Hydrogen production by *Sulfurospirillum* species enables syntrophic interactions of Epsilonproteobacteria

Stefan Kruse¹, Tobias Goris¹, Martin Westermann², Lorenz Adrian³,⁴ & Gabriele Diekert¹

Hydrogen-producing bacteria are of environmental importance, since hydrogen is a major electron donor for prokaryotes in anoxic ecosystems. Epsilonproteobacteria are currently considered to be hydrogen-oxidizing bacteria exclusively. Here, we report hydrogen production upon pyruvate fermentation for free-living Epsilonproteobacteria, *Sulfurospirillum* spp. The amount of hydrogen produced is different in two subgroups of *Sulfurospirillum* spp., represented by *S. cavolei* and *S. multivorans*. The former produces more hydrogen and excretes acetate as sole organic acid, while the latter additionally produces lactate and succinate. Hydrogen production can be assigned by differential proteomics to a hydrogenase (similar to hydrogenase 4 from *E. coli*) that is more abundant during fermentation. A syntrophic interaction is established between *Sulfurospirillum multivorans* and *Methanococcus voltae* when cocultured with lactate as sole substrate, as the former cannot grow fermentatively on lactate alone and the latter relies on hydrogen for growth. This might hint to a yet unrecognized role of Epsilonproteobacteria as hydrogen producers in anoxic microbial communities.
Hydrogen gas (H\textsubscript{2}) is an important energy substrate for many bacteria and archaea, playing a crucial role in the anaerobic food web, e.g., in syntrophic interactions. It is produced by fermenting bacteria as a result of the disposal of excess reducing equivalents. Besides H\textsubscript{2}, also formate, similarly formed during fermentative metabolism, is an important electron carrier in e.g., syntrophic fatty acid-degrading methanogenic consortia\textsuperscript{1}. Other prokaryotes may use both H\textsubscript{2} and formate as an electron donor for e.g., sulfate respiration or methanogenesis. In syntrophic interactions, the formate-/H\textsubscript{2}-producing bacterium is dependent on the electron donor uptake by its syntrophic partner, which sustains a low H\textsubscript{2} partial pressure or low formate concentration and thus enables H\textsubscript{2}/formate production, which would otherwise thermodynamically be unfavorable\textsuperscript{2-4}. For example, butyrate, propionate or acetate-oxidizing anaerobic bacteria that form H\textsubscript{2} or formate as fermentation product are dependent on formate-/H\textsubscript{2}-oxidizing microorganisms such as methanogenic archaea\textsuperscript{5-7}. It was shown that the interspecies H\textsubscript{2} or formate transfer becomes more efficient when syntrophs and methanogens are in close physical contact\textsuperscript{8,9}. The syntrophic degradation of propionate by a coculture of Pelotomaculum thermopropionicum and Methanothermobacter thermoaerotrophicus as well as butyrate degradation coupled to organohalide respiration by Syntrophomonas wolfei and Dehalococcoides mccartyi\textsuperscript{195} resulted in aggregate formation and cell-to-cell contact of the involved organisms\textsuperscript{10,11}. Besides interspecies transfer of molecular energy carriers, electrons can be transferred directly between syntrophic partners via electroconductive protein connections in a process termed direct interspecies electron transfer\textsuperscript{12}. In addition to the importance of H\textsubscript{2} in microbial food webs, H\textsubscript{2} is considered to be an alternative energy source and biohydrogen production by microorganisms is discussed as one way to generate environmentally compatible fuels\textsuperscript{13}.

Epsilonproteobacteria are hitherto considered to be H\textsubscript{2}-consuming organisms and H\textsubscript{2}-oxidizing enzymes of only a few Epsilonproteobacteria are characterized so far, e.g., the membrane-bound uptake hydrogenases of Helicobacter pylori and Wolinella succinogenes\textsuperscript{14,15}. Sulfurosirrillum carboxydovorans was shown to produce minor amounts of hydrogen, which was finally consumed again, upon CO oxidation\textsuperscript{16}. fermentative H\textsubscript{2} production has never been shown to be performed by any Epsilonproteobacterium so far, although in recent years several Epsilonproteobacteria, especially marine, deep vent-inhabiting species, were reported to encode putative H\textsubscript{2}-evolving hydrogenases in their genomes\textsuperscript{17-25}. Sulfurosirrillum spp. are free-living, metabolically versatile Epsilonproteobacteria, many of which are known for their ability to respire toxic or environmentally harmful compounds such as arsenate, selenate or organohalides (e.g., tetrachloroethene—PCE)\textsuperscript{26,27}. The anaerobic respiration with PCE, leading to the formation of cis-1,2-dichloroethene (cDCE), was studied in detail in Sulfurosirrillum multivorans (formerly known as Dehalosirrillum multivorans)\textsuperscript{28,29}. Several Sulfurosirrillum spp. were found in contaminated sediments, wastewater plants, marine environments or on bio- cathodes\textsuperscript{16,22,26,30}. The role of Sulfurosirrillum in such environments is unclear.

In previous studies, four gene clusters, each encoding a [NiFe] hydrogenase, were found in the genome of S. multivorans\textsuperscript{23} and most other Sulfurosirrillum spp.\textsuperscript{26,28}. Two of these appear to be H\textsubscript{2}-producing, the other two are potential H\textsubscript{2}-uptake enzymes as deduced from sequence similarity to known hydrogenases. Of these four hydrogenases, one of each type, H\textsubscript{2}-oxidizing and H\textsubscript{2}-producing, were previously detected in S. multivorans\textsuperscript{29,31}. The periplasmically oriented H\textsubscript{2}-oxidizing enzyme is very similar to the characterized W. succinogenes and H. pylori membrane-bound hydrogenases (MBH). It comprises three subunits, the large subunit, harboring the NiFe active site, a small subunit for electron transfer with three FeS clusters, and a membrane-integral cytochrome b. The putative H\textsubscript{2}-producing, cytoplastically oriented enzyme (Hyf) is a large, complex enzyme with eight subunits, four of them presumably membrane-integral. Regarding amino acid sequence and subunit architecture, this hydrogenase is similar to hydrogenase 4 of Escherichia coli, part of a putative second formate hydrogen lyase (FHL)\textsuperscript{32,35}. However, in S. multivorans, Hyf is unlikely to form an FHL complex since the corresponding gene cluster does not encode any formate-specific proteins as is the case for the FHL complexes in E. coli (Supplementary Figure 1).

Here, we show that several Sulfurosirrillum spp. produce H\textsubscript{2} upon pyruvate fermentation. Sulfurosirrillum caveoli was observed to produce more H\textsubscript{2} than other Sulfurosirrillum spp., which is caused by a different fermentation metabolism. To unravel the metabolism and the hydrogenase equipment of both organisms, label-free comparative proteomics was carried out. A coculture experiment of S. multivorans with the methanogenic archaon Methanothermobacter wolfei\textsuperscript{195} revealed an interspecies H\textsubscript{2} transfer between both organisms suggesting a hitherto undiscovered contribution of Sulfurosirrillum spp. and other Epsilonproteobacteria to the microbial anaerobic food web as H\textsubscript{2} producers.

Results

Adaptation of S. multivorans to pyruvate fermentation. In previous studies, S. multivorans and other Sulfurosirrillum spp. were shown to grow fermentatively on pyruvate\textsuperscript{26,33,34}. Only few data on growth behavior are available in the literature, but S. multivorans was reported to exhibit poor growth on pyruvate as sole energy source compared to respiratory growth with pyruvate and fumarate or tetrachloroethene (PCE) as electron acceptor\textsuperscript{33,35}. However, we observed an adaptation of S. multivorans to fermentative growth on pyruvate. After about 20 transfers with 10% inoculum each, a growth rate of 0.09 h\textsuperscript{-1} was observed (Fig. 1, Supplementary Figure 2). During the adaptation to pyruvate fermentation, the growth rate increased on average by 0.02 h\textsuperscript{-1} with each transfer (Supplementary Figure 3). In addition, the lag phase duration decreased from initially 40 to 5 h. After 18 transfers, no further significant increase of the growth rate was observed. This adaptation process was also observed for S. caveoli, Sulfurosirrillum deleyianum and Sulfurosirrillum arsenophilum. For further investigation of the adaptation process, S. multivorans cells not adapted to pyruvate fermentation and those adapted to pyruvate fermentation were harvested and subjected to proteome analysis (see subsection “Comparative genomics and proteomics” under the Results section).

