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# NMR-based metabolomic analysis of the physiological role of the electron-bifurcating FeFehydrogenase Hnd in *Solidesulfovibrio fructosivorans* under pyruvate fermentation

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1 **NMR-based metabolomic analysis of the physiological role of the electron-bifurcating FeFe-**  
2 **hydrogenase Hnd in *Solidesulfovibrio fructosivorans* under pyruvate fermentation**

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14

15 **Abstract**

16 *Solidesulfovibrio fructosivorans* (formely *Desulfovibrio fructosovorans*), an anaerobic sulfate-reducing  
17 bacterium, possesses six gene clusters encoding six hydrogenases catalyzing the reversible oxidation of  
18 hydrogen gas (H<sub>2</sub>) into protons and electrons. One of these, named Hnd, was demonstrated to be an electron-  
19 bifurcating hydrogenase Hnd (Kpebe et al., 2018). It couples the exergonic reduction of NAD<sup>+</sup> to the  
20 endergonic reduction of a ferredoxin with electrons derived from H<sub>2</sub> and whose function has been recently  
21 shown to be involved in ethanol production under pyruvate fermentation (Payne 2022). To understand  
22 further the physiological role of Hnd in *S. fructosivorans*, we compared the mutant deleted of part of the  
23 *hnd* gene with the wild-type strain grown on pyruvate without sulfate using NMR-based metabolomics. Our  
24 results confirm that Hnd is profoundly involved in ethanol metabolism, but also indirectly intervenes in  
25 global carbon metabolism and additional metabolic processes such as the biosynthesis of branched-chain  
26 amino acids. We also highlight the metabolic reprogramming induced by the deletion of *hndD* that leads to  
27 the upregulation of several NADP-dependent pathways.

28

29 **Highlights**

30 • Metabolomics characterized pathways of pyruvate fermentation in *S. fructosivorans*

31 • Hnd is involved in ethanol metabolism and branched-chain amino acid biosynthesis.

32 • HndD deletion leads to the upregulation of NADP-dependent pathways.

33

34 **Keywords** : metabolomics, electron-bifurcating hydrogenase, ethanol metabolism, NADP-dependent

35 pathways, *Desulfovibrio*

36

37

## 38 Introduction

39 *Desulfovibrio* is a genus of sulfate-reducing bacteria that is pervasive in a diverse range of  
40 environments. Recently, a new classification of *Desulfovibrio* bacteria has been reported and the genus  
41 *Desulfovibrio* has been subdivided into 13 new genera, but in this publication, the term *Desulfovibrio* refers  
42 to the genus in the old classification (Waite et al., 2020). *Desulfovibrio* species use sulfate as the terminal  
43 electron acceptor for the oxidation of an assortment of electron donors such as lactate, ethanol, formate, and  
44 molecular hydrogen (Rabus et al., 2013; Singleton, 1993). Hydrogen in particular plays an important role  
45 in the energy metabolism of *Desulfovibrio* species, because not only can hydrogen be used as the sole source  
46 of electrons in the presence of a suitable carbon source and electron acceptor, but hydrogen is also released  
47 during fermentative growth with certain carbon sources, to regenerate reduced electron carriers (Brandis and  
48 Thauer, 1981). Additionally, hydrogen can be consecutively produced and consumed during the degradation  
49 of organic compounds during respiration (Odom and Peck, 1981).

50 Hydrogenases are the critical enzymes implicated in this metabolism, as they catalyze the splitting  
51 or synthesis reactions of molecular hydrogen. The number of hydrogenases present varies considerably from  
52 one *Desulfovibrio* species to another, but the vast majority of the species are known to contain at least two  
53 (Baffert et al., 2019; Pereira et al., 2011). Further hydrogenase diversity in *Desulfovibrio* exists when the  
54 type (based on the metal center; [NiFe] or [FeFe]) and the cellular localization (cytoplasm, periplasm or  
55 membrane-bound) are considered, and this diversity makes the role of each hydrogenase difficult to  
56 determine. An “hydrogen cycling” model for the growth of *Desulfovibrio vulgaris* and *Desulfovibrio gigas*  
57 with lactate in the presence of sulfate was proposed (Odom and Peck, 1981). In this model, a cytoplasmic  
58 hydrogenase consumes the protons and electrons produced by lactate oxidation in order to produce H<sub>2</sub>. The  
59 H<sub>2</sub> then diffuses through the cytoplasmic membrane and is re-oxidized by a periplasmic hydrogenase,  
60 regenerating the protons and electrons. The produced protons contribute to the proton gradient across the  
61 membrane and are used for ATP synthesis. The electrons are captured by the c-type cytochrome network,  
62 and they are transferred to various membrane-bound electron-transfer complexes and used for sulfate  
63 reduction in the cytoplasm. This model also explains the multiple observations of a rapid and temporal

64 accumulation of H<sub>2</sub> known as a hydrogen burst when *Desulfovibrio* species are grown on lactate and sulfate  
65 (Brandis and Thauer, 1981; Tsuji and Yagi, 1980).

66         Since the introduction of the hydrogen cycling model forty years ago, others have suggested  
67 alternative models that minimize the importance of H<sub>2</sub> evolution on electron transport. Lupton and  
68 colleagues presented an alternative hypothesis where H<sub>2</sub> production is not obligatory, but it is used to control  
69 the redox state of internal electron carriers. In this model, the organism transports the electrons directly to  
70 sulfate through the membrane-bound electron-transfer complexes, and hydrogen evolution occurs only  
71 when the rate of electron extraction from an organic donor is greater than the rate at which the electrons are  
72 taken up by the sulfate reduction system (Lupton et al., 1984). Following experimental evidence supporting  
73 both hypotheses, Noguera and coworkers, and later, Keller and Wall proposed a unified model in which there  
74 are two electron transport mechanisms that operate in parallel: one route that is like the hydrogen-cycling  
75 model which involves H<sub>2</sub> evolution and another route that does not require H<sub>2</sub> as an intermediate (Keller  
76 and Wall, 2011; Noguera et al., 1998). The presence of multiple electron transport mechanisms is thought  
77 to promote rapid adaptation of *Desulfovibrio* species to different environments.

