Function of formate dehydrogenases in *Desulfovibrio vulgaris* Hildenborough energy metabolism

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The genome of the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough encodes three formate dehydrogenases (FDHs), two of which are soluble periplasmic enzymes (FdhAB and FdhABC<sub>3</sub>) and one that is periplasmic but membrane-associated (FdhM). FdhAB and FdhABC<sub>3</sub> were recently shown to be the main enzymes present during growth with lactate, formate or hydrogen. To address the role of these two enzymes, ∆fdhAB and ∆fdhABC<sub>3</sub> mutants were generated and studied. Different phenotypes were observed in the presence of either molybdenum or tungsten, since both enzymes were important for growth on formate in the presence of Mo, whereas in the presence of W only FdhAB played a role. Both ∆fdhAB and ∆fdhABC<sub>3</sub> mutants displayed defects in growth with lactate and sulfate providing the first direct evidence for the involvement of formate cycling under these conditions. In support of this mechanism, incubation of concentrated cell suspensions of the mutant strains with lactate and limiting sulfate also gave elevated formate concentrations, as compared to the wild-type strain. In contrast, both mutants grew similarly to the wild-type with H₂ and sulfate. In the absence of sulfate, the wild-type *D. vulgaris* cells produced formate when supplied with H₂ and CO₂, which resulted from CO₂ reduction by the periplasmic FDHs. The conversion of H₂ and CO₂ to formate allows the reversible storage of reducing power in a much more soluble molecule. Furthermore, we propose this may be an expression of the ability of some sulfate-reducing bacteria to grow by hydrogen oxidation, in syntrophy with organisms that consume formate, but are less efficient in H₂ utilization.

INTRODUCTION

Formate is an important metabolite in anoxic ecosystems. It is formed by fermentative organisms in the degradation of complex organic matter, and it is a common growth substrate for many bacteria and archaea. It is also a cellular intermediate in processes like acetogenesis or methylotrophy and a possible substrate for methanogenesis (Chistoserdova et al., 2007; Müller, 2003; Stams & Plugge, 2009; Thauer & Shima, 2008). Together with hydrogen, formate is also involved in interspecies electron transfer by syntrophic communities of bacteria and archaea in methanogenic or sulfate-reducing environments (McInerney et al., 2009; Müller et al., 2010; Schink & Stams, 2006; Stams & Plugge, 2009). The ability to use formate depends on formate dehydrogenases (FDHs), the enzymes that catalyse the reversible conversion of formate to CO₂. FDHs display a large diversity in quaternary structure, cofactor composition, cellular localization and electron donors and acceptors, reflecting the different physiological roles of formate (Ferry, 1990; Vorholt & Thauer, 2002). Most FDHs contain a molybdenum or tungsten pterin cofactor, but some aerobic bacteria possess nicotinamide adenine dinucleotide (NAD⁺)-dependent FDHs that lack a prosthetic group (Gonzalez et al., 2013; Popov & Lamzin, 1994; Vorholt & Thauer, 2002).

