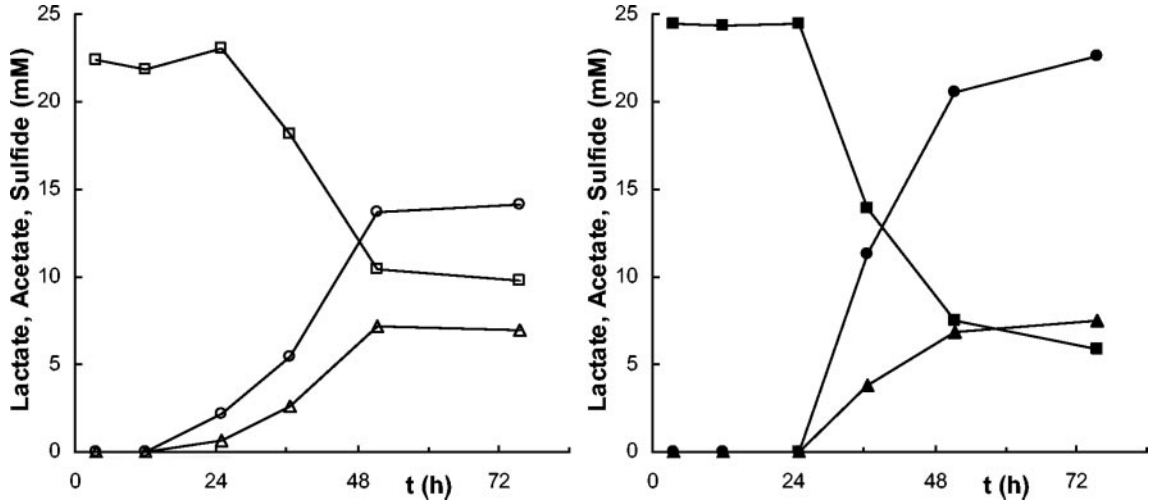

FIG. 2. Reduction of sulfite (10 mM) with lactate (20 mM) as an electron donor by pure cultures of *C. hydrogenoformans* (open symbols) and *T. ferrireducens* (closed symbols). Lactate (squares) was oxidized to acetate (circles), and sulfite (not analyzed) was reduced to sulfide (triangles). t, time.

performed to determine whether further reduction of nitrite does occur. Cultures were started with lower nitrate concentrations in these experiments since the resazurin turned pink upon nitrate reduction in cultures with initial nitrate concentrations of 10 mM. This indicates relative high, possibly unfavorable, redox conditions of the medium. During experiments with 2 mM nitrate, slight increases in ammonium were detected in cultures of *C. hydrogenoformans.* However, when extra nitrate was added to these cultures, no further reduction was observed (data not shown). Because of poor reproducibility of ammonium formation and the absence of ammonium formation in cultures of *T. ferrireducens*, nitrate concentrations were further decreased. In experiments where 0.5 mM nitrate was added at 24-h intervals, increased ammonium concentrations were detected in cultures of both *C. hydrogenoformans* and *T. ferrireducens* (Fig. 3). Cultures of *C. hydrogenoformans* converted nitrate completely to ammonium in the first 48 h of cultivation. After 48 h, residual nitrite accumulated to a final concentration of 0.2 mM at 120 h. Nitrate was not fully converted to ammonium in cultures of *T. ferrireducens*. Ammonium and nitrite accumulated simultaneously during the first 74 h in these cultures, resulting in lower final concentrations of ammonium than those in cultures of *C. hydrogenoformans* (Fig. 3). Based on these results, we conclude that nitrate can be reduced to ammonium by *C. hydrogenoformans* and *T. ferrireducens*, provided that nitrite concentrations remain low. Conversion of nitrate to ammonium is apparently slower in *T. ferrireducens* than *C. hydrogenoformans*.

Phase-contrast microscopy, selective culturing, and DGGE of 16S rDNA were applied to check the purity of used cultures. Microscopical observations and the growth on selective medium did not reveal the presence of contaminants. *C. hydrogenoformans* and *T. ferrireducens* share a similar rod-shaped morphology. Contamination of *T. ferrireducens* cultures with *C. hydrogenoformans* was excluded by the inability of these cultures to form H_2 from CO. Figure 4 shows the result of DGGE analysis of amplified 16S rDNA sequences from cultures of *C. hydrogenoformans* and *T. ferrireducens* grown with different substrates. It is expected that abundant contaminants lead to additional bands in the DGGE gel. Lanes 1 to 4 contain PCR samples of *C. hydrogenoformans* cultures; one band is visible for each tested condition. Lanes 6 to 8 contain PCR samples of *T. ferrireducens* cultures. Each growth condition shows two bands, which corresponds to the number of 16S rRNA genes of *T. ferrireducens* in the GenBank database (accession no. U76363 and U76364). Both bands vary equally in intensity under the different growth conditions, which is not expected in the case of contaminants. These results confirmed the purity of the utilized cultures of *C. hydrogenoformans* and *T. ferrireducens* and exclude cross-contamination of *C. hydrogenoformans* cultures with *T. ferrireducens*.

