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## The electron-bifurcating FeFe-hydrogenase Hnd is involved in ethanol metabolism in *Desulfovibrio fructosovorans* grown on pyruvate

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1 The electron-bifurcating FeFe-hydrogenase Hnd is involved in ethanol metabolism in *Desulfovibrio*  
2 *fructosovorans* grown on pyruvate

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23

24 **Abstract**

25 *Desulfovibrio fructosovorans*, a sulfate-reducing bacterium, possesses six gene clusters encoding six  
26 hydrogenases catalyzing the reversible oxidation of H<sub>2</sub> into protons and electrons. Among them, Hnd is an  
27 electron-bifurcating hydrogenase, coupling the exergonic reduction of NAD<sup>+</sup> to the endergonic reduction of  
28 a ferredoxin with electrons derived from H<sub>2</sub>. It was previously hypothesized that its biological function  
29 involves the production of NADPH necessary for biosynthetic purposes. However, it was subsequently  
30 demonstrated that Hnd is instead a NAD<sup>+</sup>-reducing enzyme, thus its specific function has yet to be  
31 established. To understand the physiological role of Hnd in *D. fructosovorans*, we compared the *hnd*  
32 deletion mutant with the wild-type strain grown on pyruvate. Growth, metabolites production and  
33 consumption, and gene expression were compared under three different growth conditions. Our results  
34 indicate that *hnd* is strongly regulated at the transcriptional level and that its deletion has a drastic effect  
35 on the expression of genes for two enzymes, an aldehyde ferredoxin oxidoreductase and an alcohol  
36 dehydrogenase. We demonstrated here that Hnd is involved in ethanol metabolism when bacteria grow  
37 fermentatively and proposed that Hnd might oxidize part of the H<sub>2</sub> produced during fermentation  
38 generating both NADH and reduced ferredoxin for ethanol production via its electron bifurcation  
39 mechanism.

40

41

42

## 43 Introduction

44 Sulfate reducers belonging to the  $\delta$ -Proteobacterial genus *Desulfovibrio* are ubiquitous in anoxic  
45 habitats and employ a respiratory metabolism with sulfate as the terminal electron acceptor. Molecular  
46 hydrogen plays an important role in the energy metabolism of these bacteria which can switch from  
47 respiration to fermentation. Depending on the growth conditions, H<sub>2</sub> can be used as the sole energy source  
48 during respiration or produced in order to oxidize reduced electron carriers to maintain the continuous  
49 conversion of the substrates during fermentation (Thauer *et al.*, 2007, Fauque *et al.*, 1988, Rabus *et al.*, 2013).  
50 This ability of *Desulfovibrio* to consume and produce hydrogen is exploited during syntrophic associations  
51 anaerobic environments (Bryant *et al.*, 1977, Stolyar *et al.*, 2007, Meyer *et al.*, 2013a, Meyer *et al.*, 2013b).

52 Hydrogenases, the key enzymes involved in the metabolism of molecular hydrogen, catalyze the  
53 reversible oxidation of H<sub>2</sub> into protons and electrons. Most of the known hydrogenases are iron-sulfur proteins  
54 and are classified into two main distinct families according to the two metal atoms at their active site, the  
55 [NiFe]-hydrogenases (Volbeda *et al.*, 1995, Higuchi *et al.*, 1997) and the [FeFe]-hydrogenases (Peters *et al.*,  
56 1998, Nicolet *et al.*, 1999). These enzymes are widely distributed in *Bacteria* and *Archaea* as well as in  
57 unicellular eukaryotes (Vignais *et al.*, 2001, Vignais & Billoud, 2007, Benoit *et al.*, 2020).

58 The versatility of the *Desulfovibrio* H<sub>2</sub> metabolism is due to a complex hydrogenase system composed  
59 of several different enzymes located in different cellular compartments (Thauer *et al.*, 2007, Pereira *et al.*,  
60 2011, Baffert *et al.*, 2019). The number, the type and the cellular location of these enzymes vary considerably  
61 from one *Desulfovibrio* species to another (Pereira *et al.*, 2011, Baffert *et al.*, 2019) and this diversity makes the  
62 role of these various hydrogenases difficult to determine. Forty years ago, a model for the growth of  
63 *Desulfovibrio* in lactate medium in the presence of sulfate, the “hydrogen cycling model”, was proposed (Odom  
64 & Peck, 1981). In this model, electrons and protons produced by lactate oxidation are used by a cytoplasmic  
65 hydrogenase to produce H<sub>2</sub>. After the diffusion through the cytoplasmic membrane, H<sub>2</sub> is re-oxidized by a  
66 periplasmic hydrogenase contributing to the proton gradient across the membrane used for ATP synthesis. The  
67 electrons generated by this oxidation are then returned to the cytoplasm through several integral membrane  
68 complexes where they are used for sulfate reduction. Since then, several models for energy conservation in

69 *Desulfovibrio* have been proposed in which, in addition to the H<sub>2</sub> recycling pathway, there is another H<sub>2</sub>-  
70 independent pathway involving a direct electron transfer from the donor to the acceptor (Keller & Wall, 2011,  
71 Noguera *et al.*, 1998, Sim *et al.*, 2013).

72 In *Desulfovibrio fructosovorans*, our biological model, three hydrogenases have been already  
73 characterized: Hyn, a periplasmic [NiFe]-hydrogenase (Hatchikian *et al.*, 1990, Rousset *et al.*, 1990); Hyd, a  
74 periplasmic [FeFe]-hydrogenase (Casalot *et al.*, 1998) and Hnd, a cytoplasmic [FeFe]-hydrogenase (Malki *et al.*,  
75 1995, de Luca *et al.*, 1998, Kpebe *et al.*, 2018). *D. fructosovorans* genome analysis revealed the presence of  
76 three genes clusters encoding three additional putative hydrogenases (Baffert *et al.*, 2019): a membrane-  
77 bound Ech-type hydrogenase, a cytoplasmic trimeric [FeFe]-hydrogenase Hnt similar to the electron-bifurcating  
78 hydrogenase from *Thermotoga maritima* (Schut & Adams, 2009), and a [FeFe] cytoplasmic hydrogenase  
79 showing similarity with the putative sensory [FeFe]-hydrogenase HydS from *T. maritima* (Chongdar *et al.*, 2018)  
80 or the Hfs hydrogenase from *Thermoanaerobacterium saccharolyticum* (Shaw *et al.*, 2009). The specific  
81 function of these six enzymes is not yet clearly established in *D. fructosovorans*. Marker-exchange mutagenesis  
82 was performed to determine the role of hydrogenases in the energy metabolism of the bacterium (Malki *et al.*,  
83 1997, Casalot *et al.*, 2002a, Casalot *et al.*, 2002b). Results clearly indicated an effect of the depletion of  
84 hydrogenases on the growth behavior of *D. fructosovorans*. However, it was difficult to hypothesize on the  
85 physiological implications of each hydrogenase due to enzymatic compensation processes as the result of the  
86 deletion of the genes encoding one, two or three hydrogenases (Malki *et al.*, 1997, Casalot *et al.*, 2002a,  
87 Casalot *et al.*, 2002b, Rousset *et al.*, 1991).

88 We recently produced and purified a recombinant form of Hnd and showed that it is an electron-  
89 bifurcating hydrogenase that simultaneously reduces two different electron acceptors, NAD<sup>+</sup> and a  
90 ferredoxin in the presence of H<sub>2</sub> (Kpebe *et al.*, 2018), despite it being previously described as an NADP<sup>+</sup>-  
91 reducing enzyme (Malki *et al.*, 1995, de Luca *et al.*, 1998). Flavin-based electron bifurcation is an important  
92 mechanism of energy conservation, mainly found in anaerobic microorganisms, coupling an endergonic  
93 redox reaction to an exergonic redox reaction (Buckel & Thauer, 2018a, Buckel & Thauer, 2018b). This  
94 tetrameric enzyme is encoded by the *hnd* operon, in which *hndA* and *hndC* encode the NAD-dependent unit

95 and *hndD* encodes the [FeFe]-hydrogenase subunit, which is closely related to the hydrogenase Cpl from  
96 *Clostridium pasteurianum* (Malki *et al.*, 1995). The function of the product of the *hndB* gene is unknown.  
97 This cytoplasmic Hnd complex catalyzes, *in vitro*, the coupled endergonic reduction of ferredoxin and the  
98 exergonic reduction of NAD<sup>+</sup> by the oxidation of H<sub>2</sub> (Kpebe *et al.*, 2018).