The cellular location of FDHs is linked to their function. The FDHs that function mainly as CO₂-reductases are cytoplasmic enzymes found in acetogens, in symbionts and other fermentative organisms, where they produce formate in the first step of the Wood–Ljungdahl pathway for the biosynthesis of C₁ compounds (Ragsdale, 2008). In methanogens, cytoplasmic FDHs oxidize formate as an energy substrate, which is converted to CO₂ to be used in the methanogenic pathway (Jones & Stadtman, 1981; Schauer & Ferry, 1982; Wood et al., 2003). In *Escherichia coli* a cytoplasmic FDH...
(FDH-H) associates with a membrane-bound hydrogenase forming a formate hydrogen-lyase complex (FHL) (Sawers, 2005). FDH-H is expressed during fermentative growth of E. coli. When the formate concentration builds up it oxidizes formate to CO₂, coupled to reduction of protons to H₂ by the hydrogenase partner. Periplasmic FDHs generally act to oxidize formate and are usually membrane-bound through an integral membrane subunit that transfers electrons to the quinone pool (Jormakka et al., 2002; Kröger et al., 1979). In most micro-organisms this subunit is a b-type cytochrome that may be involved in a redox loop mechanism with another membrane-bound enzyme, resulting in electron transfer to the cytoplasm and proton release in the periplasm, thus contributing to the proton motive force and, consequently, energy conservation (Simon et al., 2008). However, in the deltaproteobacterial sulfate reducers most periplasmic FDHs (and hydrogenases) are soluble, and transfer electrons to cytochrome c₃ (Matias et al., 2005; Pereira et al., 2011). These FDHs either include a dedicated cytochrome c₃ subunit (as in FdhABC₃) (Costa et al., 1997; Sebän et al., 1995), or have only two subunits (FdhAB) (Almendra et al., 1999; Raaijmakers et al., 2002), and transfer electrons to the periplasmic cytochrome c network and from this to membrane-bound complexes such as Qrc, which reduce the menaquinone pool (Venceslau et al., 2010). Sulfate-reducing bacteria (SRB) are metabolically versatile and in the absence of sulfate can also grow syntrophically with other organisms, which consume their fermentation products: H₂, CO₂, acetate or formate (Bryant et al., 1977; Muyzer & Stams, 2008; Plugge et al., 2011; Schink & Stams, 2006; Stams & Plugge, 2009; Stolyar et al., 2007). A high number of FDH genes is usually present in SRB genomes (Pereira et al., 2011), which agrees with formate playing an important role as a syntrophic intermediate. In addition, intracellular cycling of formate has also been suggested to contribute to energy conservation in SRB (Pereira et al., 2008; Voordouw, 2002). Here, formate formed intracellularly by the pyruvate-formate lyase (PFL) would be transported to the periplasm where it would be oxidized by FDHs, and electrons and channelled back to the cytoplasm for sulfate reduction leading to a proton motive force across the membrane. However, this proposal has not yet been confirmed.

To further study the function of FDHs and the role of formate in SRB metabolism we chose the model organism Desulfovibrio vulgaris Hildenborough. This bacterium has three periplasmic FDHS, two of which are soluble (FdhABC₃ and FdhAB), and one that is associated with the membrane (da Silva et al., 2011; Sebän et al., 1995). No cytoplasmic FDHs are encoded in the genome. The FdhABC₃ and FdhAB are the two main FDHs detected in D. vulgaris, and they are differentially regulated by the metals Mo and W (da Silva et al., 2011). Increased expression of both FDHs is observed during growth with formate/sulfate (relative to lactate/sulfate) (da Silva et al., 2011; Zhang et al., 2006). In addition, increased transcription of all FDHs was observed during growth with H₂/CO₂/sulfate (Pereira et al., 2008), and in particular for the W-FdhAB (da Silva et al., 2011). The function of FDHs during growth of D. vulgaris with H₂/CO₂/sulfate is not immediately obvious. During this type of growth CO₂ and acetate are also supplied as carbon sources and one or more of the FDHs may be acting to reduce CO₂ to formate. The formate may then act as a reservoir of stored hydrogen, which can be released by reversible conversion of formate to H₂ and CO₂. Alternatively, the formate is transported into the cell and converted to pyruvate by the PFL, acting in reverse. In order to get further insight on the role of formate in SRB metabolism, we constructed deletion mutants for the two main enzymes detected in D. vulgaris, FdhAB (ΔfdhAB) and the FdhABC₃ (ΔfdhABC₃), and studied their physiology under different growth conditions.