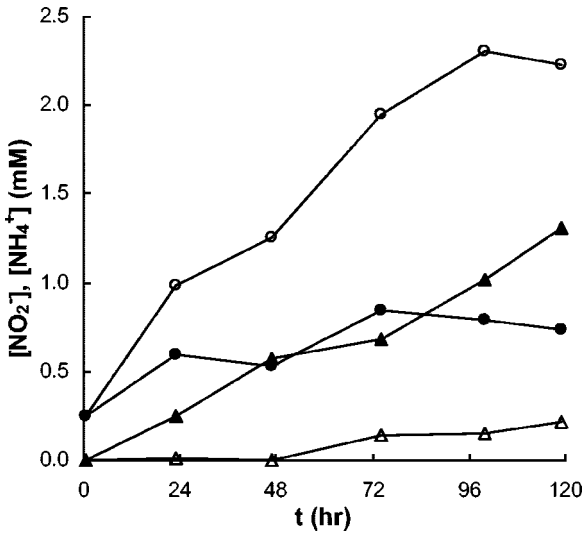

FIG. 3. Reduction of nitrate to nitrite (triangles) and ammonium (circles) by pure cultures of *C. hydrogenoformans* (open symbols) and *T. ferrireducens* (closed symbols). Nitrate was added after 0.5, 23, 47, 74, and 100 h in aliquots corresponding to a final concentration of 0.5 mM. Lactate (5 mM) served as an electron donor. t, time.

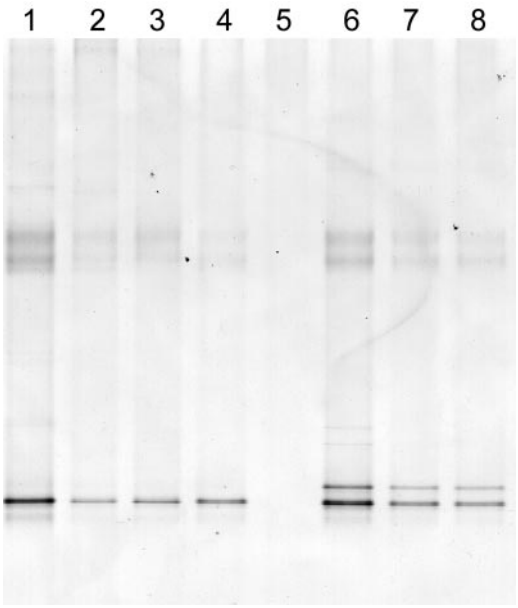

FIG. 4. DGGE of PCR-amplified V6-to-V8 region of 16S rDNA from cultures of *C. hydrogenoformans* (lanes 1 to 4) and *T. ferrireducens* (lanes 6 to 8) grown on different substrates. The substrates were CO (lane 1), CO plus nitrate (lane 2), CO plus fumarate (lane 6), lactate plus nitrate (lanes 3 and 7), and lactate alone (lanes 4 and 8).

Evidence for a close phylogenetic relation between *C. hydrogenoformans* and *T. ferrireducens* based on 16S rDNA similarity exists (11). This similarity prompted us to reexamine the metabolic capacities of *C. hydrogenoformans*. Pyruvate and CO were the only substrates reported to support growth of *C. hydrogenoformans*. *T. ferrireducens* on the other hand grows with multiple electron donors and acceptors. The presented results show that *C. hydrogenoformans* can grow with formate, lactate, glycerol, H_2, CO, AQDS, sulfite, thiosulfate, sulfur, nitrate, and fumarate. The comparable metabolisms of *C. hydrogenoformans* and *T. ferrireducens* with these substrates and in incubations with CO as an electron donor and fumarate as an electron acceptor are consistent with the close phylogenetic relationship between both microorganisms. The results also indicate that *C. hydrogenoformans* is not solely dependent on the presence of CO to survive in its habitat.

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