99 Initially, it was hypothesized that the biological function of Hnd involves the production of NADPH  
100 necessary for biosynthetic purposes (de Luca *et al.*, 1998) but because it was subsequently determined that  
101 Hnd is in fact a NAD<sup>+</sup>-reducing enzyme (Kpebe *et al.*, 2018), its specific function has yet to be established. In  
102 order to understand the involvement of the electron-bifurcating Hnd hydrogenase in the energy-generating  
103 metabolism in *D. fructosovorans*, we compared the *hndD* deletion mutant strain (SM4) (Malki *et al.*, 1997)  
104 with the wild-type strain grown on different pyruvate-containing media. Our results indicate that Hnd is not  
105 essential for the bacterial growth when pyruvate is used as the carbon source, and that this hydrogenase is  
106 involved in ethanol metabolism when bacteria grow fermentatively.

107

## 108 **Results**

### 109 *Growth of D. fructosovorans on pyruvate-containing media*

110 The growth of the wild-type strain and the SM4 strain, in which the *hndD* gene encoding the  
111 hydrogenase catalytic subunit of Hnd is deleted (Malki *et al.*, 1997) was measured in batch cultures (Figure  
112 1). Two different growth conditions were compared, both containing 25 mM pyruvate as the only source of  
113 energy: a medium containing 20 mM sulfate as electron acceptor (PS20 medium, respiratory condition) and  
114 a medium without any electron acceptor (PS0 medium, fermentative condition). Since growth during  
115 fermentation is very slow compared to sulfate respiration, the cells were also grown under a third condition  
116 in which they grow faster on a medium that contains a limiting concentration of sulfate (PS2 medium  
117 containing 2mM sulfate, mixed culture condition). In each condition, there were no striking differences  
118 among the growth curves of the two strains (Figure 1). However, growth yields as well as doubling times of  
119 both strains are significantly different under the three conditions (Table 1). In the fermentative condition,

120 the growth is slow for both strains but even slower for the mutant strain, with a doubling time of 369 h for  
121 the WT compared to 519,5 h for the mutant. However, the overall growth yield of the mutant is unaffected,  
122 because a similar maximum absorbance around 0.70 at 600 nm is reached for the two strains that  
123 correspond to 6.7/6.9. 10<sup>9</sup> cells/mL. In the respiratory condition (PS20), the growth is faster and both  
124 strains behave similarly: the growth reaches a maximum absorbance of 1.3-1.4 at 600 nm (1.33-1.41. 10<sup>9</sup>  
125 cells/mL) and the generation time is around 20 h for the WT strain and 15h for SM4. Under sulfate-limited  
126 conditions, the growth exhibits two different phases, a rapid one (phase 1, until around 40 h) before the  
127 sulfate is exhausted (see Figure S1 for the quantification of sulfate) and a slow one (phase 2, from around  
128 40 h to 110 h) in which there is no more sulfate in the medium. While the generation times of phase 1  
129 measured for both strains are similar to those of the respiratory condition, those of phase 2 are much  
130 faster than that of the fermentation condition and in this phase, the growth of the mutant strain is slightly  
131 slower than that of the WT strain. Taken together, these results indicate that Hnd is not essential for  
132 bacterial growth under either fermentative or respiratory conditions when pyruvate is used as the carbon  
133 source and that in the SM4 mutant, the generation time is affected only when there is no sulfate in the  
134 medium (fermentative condition or phase 2 of the mixed condition). On the other hand, biomass yields  
135 (maximum cell counts/mL) are not affected at all in the mutant regardless of the growth conditions.

136

### 137 *Impact of hnd deletion on metabolite production and consumption*

138 To evaluate the impact of *hndD* deletion on *D. fructosovorans* physiology, we compared pyruvate  
139 consumption as well as acetate, ethanol and H<sub>2</sub> production (Figure 2). The pyruvate is completely  
140 consumed, with the rate depending on the initial sulfate concentration in the medium: the more sulfate  
141 there is, the faster the consumption. Pyruvate consumption and acetate production are not significantly  
142 different between the two strains on PS20 and PS2 media whereas, in the absence of sulfate, both pyruvate  
143 and acetate are respectively consumed or produced slowly by the mutant than by the WT strain. However,  
144 there is a striking difference between the two strains in terms of the production of ethanol (Figure 2). Both  
145 strains produce ethanol only during mixed and fermentation conditions. In both conditions, the mutation

146 significantly affects the ethanol formation, since the SM4 mutant produces less ethanol than the WT strain  
147 (4 times less in fermentation condition and 10 times less in mixed growth condition). The highest level of  
148 produced ethanol is reached under fermentation conditions for both strains.

149 There is also a notable difference in H<sub>2</sub> production between the two strains (Figure 2). There is no  
150 H<sub>2</sub> production in respiratory conditions for either strain. Under fermentation conditions, however, H<sub>2</sub>  
151 production is more than twice as high in the mutant as in the WT strain. Under mixed conditions, the  
152 amount of H<sub>2</sub> in the headspace at the end of the exponential phase is 1.5 times higher for SM4 than for the  
153 WT strain. However, the H<sub>2</sub> amount at the end of the growth is identical for both strains due to differences  
154 of production kinetics. Collectively, these results show that the SM4 mutant strain is affected under mixed  
155 and fermentation conditions but not under respiratory conditions, suggesting that Hnd has a role in  
156 fermentative metabolism. Under fermentative conditions, the mutant produces more H<sub>2</sub> and less ethanol  
157 than the WT strain, indicating that H<sub>2</sub> and ethanol production pathways are therefore affected in the  
158 mutant. In this study, all subsequent analyses were performed on strains grown under respiration and  
159 mixed conditions because under fermentation conditions, growth is very slow and very little cellular  
160 material is obtained.

161

#### 162 *Impact of hnd deletion at the protein level*

163 Coomassie-blue-stained SDS-PAGE was used to compare the soluble protein pattern of the WT and  
164 SM4 strains grown under mixed or respiratory conditions. At the end of the exponential phase of growth,  
165 total soluble proteins were prepared and migrated on SDS-PAGE (Figure 3A). The bands indicating  
166 significant differences in protein levels between the two strains were cut out from the gel and the proteins  
167 were identified by mass spectrometry (Figure 3B and Table S1 for complete list of identified proteins). As  
168 shown in Figure 3, the protein profiles are different for the two strains. Three bands (1, 2 and 3) that were  
169 significantly more intense in the WT strain than in the mutant strain were analyzed. Results are reported in  
170 Figure 3B in which only proteins identified with the highest score in each gel band are reported (for a



171 complete list of identified proteins, see Table S1). The most striking difference concerns band 1  
172 corresponding to a protein whose gene has been annotated as iron-containing alcohol dehydrogenase  
173 (Adh) (locus tag: DesfrDRAFT\_3929). The intensity of this band suggests that this protein is very strongly  
174 produced in the WT strain whereas it is almost absent in the mutant strain under both growth conditions.  
175 Similarly, the analysis of band 3 (Figure 3) reveals the presence of a protein whose gene is annotated as  
176 aldehyde ferredoxin oxidase (Aor) (locus tag: DesfrDRAFT\_2487). Band 2 (Figure 3) corresponds to the  
177 HndC subunit of Hnd. The intensities of the Adh and Aor bands in Figure 3 suggest that the amount of these  
178 two enzymes is greater in the WT strain grown in PS2 medium than in PS20.