**METHODS**

**Construction of mutant strains.** The 500 bp regions located within and downstream of the gene encoding the catalytic subunit of FdhABC₃ (fudG₃; DU2812) were PCR-amplified using primer pairs p290f/p291r and p292f/p293r, respectively (Table 1). The amplified fragments were cloned sequentially into pNOT19 (Schweizer, 1992) by digestion with SacI and BamHI and pStI and BamHI and ligation to obtain pNOTAfndG₃. The latter was cleaved with BamHI and ligated to the cat gene-containing 1.4 kb BamHI fragment from pUC19Cm to obtain pNOTAfndG₃Cm. NotI digestion of the latter and insertion of the 4.2 kb NotI fragment from pMOB2 (Schweizer, 1992) gave pNOTAfndG₃CmMob, which was transformed into E. coli S17-1. Following conjugation of E. coli S17-1 (pNOTAfndG₃CmMob) and D. vulgaris, single crossover integrants were obtained on medium E plates containing chloramphenicol (Cm), as described elsewhere (Fu & Voordouw, 1997). Growth of the integrant in defined medium containing Cm and 5 % (w/v) sucrose produced the double crossover mutant D. vulgaris ΔfdhABC₃ in which 1029 bp of the 3’ end of the 3039 bp fudG₃ coding region were replaced with the cat gene.

Likewise, the 500 bp regions located upstream and downstream of the fdhAB genes (DUV0587 and DUV0588) were PCR-amplified using primer pairs p411f/p412r and p413f/p414r, respectively (Table 1) and cloned following digestion with HindIII and BamHI and KpnI and BamHI, respectively. The procedures were similar to those for construction of the ΔfdhABC₃ mutant, except that the nptII gene, encoding kanamycin (Km) and G418 resistance, was used as the selectable marker (Bender et al., 2007; Caffrey et al., 2007). This resulted in construction of the suicide plasmid pNOTAfdbhABkmMob and the double crossover mutant D. vulgaris ΔfdhAB, which had the fdhAB genes replaced with the nptII gene.

**Table 1.** PCR primers used for mutant construction

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Culture media and growth conditions. *D. vulgaris* wild-type (wt) and mutant strains were grown in modified Postgate medium C containing 0.2 g yeast extract 1 M, 25 μM iron, 1 μM nickel, 1 μM selenium and 38 mM sulfate. Either molybdenum or tungsten were added at a final concentration of 0.1 μM. Electron donors used were lactate, formate or hydrogen. For organic acids, a final concentration of 40 mM was used. When hydrogen was the electron donor a mixture of 80% hydrogen/20% CO₂ was used as the gas phase, to a final overpressure of 1 bar (10⁵ Pa). Acetate (10 mM) was also present in formate- or hydrogen-containing media. Growth was performed under anaerobic conditions in 500 ml flasks half filled with culture medium. The cells were grown at 37 °C and optical density was measured at 600 nm with a Shimadzu spectrophotometer. All optical density measurements are the mean of two biologically independent experiments.

**Analytical procedures.** For metabolite analysis 1 ml samples of culture were taken at regular intervals, immediately centrifuged for 10 min at 10000 g and the supernatants frozen at −20 °C. Lactate, formate and acetate concentrations were determined by high pressure liquid chromatography (HPLC), with a Waters chromatograph and an LKB 2142 Differential Refractometer (LKB Bromma) detector. An Aminex HPX-87H column (Bio-Rad) was used at 60 °C and data were collected with the Millenium 32 software, version 3.05.01 (Waters). Samples (20 μl) were eluted isocratically at a flow rate of 0.5 ml min⁻¹, with 2.5 mM H₃SO₄. Sulfate concentrations were determined with a PRP-x100 column (Hamilton) on a Waters Acuity Ultra-Performance Liquid Chromatograph with indirect UV detection at 310 nm, and data were collected and processed by Empower 2 software (Waters). Samples (10 μl) were injected at 25 °C and eluted isocratically at a flow rate of 2 ml min⁻¹, with a mobile phase of 3% (v/v) methanol and 97% (v/v) of 6 mM hydroxybenzoic acid (pH 10).