Fermentative growth and H\textsubscript{2} production by Sulfurosirrillum. To get deeper insight into the fermentation pathways and H\textsubscript{2} production capabilities of Sulfurosirrillum spp., several species were cultivated with pyruvate as sole substrate. Six species were tested for pyruvate fermentation, of which Sulfurosirrillum barnesi and Sulfurosirrillum halorespirans were not able to grow even after cultivation for several months. S. caveoli, S. deleyianum, and S. arsenophilum grew on pyruvate alone at slower rates than S. multivorans (0.03, 0.06, and 0.004 h\textsuperscript{-1}, respectively, Fig. 2, Supplementary Figures 4 and 5). H\textsubscript{2} production was measured for all fermentatively growing Sulfurosirrillum spp., but the produced amount differed, depending on the species. S. caveoli produced the highest amount of H\textsubscript{2} followed by S. arsenophilum. S. deleyianum, and S. multivorans produced about 100 μmol H\textsubscript{2} per 100 mL culture. Desulfitobacterium hafniense DCB-2, a known
pyruvate-fermenting organohalide-respiring bacterium, grows similar to Sulfurospirillum spp. (Fig. 2a) but produced only minor amounts of H2 (20 µmol) (Fig. 2b). Fermentative growth on lactate was not observed for any of the organisms including D. hafniense DCB-2 even after cultivation for several months.

Fermentative metabolism of S. multivorans and S. cavolei. To unravel the fermentative metabolism of two Sulfurospirillum spp. showing different H2 production patterns during growth on pyruvate, S. multivorans and S. cavolei were cultivated in a fermentation apparatus in which the gas phase of the Schott bottle was connected to CO2 and H2 traps (see Supplementary Figure 6) to avoid increasing gas partial pressures and hence a possible product inhibition on H2 production or growth (see also subsection “Product inhibition by H2 on fermentation of Sulfurospirillum”). Enhanced H2 evolution was measured when compared to the serum bottle experiment, with up to hundred times more H2 produced, while the growth was slower than in the previous setup (Fig. 3a, Supplementary Figure 7A and 8A). After consumption of 40 mM pyruvate, 27 mM acetate, 10 mM lactate, 3 mM succinate, 10 mM H2, and 28 mM CO2 were measured as fermentation products of S. multivorans (Fig. 3a). S. cavolei showed slower growth than S. multivorans and a much higher amount of H2 evolved. During growth, which took 8–10 days, pyruvate (40 mM) was used up completely and 38 mM acetate, 36 mM H2, and 38 mM CO2 were the only products detected (Fig. 3b, Supplementary Figures 7B and 8B). No other organic acids such as formate or alcohols, e.g. ethanol, were detected for both. S. deleyianum showed similar fermentation products as observed with S. multivorans (Supplementary Figure 9).

The stoichiometry of the fermentation was verified by calculating the carbon recovery and an oxidation/reduction balance (Supplementary Table 1, Eqs. (1) and (2)). In S. multivorans, the amount of reducing equivalents generated from pyruvate oxidation was calculated to be 54 [H], which fits to the amount of used reducing equivalents for the production of molecular hydrogen, lactate and succinate (52 [H], Supplementary Table 1). In S. cavolei, pyruvate oxidation leads to the generation of 76 [H], which were almost exclusively (72 [H]) used for proton reduction to H2. In addition, the carbon recovery is in...
agreement with the theoretical values and is 102.5% for \textit{S. multivorans} and 95% for \textit{S. cavolei}. The anabolic assimilation of the carbon source is minor with approximately 2.5 mM for \textit{S. multivorans} and 2 mM for \textit{S. cavolei} as calculated from OD and dry weight.

\begin{align*}
1.0 \text{ Pyruvate} &\rightarrow 0.7 \text{ Acetate} + 0.25 \text{ Lactate} + 0.075 \text{ Succinate} \\
&+ 0.25 \text{ H}_2 + 0.7 \text{ CO}_2 \\
(1) \\
1.0 \text{ Pyruvate} &\rightarrow 0.95 \text{ Acetate} + 0.9 \text{ H}_2 + 0.95 \text{ CO}_2 \\
(2)
\end{align*}

**Product inhibition by H\textsubscript{2} on fermentation of \textit{Sulfurospirillum}**.

The different amount of H\textsubscript{2} produced in the growth experiments in serum bottles and the fermentation apparatus imply a product inhibition of H\textsubscript{2} on H\textsubscript{2} production. To investigate the effect of H\textsubscript{2} in the gas phase on the fermentative growth of \textit{S. multivorans} and \textit{S. cavolei}, both organisms were cultivated in serum bottles with a gas phase of 100% H\textsubscript{2} or 100% nitrogen (Fig. 4, Supplementary Figures 10 and 11). With nitrogen as gas phase, \textit{S. multivorans} and \textit{S. cavolei} showed similar growth and production rates of organic acids as observed in the fermentation apparatus. A strong negative effect on growth was observed with 100% H\textsubscript{2} in the gas phase. \textit{S. multivorans} was still able to ferment pyruvate but showed an inhibited growth and a lower cell density compared to the culture without H\textsubscript{2} in gas phase, while \textit{S. cavolei} was almost completely inhibited (Fig. 4a). The restricted growth is also reflected by a lower pyruvate consumption rate (Fig. 4b). In addition, the formation of fermentation products shifted from acetate production to lactate and succinate formation in \textit{S. multivorans} (Fig. 4c–e). \textit{S. cavolei} produced neither lactate nor succinate and only minor amounts of acetate.

**Hydrogenase activities by \textit{Sulfurospirillum} cell suspensions.**

The H\textsubscript{2} production and oxidation capability of cell suspensions of \textit{S. multivorans} and \textit{S. cavolei} was analyzed to obtain further evidence about the hydrogenase involved in the production and oxidation reaction. Transcriptional and proteomic studies revealed the presence of two [NiFe] hydrogenases in \textit{S. multivorans} \textsuperscript{31}: a hydrogen-oxidizing periplasmically oriented MBH and a putative H\textsubscript{2}-producing cytoplasmically oriented MBH \textsuperscript{(Hyf)}. These two hydrogenases might be distinguished by their different subcellular localization and thus their accessibility to redox mediators like viologens in hydrogenase activity assays. Photometrically measured H\textsubscript{2}-oxidizing activity was detected in whole cell suspensions as well as in membrane and soluble fractions (Table 1). In contrast, H\textsubscript{2}-producing activity, as monitored by GC, was only measured with membrane fractions but not in whole cell suspensions of \textit{S. multivorans} and \textit{S. cavolei} with approximately 1.5-fold higher activity in \textit{S. cavolei} (Table 1). The membrane fractions of \textit{S. multivorans} and \textit{S. cavolei} cells grown on pyruvate as sole energy source were about 2-fold more active in H\textsubscript{2}-production than those of cells cultivated under respiratory growth conditions with pyruvate plus fumarate, while the latter exhibited slightly more H\textsubscript{2} oxidation activity. \textit{Clostridium pasteurianum} DSM 525, which is known to harbor a cytoplasmic soluble H\textsubscript{2}-producing hydrogenase, exhibited hydrogenase activity only in the soluble fractions and showed no H\textsubscript{2} producing activity in cell suspensions with methyl viologen as electron donor (Supplementary Table 2), thus serving as a control for the hydrogenase localization experiment.