78         In *Solidesulfovibrio fructosivorans* (formerly *Desulfovibrio fructosovorans*), our model organism,  
79 three hydrogenases have already been characterized biochemically: Hyn, a periplasmic [NiFe] hydrogenase  
80 (Hatchikian et al., 1990); Hyd, a periplasmic [FeFe]-type (Casalot et al., 1998) and Hnd, a tetrameric,  
81 cytoplasmic [FeFe] hydrogenase (De Luca et al., 1998a, 1998b; Dermoun et al., 2002). In addition to these  
82 three, genomic analysis of *S. fructosivorans* revealed genes for three other putative hydrogenases (Baffert  
83 et al., 2019): a trimeric, cytoplasmic [FeFe]-type that is structurally similar to the electron-bifurcating  
84 hydrogenase Hyd found in *Thermotoga maritima* (Schut and Adams, 2009), a membrane-bound Ech-type  
85 (Schoelmerich and Müller, 2020), and a cytoplasmic, [FeFe]-type that has homology with putative  
86 phosphatase-linked sensory hydrogenases like Hfs from *Thermoanaerobacter saccharolyticum* (Eminoğlu  
87 et al., 2017; Shaw et al., 2009) and HydS from *Ruminococcus albus* (Zheng et al., 2014) or *T. maritima*  
88 (Chongdar et al., 2018). Marker-exchange mutagenesis demonstrated an influence of each of these  
89 hydrogenases on the growth parameters of the bacterium but failed to establish a specific role for each of

90 the hydrogenases in the energy metabolism (Casalot et al., 2002; Malki et al., 1997; Rousset et al., 1991),  
91 due to a probable compensation mechanism between the different hydrogenases (Casalot et al., 2002).

92 We recently established that Hnd functions with a flavin-based electron bifurcation mechanism  
93 (Kpebe et al., 2018), a method of energy coupling generally found in anaerobic prokaryotes that was first  
94 described in 2008 (Li et al., 2008). Hnd couples the reduction of two different electron acceptors: NAD<sup>+</sup>,  
95 which has a high redox potential and a ferredoxin which has a relatively low redox potential (Kpebe et al.,  
96 2018). Reduction of the ferredoxin is thermodynamically unfavorable and would not proceed if the two  
97 reactions were not coupled. The “energy rich” reduced ferredoxin can then be used as a source of low  
98 potential electrons for other reactions (Buckel and Thauer, 2018; Müller et al., 2018).

99 Hnd was characterized as an NAD-dependent hydrogenase (Kpebe et al., 2018). Although putative  
100 NAD-dependent electron-bifurcating hydrogenases are abundant in anaerobes (Greening et al., 2016), very  
101 few have been characterized biochemically and little is known about their metabolic function in vivo.  
102 Among the genus *Desulfovibrio*, however, a relatively small number of strains contain genes for putative  
103 electron-bifurcating hydrogenases (Baffert et al., 2019; Kpebe et al., 2018). It was recently determined that  
104 Hnd plays a key role in the ethanol metabolism of *S. fructosivorans*, as a strain deleted of *hndD* produces  
105 only trace amounts of ethanol during pyruvate fermentation compared to the wild-type strain (Payne et al.,  
106 2022). Under this condition, we proposed that Hnd oxidizes part of the H<sub>2</sub> produced, probably by Ech,  
107 during fermentation generating both NADH and reduced ferredoxin for ethanol production via its electron  
108 bifurcation mechanism. Nevertheless, many questions remained about the role of Hnd in the global  
109 metabolism of *S. fructosivorans*.

110 In this context, we decided to explore the function of Hnd in *S. fructosivorans* metabolism using an  
111 NMR-based metabolomics approach. Indeed, metabolomics was demonstrated to provide valuable insights  
112 regarding the physiological role of specific enzymes in bacteria through comparison between the wild-type  
113 and the mutant metabolism (Hubert and de Carvalho, 2022). We report here the extracellular and  
114 intracellular metabolic fingerprinting of *S. fructosivorans* wild-type (Ollivier et al., 1988) and SM4 ( $\Delta hndD$ )  
115 (Malki et al., 1997) strains grown on pyruvate via liquid state and High Resolution Magic Angle Spinning  
116 (HRMAS) NMR-based metabolomics techniques, respectively. Our results demonstrate that the electron

117 bifurcating reaction catalyzed by Hnd is important for the generation of reducing equivalents (reduced  
118 ferredoxin and NADH), which intervene in a variety of metabolic pathways, even those outside the expected  
119 processes of carbon metabolism and energy generation.

120

## 121 **Material and Methods**

### 122 **Bacterial Strains and Growth Conditions**

123 *S. fructosivorans* DSM3604 (wild-type strain) (Ollivier et al. 1988) and *S. fructosivorans* SM4 ( $\Delta hndD$ ,  
124 Cm<sup>R</sup>) (Malki et al., 1997) were grown in a culture medium containing 40 mM of pyruvate in absence of  
125 sulfate (PS0 medium), as described previously (Payne et al., 2022). Strain SM4 was grown with  
126 thiamphenicol 35  $\mu\text{g}\cdot\text{mL}^{-1}$  (dissolved in methanol) to avoid contamination from other bacteria. Both strains  
127 were grown anaerobically at 37 °C in volumes of 100 mL contained in 120 mL glass serum bottles sealed  
128 with a butyl rubber stopper and an aluminum crimp top. The medium was inoculated with 5% (v/v) of fresh  
129 cultures grown in a pyruvate 40 mM, sulfate 2mM culture medium (PS2 medium) as previously described  
130 (Payne et al., 2022). Six biological replicates were inoculated for each strain, with the inoculum originating  
131 from the same culture in order to maximize reproducibility. Growth was monitored by OD<sub>600</sub> measurements.  
132 In addition, SM4 strains (n=3) was also grown in a medium exempt of antibiotics following the same  
133 protocol to assess the innocuity of the antibiotics on the overall metabolism of the bacteria.