**Formate quantification in cell suspensions.** Cells grown with either lactate or H₂ were collected at mid-exponential phase, centrifuged for 15 min at 6000 g and suspended in a 10-fold smaller volume of fresh culture medium containing a limiting concentration of sulfate (10 mM). Lactate (40 mM) or H₂ [1 bar (10⁵ Pa) overpressure; 80% H₂, 20% CO₂] were provided as electron donors. Cells were incubated at 37 °C and samples taken at several intervals and treated as described above. For formate quantification, 20 μl of supernatant from each sample was dispensed per well in a 96-well plate (Greiner). The reaction was started by adding a solution containing 1 mM NAD⁺, 40 mM Tris/Cl buffer pH 8 (at 25 °C) and 0.5 U of *Candida boidinii* formate dehydrogenase (Sigma) to a final volume of 200 μl per well. Absorbance was read at 340 nm at the start and after 1 h of incubation at 37 °C, in a 96-well plate reader (BioTek). When necessary the samples were appropriately diluted.

**RESULTS**

In a previous study, we showed that FdhABC₃ is the main FDH present when Mo is available, and is particularly upregulated during growth with formate and sulfate (da Silva et al., 2011). On the other hand, FdhAB is the main FDH in the presence of W, and ΔfdhAB is particularly upregulated during growth with H₂ and sulfate, while W causes repression of the fdhABC₃ genes. The metals have no effect on growth rate during growth with H₂ and sulfate, whereas a small increase in growth rate was observed in formate/sulfate and lactate/sulfate in the presence of W relative to Mo. These results, obtained with wild-type (wt) cells, were again confirmed in the present work.

**Growth with formate/sulfate**

When formate (40 mM) was used as electron donor with excess sulfate (38 mM) significant differences were observed between wt and mutant strains, and strikingly, different phenotypes were revealed in the presence of Mo or W (Fig. 1). In the presence of Mo (Fig. 1a, b) the wt and ΔfdhABC₃ strains had similar initial growth rate, but this slowed down over time for the mutant, and its final cell density was considerably lower than for wt. The ΔfdhAB strain had an even lower growth rate but the final cell density was not much different from that of the ΔfdhABC₃ mutant. In the presence of W (Fig. 1c, d), the ΔfdhABC₃ strain had only a slightly lower growth rate than the wt, and the final cell density was similar for both, while growth of the ΔfdhAB strain was severely impaired. Metabolite analysis showed that four formate molecules were consumed per sulfate, as expected. Formate consumption (Fig. 1b, d) and sulfate reduction (data not shown) followed the growth pattern in all experiments including for the ΔfdhAB mutant in the presence of W, where practically no growth occurred.

*D. vulgaris* does not possess all the enzymes necessary for the complete Wood–Ljungdahl pathway, namely a formyltetrahydrofolate synthase, which would allow the use of CO₂ or formate as carbon sources for C₁ metabolism (Maden, 2000). For this reason, when growing with formate or H₂ as electron donors, acetate or acetate and CO₂ have to be added as carbon sources. Acetate is activated to acetyl-CoA, which can be reductively carboxylated to pyruvate (Tang et al., 2007), and is only used for anabolic reactions, with 70% of cell carbon being derived from acetate and 30% from CO₂ (Badziong et al., 1979). Acetate was not detectably metabolized and remained constant (data not shown), due to the low biomass produced.

**Growth with lactate/sulfate**

When lactate (40 mM) was used as an electron donor with excess sulfate (38 mM) both mutant strains, ΔfdhAB and ΔfdhABC₃, grew at a lower rate than the wt and reached a lower final cell density. Both effects were more pronounced for the ΔfdhABC₃ strain, and this behaviour was identical irrespective of whether Mo or W were present in the medium (Fig. 2a, c). These results indicate that the FDHs play a role in energy metabolism during growth on lactate/ sulfate. Metabolite analysis showed that, in cultures of the wt, 40 mM lactate and 20 mM sulfate were consumed, in accordance with the expected stoichiometry. In contrast, the mutants were not able to metabolize all lactate present in the medium (Fig. 2b, d). Both mutants consumed only 30 mM lactate and reduced 15 mM of sulfate, in the presence of Mo or W. Most of the lactate used was oxidized to acetate and CO₂ for energy generation, and only a small part was incorporated into biomass (Stolyar et al., 2007; Tang et al., 2007). No extracellular formate was detected during growth.
Growth with H₂/sulfate