**Comparative genomics and proteomics.**

To unravel the cause of the different fermentative metabolisms of the two \textit{Sulfurospirillum} sp., a comparative genomic analysis was done with the RAST sequence comparison tool \textsuperscript{35}. Additionally, proteomes of \textit{S. cavolei} NBRCC109482 and \textit{S. multivorans} cultivated under fermentating and respirating conditions with fumarate as electron acceptor were analyzed. Bidirectional blast hits with more than 50% amino acid sequence identity were considered as orthologs, proteins putatively fulfilling the same functions in both organisms. The genomes were overall similar, with 2057 of 2768 of the encoded proteins in \textit{S. cavolei} being orthologs. Only few of the nonorthologous proteins in \textit{S. cavolei} could be considered to play a role in the fermentation based on their annotation and putative involvement in one of the pathways connected to fermentative catabolism. Among the proteins encoded in the \textit{S. cavolei} genome (annotated RefSeq WGS accession number NZ_BBQE01000001.1), which do not have an ortholog in \textit{S. multivorans}, we found a cluster encoding an [FeFe] hydrogenase (Supplementary Figure 12) known to contribute to fermentative H\textsubscript{2} production in many bacteria, e.g. \textit{Clostridia}. A nearly identical gene cluster is found in the other two genomes of \textit{S. cavolei} strains UCH003 (NZ_AP014724.1) and MES (JSEC00000000.1), the latter of which was assembled from a metagenome \textsuperscript{22}. The large [FeFe] hydrogenase catalytic subunit gene, \textit{hydA}, is disrupted by a stop codon resulting from a nucleotide insertion only in \textit{S. cavolei}. The mutation was confirmed by PCR and Sanger sequencing. RT PCR analysis suggested that \textit{hydA} was transcribed under pyruvate-fermenting growth conditions (Supplementary
However, the [FeFe] hydrogenase was not identified in the proteome of *S. cavolei*. Apart from the [FeFe] hydrogenase, the four [NiFe] hydrogenase gene clusters were highly similar in the genomes of both *S. multivorans* and *S. cavolei*. The Hyf hydrogenase was found in high abundances especially in the proteome of *S. multivorans* cultivated with pyruvate alone. Here, four out of eight of the structural subunits were found in the 10% of the most abundant proteins, while none were found in the top 10% under respiratory conditions. In *S. cavolei*, the hydrogenase 4 subunits were not as abundant as in *S. multivorans* with only two out of six quantified subunits in the top 20% (Supplementary Data 1 and 2). In both organisms, a significantly higher amount of Hyf subunits was quantified under fermentative growth conditions (*S. multivorans*: 4- to 27-fold for the structural subunits HyfA-HyfI, all p values are <0.001, *S. cavolei*: 2- to 5-fold for HyfA-HyfI, all p values are <0.05; Fig. 5, Supplementary Table 3, Supplementary Data 2). Interestingly, the *Hyf* gene cluster is disrupted at one site in *S. halorespirans*, which cannot grow on pyruvate alone. Genome sequencing36 (CP017111.1) revealed a transposase insertion at *hyfB* which might result in a nonfunctional gene *S. halorespirans*. The transposon insertion was confirmed by PCR using *hyfB*-specific primers flanking the transposase (Supplementary Figure 14, Supplementary Table 4). The membrane-bound subunits

| Cellular fraction     | Hydrogenase activity (nkat mg⁻¹) | *S. multivorans* | *S. cavolei* |
|-----------------------|----------------------------------|------------------|--------------|
|                       |                                  | MV → H₂ | H₂ → BV | H₂ → MV | MV → H₂ | H₂ → BV | H₂ → MV |
| Cell suspensions      |                                  | <0.01   | 4.1 ± 0.5 | 0.7 ± 0.3 | <0.01   | 5.5 ± 0.6 | 1.4 ± 0.3 |
| Membrane fractions    |                                  | 12.3 ± 2.4 | 23.5 ± 2.1 | n.d. | 20.6 ± 3.7 | 10.1 ± 0.5 | n.d. |
|                      | Pyr                              | 5.7 ± 1.5 | 36.6 ± 3.3 | n.d. | 10.1 ± 2.4 | 13.5 ± 1.6 | n.d. |
|                      | Pyr + Fum                        | <0.01   | n.d. | n.d. | <0.01 | n.d. | n.d. |
| Soluble fractions     |                                  | <0.01   | 2.3 ± 0.3 | n.d. | <0.01 | 1.9 ± 0.3 | n.d. |
|                      | Pyr                              | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

MV → H₂ indicates H₂ formation activity, H₂ → BV/MV indicates H₂ oxidation. Data are derived from three independent biological replicates and show means ± standard deviation. MV methyl viologen, BV benzyl viologen, Pyr pyruvate, Fum fumarate, n.d. not determined.
HyIE and HyIF were found in fermenting cells of *S. multivorans* exclusively. Sequence comparison of the Hyf hydrogenase of *S. multivorans* shows similarities to the proton-pumping complex I of *Thermus thermophilus* (Supplementary Figure 15). An analysis regarding the potential proton-pumping capabilities of the *S. multivorans* Hyf deduced from conserved amino acids which are responsible for proton pumping in complex I of *T. thermophilus* and a comparison to the *E. coli* FHL is given in the Supplementary information (Supplementary Note 1, Supplementary Table 5 and Supplementary Figures 16–18). Important conserved amino acids which are likely involved in proton pumping of complex I are fully conserved in one of the four Hyf membrane subunits, namely in HyfF.

A search for the *hyf* gene cluster in genomes of Epsilonproteobacteria shows that it is ubiquitous in, but not limited to, *Sulfurospirillum* spp. (Supplementary Table 6). Four out of 15 *Sulfurospirillum* spp. genomes harbor a second *hyf* gene cluster colocalized with genes encoding a formate transporter and a formate dehydrogenase (Supplementary Figure 1). In *Arcobacter* spp. and the marine species *Caminibacter mediatlanticus* and *Lebetimonas* spp., only the latter gene cluster encoding a putative FHL complex is found. In several *Campylobacter* spp. including *Campylobacter concisus*, a *hyf* gene cluster with a formate transporter gene was identified (Supplementary Figure 1), while a second group of *Campylobacter* (including *Campylobacter fetus*) does not encode any formate-related proteins (Supplementary Table 6).

The subunits of the MBH were quantified in either unsignificantly lower amounts (HydAB, approximately 2-fold, p values 0.40 and 0.07, Fig. 5) or slightly higher amounts (HydC, approximately 2-fold, p value 0.01) under fermenting conditions for *S. multivorans*. In contrast, HydABC were found in significantly lower amounts in *S. cavolei* when grown fermentatively (Fig. 5). Of the cytoplasmic H$_2$-producing hydrogenase (Ech-like), only one subunit (present in the lower 50% abundant proteins) was quantified in *S. multivorans* grown with pyruvate alone. In *S. cavolei*, five of six Ech-like hydrogenase subunits were quantified in cells cultivated with pyruvate alone and two of six subunits in pyruvate/fumarate-cultivated cells, all of them in the lowest third abundant proteins. No subunit of the cytoplasmic uptake hydrogenase (HupSL) was found in any of the proteomes.

Of the proteins related to pyruvate metabolism, a pyruvate, water dikinase (phosphoenolpyruvate [PEP] synthetase) is encoded in the genome of *S. multivorans* (encoded by SMUL_1602), but not in *S. cavolei*. This enzyme is responsible for the ATP-dependent synthesis of phosphoenolpyruvate from pyruvate in gluconeogenesis (Supplementary Figure 19). The PEP synthetase was found in 6.3-fold higher abundance (p value 0.02) in the proteome of fermentatively cultivated *S. multivorans* cells (Supplementary Table 3). In *S. cavolei*, PEP might be formed from pyruvate via oxaloacetate by two reactions catalyzed by pyruvate carboxylase and PEP carboxykinase. These two enzymes are encoded in one gene cluster (SCA02S_RS02520 and SCA02S_RS02525, respectively, Supplementary Figure 20). In *S. multivorans* these proteins (SMUL_0789 and SMUL_0791) cluster with a gene encoding a subunit similar to the membrane subunit of a putative Na$^+$-translocating oxaloacetate decarboxylase (SMUL_0790), of which an ortholog is not encoded in *S. cavolei* (Supplementary Figure 20). Both pyruvate carboxylase/oxaloacetate decarboxylase and PEP carboxykinase were found in the proteomes of both organisms in slightly higher amounts in cells grown with pyruvate only (Supplementary Data 2). Similar to *S. cavolei*, also *S. arsenophilum*, producing larger amounts of H$_2$ than *S. multivorans* (Fig. 2), lacks the putative oxaloacetate decarboxylase subunit gene.