134

### 135 **Sample Collection**

136 Samples from each culture were collected after 164 h of culture. To normalize the signals based on the  
137 number of cells, the OD<sub>600</sub> was measured for each culture and a sample of each containing 10<sup>10</sup> cells was  
138 taken (10<sup>10</sup> cells=10 mL culture @OD<sub>600</sub>=1). All the following manipulations were performed at 4°C in a  
139 cold room. The cells were collected by centrifugation for 5 min at 12,000 g. The supernatant was removed  
140 and filtered (0.2  $\mu\text{m}$  pore size cellulose filters) and frozen in liquid N<sub>2</sub>. The pellets were washed one time  
141 with sterile PBS (137 mM NaCl, 2.7 mM KCl, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4), and centrifuged  
142 for 5 min at 12,000 g. The pellets were frozen in liquid N<sub>2</sub>, and the frozen supernatants and pellets were  
143 stored at -80°C until analysis with NMR.

**145 <sup>1</sup>H liquid-state NMR spectroscopy and data processing**

146 Five hundred microliters of each supernatant sample were mixed with 100  $\mu$ L of D<sub>2</sub>O containing 1.68 mM  
147 trimethylsilylpropanoic acid (TSP) to act as an internal reference. The samples were then placed into a 5  
148 mm NMR tube. Experiments were carried out on a Bruker Avance III spectrometer operating at 600 MHz  
149 for the <sup>1</sup>H frequency equipped with a TXI probe. A NOESY 1D pulse sequence experiment with a relaxation  
150 time of 17.5 s associated with a water presaturation pulse of 2.5 s and an acquisition time of 2.7 s was run  
151 on each sample at a temperature of 300 K. The mixing time was set to 10 ms. For each spectrum, 128 free  
152 induction decays (FID) of 65K data points were collected using a spectral width of 12,000 Hz. The FIDs  
153 were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz and  
154 zero-filled once prior to Fourier transformation. <sup>1</sup>H NMR spectra were imported into NMRProcFlow v1.4  
155 online (Jacob et al., 2017) and divided into buckets using the intelligent bucketing tool with a resolution  
156 factor of 0.5 and a signal-to-noise threshold of 1, leading to 345 buckets. The region between 4.50 and 5.20  
157 ppm was excluded in order to remove the effect of the water signal presaturation. Signals corresponding to  
158 the antibiotic were also removed.

**159 <sup>1</sup>H HRMAS NMR spectroscopy and data processing**

160 Pellets containing 10<sup>10</sup> cells were resuspended with 10  $\mu$ L of D<sub>2</sub>O containing 1.68 mM TSP, to  
161 provide an internal reference, then added to a 4 mm ZrO<sub>2</sub> HRMAS rotor with a cylindrical insert. All  
162 HRMAS experiments were carried out on a Bruker Avance spectrometer operating at 400 MHz for the <sup>1</sup>H  
163 frequency equipped with a <sup>1</sup>H/<sup>13</sup>C/<sup>31</sup>P HRMAS probe. Spectra were acquired at 277 K with a spin rate of 4  
164 kHz. A Carr–Purcell–Meiboom–Gill (CPMG) NMR spin echo sequence ([presat-90°-( $\tau$ -180°- $\tau$ )n]) with an  
165 overall spin echo time of 25 ms, preceded by a water presaturation pulse during the relaxation delay of 2 s,  
166 was used to reduce the signal intensities of lipids and macromolecules. For each spectrum, 256 free  
167 induction decays (FID) of 32 k data points were collected using a spectral width of 8000 Hz. The FIDs were  
168 multiplied by an exponential weighting function corresponding to a line broadening of 0.5 Hz and zero-  
169 filled once prior to Fourier transformation.



170 <sup>1</sup>H NMR spectra were manually phased and referenced to the TSP signal ( $\delta = 0$  ppm), and  
171 automatically baseline corrected and using Topspin 3.5 (Bruker BioSpin GmbH, Karlsruhe, Germany). The  
172 spectra were imported into NMRProcFlow v1.4 online (Jacob et al., 2017) and divided into buckets using  
173 the intelligent bucketing tool with a resolution factor of 0.5 and a signal-to-noise threshold of 3, leading to  
174 122 buckets. The region between 4.85 and 5.20 ppm was excluded in order to remove the influence of  
175 variations in the water presaturation efficiency. The spectral buckets were then normalized to total spectrum  
176 intensity using MetaboAnalyst v 3.0.3 (Pang et al., 2020).

### 177 **Statistical analysis**

178 Statistical analysis was carried out in R v 4.0.3 (R Core Team, 2021) using the mixOmics package (Le Cao  
179 et al., 2021). Partial least squares regression (PLS) in canonical mode, an unsupervised statistical method,  
180 was performed to relate the intracellular metabolome with the extracellular one. The intra- and extracellular  
181 data sets, respectively, the X- and Y- matrices, were integrated and partial least squares regression (PLS)  
182 was used to maximize the covariance between both matrix variables. With this analysis, latent variables,  
183 linear combinations of the NMR variables from both blocks were calculated, so that NMR variables sharing  
184 the same pattern participate equally in the discrimination between the samples.

### 185 **Metabolite quantification**

186 Relevant metabolites from the culture supernatant samples were quantified using Chenomx NMR Suite  
187 9.0 software (Chenomx, Inc; Edmonton, AB, CA). Using the Processor tool, the spectra were calibrated  
188 using the internal standard of 0.28 mM TSP as the reference. The reference metabolite spectra were then  
189 fitted to the sample spectra using the Profiler tool. The absolute concentration values were then normalized  
190 according to the OD600 for each sample, i.e. according to the final number of cells in each bottle.