179

#### 180 *Expression of hnd on pyruvate medium*

181 Metabolite production analysis suggests that Hnd has a role in fermentative metabolism.  
182 Expression of *hndD* was determined in the WT strain using RT-qPCR under both mixed and respiratory  
183 conditions. Figure 4 shows that *hndD* expression is much higher (by a factor of 80) in cells grown in PS2  
184 than in PS20. Hnd production was followed by western blot (Figure 5A). While the enzyme is detected in  
185 bacteria that grew under mixed conditions (PS2), it is not detected in those that grew in respiration  
186 condition (PS20). The same profile of Hnd production is obtained when *D. fructosovorans* used a limiting  
187 concentration of fumarate or sodium thiosulfate as electron acceptor instead of sulfate (Figure S2).

188 Cytoplasmic extracts of the WT strain at the early stationary phase grown in PS2 or PS20 medium were  
189 migrated on a Blue-Native gel electrophoresis followed by in-gel detection of hydrogenase activity (Figure  
190 5B). An activity band, corresponding to Hnd (Kpebe *et al.*, 2018), is visible when the strain grew under  
191 mixed condition but not under respiratory condition, which is consistent with the higher *hndD* gene  
192 expression and the higher protein production under mixed condition than under respiration. Taken  
193 together, these results demonstrate that the expression of *hnd* operon is not constitutive and is strongly  
194 regulated at the transcriptional level, and that *hnd* expression is much higher under mixed condition than  
195 respiratory condition suggesting that the enzyme has a role in fermentative metabolism.

196

197 *aor and adh gene expression*

198           The expression of *adh* and *aor* genes was analyzed by RT-qPCR to determine if the difference in the  
199 amount of the two enzymes in both strains was due to a difference in their gene expression (Figure 4). In  
200 the WT strain, as for the *hnd* gene, *adh* and *aor* expressions are much higher when bacteria grow in mixed  
201 condition than in respiration (increased by a factor of 13 for *adh* and 10 for *aor*) indicating a transcriptional  
202 regulation. However, the expression of both genes is strongly decreased in the mutant strain, with almost  
203 no expression of either gene being detected. Gene expression analysis shows that the deletion of the *hndD*  
204 gene drastically impacts the expression of both *adh* and *aor* genes, which is consistent with the findings of  
205 the proteomics analysis.

206

207 *Adh level in the periplasmic FeFe-hydrogenase deletion strain*

208           Haveman *et al.* pointed out that, in DvH, in the mutant strain deleted of the periplasmic FeFe-  
209 hydrogenase, the *adh* gene is strongly downregulated (Haveman *et al.*, 2003). We compared, using SDS-  
210 PAGE, the production of Adh by the *D. fructosovorans* WT strain with the mutant strain that is deleted of its  
211 periplasmic FeFe-hydrogenase (SF mutant) (Casalot *et al.*, 2002b) to verify whether, as in DvH, the deletion  
212 of this hydrogenase in *D. fructosovorans* has an impact on the level of Adh (Figure S3). In contrast to what  
213 was observed in the DvH hydrogenase mutant (Haveman *et al.*, 2003), the amount of Adh in the SF strain is  
214 comparable to that present in the WT strain. Furthermore, the SF strain grows as well as the WT strain on  
215 PS2 medium (Figure S4). These results suggest that the regulatory mechanisms involved are different in the  
216 two *Desulfovibrio* species.

217

218 *Complementation of the hnd deletion*

219 In order to confirm that the phenotype of the SM4 deletion strain resulted from the absence of the  
220 Hnd hydrogenase, complementation analyses were performed. The growth of the complementation strain  
221 SM4/pBGhnd6 on PS2 medium is similar to that of WT and SM4 strains (Figure S3). The *hnd* operon is  
222 expressed from the pBGhnd6 plasmid in the SM4 strain, as can be observed in Figure 6A showing the  
223 relative quantification of *hndD* mRNA levels measured by RT-qPCR. However, in the SM4/pBGhnd6 strain,  
224 the expression level does not reach that of the WT strain. Western blot analysis indicates that whereas the  
225 level of HndD is very high in the WT strain grown under mixed culture conditions, it is lower in the  
226 complementation strain (Figure 5A). Quantification of the intensity of the band corresponding to HndD for  
227 the complementation strain on PS2 is almost 2.5 times less intense than for the WT strain. Similarly, the  
228 complementation strain produces an intermediate amount of ethanol between that produced by the WT  
229 and SM4 strains (Figure 6B). Taken together, these results indicate first that the expression of *hnd* from the  
230 pBGhnd6 plasmid does not fully complement the SM4 strain but also that the low expression of *hnd*  
231 partially restores ethanol production in this strain.

232

## 233 Discussion

234 In this study, *D. fructosovorans* WT strain was compared to the SM4 mutant strain lacking Hnd ( $\Delta hndD$ )  
235 (Malki *et al.*, 1997) in order to get new insights into the physiological role of this electron-bifurcating  
236 hydrogenase when the bacteria grow on pyruvate as a carbon and energy source, under respiratory (PS20  
237 medium) and fermentative (PS0 medium) conditions. We have also tested a mixed culture condition (PS2  
238 medium) using a limiting sulfate concentration, making it possible to reach a higher growth rate as compared  
239 to the fermentative condition. After sulfate depletion, the metabolism switches from respiration to  
240 fermentation. Under this condition, the *hnd* operon is much more expressed than in the respiratory condition  
241 (PS20 medium) (Figure 4). The increase in expression level is followed by an increase in enzyme production as  
242 revealed by western blot and in-gel hydrogenase activity detection (Figure 5). This suggests that Hnd  
243 hydrogenase plays a role under fermentative conditions.

244 Deletion of the *hndD* gene leads to a drastic decrease in the expression of the two genes *adh*  
245 (*DesfrDRAFT\_3929*) and *aor* (*DesfrDRAFT\_2487*) on PS2 medium, coding respectively for proteins annotated as  
246 iron-containing alcohol dehydrogenase (Adh) and aldehyde ferredoxin oxidoreductase (Aor) (Figures 3 and 4).  
247 Alcohol dehydrogenases are a group of oxidoreductases that catalyze the interconversion of alcohols to  
248 aldehydes or ketones. They are ubiquitous and most of them are NAD- or NADP-dependent enzymes. The size  
249 and metal content of Adh vary (for reviews, see (Gaona-Lopez *et al.*, 2016, Radianingtyas & Wright, 2003, Reid  
250 & Fewson, 1994)). Adh3929 from *D. fructosovorans* shows high sequence identity with iron-containing alcohol  
251 dehydrogenases from various bacteria (52 % with the iron-containing Adh (GI 56552492) from *Zymomonas*  
252 *mobilis* for example) and contains a conserved iron-binding motif (GX<sub>2</sub>HX<sub>2</sub>AHX<sub>2</sub>GX<sub>5</sub>PHG where X denotes any  
253 amino acid). Adh3929 is highly expressed in *D. fructosovorans* (Figures 3 and 4) and it has been shown  
254 previously that it is one of the most abundant enzyme in *Desulfovibrio* (Haveman *et al.*, 2003, Ramos *et al.*,  
255 2015). Ramos *et al.* purified Adh1 (DVU2405) from *D. vulgaris* Hildenborough (DvH), which has 81 % sequence  
256 identity to Adh3929, and showed that this enzyme is NAD-dependent and uses ethanol as substrate (Ramos *et*  
257 *al.*, 2015). Aor are tungsten-containing enzymes sensitive to O<sub>2</sub>. They catalyze the reversible oxidation of an  
258 aldehyde to the corresponding acid using a ferredoxin. Aor2487 from *D. fructosovorans* shows 37% sequence  
259 identity with Aor from *Pyrococcus furiosus* whose crystallographic structure has been solved (Chan *et al.*,  
260 1995). These two enzymes Adh and Aor can therefore be involved in the same metabolic pathway since the  
261 product of one can be the substrate of the other. Taken together, our data reveal that Hnd is involved in  
262 ethanol metabolism when *D. fructosovorans* grows in pyruvate under mixed conditions, which led us to  
263 propose a model in which the hydrogenase Hnd would oxidize part of the H<sub>2</sub> produced during fermentation  
264 generating both NADH and reduced ferredoxin as a result of its electron bifurcation mechanism (Figure 7). H<sub>2</sub>  
265 production results from the release of reducing power in the absence of terminal electron acceptors, enabling  
266 the oxidation of pyruvate. In this model, pyruvate would be converted into acetate by the two enzymes  
267 pyruvate:ferredoxin oxidoreductase and acetyl-CoA synthase. H<sub>2</sub> could be produced by the membrane  
268 hydrogenase Ech from reduced ferredoxin, which would contribute to the electrochemical proton gradient for  
269 ATP synthesis. This model proposes that the role of the Hnd hydrogenase would be to produce reducing  
270 equivalents (NADH and reduced ferredoxin) from H<sub>2</sub>, allowing the production of ethanol by Adh3929 and