A pyruvate:ferredoxin oxidoreductase (PFOR) and a ferredoxin (Fd) showed also a higher abundance in both *Sulfurospirillum* sp. under fermenting conditions (*S. multivorans*: PFOR 2-fold, Fd 6-fold, *S. cavolei*: PFOR 4-fold, Fd 2-fold, all p values <0.01; Fig. 5, Supplementary Table 3). A second pyruvate-oxidizing enzyme, a quinone-dependent pyruvate dehydrogenase encoded exclusively in the genome of *S. multivorans*, was significantly lower abundant during pyruvate fermentation (7-fold, p value 0.02). The enzymes responsible for ATP generation via substrate-level phosphorylation, phosphotransacetylase and acetate kinase are slightly higher abundant during pyruvate fermentation in both *Sulfurospirillum* sp. (approximately 2-fold for both enzymes in *S. multivorans*, p values <0.01 and approximately 3-fold in *S. cavolei*, p values <0.001, Supplementary Table 3). The malic enzyme is higher abundant during fermentation in *S. multivorans* (3.7-fold, p value <0.001, Supplementary Table 3) and not quantified in any proteome of *S. cavolei* (Supplementary Table 3).

A putative lactate dehydrogenase (SMUL_0438, SCA02S_RS08360) with 35% amino acid sequence identity to a characterized lactate-producing lactate dehydrogenase from *Selenomonas ruminantium* was not detected in these proteomes. This is in accordance to the lack of pyridine dinucleotide-dependent lactate-oxidizing or pyruvate-reducing activities in cell extracts of *S. multivorans* (methods described in Supplementary Note 2). Several candidates for pyridine dinucleotide-independent lactate dehydrogenases (ILDH) are encoded in the genome of *S. multivorans*. Since *S. deleyianum* shows also lactate production during pyruvate fermentation, only genes present as orthologs in both genomes were considered to be responsible for lactate production in *Sulfurospirillum* spp. Functionally characterized ILDHs are flavin and FeS-cluster-containing oxidoreductases or

---

**Fig. 5** Comparative proteomics of proteins possibly involved in pyruvate fermentation of *S. multivorans* and *S. cavolei*. Comparison of cells grown with pyruvate alone was done with cells grown with pyruvate/fumarate. For quantified proteins the protein intensity ratio is given as colored squares. Nonsignificantly altered proteins levels are marked with an equal sign (p values >0.05). Proteins exclusively found in pyruvate-fermenting cells are colored dark gray. All data were obtained from three independent biological replicates. Hyf-like hydrogenase (SMUL_2383-2392; SCA02S_RS01920-RS01965), MBH membrane-bound hydrogenase (SMUL_1423-1425; SCA02S_RS01350-RS01360), Fd ferredoxin (SMUL_0303; SCA02S_RS12260), PFOR pyruvate:ferredoxin oxidoreductase (SMUL_2630; SCA02S_RS04525), Pyr pyruvate, Fum fumarate.
enzymes related to malate:quinone oxidoreductase. Only two candidates of the former class were identified in the genome, encoded by SMUL_1449 and SMUL_2229. Of these, only the latter gene product was detected in the proteome, however, not in altered amounts under fermentative conditions when compared to respiratory cultivation. To detect proteins involved in lactate oxidation, we compared the proteomes of S. multivorans cells cultivated with lactate and fumarate to that of pyruvate/fumarate-cultivated cells. While we did not observe significant changes in protein abundances of any proteins putatively involved in lactate oxidation, we observed a high abundance of a membrane-bound flavin and Fe-S cluster-containing protein (encoded by SMUL_0787, among the 15 highest abundant proteins in both proteomes) and lactate utilization proteins ABC (encoded by SMUL_1033–1035, in the top 40% abundant proteins) described to oxidize lactate in the related Campylobacter jejuni. The protein encoded by SMUL_0438 was detected in the proteomes of lactate/fumarate and pyruvate/fumarate-cultivated cells, but quantified to be only minor abundant and unsignificantly more abundant in lactate-cultivated cells (lowest 20% abundant proteins, Supplementary Data 4).

Cells adapted to pyruvate fermentation showed not many altered abundances of the aforementioned proteins involved in the fermentative physiology of S. multivorans. However, a molybdoenzyme encoded by SMUL_2101, was one of the ten most abundant during pyruvate fermentation (142-fold more abundant compared to fumarate-respiring cells). This noncharacterized molybdoenzyme oxidoreductase of the aldehyde/xanthine oxidoreductase family was also significantly more abundant (16.5-fold) in pyruvate fermentation-adapted S. multivorans cells (Table 2, Supplementary Data 5). A highly similar aldehyde oxidoreductase is also higher abundant during fermentation of S. cavolei. A BlastP query against the Genbank nr database revealed that orthologs of this protein are conserved in all Sulfospirillum spp., but not in other Epsilonproteobacteria. Closely related proteins (>70% amino acid sequence identity over the whole sequence length) are encoded, among others, in several Clostridium spp. and in Desulfovibrio spp. known for their fermentative metabolism (~60% sequence identity). Additionally to this putative aldehyde oxidoreductase, three other proteins were observed to be more abundant in S. multivorans cells adapted to pyruvate fermentation and at the same time significantly more abundant in S. multivorans and S. cavolei cells during pyruvate fermentation. These include a membrane-integral cytochrome-like protein, a secreted type II asparaginase and a NosL family protein (Table 2, Supplementary Data 5).

**Table 2** Significantly altered protein abundances in the proteomes of pyruvate-adapted cells compared to that of nonadapted cells

| Sm Locus | Protein                        | Lg Pyr | Lg Pyr Ad | Ratio Pyr Ad/Pyr | Ratio Sm Ferm | Ratio Sc Ferm |
|----------|--------------------------------|--------|-----------|------------------|---------------|---------------|
| SMUL_0150 | cytochrome c                  | 6.4    | 8.0       | 39.5             | 8.1           | 7.1           |
| SMUL_2101 | Aldehyde oxidoreductase       | 7.7    | 9.0       | 16.5             | 141.6         | 6.6           |
| SMUL_2819 | Asparaginase                  | 8.5    | 9.3       | 6.1              | 4.1           | 3.8           |
| SMUL_3232 | NosL family protein           | 7.2    | 8.7       | 31.4             | 5.9           | 6.1           |

Shown are the proteins which at the same time are significantly altered in the S. multivorans and S. cavolei proteomes of fermentatively cultivated cells

Syntrophy of Sulfospirillum multivorans with Methanococcus voltae. To unravel the potential role of S. multivorans in a syntrophic partnership as H2 producer, a coculture with M. voltae was prepared. M. voltae is a methanogenic archaean dependent on either H2 or formate as electron donor and CO2 as electron acceptor. To investigate the syntrophic interaction of the two organisms, the coculture was cultivated with lactate, which could not serve as a fermentation substrate for pure S. multivorans cultures. A syntrophic, hydrogen-consuming partner keeping H2 concentration at a low level in cocultures might render lactate fermentation by S. multivorans thermodynamically feasible in a coculture. A medium optimized for M. voltae was used for the coculture (see Methods section). This medium contained a number of organic substances (i.e. the amino acids leucine and isoleucine, as well as casamino acids) not present in the medium of S. multivorans and several controls with S. multivorans pure cultures were performed to exclude any unprecedented growth effects potentially caused by this medium. The growth behavior of S. multivorans in the modified M. voltae medium with pyruvate alone as substrate was similar to that in the medium originally used for cultivation of S. multivorans, albeit a bit slower (Supplementary Figure 21A). The morphology of S. multivorans cells was unaltered in the M. voltae medium and independent from the type of cultivation—fermentatively or respiratory (Supplementary Figure 21B, C). The new medium with lactate as sole growth substrate could not promote growth for pure S. multivorans cultures. In the corresponding coculture, 15 mM lactate was consumed in approximately 2 weeks while methane was formed, indicating lactate fermentation by S. multivorans and H2 transfer to M. voltae as syntrophic partner (Fig. 6a, b). To compare this result to a coculture with S. multivorans cells not adapted to fermentation, we cocultivated the nonadapted cells with M. voltae under otherwise identical conditions. Lactate was consumed in this coculture only slightly slower, taking about 20 days (Supplementary Figure 22). Electron microscopic analyses of the coculture revealed cell aggregates with sizes between 50 and 600 µm (Fig. 6c, Supplementary Figure 23). These aggregates showed a compact network of the rod-shaped S. multivorans and cocoidal M. voltae with net-forming flagellum-like structures surrounding the organisms. The cells in the aggregates were embedded in extracellular polymeric substances (EPS)-like structures, which might aid cell-to-cell contact.