When hydrogen was used as electron donor, a headspace overpressure of 1 bar (10⁵ Pa) of H₂/CO₂ was maintained during growth and acetate was again present to be used as an additional carbon source. No significant differences in growth rate or final cell densities were observed between wt and mutant strains, except for a small delay to start growth, and a slightly reduced cell density in the ΔfdhABC₃ mutant.
This suggests that the FDHs are not essential for growth in H₂/CO₂, despite the increase in their expression levels under these conditions (da Silva et al., 2011; Pereira et al., 2008). Approximately 10 mM sulfate was consumed by all strains before growth stopped, which was possibly due to inhibition of hydrogenases caused by the accumulation of sulfide (Fig. 3a, c). As with growth in lactate/sulfate medium, no extracellular formate was detected during growth on H₂/sulfate medium.

**Formate quantification in cell suspensions**

The growth phenotype of the FDH mutants in lactate/sulfate medium suggests that intracellular formate cycling is occurring under these conditions. In contrast, no phenotype was observed for growth with H₂/CO₂, which upregulates FdhAB expression. The fact that extracellular formate is not being detected in either growth condition does not mean that it is not being formed and consumed by the cells at significant rates. The excreted concentration could be so low that it is below the HPLC detection limit. In order to try to detect low levels of formate, we carried out experiments with concentrated cell suspensions, and measured formate enzymically, which is a more sensitive method. In these experiments sulfate was present in limiting amounts (10 mM) relative to the substrate.

With lactate as electron donor (40 mM) sulfate was consumed in the first three hours of incubation by the wt and ΔfdhABC₃ mutant, whereas it took longer for the ΔfdhAB mutant to consume all the sulfate (Fig. 4). For reduction of 10 mM sulfate, about 20 mM of lactate was consumed and 20 mM of acetate was produced (data not shown), according to the expected stoichiometry. After sulfate is depleted, lactate can be fermented, to acetate, CO₂ and H₂ (Pankhania et al., 1988; Voordouw, 2002), but formate can also be produced by PFL. In fact, very low levels of formate were detected transiently after sulfate was consumed. These levels were higher in the mutants than in the wt, which agrees with a role of the FDHs in oxidizing formate under these conditions, pointing to a cycling mechanism during growth with lactate and sulfate.

With H₂ as the electron donor, both wt and mutants took longer to consume sulfate than with lactate as the electron donor, but it was eventually depleted after 40 h for wt in medium with Mo, and was reduced slightly faster in medium with W (Fig. 5b, d). In medium with Mo, a low level of formate started to be detected at 20 h incubation, both in wt and in ΔfdhAB cells, and at 40 h in ΔfdhABC₃ cells (Fig. 5a). After sulfate was depleted, formate started to accumulate, and at the end of the incubation period the formate concentration was higher for the wt (10 mM) than for both mutants, indicating that FdhAB and FdhABC₃ were functioning as CO₂-reductases under these conditions. The rate of CO₂ reduction was similar for wt and ΔfdhABC₃, but was considerably slower for the ΔfdhAB strain. ΔfdhABC₃ cells accumulated more formate than ΔfdhAB cells (8 and 5 mM, respectively). In medium with W, formate started to accumulate at 20 h incubation for both wt and ΔfdhABC₃, and at a similar rate in both (Fig. 5c). At the end of the incubation there was 11 mM formate.

Fig. 3. Growth curves (a, c) and sulfate consumption (b, d) of the wt, ΔfdhAB and ΔfdhABC₃ strains in 1 bar (10⁵ Pa) overpressure of H₂/CO₂ (80%/20%) and sulfate (38 mM), in the presence of either Mo (0.1 μM; a and b) or W (0.1 μM; c and d). Data are means from duplicate experiments; error bars indicate se.
for the wt and 10 mM for the ΔfdhABC3 mutant. In contrast, only 0.4 mM formate accumulated in the incubation with ΔfdhAB cells.