271 Aor2487. The lower H<sub>2</sub> production of the WT compared to the SM4 mutant strains is also in agreement with  
272 the hypothesis that the physiological role of Hnd when pyruvate is present is thus to consume H<sub>2</sub>.

273 The determination of the hydrogenase activity, both H<sub>2</sub>-production and H<sub>2</sub>-oxidation, on total soluble extracts  
274 of WT and SM4 mutant strains grown under mixed condition (PS2) shows that both activities are strongly  
275 decreased in the mutant strain (Table S2). It is our assumption that the increased H<sub>2</sub> release in strain SM4  
276 (Figure 2) is due to a lack of consumption by Hnd, not an overproduction by Ech. Preliminary proteomic results  
277 demonstrate that Ech expression does not vary based on strain or growth condition (data not shown), and  
278 there is no indication that its activity would differ between the two strains. In strain SM4, when Hnd is not  
279 present, the H<sub>2</sub>-production activity measured in the soluble fraction is 44 times less than when it is present in  
280 the WT strain (Table S2). Moreover, H<sub>2</sub>-oxidation activity shows that strain SM4 consumes 18 times less H<sub>2</sub> than  
281 the WT (Table S2) most likely due to the lack of Hnd in this strain.

282 The recently characterized electron-bifurcating hydrogenases were isolated from the anaerobic bacteria  
283 *Thermotoga maritima* (Schut & Adams, 2009), *Acetobacterium woodii* (Schuchmann & Müller, 2012), *Moorella*  
284 *thermoacetica* (Wang et al., 2013), and *Ruminococcus albus* (Zheng et al., 2014). In the acetogenic bacteria *A.*  
285 *woodii* and *M. thermoacetica* which can produce acetate from CO<sub>2</sub> with H<sub>2</sub> as the reductant, these enzymes  
286 were found to catalyze the reduction of ferredoxin and NAD<sup>+</sup> with H<sub>2</sub>. NADH and reduced ferredoxin are used  
287 for CO<sub>2</sub> reduction and to contribute to the ion gradient across the membrane (Schuchmann & Müller, 2012,  
288 Wang et al., 2013). In *R. albus* and *T. maritima*, which are glucose fermenting bacteria, trimeric HydABC  
289 hydrogenase mediates H<sub>2</sub>-production by coupling the endergonic reduction of protons with NADH to the  
290 exergonic reduction of protons with reduced ferredoxin (Schut & Adams, 2009, Zheng et al., 2014). *R. albus*, a  
291 rumen bacterium, fermentatively produces acetate, ethanol, CO<sub>2</sub> and H<sub>2</sub> in pure culture and in this high H<sub>2</sub>-  
292 condition, H<sub>2</sub> is mainly generated via a ferredoxin-dependent hydrogenase (HydA2). However, *R. albus*  
293 redirects carbon flux from ethanol production to the more energetically efficient pathway of acetate  
294 production when it grows in co-culture with a hydrogenotrophic bacterium that keeps the H<sub>2</sub> partial pressure  
295 sufficiently low through interspecies hydrogen transfer. Under this low H<sub>2</sub>-condition, only acetate, CO<sub>2</sub> and H<sub>2</sub>  
296 are produced. The acetate-only pathway generates more ATP per mole of glucose and equimolar amounts of

297 NADH and reduced ferredoxin, which are then re-oxidized *via* the electron-confurcating HydABC hydrogenase  
298 to produce H<sub>2</sub> (Zheng *et al.*, 2014, Greening *et al.*, 2019). *D. fructosovorans*, like *R. albus*, also produces acetate,  
299 H<sub>2</sub>, ethanol and CO<sub>2</sub> (not measured in this study) when growing on pyruvate in the absence of an electron  
300 acceptor. In DvH, ethanol is also a minor product of pyruvate fermentation (Ramos *et al.*, 2015, Traore *et al.*,  
301 1981) and the electron flow is redirected towards ethanol when H<sub>2</sub> accumulates at the expense of the  
302 energetically more favourable production of acetate (Ramos *et al.*, 2015). However, in *R. albus*, HydABC is  
303 responsible for the production of H<sub>2</sub> when the *p*H<sub>2</sub> is kept very low whereas in *D. fructosovorans*, under  
304 fermentative conditions, Hnd is involved in the consumption of H<sub>2</sub> when H<sub>2</sub> accumulates allowing the reduction  
305 of NAD<sup>+</sup> for ethanol production.

306 In the WT strain, the expression profile of the three genes *hndD*, *adh* and *aor* is conserved in both PS2 and  
307 PS20 media (although *adh* has an expression level that is about twice that of the two genes in PS2) (Figure  
308 4) and shows that their expression is much higher in the mixed condition than in the respiration condition.  
309 Adh and Aor are thus overproduced when Hnd is overproduced. Their expression is thus strongly regulated  
310 at the transcriptional level, but the mechanism of regulation involved remains to be elucidated. Likewise,  
311 the molecular basis of the strong drop in *adh* and *aor* expression in the mutant has not yet been  
312 deciphered. The drop of Adh level in the SM4 mutant is specific for the deletion of *hnd* in *D. fructosovorans*  
313 and is not observed when another FeFe-hydrogenase gene is deleted (in the SF mutant, Figure S3) whereas  
314 in DvH, in the periplasmic FeFe-hydrogenase deleted strain, *adh* expression decreases drastically. It should  
315 be noted that DvH does not contain any electron-bifurcating hydrogenases.

316 We propose that the regulation of *hnd* expression might be under the control of Rex, which has been  
317 described as a global redox-sensing transcriptional repressor that is controlled by the intracellular NAD<sup>+</sup>/NADH  
318 ratio, a critical indicator for the redox state of the cell. NADH disrupts the interaction of Rex with its respective  
319 binding sites, proposed to be induced by a conformational change in the N-terminal domains of the Rex  
320 homodimer depending on NAD<sup>+</sup> or NADH binding (Park *et al.*, 2018, Christensen *et al.*, 2015, Brekasis & Paget,  
321 2003). Rex has been shown in several bacteria to regulate genes for central metabolism, for proteins involved in  
322 energy conversion and in a variety of metabolic processes (Ravcheev *et al.*, 2012). The presence of a predicted