**Discussion**

In this study, production of H2 was observed for several Sulfospirillum species during pyruvate fermentation, which is the first evidence of fermentative H2 production for Epsilonproteobacteria, which hitherto were generally regarded as H2 oxidizers. Specifically, we report H2 production for S. multivorans, S. cavolei, S. arsenophilum, and S. deleianum during fermentative growth on pyruvate. Sequential subcultivation on pyruvate alone revealed a continuous adaptation of Sulfospirillum spp. to a fermentative metabolism. A comparison of the proteomes of cells adapted to pyruvate fermentation with nonadapted cells revealed four proteins which might have a positive effect on the growth rate during pyruvate fermentation of S. multivorans. The proposed role of these four proteins, namely an aldehyde oxidoreductase, a cytochrome, an asparaginase, and a NosL family protein, is discussed later in this chapter.
the complete mechanism behind this long-term adaptation process in *Sulfurospirillum* spp. remains largely unresolved for now and might include also genomic rearrangements and/or population dynamics. An unresolved long-term regulatory effect similar to the one observed for *S. multivorans* after continuous transfer without PCE as electron acceptor might play a role as well. The low adaptation to fermentative metabolism in *Sulfurospirillum* might also be an effect of the long-term respiratory cultivation of the organisms in our laboratory and cell culture collections. Whether *Sulfurospirillum* spp. in natural habitats shows an equally long adaptation time to fermentative metabolism is likely dependent on the type and concentration of electron donor and electron acceptor in the environment.

Two different fermentation balances were observed for the different *Sulfurospirillum* spp. tested. While *S. cavolei* showed the highest H₂ production rate and produced, besides hydrogen, acetate and CO₂, *S. deleyianum* and *S. multivorans*, displaying lower H₂ production, additionally produced succinate and lactate. Pyruvate is most likely oxidatively decarboxylated to acetyl-CoA by the pyruvate:ferredoxin oxidoreductase, which showed an upregulation in the proteome of fermentatively cultivated compared to fumarate-respiring cells in both *S. multivorans* and *S. cavolei*. In contrast, the quinone-dependent pyruvate dehydrogenase (PoxB), which could transfer electrons generated upon pyruvate fermentation to menaquinone, is downregulated in fermenting cells and therefore most likely does not contribute significantly to pyruvate oxidation under this condition. A pyruvate formate lyase is not encoded in any *Sulfurospirillum* spp., which, in addition to the low protein abundance of a cytoplasmic formate dehydrogenase in *S. multivorans* and *S. cavolei*, argues against the role of the Hyf in an FHL complex as opposed to the suggested function for Hyf in *E. coli*. The generated acetyl-CoA is used to generate acetate and one mol ATP per mol pyruvate via substrate-level phosphorylation.

The high abundance of an aldehyde oxidoreductase similar to the extensively studied molybdoenzyme of *D. gigas* in the proteome of pyruvate-fermenting cells and especially those adapted to pyruvate fermentation points to the possible formation of an aldehyde as an intermediate during pyruvate fermentation. This aldehyde intermediate could be oxidized for energetic

---

**Fig. 6** Syntrophic coculture of *S. multivorans* and *Methanococcus voltae*. a Scheme of syntrophic interactions and exchange of metabolites and b lactate concentration in *S. multivorans* pure culture and coculture of *S. multivorans* and *M. voltae*. c Electron microscopic images of aggregates. Magnifications, ×150 (whole aggregate, upper row left, scale bar 200 µm), ×5000 (upper right, scale bar 3 µm), ×10,000 (lower images, scale bar 2 µm). Sections of the lower images were obtained from different areas of the aggregate. White arrows indicate EPS-like structures. Cultivation experiments included three biological replicates in which similar aggregates were formed. S.m. *S. multivorans*. Pure *S. multivorans* and the coculture were cultivated in a medium originally optimized for *M. voltae* modified as described in the Methods section.
The corresponding reaction is unlikely to occur. The biosynthesis of fumarate reductase are also present in S. multivorans fumarate hydratase and fumaric reductase are also present in S. multivorans. Hyf might pump protons via its membrane-integral subunits (Supplementary Note 1, Supplementary Table 6 and Supplementary Figures 10-13) which could lead to additional ATP production via chemiosmotic coupling. The reaction products are highlighted in light blue boxes. Protein abundance ratios (pyruvate alone versus pyruvate/fumarate) are indicated by colored squares (S. multivorans) and circles (S. cavolei) at the protein abbreviations. Color code of the ratios is given in the box at the lower right. Hyf Hyf-like hydrogenase (SMUL_2383-2392; SCA02S_RS01920-RS01965), PFO pyruvate:ferredoxin oxidoreductase (SMUL_2630; SCA02S_RS04525), PTA phosphotransacetylase (SMUL_1483; SCA02S_RS00245), AK acetate kinase (SMUL_1484; SCA02S_RS00240), ME malic enzyme (SMUL_3158; corresponding enzyme in S. cavolei is not present), FH fumarate hydratase (SMUL_1459, SMUL_1679-1680; SCA02S_RS00615-RS00620), FR fumarate reductase (SMUL_0550-0552; SCA02S_RS07735-RS07740)

Fig. 7 Tentative scheme of pyruvate fermentation metabolism in S. multivorans and S. cavolei. Reactions represented by solid arrows belong to the core pyruvate metabolism and are catalyzed by both organisms. Reactions with dashed arrows are solely catalyzed by S. multivorans fumarate hydratase and fumarate reductase are also present in S. cavolei. Hyf might pump protons via its membrane-integral subunits (Supplementary Note 1, Supplementary Table 6 and Supplementary Figures 10-13) which could lead to additional ATP production via chemiosmotic coupling. Question marks indicate enzymes not identified. Fermentation products are highlighted in light blue boxes. Protein abundance ratios (pyruvate alone versus pyruvate/fumarate) are indicated by colored squares (S. multivorans) and circles (S. cavolei) at the protein abbreviations. Color code of the ratios is given in the box at the lower right. Hyf Hyf-like hydrogenase (SMUL_2383-2392; SCA02S_RS01920-RS01965), PFO pyruvate:ferredoxin oxidoreductase (SMUL_2630; SCA02S_RS04525), PTA phosphotransacetylase (SMUL_1483; SCA02S_RS00245), AK acetate kinase (SMUL_1484; SCA02S_RS00240), ME malic enzyme (SMUL_3158; corresponding enzyme in S. cavolei is not present), FH fumarate hydratase (SMUL_1459, SMUL_1679-1680; SCA02S_RS00615-RS00620), FR fumarate reductase (SMUL_0550-0552; SCA02S_RS07735-RS07740)

reasons or for detoxification, which both could explain the faster growth after adaptation. However, aldehyde formation in Sulfospirillum spp. is obscure. The most obvious source of aldehyde formation would be pyruvate decarboxylation by a pyruvate decarboxylase as observed in Gluconobacter oxydans45. Since such a decarboxylase is not encoded in the genome of any Sulfospirillum spp., the corresponding reaction is unlikely to occur. Alternatively, acetdehyde might be produced from acetate via acetyl-CoA and acetaldehyde oxidoreductase, possibly because of the high levels of acetate in the medium in the late phase of fermentation. Another enzyme also higher abundant in cells adapted to pyruvate is a type II asparaginase, which is secreted into the periplasm and suggested to play a role in generating the energy conservation of this hydrogenase a possible scenario. The involvement of an Hyf in the hydrogen-producing hydrogenase since methyl viologen should not have access to the cytoplasm. A cytoplasmic localization was also suggested previously for Hyf of S. multivorans based on the lack of a signal peptide in any of the corresponding subunit amino acid sequences23,31. The involvement of an Hyf in H2 production via pyruvate oxidation was also observed for a group 4 hydrogenase from Pyrococcus furiosus48 and a genetically modified E. coli strain49. The structure and subunit composition of several group 4 hydrogenases suggested their involvement in the generation of a proton motive force, thereby contributing to ATP formation50,51. A thorough alignment analysis of the subunits of Sulfospirillum spp. Hyf indicated that most of the important residues in the membrane helices are conserved, thus making a role in energy conservation of this hydrogenase a possible scenario. The difference in the amount of H2 produced with S. cavolei producing more H2 than S. multivorans can be explained by two different fermentation metabolism types. Opposed to S. cavolei, reducing equivalents can be channeled into the production of lactate and succinate by S. multivorans (as was also observed for S. deleyianum) upon pyruvate fermentation. Succinate might be produced from fumarate (fumarate reductase) via malate (fumarase), which could be formed from pyruvate via reductive decarboxylation to malate by the malic enzyme (Fig. 7). This enzyme, which often functions in the reverse direction e.g. in C4 plants, is upregulated in S. multivorans under fermentative conditions. This finding supports the involvement of the malic enzyme in conversion of pyruvate to malate. The malic enzyme was not detected in the proteomes of S.
cavolei, which might at least partially explain the different fermentation balances.