191 Concerning the quantification of the intracellular metabolites, the TSP signal cannot be used directly for  
192 quantification since TSP binds to proteins and other macromolecules, which affects the intensity of its  
193 signal. The ERETIC method, calibrated on a sample of 1.06 mM 2-oxoglutarate, was thus used to quantify  
194 the free-TSP signal (Akoka et al., 1999). Metabolites were then quantified using Chenomx NMR suite 9.0  
195 as explained previously. For each quantified metabolite, two-tailed Student t-test was used to calculate the  
196 p-value of the discrimination between WT and SM4 samples. The fermentation balance was calculated as

197 the ratio between the produced metabolite concentration and the consumed pyruvate concentration. It is  
198 expressed in mol/mol pyruvate.

### 199 **Data availability**

200 Acquired NMR spectra can be found in the following Mendeley dataset: <https://doi.org/10.17632/t99vf4w5tf.1>

202

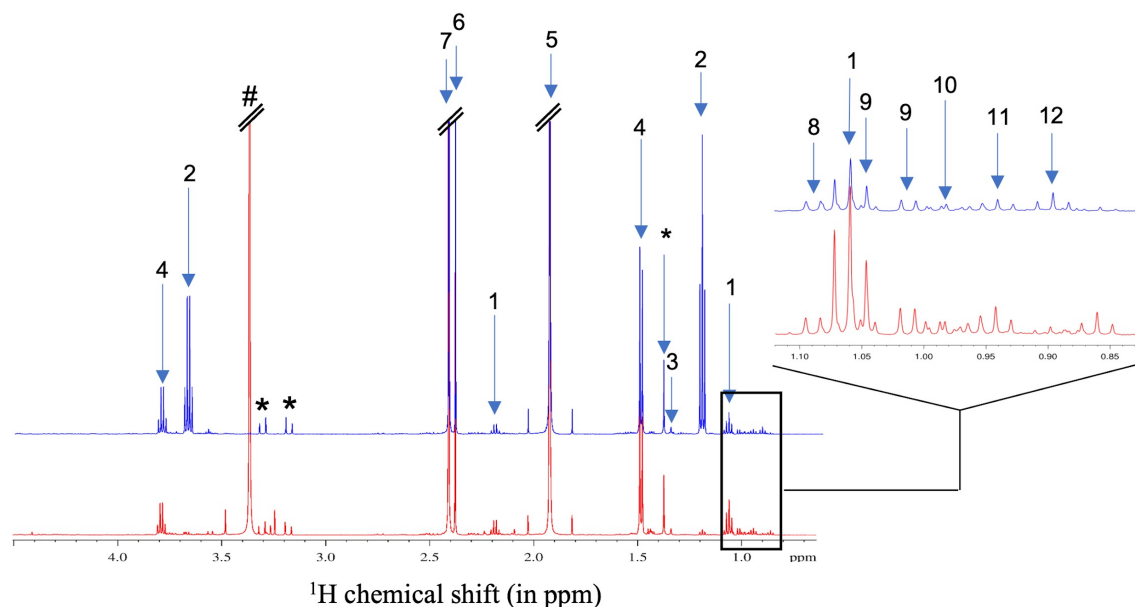
## 203 **Results**

### 204 **Identification of intra- and extracellular metabolites under pyruvate fermentation using NMR** 205 **spectroscopy**

206 WT and *hndD* deletion mutant (SM4) strains were grown in a sulfate-free medium containing pyruvate as  
207 the sole source of carbon (PS0 medium). As presented on Figure S1, the growth rates were 0.006 h<sup>-1</sup> and  
208 0.005 h<sup>-1</sup> for WT and SM4 strains, respectively. Pyruvate was consumed at a rate of 0.62 +/- 0.01 mM/h for  
209 both strains. Hnd does not appear to be crucial for *S. fructosivorans* growth, as both strains reach similar  
210 final OD<sub>600</sub> values (Figure S1). The <sup>1</sup>H NMR spectra of the spent culture media from WT (n=3), SM4 grown  
211 with antibiotics (n=3) and SM4 without antibiotics (n=3) samples was also recorded and analyzed using  
212 principal component analysis (PCA). The PC2 vs PC3 score plot, presented in Figure S2, shows a clear  
213 clustering of all SM4 samples without discrimination between the ones exposed to the antibiotics and the  
214 others. This validates the innocuity of the antibiotics on the overall metabolism of the SM4 strains.

215 To better understand the physiological role of Hnd during fermentative H<sub>2</sub>-production, the metabolism of  
216 wild type strain was compared with the one of the SM4 mutant strain for which *hndD* encoding the catalytic  
217 subunit of the Hnd hydrogenase was deleted. The modulations of the global bacterial metabolism, i.e.  
218 extracellular (culture medium) and intracellular metabolites (bacterium cells), were analyzed using NMR-  
219 based metabolomics.

220 The extracellular and intracellular metabolic <sup>1</sup>H NMR profiles of the WT and SM4 samples were recorded  
221 using liquid-state and HRMAS NMR spectroscopy, respectively. Representative spectra of extracellular and  
222 intracellular metabolites for both strains are presented in Figure 1 and S3, respectively.



223

224 **Fig. 1** Representative  $^1\text{H}$  NMR spectra of *S. fructosivorans* WT (blue) and SM4 (red) PS0 spent culture medium (after  
 225 164 h growth). \* = pyruvate dimer signals; # = methanol from antibiotic. Assignments: 1: propionate; 2: ethanol; 3:  
 226 lactate; 4: alanine; 5: acetate; 6: pyruvate; 7: succinate; 8: methylsuccinate; 9: valine; 10: 2-aminobutyrate; 11:  
 227 isoleucine; 12: 3-methyl-2-oxovalerate. Only major metabolites are represented on the figure. Assignments of all  
 228 identified metabolites are reported in Table S1.