323 Rex-binding site in the upstream untranslated region of the *hnd* operon (Figure S5) suggests that its expression  
324 might be controlled by the intracellular NAD<sup>+</sup>/NADH redox state. In DvH, Rex was demonstrated to be a  
325 transcriptional repressor of *sat*, a gene encoding for a sulfate adenylyl transferase which is involved in sulfate  
326 respiration. Surprisingly, *sat* transcript levels were highest when the cells were grown with pyruvate in the  
327 absence of sulfate, with the highest levels being found in the  $\Delta$ *rex* strain, suggesting that Rex induces the  
328 expression of genes encoding for proteins that are involved in scavenging sulfate and those that are implicated  
329 when the preferred electron acceptor is limiting (Christensen *et al.*, 2015). In *D. fructosovorans*, a putative *rex*  
330 gene (DesfrDRAFT\_2623) encodes a 24.8 kDa protein that shows 47 % sequence identity with the Rex repressor  
331 from *Thermotoga maritima* whose crystallographic structure has been solved (Park *et al.*, 2018). In the  
332 anaerobic hyperthermophile bacterium *Caldicellulosiruptor bescii*, structural genes for the NiFe Ech-type  
333 hydrogenase and the FeFe Hnd-type hydrogenase are members of the Rex regulon (Sander *et al.*, 2019,  
334 Ravcheev *et al.*, 2012). This suggests that Rex may play an important role in H<sub>2</sub> metabolism in this bacterium. In  
335 *D. fructosovorans*, the involvement of the putative Rex regulator remains to be determined experimentally. In  
336 contrast, a Rex binding site was not found in the promoter of *aor2487* and *adh3929*, suggesting that although  
337 the expression profile of these three genes is similar, their expression is not under the control of the same  
338 regulators.

339 As already mentioned, *D. fructosovorans* harbors a gene cluster for a putative H<sub>2</sub>-sensing [FeFe]-  
340 hydrogenase, called Hfs or HydS (DesfrDRAFT\_3590) whose mechanism has yet to be elucidated. This type  
341 of hydrogenase appears to have a regulatory role and the hydrogenase domain containing a non-canonical  
342 active site (Chongdar *et al.*, 2018) is fused to a C-terminal PAS domain. The sensory function of HydS has  
343 been predicted based on the presence of this PAS domain (Greening *et al.*, 2016). PAS domains are widely  
344 utilized for various signal transduction and sensory functions (Henry & Crosson, 2011) and are frequently  
345 found in signal transduction cascades involving serine/threonine protein kinases and phosphatases.  
346 Transcriptional studies of *R. albus* have provided some insights into the regulatory function of HydS (Zheng  
347 *et al.*, 2014). In this bacterium, the genes encoding for HydS are co-transcribed with the transcriptional  
348 repressor Rex and the ferredoxin-dependent [FeFe]-hydrogenase HydA2, whereas ~~there is an~~ genes  
349 encoding the electron-bifurcating hydrogenase HydABC are located on a separate operon (Zheng *et al.*,

350 2014). It is likely that HydS, in tandem with Rex, is sensing the NADH/NAD<sup>+</sup> ratio of the cell *via* the H<sub>2</sub> partial  
351 pressure, with the ratio being higher at high H<sub>2</sub> concentrations, and modulating the transcription of genes  
352 so that the NADH/NAD<sup>+</sup> ratio is balanced (Zheng *et al.*, 2014). Greening *et al.* also proposed that in *R. albus*,  
353 FeFe-hydrogenases are regulated by HydS as a result of direct H<sub>2</sub>-sensing (Greening *et al.*, 2019). It is of  
354 note that the presence of a *hydS* gene in the genome of *Desulfovibrio* species is always correlated with the  
355 presence of *hnd* genes (Baffert *et al.*, 2019). The coexistence of multimeric [FeFe]-hydrogenases and HydS  
356 is also found in other H<sub>2</sub>-forming bacteria such as in *Thermoanaerobacterium saccharolyticum*,  
357 *Thermoanaerobacter tengcongensis* and *Thermotoga maritima* (Chongdar *et al.*, 2018, Pan *et al.*, 2003,  
358 Shaw *et al.*, 2009, Soboh *et al.*, 2004) however the extent of the role of HydS in the regulatory cascade still  
359 needs to be clarified.

360 In DvH, an hexameric NADH dehydrogenase/heterodisulfide reductase complex Hdr-Flx is involved  
361 in ethanol metabolism. Strains mutated in genes that encode the proteins of this complex also produce a  
362 reduced amount of ethanol compared to the WT strain during pyruvate fermentation (Ramos *et al.*, 2015).  
363 The Hdr-Flx complex is proposed to be essential for NADH oxidation during growth on ethanol, probably  
364 using an electron bifurcation mechanism that leads to the reduction of a ferredoxin and a small protein,  
365 DsrC, involved in sulfate reduction, whereas during fermentation, the complex would function in the  
366 opposite direction reducing NAD<sup>+</sup> for ethanol production (Ramos *et al.*, 2015). In *D. fructosovorans*, the 7  
367 genes encoding this complex are present in the genome immediately downstream of the *adh* gene  
368 (*desfrDRAFT\_3930* to *3936*), suggesting that it may also be involved in ethanol metabolism but its role in *D.*  
369 *fructosovorans* remains to be determined.

370 In conclusion, we demonstrated that the electron-bifurcating hydrogenase Hnd is involved in  
371 ethanol metabolism in *D. fructosovorans* in pyruvate medium under fermentative conditions. The deletion  
372 of the *hnd* gene has a drastic effect on the expression of both *adh* and *aor* genes. Although the link  
373 between the deletion of *hnd* and the decrease in the expression of both *adh* and *aor* as well as the  
374 regulatory mechanisms at play remain to be understood, this study nevertheless provides a significant  
375 advance in the function of Hnd in *D. fructosovorans*.



376

## 377 **Experimental procedures**

### 378 *Bacterial strains and growth conditions.*

379 The WT strain of *Desulfovibrio fructosovorans* DSM 3604 (Ollivier *et al.*, 1988) and the two mutants, SM4  
380 and SF strains, which are deleted of *hndD* encoding the hydrogenase catalytic subunit of Hnd ( $\Delta hndD$ , Cm<sup>R</sup>)  
381 (Malki *et al.*, 1997) or of *hydAB* encoding the small and large subunits of the periplasmic [FeFe]-  
382 hydrogenase ( $\Delta hydAB$ , Gm<sup>R</sup>) (Casalot *et al.*, 2002b), respectively, were used in this study. To complement  
383 the deletion strain SM4, the pBGhnd6 plasmid containing the entire *hnd* operon including the promoter  
384 region (600 bp) was constructed and used to transform the SM4 strain as previously described (Kpebe *et*  
385 *al.*, 2018) to produce the SM4/pBGhnd6 strain.

386 Strains were grown anaerobically at 37°C in different media. The basal medium (pH 6.8) contains 25 mM  
387 sodium pyruvate, 8 mM NH<sub>4</sub>Cl, 1.3 mM CaCl<sub>2</sub>, 17.1 mM NaCl, 6.7 mM KCl, 2.1 mM MgCl<sub>2</sub> and trace  
388 elements (Rousset *et al.*, 1998) and corresponds to the PS0 medium. Na<sub>2</sub>SO<sub>4</sub> was added to the basal  
389 medium to obtain the PS20 medium containing 20 mM sulfate. MgCl<sub>2</sub> and CaCl<sub>2</sub> in the basal medium were  
390 replaced by 1 mM CaSO<sub>4</sub> and 1 mM MgSO<sub>4</sub> to obtain the PS2 medium containing 2 mM sulfate. Prior to  
391 inoculation, 20 mM KH<sub>2</sub>PO<sub>4</sub> pH 6.8, 2.5 mM Na<sub>2</sub>S and 10 mM NaHCO<sub>3</sub> were added. Thiamphenicol (35  
392 µg/mL) or gentamicin (20 µg/mL) were added to the SM4 or SF mutant culture media. The media were  
393 inoculated with 5% (v/v) of fresh cultures.