The origin of lactate in *S. multivorans* is not clear. A protein annotated as NAD⁺-dependent lactate dehydrogenase was not detected or was in low abundance in the proteomes and no NAD (P)⁺-dependent lactate production could be measured. Most likely, the NAD(P)(H)-dependent lactate dehydrogenase is mis-annotated in the genome of *S. multivorans*, as reported for the corresponding protein of the related Epsilonproteobacterium *C. jejuni*52. A role of the *S. multivorans* “lactate dehydrogenase” in respiratory lactate oxidation was also unlikely as its low abundance in the proteome of lactate-grown cells suggest. Instead, lactate is likely oxidized in *S. multivorans* by orthologs of the NAD⁺-independent enzymes recruited by *C. jejuni* for lactate oxidation, a flavin Fe-S cluster-containing enzyme and a three-partite lactate utilization protein52. These proteins were shown to be not substrate-inducible in *C. jejuni*, which is in line with the observed similar abundance of both proteins in lactate and pyruvate-cultivated *S. multivorans* cells. A possible source of lactate in pyruvate fermentation of *S. multivorans* could be the reduction of pyruvate via one of these or another NAD⁺-independent lactate dehydrogenase (iLDH), which are mostly characterized to be functional in the direction of lactate oxidation53,54. In *Sulfospirillum* spp., one could act in the reverse direction to produce lactate, possibly with reduced ferredoxin as electron donor. Of the several candidates of iLDHs of *S. multivorans*, only one of them shows a slight upregulation on pyruvate alone. A corresponding gene cluster is not encoded in the lactate-producing *S. delleyianum*, making it an unlikely candidate for lactate production. A glycolate oxidase was shown to be responsible for lactate oxidation in *Pseudomonas putida*38,55 and a homolog is encoded in both lactate-producing *Sulfospirillum* spp. This protein, however, is not upregulated upon pyruvate fermentation and further studies are needed to identify the lactate-producing enzyme in *S. multivorans*.

The different disposal of excess reducing equivalents during fermentation enables *S. multivorans* to grow with pyruvate even with 100% H₂ in the gas phase, whereas the growth of *S. cavolei* was nearly completely abolished under these conditions. This correlates with a shift towards a higher production of lactate and succinate and a lower acetate and H₂ production of *S. multivorans* under these conditions. H₂ production via Hyf is obviously subject to product inhibition and *S. multivorans* is able to circumvent this by using alternative cytoplasmic electron sinks upon fermentation.

The inability of *Sulfospirillum* spp. to use lactate as sole substrate in pure cultures is most probably due to the thermodynamically unfavorable lactate oxidation to pyruvate upon H₂ production. To test the ecological significance of our observation, we established a lactate-consuming syntrophic partnership of *S. multivorans* with a hydrogen-consumer, *M. voltae*. Indeed, this coculture enabled lactate utilization by *S. multivorans* and the formation of large cell aggregates of the two organisms presumably via the formation of EPS was observed. Since *M. voltae* is not able to thrive in minimal medium40, we cultivated the coculture not in the medium used for *S. multivorans* cultivation, but in a medium for *M. voltae* which included several organic substances. The influence of this medium on *S. multivorans* cultivation was negligible, so that the observed lactate degradation and aggregate formation can be unequivocally linked to the syntrophic coculture. The interspecies electron carrier in the syntrophic relationship of *S. multivorans* and *M. voltae* is hydrogen based on the metabolite analysis of pure *S. multivorans* cultures, where only hydrogen was found as a product of pyruvate fermentation. However, lower levels of formate (<0.5 mM) could not be detected in our analysis and therefore we cannot completely exclude formate as a second electron carrier of lower importance here. Production of formate from CO₂ in *S. multivorans* could be mediated by a cytoplasmic formate dehydrogenase (SMUL_0079), for which the electron donor and catalytic bias is yet unknown. The three periplasmic formate dehydrogenases of *S. multivorans* are putatively formate-oxidizing and most probably connected to the menaquinone pool via a cytochrome b subunit and therefore the production of formate by these enzymes would be thermodynamically unfavorable, since menaquinones have a relatively positive midpoint potential of around −100 mV. Direct interspecies electron transfer is also unlikely to proceed in this syntrophic interaction, since besides none of the typical nanowire structures were visible in electron microscopy, no pili or extracellular cytochromes56, usually used for interspecies electron transfer of e.g. *Shewanella* spp. and *Geobacter* spp.57,58, were found in the genome of any *Sulfospirillum* spp. Both *S. multivorans* cells adapted and not adapted to pyruvate fermentation supported the growth of the coculture, which strengthens our suggested role of *Sulfospirillum* spp. as H₂ producers in anaerobic food webs. Additionally, this role as a potential H₂ producer is most likely not limited to this genus. In a genome mining approach, hyf gene clusters were found among several genera of Epsilonproteobacteria inhabiting a wide range of habitats. Some *Campylobacter* spp. known to be opportunistic or food-borne pathogens encode the same Hyf as *Sulfospirillum* spp., while hyf gene clusters containing either a formate channel gene in different *Campylobacter* spp. or additionally a cytoplasmic formate dehydrogenase in other phyla might indicate the formation of an FHL complex. Since a PFL is missing in these bacteria, it might be presumed that extracellular formate might aid growth in these bacteria as reported for *Thermococcus* spp.59. Some *Sulfospirillum* spp. even encode for both an FHL-independent Hyf and one presumably forming an FHL complex, pointing towards separate regulation and roles of both hydrogenases and thus for even more physiological diversity in this genus.

Taken together, our results show that several Epsilonproteobacteria have to be considered as H₂ producers and serve as syntrophic partners in e.g. the presence of lactate, which is a widely distributed organic electron donor in natural habitats. H₂ production in *Sulfospirillum* spp. under the tested conditions relies on Hyf, a multisubunit, membrane-bound and cytoplasmically oriented group 4 NiFe hydrogenase similar to the one used in a second *E. coli* FHL complex and probably functioning as a proton pump. Adaptation to fermentative conditions seems to be common in *S. multivorans* and related strains, although the underlying mechanism of this process is still unclear. Two separate clades of *Sulfospirillum* spp. have different fermentation pathways, the *S. cavolei* clade producing more H₂ and exclusively one organic acid, namely acetate, in comparison to *S. multivorans*, which additionally produces lactate and succinate. All these findings imply an even higher versatility for Epsilonproteobacteria than previously thought, although it should be noted here that most of them primarily rely on a respiratory metabolism. Still, our established coculture with a methanogen suggests a new ecological role for *Sulfospirillum* spp., which inhabit a large range of environmentally or biotechnologically important habitats such as wastewater plants, oil reservoirs, bioelectrodes, contaminated sediments or marine areas.