229

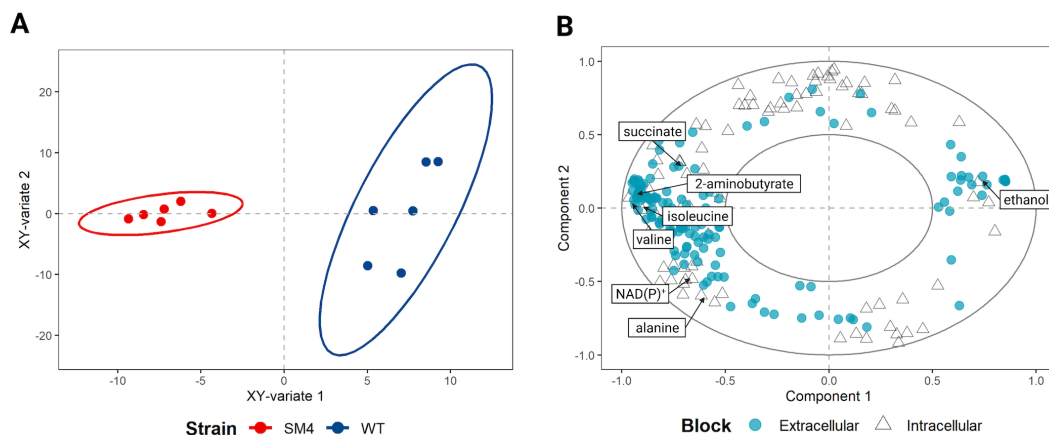
230 The assignments of all the identified metabolites (intra- and extracellular compounds) are reported in Table  
 231 S1. The intracellular NMR profile of the *S. fructosivorans* WT strain was dominated by the signals of  
 232 trehalose, alanine, lysine, ethanol, succinate, acetate and an intense unknown signal at 3.72 ppm. Less  
 233 concentrated metabolites such as  $\text{NAD(P)}^+$ , adenosine monophosphate, glutamate, 2-hydroxyisobutyrate  
 234 and inosine were also identified. Some metabolites, mainly amino acids, short chain fatty acids and their  
 235 derivatives and other organic acids were also identified in the spent culture medium.

236

### 237 **Metabolic profiling of *S. fructosivorans* under pyruvate fermentation conditions**

238 To identify the metabolites discriminating between WT and SM4 samples, unsupervised canonical PLS  
 239 analysis was then performed using intracellular and extracellular NMR data as X- and Y-matrices,  
 240 respectively. The resulting score plot displayed a clear discrimination between both strains and discriminant

241 extracellular and intracellular metabolites are presented on the loading plot (Figure 2A and 2B). Since the  
242 PLS analysis is an unsupervised method, no validation of the model robustness was necessary.



243

244 **Fig. 2** PLS score (a) and loadings (b) plots performed on <sup>1</sup>H NMR spectra of PS0 WT and SM4 samples. Each point  
245 on the score plot represents an individual sample. Each point on the loadings plot represents a spectral bucket. The  
246 most discriminant metabolites are mentioned on the loading plot.

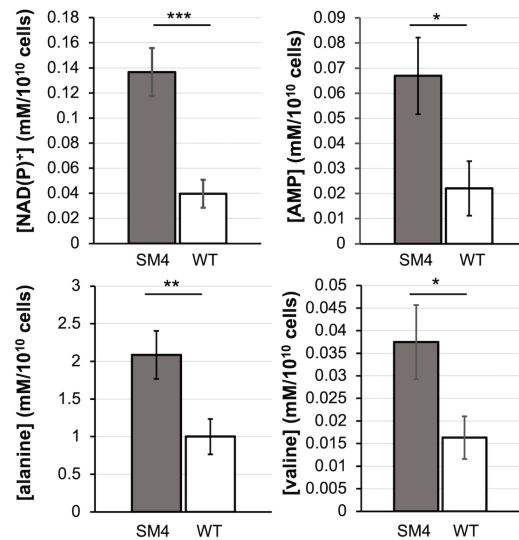
247

248 The loss of Hnd in *S. fructosivorans* led to the accumulation of intracellular NAD(P)<sup>+</sup>, AMP, alanine and  
249 valine in the mutant strain compared to the WT strain. Quantification of these four significant metabolites  
250 were performed using the ERETIC method (Fig. 3). NAD(P)<sup>+</sup> and AMP levels were 3 times higher in the  
251 mutant than in the WT strain when alanine and valine levels were increased by 2-fold (Table S2).

252

253

254

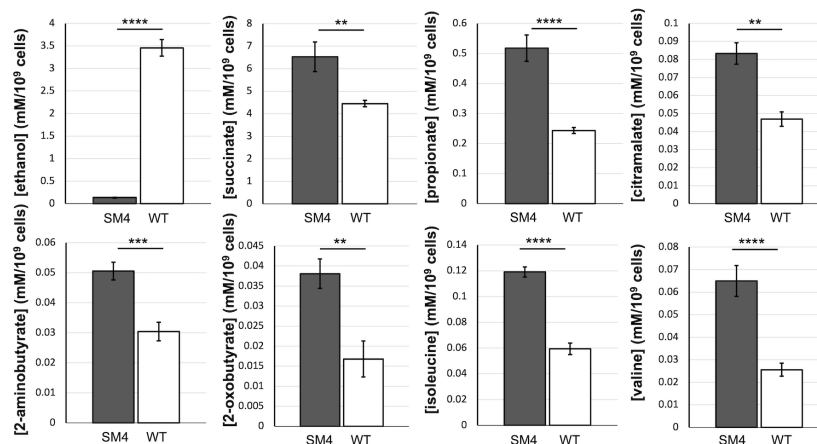


255

256 **Fig. 3** Absolute concentrations (in mM) of the intracellular discriminant metabolites between *S. fructosivorans* WT  
 257 and SM4 mutant strains grown under pyruvate fermentation conditions. Normalization was not necessary since the  
 258 number of cells is identical for each sample. \*\*\*: p-value <0.001, \*\*: p-value <0.01; \*: p-value <0.05

259

260 The PLS results indicated also that the deletion of *hnd* affected the levels of the excreted metabolites (Fig.  
 261 4).