**DISCUSSION**

Many bacterial and archaean genomes code for multiple FDHs. Isoenzymes with different functions, expressed
under different conditions, with different cell locations, or incorporating different metals in the active site often coexist in the same organism (Chistoserdova et al., 2007; Matson et al., 2010; Pierce et al., 2008; Sawers, 2005; Wood et al., 2003). This allows the use of different pathways for energy conservation and adaptation to environmental constrains, such as substrate or metal availability. The *D. vulgaris* genome codes for three periplasmic FDHs, suggesting a function mainly in formate oxidation. The two main enzymes detected (FdhABC3 and FdhAB) (da Silva et al., 2011) are soluble and interact with the cytochrome c pool. In the present work we constructed deletion mutants of each of these enzymes to gain insight into their role in energy metabolism during growth with formate, but also with lactate or hydrogen, two of the main energy substrates for SRB. In interpreting the results it is important to keep in mind that these substrates, as well as the metals Mo and W, are involved in regulating FDH expression (da Silva et al., 2011). In the presence of Mo, the main FDH expressed is FdhABC3, and this is significantly upregulated by formate. In the presence of W the FdhABC3 is downregulated and Fdhab is the main enzyme, and this is upregulated with both formate and H2. Similar observations have been made for FDHs from *Desulfovibrio alaskensis* (Mota et al., 2011).

With formate as energy source, the role of FDHs is rather straightforward. They oxidize formate to CO2, releasing protons in the periplasm and electrons that are transferred through membrane-bound carriers to the cytoplasm to reduce sulfate, thus generating a proton motive force. Upon growth with formate the two mutants revealed different phenotypes in medium with Mo or W. ΔfdbhAB was most impaired, showing a lower growth rate than wt and the ΔfdhABC3 mutant in medium with Mo, whereas in the presence of W this mutant was unable to grow with formate. This is due to the downregulation of FdhABC3 by W and its inability to incorporate this metal. Thus, FdhAB is essential for growth in the presence of W. However, it also plays a role in Mo conditions, since the ΔfdhAB mutant did not grow identically to the wt in the presence of Mo.

The ΔfdhABC3 mutant grew slower than the wt in Mo conditions, and the final biomass yield was considerably lower, which agrees with the fact that FdhABC3 is the main FDH present in formate/Mo conditions (da Silva et al., 2011). In the presence of W, the ΔfdhABC3 mutant grew at the same rate as and with similar cell yield to the wt, indicating that FdhABC3 does not play a role in these conditions, where FdhAB is the main FDH present. Thus, when Mo is available both soluble FDHs play a role during growth with formate, whereas only Fdhab is involved in the presence of W. These results highlight the importance of considering the effect of trace elements in the phenotypic analysis of mutants. This effect is often neglected, but as shown here can be very significant if these elements are involved in regulating the expression of the deleted genes.

When lactate is used as an electron donor for sulfate reduction, substrate level phosphorylation alone is not sufficient to generate energy, since the same number of ATP molecules is produced through this mechanism as is consumed for sulfate activation. Alternative mechanisms involving the cycling of intermediates, such as hydrogen (Odom & Peck, 1981), CO (Voordouw, 2002) or formate (Heidelberg et al., 2004; Pereira et al., 2008; Voordouw, 2002), were proposed. Lactate is first oxidized to pyruvate, whose metabolism can proceed via pyruvate:ferredoxin oxidoreductase (PFOR) or via the PFL. PFL produces formate that can be transported across the membrane and oxidized to CO2 by the periplasmic FDHs. Both mutants showed impaired growth in lactate and sulfate in the presence of either Mo or W, in agreement with the less pronounced regulatory effect of these metals upon lactate growth (da Silva et al., 2011). This result shows for the first time that both FdhAB and FdhABC3 play a role during growth on this substrate, which provides strong evidence for the formate cycling hypothesis during growth with lactate and sulfate. This hypothesis is further supported by the observation that in *D. alaskensis* G20 (formerly *Desulfovibrio desulfuricans* G20) a mutant containing a deletion of the gene coding for cytochrome c5, an electron acceptor for the periplasmic FDHs, accumulates formate during growth with lactate and sulfate (Li et al., 2009).