Methods

Cultivation of bacteria. *S. multivorans* was cultivated under anaerobic conditions at 28 °C in a defined mineral medium without vitamin B₁₂ (cyanocobalamin) as follows. The basal medium contained per L: 70 mg Na₂SO₄, 200 mg KH₂PO₄, 250 mg NH₄Cl, 1 g NaCl, 400 mg MgCl₂, 6 H₂O, 500 mg KCl, and 150 mg CaCl₂, 2 H₂O. Medium was made anoxic (under N₂ (150 kPa)) and autoclaved and
et al.40. Cocultures were grown in rubber-stoppered serum bottles with a ratio of aqueous to gas phase of 1:1. If not stated otherwise, the gas phase was N2/CO2 (80:20 (v/v); 150 kPa) and the gas phase of the washing flask was further connected to a water-filled measuring cylinder placed upside down in a water bath. The amount of H2 was determined volumetrically via the displaced volume of water in the measuring cylinder that correlates with the amount of H2 produced. The gas volume was adjusted to standard temperature (25 °C) and pressure (1 bar). The concentration was calculated using the ideal gas equation. The adaptation step was performed in the absence of oxygen, and the gas phase of the Schott bottle was connected to anoxic, sterile solutions of dissolved fermentation products (Supplementary Table 1). The injection volume was 20 µL per 10 mL sample. Organic acids were separated by HPLC at 50°C on an AMINEX HPX-87H column (7.8 x 300 mm, Bio-Rad, Munich, Germany) with a cation guard pre-column using 5 mM H2SO4 as mobile phase at a flow rate of 0.7 mL min-1. The injection volume was 20 µL per sample. All acids (e.g. pyruvate, acetate, lactate, succinate, and fumarate) were monitored by their absorption at 210 nm. Retention times were compared to known standards and concentrations were calculated using calibration curves. The buffer was used at pH 8.0. Protein concentration was determined according to the method of Bradford49. Hydrogenase enzyme activities are given in nanokatal units (1 nmol H2 evolved per second).

Measurement of hydrogenase activity. H2-oxidizing activity was measured in H2-saturated buffer (50 mM Tris-HCl, pH 8.0) with 1 mM benzyl viologen (BV) or methanol (MV) at 30°C as artificial electron acceptors. The redox of the redox dyes was followed at 578 nm using a Cary 100 spectrophotometer (Agilent Technologies, Waldbronn, Germany). H2-evolving activities of cell extracts were determined gas chromatographically with 1 mM MV as electron donor; MV was reduced with 20 mM sodium dithionite in an anoxic buffer system (50 mM Tris-HCl, pH 8.0). Protein concentration was determined according to the method of Bradford49. Hydrogenase enzyme activities are given in nanokatal units (1 nmol H2 evolved per second).

洗涤步骤的占位符

Reversed transcription and polymerase chain reaction. Total RNA from three independent S. cavoli cultures was isolated from cells in the mid-expansion growth phase using the RNeasy mini kit (Qiagen, Hilden, Germany). Residual genomic DNA (gDNA) in the RNA samples was removed with DNase I (RNase free; Roche, Mannheim, Germany). RNA quality was checked by visual inspection of agarose gel electrophoresis. One microgram of RNA was cross-linked with 50 µg cyanogen bromide (CNBr, Sigma-Aldrich, Munich, Germany) with a cation H guard pre-column using 5 mM H2SO4 as reaction buffer and PCR-grade water (Fermentas, St. Leon Rot, Germany) was added (per 10 µL reaction mixture) and 1.5 µL enzyme (1 U/reaction). The injection volume was 20 µL per sample. All acids (e.g. pyruvate, acetate, lactate, succinate, and fumarate) were monitored by their absorption at 210 nm. Retention times were compared to known standards and concentrations were calculated using calibration curves. H2 was measured gas chromatographically with 99.999% argon as the carrier gas using a thermal conductivity detector (AutoSystem, Perkin Elmer, Berlin, Germany). Some aspects of the gas phase analyses were automated with a computer program (Hamilton, Bonaduz, Switzerland). Concentrations were calculated using calibration curves. CO2 formed during the cultivation was determined gravimetrically. To 15 mL of the solution of the CO2 trap 7.5 mL NH4Cl (1 M) and 15 mL BaCl2 (1 M) were added and the pH was adjusted to 9 with concentrated HCl (37%). After 2 h at room temperature or 2 h at room temperature and resuspension in two volumes (2 mL per g cells) of the same buffer containing DNase I (AppliChem, Darmstadt, Germany) and protease inhibitor (one tablet for 10 mL buffer; complete Mini, EDTA-free; Roche, Mannheim, Germany). The resuspended cells were disrupted using a bead mill (10 min at 25 Hz; MixerMill MM400, Retsch GmbH, Haan, Germany) with an equal volume of glass beads (0.25–0.5 mm diameter; Carl Roth GmbH, Karlsruhe, Germany). The crude extracts were separated from the glass beads by centrifugation (14,000 x g, min 20 x at 4°C). The obtained supernatants were considered as soluble fractions (SF). The pellets were washed twice with 50 mM Tris-HCl (pH 8.0) including protease inhibitor (one tablet for 10 mL buffer; Complete Mini, EDTA-free; Roche, Mannheim, Germany) and resuspended in the same buffer. The suspension was used as membrane fraction (MF).

Analytical methods. Liquid samples were taken anaerobically, filtered with 0.2 µm syringe filters (Minisart RBC, Sartorius, Göttingen, Germany) with 100 mM NaHCO3 solved in 50 mM (v/v) H2SO4, and filtered with concentrated H2SO4 (2.5 µL·mL−1 sample volume). Organic acids were separated by HPLC at 50°C on an AMINEX HPX-87H column (7.8 x 300 mm, Bio-Rad, Munich, Germany) with a cation guard pre-column using 5 mM H2SO4 as mobile phase at a flow rate of 0.7 mL min-1. The injection volume was 20 µL per sample. All acids (e.g. pyruvate, acetate, lactate, succinate, and fumarate) were monitored by their absorption at 210 nm. Retention times were compared to known standards and concentrations were calculated using calibration curves. H2 was measured gas chromatographically with 99.999% argon as the carrier gas using a thermal conductivity detector (AutoSystem, Perkin Elmer, Berlin, Germany). Some aspects of the gas phase analyses were automated with a computer program (Hamilton, Bonaduz, Switzerland). Concentrations were calculated using calibration curves. CO2 formed during the cultivation was determined gravimetrically. To 15 mL of the solution of the CO2 trap 7.5 mL NH4Cl (1 M) and 15 mL BaCl2 (1 M) were added and the pH was adjusted to 9 with concentrated HCl (37%). After 2 h at room temperature or 2 h at room temperature and resuspension the precipitated barium carbonate was filtered with filter circles and dried overnight at 80°C.
electrophoresis. The PCR reaction mixture contained 1 μg DNA, 2.5 μL forward and reverse primer, 1.5 μL 10 mM dNTP mix, 5 μL HF reaction buffer and 0.5 μL Phusion DNA polymerase (2 U μL \(^{-1}\)). Thermo Scientific, Schwerte, Germany). The mix was filled up to 25 μL with PCR-grade water (Fermentas, St. Leon Rot, Germany). The PCR program included following steps: 96 °C for 5 min, 30 cycles of 96 °C for 1 min, 60 °C for 30 s, 72 °C for 30 s and final elongation at 72 °C for 10 min in a thermo cycler (Mastercycler, Personal, Eppendorf, Hamburg). Used primer pairs are listed in Supplementary Table 4.

**Field emission-scanning electron microscopy.** Field emission-scanning electron microscopy (FE-SEM) was performed using a Jeol JSM 6700F microscope (Carl Zeiss, Oberkochen, Germany). The samples were mounted on aluminum stubs and sputter coated with gold. The samples were observed at a voltage of 2 kV.

**Protein sample preparation and proteomics.** Protein concentration of extracted proteins was determined using a Bradford reagent (Bio-Rad, Munich, Germany) with bovine serum albumin as standard. For protein identifications, 20 μg of crude extracts were first cleaned from cations and cell debris by running shortly into an SDS gel. For this, the gel was run at 13 mA until the proteins entered the separating gel at a depth of about 3–5 mm. Then the protein band was cut out, reduced, alkylated and proteolytically digested with trypsin (Promega, Madison, WI, USA) and subsequently desalted and concentrated with C18 ZipTip pipette tips (Merck Millipore).