262

263 **Fig. 4** Absolute concentrations (in mM) of the extracellular discriminant metabolites between *S. fructosivorans* WT  
 264 and SM4 mutant strains grown under pyruvate fermentation. Concentrations were normalized according to the number  
 265 of cells at the time of collection. \*\*\*\*: p-value <0.0001, \*\*\*: p-value <0.001; \*\*: p-value <0.01

266

267 The levels of propionate, 2-aminobutyrate, 2-oxobutyrate, citramalate, valine, isoleucine, and succinate  
 268 were higher in the mutant while, as expected, the level of excreted ethanol was dramatically reduced. The

269 fold changes and p-values for each quantified metabolite are reported in Table S3. The quantification of  
 270 these metabolites showed that pyruvate was mainly fermented into acetate, ethanol and succinate. To clarify  
 271 the stoichiometry of pyruvate fermentation into these metabolites, we calculated the fermentation balance,  
 272 i.e. the amount of produced metabolites in mole *per* mole of fermented pyruvate, which is reported in table  
 273 1.

274 **Table 1.** Fermentation balance for the major products of pyruvate fermentation

Strains	Acetate (mol/mol pyruvate)	Ethanol (mol/mol pyruvate)	Succinate (mol/mol pyruvate)
WT	0.77	0.09	0.12
SM4	0.79	0.004	0.18

275

276 The fermentation balance shows that the discrepancy of ethanol production in the SM4 strain led to a  
 277 diversion of the metabolic fluxes towards mainly the production of succinate, and towards the production  
 278 of acetate to a lesser extent.

## 279 Discussion

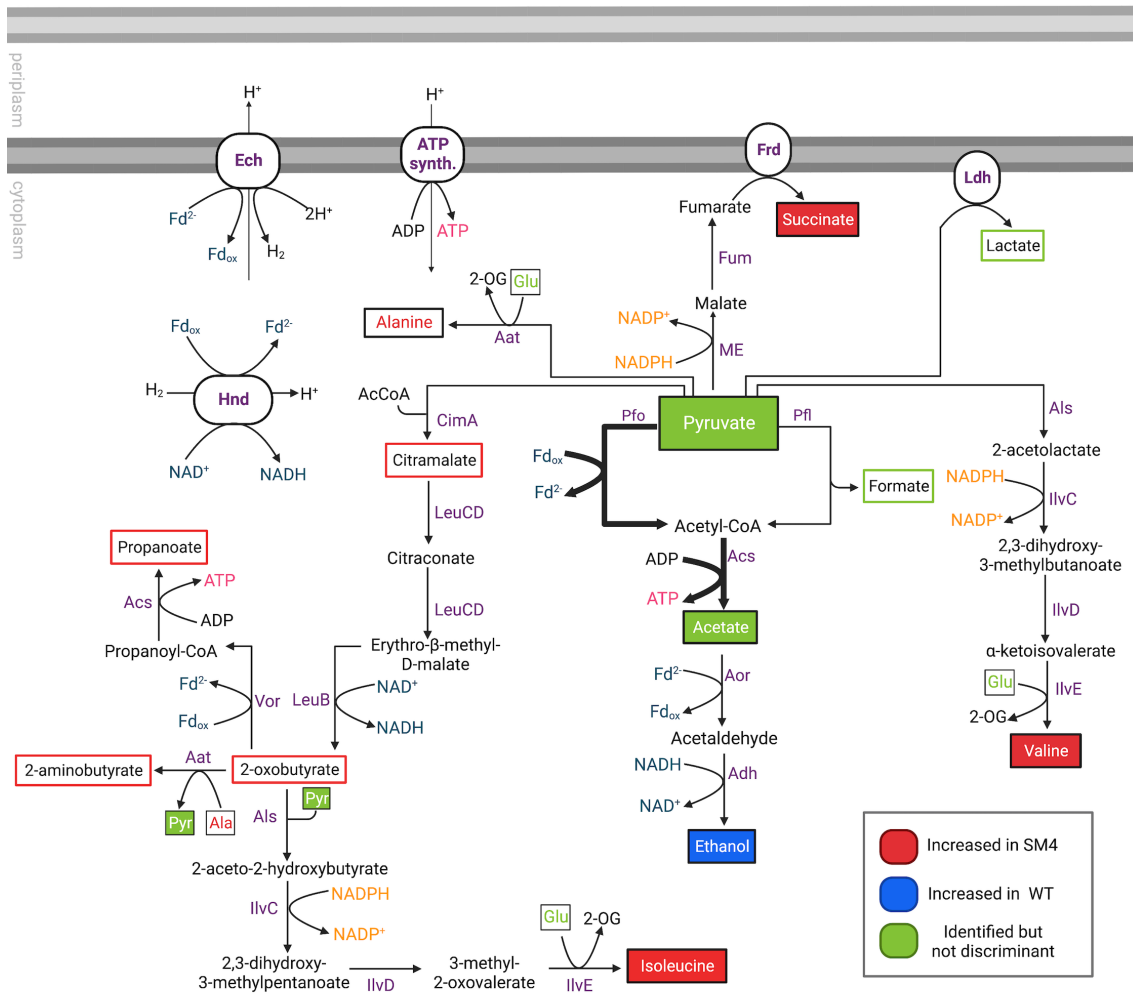
280 Our group previously demonstrated that Hnd is involved in ethanol production during pyruvate fermentation  
 281 (Payne et al., 2022). However, this involvement is non-essential, as the growth rate and the rate of pyruvate  
 282 consumption remained similar for both strains. This raised further questions about other pathways in which  
 283 Hnd could potentially be involved. To further clarify the role of Hnd in *S. fructosivorans* metabolism, a  
 284 metabolomics approach was carried out on WT and SM4 strains.