### 394 *Hydrogen production measurement*

395 Cells were grown in 100 mL bottles containing 90 mL of PS0, PS2 or PS20 medium. The media were  
396 inoculated with fresh cultures of WT or SM4 strains grown on PS2 medium. Each culture was done in  
397 triplicate. For H<sub>2</sub> quantification, 100 µL of headspace gas was removed periodically using a gastight syringe  
398 and injected into a gas chromatography system (Agilent 7820A) equipped with a thermal conductivity  
399 detector and a GS-Carbonplot 115-3133 column (30 m, 530 µm, 3 µm), using nitrogen as the carrier gas, at

400 a flow rate of 8.2 ml/min, an oven temperature of 30 °C and a detector temperature of 200 °C. The H<sub>2</sub>  
401 production is expressed as micromols of H<sub>2</sub> accumulated in the headspace.

#### 402 *HPLC analysis*

403 Acetate production, pyruvate and ethanol consumption were followed by HPLC. The analysis was  
404 performed on the same *D. fructosovorans* cultures as those used to measure H<sub>2</sub> production and as those  
405 used for the growth curves. 1 mL of culture samples were collected at different growth phase, centrifuged  
406 at 14 100 g for 6 min to remove the cells, filtered through a 0.22 µm filter and stored at -20°C until use.  
407 Immediately before the analysis, 10 µL of H<sub>2</sub>SO<sub>4</sub> 0,5 M were added to 490 µL of sample to obtain a final  
408 concentration of 10 mM of H<sub>2</sub>SO<sub>4</sub> and the samples were centrifugated for 5 min. The analysis was carried  
409 out on an Agilent 1200 HPLC system equipped with a refractometer (Agilent Technologies). Acetate,  
410 ethanol and pyruvate separation was achieved with an Agilent Hi-Plex H column (300 x 7.7mm) set at 50 °C.  
411 25 µL of samples were injected. The compounds were eluted with 10mM sulfuric acid mobile phase at a  
412 flow rate of 0.6 mL.min<sup>-1</sup>.

#### 413 *Ethanol quantification with an enzymatic kit*

414 When indicated, ethanol production in the growth medium was determined with an enzymatic kit  
415 from Boehringer Mannheim/R-Biopharm according to the manufacturer instructions. This method is based  
416 on quantifying NADH formed from ethanol through the combined action of Adh and aldehyde  
417 dehydrogenase.

#### 418 *Sulfate determination*

419 The concentration of sulfate was measured using a turbidimetric method previously described  
420 (Marietou *et al.*, 2009), based on the reaction of sulfate with barium chloride, resulting in the precipitation  
421 of barium sulfate. At different growth times, 5 mL culture samples were harvested, 0.22 µm filter-sterilized  
422 and stored at 4°C. 100 µL of these samples were added to 900 µL of conditioning agent (10.2 mM glycerol,  
423 6 mM concentrated HCl, 22.3 mM NaCl, 1.7 % ethanol) and 100 µL of 1 M barium chloride. The solution was  
424 then vortexed for 30 s and incubated at room temperature for 45 min to allow the precipitation of barium

425 sulfate. The absorbance of the solution at 420 nm was determined and the concentration of sulfate in the  
426 samples was calculated from the calibration curve prepared using standard solutions of sodium sulfate.

#### 427 *Separation of proteins by SDS-PAGE*

428 *D. fructosovorans* cells were grown in 1L bottles containing 900 mL medium, harvested at the end  
429 of exponential phase of growth by centrifugation. The cell pellet was resuspended in buffer Tris-HCl 20 mM  
430 pH 7.6 supplemented with protease inhibitors (SIGMAFAST™ Protease Inhibitor Cocktail Tablet, Sigma-  
431 Aldrich), DNase I (Roche) and Lysozyme (Sigma-Aldrich). Cells were disrupted by two passages through a  
432 chilled French press cell at a pressure of 100 MPa. After an ultracentrifugation at 200 000 g for 45 min at  
433 4°C, proteins were separated on 10% polyacrylamide denaturing gels (SDS-PAGE) using a mini VE  
434 electrophoresis apparatus (Amersham Pharmacia). After migration, gel was stained with Coomassie blue  
435 staining solution (20% ethanol, 10% acetic acid and 0.15% Coomassie blue R250) and destained by soaking  
436 for at least two hours in a solution of ethanol/acetic acid/water (20:10:70 vol/vol/vol).

#### 437 *Blue-Native gel (BN-gel) electrophoresis and in-gel hydrogenase activity detection*

438 *D. fructosovorans* cells were grown in 1L bottles containing 900 mL medium, harvested at the end  
439 of exponential phase of growth by centrifugation. Periplasmic extract was removed from the soluble  
440 sample to avoid periplasmic hydrogenase activity bands on the BN gel. The cell pellet was resuspended in 5  
441 mL TRIS-HCL 50 mM pH 9, sodium carbonate 170 mM, EDTA 50 mM and incubate 30 min at 30°C under  
442 gentle agitation (100 RPM). After centrifugation for 20 min at 20000 g, the incubation step was repeated  
443 another time in the same condition. The cell pellet was then used to prepare the soluble extract as  
444 described for the SDS-PAGE. BN gels were performed on 5-13% acrylamide gradient gel, according to the  
445 method of Schägger (Schägger *et al.*, 1994, Schägger & von Jagow, 1991), as described by Le Laz *et al.*  
446 (2014) (Le Laz *et al.*, 2014). In-gel hydrogenase activity was detected as described previously by Kpebe *et al.*  
447 (2018) (Kpebe *et al.*, 2018) using methyl viologen as electron acceptor.

#### 448 *Western immunoblots*

449 *D. fructosovorans* cells were grown in 100 mL bottles containing 90 mL of PS2 or PS20 medium.  
450 When indicated, sulfate was replaced by 2 or 20 mM sodium thiosulfate, or 4 or 40 mM sodium fumarate.  
451 Cells were harvested at late exponential phase or stationary phase of growth. Cells were disrupted at 100°C  
452 for 10 min in a loading buffer in the presence of 215 mM  $\beta$ -mercaptoethanol, 15 mM DTT and 2 % SDS and  
453 the extracts were centrifuged at 14 000g for 10 min. Soluble proteins in the supernatant were quantified  
454 with the Bio-Rad protein assay based on Bradford dye binding method, using bovine serum albumin (BSA)  
455 as a standard. Proteins were separated by 10% SDS-PAGE and electrotransferred to a nitrocellulose  
456 membrane (Fastblot, Biometra). The membrane was blocked for 1 h in TBST (500 mM Tris-HCl pH 7.5, 1.5 M  
457 NaCl, 0.2% Tween 20) containing 5% skim milk and incubated overnight at 4°C with a 5000-fold dilution of  
458 the primary rabbit anti-HndD antibody in the same buffer. After three 10 min washes in TBST, the  
459 membrane was developed using a 1/20 000 dilution of horseradish peroxidase-conjugated goat anti-rabbit  
460 secondary antibody for 1 h and the ECL western blotting detection reagents (Amersham). The primary anti-  
461 HndD antibody was generated by Eurogentec from a rabbit immunized with gel purified his-tagged HndD  
462 protein. IgG from serum were purified by affinity chromatography (protein G Sepharose, GE Healthcare).  
463 Protein loading was controlled by performing two identical SDS-PAGE in parallel. The first one was used for  
464 western immunoblotting experiment while the second one was stained with Coomassie blue staining  
465 solution. Protein quantification was done with Image J free software (NIH, LOCI, University of Wisconsin)  
466 (Schneider *et al.*, 2012).

#### 467 *Protein Identification by In-gel Digestion and Mass Spectrometry*

468 Gel electrophoresis bands of proteins from SDS-PAGE were put into a 96-well microplate (Greiner)  
469 and in-gel trypsin digestion of proteins was performed according to Guiral *et al.* (2009) (Guiral *et al.*, 2009).  
470 Proteins were identified by LC-MSMS as described previously by Guiral *et al.* (2009) (Guiral *et al.*, 2009)  
471 except that the peptides were separated by one dimension liquid chromatography on the reversed-phase  
472 column C18 only, and that the proteins analysed here are soluble proteins instead of membrane proteins.  
473 The database *Desulfovibrio fructosovorans* (TxID 878, 4701 entries) used was extracted from NCBI.