When lactate is present in excess, as in the experiments with concentrated cell suspensions, cells turn to a fermentative metabolism after sulfate is consumed. Here, we could barely detect accumulation of formate, which agrees with previous reports indicating that H2 rather than formate is produced in lactate fermentation, and is the main metabolite responsible for interspecies electron transfer in syntrophic growth (Pankhania et al., 1988; Stolyar et al., 2007). However, formate also plays a role, albeit less important, as indicated by reduced growth of a syntrophic association between *D. vulgaris* and a mutant of *Methanococcus maripaludis* containing a deletion of two FDHs (Stolyar et al., 2007), or by the reduced ability for syntrophic growth on lactate of a PFL deletion mutant of *D. alaskensis* G20 (Li et al., 2011).

Regarding growth in H2/CO2, it was previously shown that expression of FDHs and PFL activating enzyme is higher in *D. vulgaris* when cells are grown with H2/CO2 relative to lactate (Pereira et al., 2008), suggesting that in these conditions FDHs could also be involved in energy conservation. However, the role played by FDHs during growth with H2/CO2 is not clear. No significant differences in growth were observed between the mutants and the wild-type during growth in H2/sulfate, indicating that the FDHs are not essential. If FDHs are functioning as CO2-reductases, then less formate would be expected to accumulate in the mutants versus the wt. During growth with H2/CO2/sulfate, no formate production was detected, but with concentrated cell suspensions placed under H2/CO2, formate accumulation was observed as sulfate became depleted, and this accumulation was faster and reached
higher concentrations in wt cells, indicating that formate is indeed being produced by the FDHs from reduction of CO₂. When sulfate becomes depleted, an excess of reductive power accumulates. In this situation, the FDHs may function in reverse to reduce CO₂, which replaces sulfate as electron acceptor, providing an electron and proton sink for H₂ oxidation. The results indicate that the main FDH acting as CO₂-reductase in these conditions is FdhAB, although in Mo the FdhABC₃ also plays a role. Previously, we tested both purified enzymes for CO₂ reduction activity and could not detect activity for FdhAB, while a low activity was detected for FdhABC₃ (da Silva et al., 2011). However, the present results indicate that in vivo, FdhAB does indeed function as CO₂-reductase. W-containing FDHs are proposed to function mainly as CO₂-reductases because of the lower redox potential of W(V)/W(VI) compared to Mo(V)/Mo(VI), and many of them are extremely sensitive to oxygen, although Desulfovibrio gigas FdhAB appears to be an exception (Almendra et al., 1999; Hagen et al., 2009). Most likely the CO₂-reductase activity of isolated D. vulgaris FdhAB was lost upon purification.

The observed production of formate from hydrogen and CO₂ may have biological and environmental significance. When growing syntrophically, SRB ferment organic acids and alcohols, as long as the end products (acetate, CO₂, H₂ and possibly formate) are consumed by other organisms, keeping their concentration low (Bryant and possibly formate) are consumed by other organisms, keeping their concentration low (Bryant et al., 1977; Muyzer & Stams, 2008). Although SRB are generally considered to provide enough energy for microbial growth. In conclusion, this work provides evidence for the occurrence of intracellular formate cycling during growth of D. vulgaris Hildenborough with lactate and sulfate, as one of several metabolic pathways the organism uses for energy conservation. Both soluble FDHs were found to play a role in this process. During growth with H₂ and sulfate (in the presence of CO₂ and acetate), the FDHs are apparently non-essential, but when sulfate is exhausted they reduce CO₂ to formate, in what may be a newly recognized pathway to allow syntrophic growth of SRB on hydrogen. Further work will be required to test this hypothesis.

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