285 To elucidate the secondary pathways influenced by Hnd, we characterized first the metabolism of *S.*  
 286 *fructosivorans* WT strain during pyruvate fermentation using NMR spectroscopy. One of the major  
 287 intracellular metabolites identified was  $\alpha,\alpha$ -trehalose, a disaccharide consisting of two glucose monomers.  
 288 Several *Desulfovibrio* species have been shown to synthesize polyglucose molecules which serve as a  
 289 storage polymer, and the conditions inducing polyglucose accumulation vary between species (Fareleira et  
 290 al., 1997; Santos et al., 1993; Stams et al., 1983; Van Niel and Gottschal, 1998). For example, in *D. vulgaris*  
 291 str. Hildenborough, polyglucose accumulation is caused by limiting  $\text{Fe}^{2+}$  and  $\text{NH}_4^+$  in the growth medium,

292 whereas in *D. gigas*, high levels of polyglucose are accumulated regardless of the growth condition (Stams  
293 et al., 1983). In *D. gigas* (now *Megalodesulfobivrio gigas*), the degradation of polyglucose has been shown  
294 to provide reducing potential to produce nucleoside triphosphates under both fermentative and respiratory  
295 conditions, and to aid in survival after exposure to O<sub>2</sub> (Fareleira et al., 1997). Trehalose specifically was  
296 accumulated in *D. halophilus* (now *Pseudodesulfobivrio halophilus*) in response to increasing medium  
297 osmolarity (Welsh et al., 1996). However, in our study,  $\alpha,\alpha$ -trehalose content was not affected by the  
298 deletion of *hndD* suggesting that it may not be involved in the pyruvate fermentation process. This was also  
299 demonstrated by van Niel et al. in *D. salexigens* Mast1 (now *Maridesulfobivrio salexigens*) for which very  
300 little polyglucose was accumulated during pyruvate fermentation (Van Niel et al., 1996).

301 The other major identified intracellular metabolites were the markers of pyruvate fermentation, such as  
302 acetate, succinate, and ethanol. These metabolites were also detected in high concentration in the spent  
303 culture medium, which would imply that they are first accumulated in the cells before being excreted in the  
304 medium. Other end-products of the pyruvate fermentation, such as lactate, propanoate, formate, butyrate,  
305 and butanol, could only be detected in the spent culture medium, meaning that these are transient  
306 intracellular metabolites.

307 In addition, we identified intracellular amino acids such as alanine, isoleucine, valine, glutamate, and lysine,  
308 which were all detected in the spent culture medium as well, except for glutamate and lysine. Furthermore,  
309 the examination of the spent culture medium enabled us to identify other metabolites such as citramalate,  
310 2-oxobutyrate and 3-methyl-2-oxovalerate. Except for lysine, all these metabolites belong to interconnected  
311 biosynthesis pathways in which pyruvate plays a central role (Fig. 5).



312

313 **Fig.5** *S. fructosivorans* metabolic pathways active during pyruvate fermentation. One of the major produced metabolite  
 314 is acetate formed from the one-step conversion of acetyl-CoA through acetyl-CoA synthase (Vita et al., 2015). In the  
 315 WT strain, ethanol is formed as a minor fermentation product with acetate and acetaldehyde as intermediary  
 316 metabolites. When *hnd* is deleted, in strain SM4, ethanol is not formed, and flux is redirected to form higher  
 317 concentrations of succinate and the branched-chain amino acids isoleucine and valine. 2-oxobutyrate, a cytotoxic  
 318 intermediate in L-isoleucine biosynthesis, is metabolized into 2-aminobutyrate and propanoate. All detected  
 319 metabolites are framed. Colored letters framed in black indicates the metabolite is exclusively intracellular; black  
 320 letters framed in color indicates the metabolite is exclusively extracellular; white letters with a filled frame in color  
 321 indicates the metabolite is both intra- and extracellular. The color indicates in which strain the metabolite concentration  
 322 is increased: red = increased in SM4; blue = increased in WT; green = metabolite was identified but not discriminant.  
 323 Bold arrows indicate the pathways with increased flux in SM4. The names of the enzymes catalyzing each reaction are  
 324 indicated in purple. Aat: alanine aminotransferase; Acs: acetyl-CoA synthase; Adh: alcohol dehydrogenase; Als:  
 325 acetolactate synthase; Aor: aldehyde ferredoxin oxidoreductase; Cim: citramalate synthase; Ech: energy-converting



326 hydrogenase; Frd: fumarate reductase; Fum: fumarase; Hnd: tetrameric electron-bifurcating hydrogenase; IlvC: ketol-  
327 acid reductoisomerase; IlvD: dihydroxy-acid dehydratase; IlvE: branched-chain amino acid aminotransferase; Kor:  
328 ketoisovalerate oxidoreductase; Ldh: lactate dehydrogenase; LeuB: 3-isopropylmalate dehydrogenase; LeuCD: 3-  
329 isopropylmalate dehydratase/isomerase; ME: NADP-malic enzyme; Pfl: pyruvate formate-lyase; Por: pyruvate  
330 ferredoxin oxidoreductase. AcCoA: acetyl-CoA; Ala: alanine; Fd: ferredoxin; Glu: glutamate; 2-OG: 2-oxoglutarate  
331

332 Pyruvate reacts with glutamate and is converted into L-alanine and 2-oxoglutarate through the action of an  
333 alanine aminotransferase. L-valine is also synthesized from pyruvate, with the NADPH-dependent ketol-  
334 acid reductoisomerase acting in an intermediate step. Interestingly, the presence of citramalate, 2-  
335 oxobutyrate, and 3-methyl-2-oxovalerate suggests that in *S. fructosivorans*, isoleucine is formed from an  
336 alternative biosynthesis pathway from pyruvate and acetyl-CoA instead of threonine. This pathway was  
337 introduced as the primary pathway to produce isoleucine in *Geobacter sulfurreducens* (Risso et al., 2008),  
338 but has never been previously described for *Desulfovibrio*. The intermediate 2-oxobutyrate was further  
339 metabolized directly to 2-aminobutyrate by the alanine aminotransferase or to propanoate with propanoyl-  
340 CoA as the intermediary metabolite.