#### 474 *Hydrogenase activity in total soluble extracts*

475 *Preparation of soluble extracts*

476 *D. fructosovorans* cells, grown in 1 L bottles containing 900 mL of PS2 medium were harvested at  
477 the end of the exponential phase and resuspended in 10 mL of 100 mM Tris-HCl pH 8 supplemented with  
478 protease inhibitors (SIGMAFAST™ Protease Inhibitor Cocktail Tablet, Sigma-Aldrich), DNase I (Roche) and  
479 Lysozyme (Sigma-Aldrich). The cells were broken in an anaerobic glovebox (Jacomex) using a sonicator  
480 (Hielscher) in 10 cycles of 1 minute of sonication then 1 minute of rest in a water bath chilled at 4°C. The  
481 cell extract was ultracentrifuged at 200 000 g for 45 min at 4°C, then the soluble fraction was recovered,  
482 and the total protein was quantified using the Bio-Rad protein assay based on Bradford dye binding  
483 method, using bovine serum albumin (BSA) as a standard.

484 *H<sub>2</sub> evolution assays*

485 H<sub>2</sub> evolution assays were carried out using dithionite-reduced methyl viologen (50 mM reduced  
486 with 23 mM sodium dithionite) as electron donor, in anaerobic 6.5 mL-serum bottles containing 1 mL  
487 reaction mixture composed of Tris-HCl 0.1 M pH8, at 30°C. The gas phase was 100% N<sub>2</sub>. The reaction was  
488 started by the addition of 26.3 µg proteins of WT *D. fructosovorans* soluble extract or 226.5 to 377.5 µg  
489 proteins of SM4 strain soluble extract. H<sub>2</sub> production was measured using gas chromatography (GC). Fifty  
490 microliters of headspace gas was removed periodically from the vials using a gastight syringe and injected  
491 into a GC system (Agilent 7820) equipped with a thermal conductivity detector and a Carbon-plot capillary  
492 column (30 m, 0.53 mm, 3 µm), using nitrogen as the carrier gas, at a flow rate of 8.15 mL/min, an oven  
493 temperature of 30 °C and a detector temperature of 200 °C. One unit of hydrogenase activity corresponds  
494 to the production of 1 µmol of H<sub>2</sub>/min.

495 *H<sub>2</sub> oxidation assays*

496 H<sub>2</sub>-oxidizing activity measurements were performed in anaerobic quartz cuvettes, under 1 bar-  
497 pressure of H<sub>2</sub>, in 800 µL of reaction mixture containing 100mM Tris-HCl pH 8.0, 2mM dithiothreitol (DTT),  
498 and 50mM methyl viologen (Sigma Aldrich) as artificial electron acceptor. The reaction was started by the  
499 addition of 0.5 to 7.5 µg proteins of WT *D. fructosovorans* soluble extract or SM4 strain soluble extract to

500 the mixture to start the reaction and methyl viologen reduction was monitored at 604 nm  
501 ( $\epsilon=13,600\text{M}^{-1}\cdot\text{cm}^{-1}$ ) using a UV-Vis spectrophotometer Lambda 25 (Perkin Elmer). One unit of hydrogenase  
502 activity corresponds to the uptake of 1  $\mu\text{mol}$  of  $\text{H}_2/\text{min}$ .

### 503 *RNA preparation*

504 *D. fructosovorans* cells, grown in 2 L bottles containing 1.8 L of PS2 or PS20 medium, were  
505 harvested at the end of the exponential phase of growth.  $10^{10}$  cells were pelleted, washed three times, and  
506 resuspended in 200  $\mu\text{L}$  of 10 mM Tris-HCl, pH 8. Total RNA was prepared using the High Pure RNA kit from  
507 Roche Diagnostics according to the manufacturer's instructions. To remove genomic DNA contamination,  
508 total RNA was then treated with the TURBO DNA-free<sup>TM</sup> kit (Life Technologies). RNA concentration and  
509 purity were determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific) and electrophoretic  
510 migration on an agarose gel was performed to verify the absence of RNA degradation. The absence of  
511 contaminant DNA was verified by PCR with the *Taq* DNA-dependent DNA polymerase (Fermentas) using  
512 *16S* specific primers (16S-F and 16S-R, Table S3).

### 513 *Real-time quantitative PCR (RT-qPCR).*

514 Reverse transcription was performed on 500 ng of total RNA by using the SuperScriptIII reverse  
515 transcriptase and random primers (Invitrogen). Real-time PCR runs were carried out on a CFX96 Real time  
516 System (Bio-Rad). Cycling parameters of the real time PCR were 98°C for 2 min, followed by 45 cycles of  
517 98°C for 5 s and 60°C for 10 s, ending with a melting curve analysis from 65°C to 95°C to determine the  
518 specificity of the amplification. To determine the amplification kinetics of each product, the fluorescence  
519 derived from the incorporation of EvaGreen into the double-stranded PCR products was measured at the  
520 end of each cycle using the SsoFast<sup>TM</sup> EvaGreen<sup>®</sup> Supermix 2X Kit (Bio-Rad). The results were analyzed using  
521 the Bio-Rad CFX Manager Software 3.0. The *16S* rRNA gene was used as a reference for normalization. The  
522 real-time quantitative PCR experiments were performed in duplicate assays with total RNA from at least  
523 three independent growth experiments. Primers used for the real time PCR are listed in Table S3.

524

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533 The authors have no conflict of interest to declare.

## 534 **Author Contributions**

535 Conception and design of the study: MB and LS; acquisition, analysis, or interpretation of the data, NP, AK,  
536 CG, CB, JR, RL and YD, LS and MB; writing of the manuscript: MB, NP and AK. All authors reviewed the  
537 manuscript.

538

## 539 **Figure legends**

540 Figure 1: Growth curves of *Desulfovibrio fructosovorans* WT and SM4 strains. Bacteria were grown in PSO  
541 (A, fermentation), PS2 (B, mixed culture condition) and PS20 (C, respiration) media containing no, 2 mM  
542 and 20 mM sulfate respectively and 25 mM pyruvate as electron donor. WT strain, black curves; SM4 strain,  
543 red curves. OD600 was followed as a function of time. OD units were converted into number of cells ( 1 mL  
544 of cells at OD600 of 1 represents  $10^9$  cells). Data represent the averages of the results of three replicate  
545 growths. Error bars correspond to standard deviations.

546 Figure 2: Pyruvate, acetate, ethanol and H<sub>2</sub> quantification. Pyruvate (open squares), acetate (closed  
547 triangles) and ethanol (closed circles) in the growth medium and H<sub>2</sub> (open diamonds) in the headspace  
548 were quantified by HPLC or GC during the growth of the *D. fructosovorans* WT strain (black curves) or SM4

549 mutant strain (red curves) under fermentation (PS0, A and D), mixed culture condition (PS2, B and E) or  
550 respiration (PS20, C and F). Analysis were performed on the same *D. fructosovorans* cultures as those used  
551 for the growth curves. Times indicated on the x-axis correspond to those in Figure 1. Data represent the  
552 averages of the results of three replicate growths. Error bars correspond to standard deviations.