341 The primary effect of the deletion of *hnd* was the difference in ethanol content in the mutant strain. This is  
342 concurrent with the results previously presented demonstrating that Hnd was involved in the process of  
343 ethanol formation from pyruvate in the absence of sulfate (Payne et al., 2022). We showed decreased levels  
344 of gene expression for two enzymes, aldehyde:ferredoxin oxidoreductase (Aor) and alcohol dehydrogenase  
345 (Adh) in strain SM4, enzymes which are responsible for the conversion of acetate into acetaldehyde, then  
346 into ethanol. Our metabolomics analysis confirmed that, in absence of Hnd, the fermentation process of  
347 pyruvate was interrupted before the formation of ethanol. Considering the increased concentration of  
348 succinate produced in strain SM4, the pyruvate that is not metabolized into ethanol appears to be redirected  
349 to the pathway of succinate formation via malate and fumarate, which is confirmed by the fermentation  
350 balance presented in table 1. The oxidation of pyruvate to malate in *Desulfovibrio* is catalyzed by the NADP-  
351 dependent malic enzyme (Kremer et al., 1989), which was shown to be critical for growth during pyruvate  
352 fermentation in *D. alaskensis* G20 (now *Oleidesulfovibrio alaskensis*) (Meyer et al., 2014). The reduction  
353 of fumarate to succinate is catalyzed by the membrane-bound fumarate reductase complex FrdABC, which

354 reduces succinate with electrons derived from the menaquinol pool (Meyer et al., 2014; Thauer et al., 2007).  
355 Both enzymes catalyze reactions that are independent of NAD and ferredoxin, thus not linked to Hnd via  
356 these redox partners. In some organisms, succinate efflux is coupled to the generation of an ion gradient,  
357 but in *S. fructosivorans*, it is still unclear what transporter is involved in succinate excretion. No homologs  
358 of known anaerobic C4-dicarboxylic acid transporters (Dcu family) (Engel et al., 1994; Kleefeld et al., 2009;  
359 Zientz et al., 1996) were found in the genome of *D. fructosivorans*, nor were homologs of the TRAP  
360 transporters (Tripartite ATP-independent periplasmic transporters) (Rosa et al., 2018) as was reported in *O.*  
361 *alaskensis* G20 (Meyer et al., 2014). The most likely candidates are two proteins with CitT-like domains  
362 (DesfrDRAFT\_1398 and 1718), but in *Escherichia coli* CitT is predicted to function as a citrate/succinate  
363 antiporter and is utilized when cells ferment citrate (Pos et al., 1998). In *O. alaskensis*, the efflux of succinate  
364 was linked to the generation of a chemiosmotic gradient due to the symport of protons (Meyer et al., 2014),  
365 but with the lack of a known succinate transporter homolog in *S. fructosivorans*, this conclusion cannot be  
366 drawn.

367 We also observed increased production of the branched-chain amino acids, valine and isoleucine, in strain  
368 SM4. The ketol-acid reductoisomerase IlvC is involved in the biosynthesis of both valine and isoleucine  
369 and is dependent on NADPH (Rane and Calvo, 1997), as is the malic enzyme. It is therefore plausible that  
370 pyruvate oxidation in strain SM4 is redirected to pathways that are dependent on NADPH, away from the  
371 ethanol production pathway which is dependent on NADH and therefore correlated to the presence of Hnd.  
372 2-oxobutyrate is a central metabolite formed in the isoleucine biosynthesis pathway, but due to its  
373 cytotoxicity (Fang et al., 2021), it is exported from the cell or metabolized to propanoate or 2-aminobutyrate.  
374 An increased concentration of intracellular alanine was also observed in strain SM4. The amino group from  
375 alanine is transferred by the alanine aminotransferase to 2-oxobutyrate to form 2-aminobutyrate, thus the  
376 increased production of alanine in SM4 could correspond with the increased production of 2-aminobutyrate.  
377 The diversion of pyruvate oxidation from ethanol production to pathways dependent on NADPH is a likely  
378 compensation mechanism due to the loss of a source of NADH when *hndD* is deleted. However, increased  
379 amounts of NADPH are needed in SM4 compared to the WT in order to sustain the flux through these  
380 pathways. A potential source of NADPH is the putative electron-bifurcating hydrogenase Hnt

381 (DesfrDRAFT\_0985-0987), identified by our team (Baffert et al., 2019). This trimeric enzyme is encoded  
382 in the same cluster as genes encoding for the malic enzyme, fumarase and fumarate reductase, which gives  
383 an indication of its function in malate and fumarate metabolism. However, no biochemical or genetic studies  
384 have been carried out on Hnt, and further works have to be performed to understand better its physiological  
385 function. It does, however, seem rational that *S. fructosivorans* would have two different bifurcating  
386 hydrogenases with specificities for different redox cofactors. In this case, Hnt would be consuming H<sub>2</sub> and  
387 producing reduced ferredoxin and NADPH, albeit at a slower rate than Hnd, because strain SM4 produces  
388 more H<sub>2</sub> compared to the WT (Payne et al., 2022).

389 Metabolomics analysis of *S. fructosivorans* WT and SM4 ( $\Delta hndD$ ) was able to confirm that Hnd was mainly  
390 involved in the pathway of ethanol production under pyruvate fermentation, which was dramatically  
391 downregulated in the SM4 strain. We also highlighted the metabolic reprogramming that took place in which  
392 several NADP-dependent pathways, including succinate production and branched-chain amino acid  
393 biosynthesis, were upregulated due to the deletion of *hndD*.

394

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#### 402 **Declaration of interest**

403 The authors have no conflict of interest to declare.

#### 404 **Author statement**

405 MB and LS conceptualized and designed the study. NP, AK, CG, CB, MB performed the microbiological  
406 experiments and cell culture. NP performed the metabolomic analysis. MM, TH, FT, MB and LS supervised  
407 the study. NP, MB and LS interpreted the results. NP and LS drafted the manuscript. All co-authors

408 participated in the critical review of the final manuscript. All authors have read and approved the revised  
409 version of the manuscript.

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