553 Figure 3: Proteomic analysis. (A) Separation by Coomassie-blue-stained SDS-PAGE of soluble proteins from  
554 WT (lanes 1 and 3) and SM4 mutant (lanes 2 and 4) strains of *Desulfovibrio fructosovorans* grown under  
555 mixed (lanes 1 and 2) or respiratory (lanes 3 and 4) conditions. Cells were harvested at the end of the  
556 exponential phase. 45 µg of soluble proteins were loaded in each lane of a 10 % SDS-PAGE. Molecular mass  
557 markers (lane 5) are indicated in kDa. (B) Proteins identified by mass spectrometry (ion trap MS/MS) in  
558 bands showing a different intensity in the two strains. Data correspond to identified proteins in bands 1, 2  
559 and 3 from WT cells grown under mixed condition (panel A, lane 1). Only proteins identified with the  
560 highest score in each gel band are reported (for the complete list of identified proteins, see Table S1). Table  
561 heading: Protein name, protein name in NCBI database. NCBI entry, accession number. Unique peptides:  
562 number of distinct peptides matching to protein sequence and unique to this protein. Sequence coverage,  
563 protein sequence coverage by the matching peptides (in %). Theor.  $M_r$ , theoretical molecular mass in kDa of  
564 the identified protein. These results are representative of several similar experiments.

565 Figure 4: Relative expression of the *adh*, *aor* and *hndD* genes quantified by RT-qPCR. *D. fructosovorans* WT  
566 and SM4 strains were grown under mixed culture condition (PS2 medium) or under respiratory condition  
567 (PS20 medium) and samples were collected at the end of the exponential phase of growth. Expression of  
568 the genes was normalized to that of the 16S rRNA gene. These results are representative of several similar  
569 experiments.

570 Figure 5: Hnd hydrogenase production and detection of its activity on BN-gel. (A) Western blot analysis of  
571 HndD production by WT (lanes 1 and 2), SM4 (lane 3) and SM4/pBGhnd6 (lane 4) cells of *D. fructosovorans*  
572 grown in medium containing 2 mM (PS2) (lanes 1, 3 and 4) or 20 mM (PS20) (lane 2) sulfate. Cells were  
573 harvested at stationary phase of growth and 25 µg of total proteins from whole-cell protein extracts were  
574 separated by 10% SDS-PAGE and subjected to Western blotting using an antibody raised against HndD. The



575 SDS-PAGE gel below the western blot is a loading control. (B) In-gel detection of hydrogenase activity in on  
576 BN-gel of cytoplasmic proteins prepared from *D. fructosovorans* WT strain grown on PS20 (lane 1) or PS2  
577 (lane 2) medium. 35 µg of cytoplasmic proteins were loaded on a 5–13% polyacrylamide gel. Molecular  
578 weight markers are indicated in kDa (lane 3).

579 Figure 6: Relative *hndD* gene expression and production of ethanol by *D. fructosovorans* WT strain and the  
580 complementation strain SM4/pBGhnd6 grown under mixed culture condition (PS2 medium). Samples were  
581 collected at the end of the exponential phase of growth. (A) Relative expression of the *hndD* gene  
582 quantified by RT-qPCR. Expression of the gene was normalized to that of the 16S rRNA gene. Error bars  
583 correspond to standard deviations. (B) Ethanol concentration in the growth medium quantified with an  
584 enzymatic kit. These results are representative of several similar experiments.

585 Figure 7: Model of the pyruvate oxidation pathway by *D. fructosovorans* under pyruvate fermentation  
586 conditions. Pyruvate is converted into acetate by the 2 enzymes Pfo and Acs. Part of the acetate is  
587 converted into ethanol by Aor and Adh. H<sub>2</sub> could be produced by the membrane NiFe-hydrogenase Ech  
588 from reduced ferredoxin, which would contribute to the electrochemical proton gradient for ATP synthesis.  
589 This model proposes that the role of the Hnd hydrogenase would be to produce reducing equivalents  
590 (NADH and reduced ferredoxin) from H<sub>2</sub>, allowing the production of ethanol. Pfo, pyruvate ferredoxin  
591 oxidoreductase; Acs, Acetyl-CoA synthase; Aor, Aldehyde ferredoxin oxidoreductase; Adh, Alcohol  
592 dehydrogenase; Ech and Hnd, Hydrogenases. Fd<sub>ox</sub>, oxidized ferredoxin; Fd<sup>2-</sup>, reduced ferredoxin. This figure  
593 was created with BioRender.com.

594 Figure S1: Growth curve, sulfate consumption and H<sub>2</sub> production by *D. fructosovorans* WT cells. Bacteria  
595 were grown in PS2 medium (mixed culture condition). Sulfate concentration in the growth medium was  
596 determined using a turbidimetric method with barium chloride. H<sub>2</sub> in the headspace was quantified using  
597 GC. H<sub>2</sub> starts to accumulate when sulfate is completely reduced.

598 Figure S2: Western blot analysis of HndD production by *D. fructosovorans* WT cells grown in medium  
599 containing 25 mM pyruvate and 20 mM thiosulfate (lane 1), 2 mM thiosulfate (lane 2), 40 mM fumarate

600 (lane 3) or 4 mM fumarate (lane 4) as electron acceptor. Cells were harvested at late exponential phase of  
601 growth and 2 µg of total proteins from whole-cell protein extracts were separated by 10% SDS-PAGE and  
602 subjected to western blotting using an antibody raised against HndD. The SDS-PAGE gel below the western  
603 blot is a loading control.

604 Figure S3: Separation by Coomassie-blue-stained SDS-PAGE of soluble proteins from WT (lane 2), SM4  
605 mutant (lane 3) and SF mutant (lane 4) strains of *Desulfovibrio fructosovorans* grown under mixed  
606 conditions (PS2). Cells were harvested at the end of the exponential phase. 45 µg of soluble proteins were  
607 loaded in each lane of a 10 % SDS-PAGE. Molecular mass markers (lane 1) are indicated in kDa.

608 Figure S4: Growth curves of *Desulfovibrio fructosovorans* WT, SM4 ( $\Delta hndD$ ), SM4/pBGhnd6 and SF  
609 ( $\Delta hydAB$ ) strains. Bacteria were grown in PS2 medium containing 25 mM pyruvate as electron donor and 2  
610 mM sulfate as electron acceptor (mixed culture condition).

611 Figure S5: Predicted Rex binding-site in the promoter region of *hnd*. (A) Sequence logo of the DNA  
612 recognition motif for all Rex sites in the order Desulfovibrionales. The sequence logo was constructed  
613 based on the alignment of 117 sites identified as Rex recognition sequences (available in the RegPrecise  
614 database). (B) Consensus sequence of the Rex binding sites in the promoter region of the tetrameric,  
615 cytoplasmic [FeFe] hydrogenases found in *Desulfovibrio*. The consensus sequence was constructed by  
616 aligning the known sites with ClustalW multiple alignment. (C) The Rex binding sites and their locations in  
617 the promoter region of the tetrameric, cytoplasmic [FeFe] hydrogenases in *Desulfovibrio* species. The  
618 promoter region sequences were taken from NCBI and the Rex-binding site was mapped. Identical residues  
619 between the species are highlighted in red.

620

621 **Table**622 Table 1: Growth parameters of WT and SM4 *D. fructosovorans* strains.

Medium		Doubling time (h)		Maximum cell count/mL <sup>†</sup>	
		WT	SM4	WT	SM4
PS0		369.0±73.5	519.5±100.7	6.90×10 <sup>8</sup> ± 4.0×10 <sup>7</sup>	6.70×10 <sup>8</sup> ± 3.0×10 <sup>7</sup>
PS2	Phase 1 0-40 hrs	17.2±0.9	17.4±5.7	1.12×10 <sup>9</sup> ± 1.0×10 <sup>7</sup>	1.16×10 <sup>9</sup> ± 2.0×10 <sup>7</sup>
	Phase 2 40-110 hrs	89.3±3.5	114.2±1.1		
PS20		20.5±0.3	15.3±1.2	1.33×10 <sup>9</sup> ± 1.0×10 <sup>7</sup>	1.41×10 <sup>9</sup> ± 4.0×10 <sup>7</sup>

623 † The highest optical density at 600 nm was converted into number of cells (1 mL of cells at OD600 of 1  
624 represents 10<sup>9</sup> cells). Data are mean values +/- standard deviation and were obtained from triplicate  
625 separated experiments.

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627 **References**

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