Mass spectrometry was performed using an Orbitrap Fusion (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a TriVersa NanoMate (Advion, Ltd., Harlow, UK). 5 μL of the peptide solution was separated with a Dionex Ultimate 3000 nano LC system (Dionex/THERMO Fisher Scientific, Idstein, Germany) using a 15 cm analytical column (Acclaim PepMap RSLC, 2 μm C18 particles, Thermo Scientific) at 35 °C. Liquid chromatography was done with a constant flow of 300 nL min\(^{-1}\) with a mixture of solvent A (0.1% formic acid) and B (80% acetonitrile, 0.08% formic acid) in a linear 90 min gradient of 4 to 5% solvent B. MS/MS scans were taken with a cycle time of 3 s in the Orbitrap mass analyzer between 350 and 2000 m/z at a resolution of 120,000, automatic gain control (AGC) target 4×10\(^5\), maximum injection time 50 ms. Data-dependent acquisition was employed selecting for highly intense ions (>5×10\(^4\)) and charge state between +2 and +7 with a precursor ion isolation windows of 1.6 m/z. Fragmentation was done via higher energy dissociation at 30% energy, and also measured in the dynamic exclusion mode.

**Data availability**

The raw proteomic data have been deposited in the PRIDE repository with the accession numbers PXD010316 (S. multivorans and S. casei) pyruvate fermentation and PXD010303 (pyruvate fermentation adaptation and lactate oxidation).

**Received:** 25 January 2018 **Accepted:** 24 October 2018

**Published online:** 19 November 2018

**References**

1. De Bok, F. A., Plugging, C. M. & Stams, A. J. Interspecies electron transfer in methanogenic propionate degrading consortia. *Water Res. 38,* 1368–1375 (2004).

2. McInerney, M. *et al.* Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. *Ann. N.Y. Acad. Sci. 1125,* 58–72 (2008).

3. Sieber, J. R., McInerney, M. J. & Gunsalus, R. P. Genomic insights into syntrophy: the paradigm for anaerobic metabolic cooperation. *Ann. Rev. Microbiol. 66,* 429–452 (2012).

4. Schink, B. Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol. Mol. Biol. Rev. 61,* 262–280 (1997).

5. Schink, B. & Stams, A. Prokaryotic communities and ecophysiology. In *The Prokaryotes* (eds Rosenberg, E. et al.) 471–494 (Springer, Berlin Heidelberg, 2013).

6. Stams, A. J. & Plugge, C. M. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nat. Rev. Microbiol. 7,* 568–577 (2009).

7. Morris, B. E., Henneberger, R., Huber, H. & Moisil-Eichinger, C. Microbial syntrophy: Interaction for the common good. *FEMS Microbiol. Rev. 37,* 384–406 (2013).

8. Shovlin, N. J. *et al.* Interspecies metabolic interactions between anaerobic bacteria in methanogenic environments. *Antonie. Van. Leeuwenhoek 66,* 271–294 (1994).

9. de Bok, F. A., Plugging, C. M. & Stams, A. J. Interspecies electron transfer in methanogenic propionate degrading consortia. *Water Res. 38,* 1368–1375 (2004).

10. Ishii, S., Kosaka, T., Hori, K., Hotta, Y. & Watanabe, K. Coaggregation facilitates interspecies hydrogen transfer between Pelotomaculum thermopropionicum and Methanothemobacter thermautotrophicus. *Appl. Environ. Microbiol. 71,* 7838–7845 (2005).

11. Mao, X., Stenuit, B., Polasko, A. & Alvarez-Cohen, L. Efficient metabolic exchange and electron transfer within a syntrophic trichloroethene-degrading coculture of *Dehalococcoides mccartyi* 195 and *Syntrophomonas wolfei*. *Appl. Environ. Microbiol. 71,* 2015–2024 (2015).

12. Lovley, D. R. Happy together: microbial communities that hook up to degrade electrons. *Isme J. 11,* 327–336 (2017).

13. Lee, H. S., Vermaas, W. F. & Rittmann, B. E. Biological hydrogen production: prospects and challenges. *Trends Biotechnol. 28,* 262–271 (2010).

14. Dross, F. *et al.* The quinone-reactive Nf/Fe-hydrogenase of *Wolinella succinogenes*. *Eur. J. Biochem. 206,* 93–102 (1992).

15. Maier, R. J. *et al.* Hydrogen uptake hydrogenase in *Helicobacter pylori*. *FEMS Microbiol. Lett. 141,* 71–76 (1996).

16. Jensen, A. & Finster, K. Isolation and characterization of *Sulfurospirillum carboxydovorans* sp nov., a new microaerophilic carbon monoxide oxidizing epsilon proteobacterium. *Antonie Van. Leeuwenhoek Int. J. General. Mol. Microbiol. 87,* 339–353 (2005).

17. Meyer, J. L. & Huber, J. A. Strain-level genomic variation in natural populations of *Lebetimonas* from an erupting deep-sea volcano. *Isme J. 8,* 867–880 (2014).

18. Handley, K. M. *et al.* The complete genome sequence for putative H₂- and S-oxidizer *Candidatus Sulfaricurvum* sp., assembled de novo from an aquifer-derived metagenome. *Environ. Microbiol. 16,* 3443–3462 (2014).

19. Nakagawa, S. *et al.* Deep-sea vent epsilon-proteobacterial genomes provide insights into emergence of pathogens. *Proc. Natl. Acad. Sci. USA 104,* 12146–12150 (2007).

20. Roalkvam, I. *et al.* Physiological and genomic characterization of *Arubobacter inopinatus* IR-1 reveals new metabolic features in Epsilonproteobacteria. *Front. Microbiol. 6,* 987 (2015).

21. Campbell, B. J. *et al.* Adaptations to submarine hydrothermal environments exemplified by the genome of *Nautilia profundicola*. *Plos Genet. 5,* e1000362 (2009).

22. Ross, D. E., Marshall, C. W., May, H. D. & Norman, R. S. Comparative physiological studies. *Environ. Microbiol. 10,* 1215–1222 (2008).

23. Goris, T. *et al.* Insights into organelle-specific respiration and the versatile catalobe of *Sulfurospirillum multivorans* gained from comparative genomics and physiological studies. *Environ. Microbiol. 16,* 3562–3580 (2014).

24. Zhang, Y. & Sievert, S. M. Pan-genome analyses identify lineage- and niche-specific markers of evolution and adaptation in *Epulosphaeraeae*. *Front. Microbiol. 5,* 110 (2014).

25. Han, Y. & Perner, M. The globally widespread genus *Sulfinimonas*: versatile energy metabolisms and adaptations to redox clines. *Front. Microbiol. 6,* 989 (2015).
26. Goris, T. & Diekert, G. The genus Sulfurospirillum. In Organohalide-respiring bacteria (eds Adrian, L. & Löfler, F.) (Springer, Berlin Heidelberg, 2016).

27. Lutjen, M. et al. Anaerobic reduction and oxidation of quinone moieties and the reduction of oxidized metals by halorespiring and related organisms. FEMS Microbiol. Ecol. 49, 145–150 (2004).

28. Neumann, A., Scholz-Muramatsu, H. & Diekert, G. Tetrachloroethene metabolism of Dehalosporillum multivorans. Arch. Microbiol. 162, 295–301 (1994).

29. Goris, T. et al. Proteomics of the organohalide-respiring Epsilonproteobacterium Sulfurospirillum multivorans adapted to tetrachloroethene and other energy substrates. Sci. Rep. 5, 13794 (2015).

30. Page, A. et al. Microbial diversity associated with a Paralvinella sulfidicina tube and the adjacent substratum on an active deep-sea vent chimney. Geobiology 2, 225–239 (2004).

31. Kruse, S., Goris, T., Wolf, M., Wei, X. & Diekert, G. The NiFe hydrogenases of the tetrachloroethene-respiring psilonproteobacterium Sulfurospirillum multivorans: biochemical studies and transcription analysis. Front. Microbiol. 8, 444 (2017).

32. Mizroboy, S., Romero-Pareja, P., Coello, M., Trchounian, A. & Trchounian, K. Evidence for hydrogenase-4 catalyzed biohydrogen production in Esherichia coli. Int. J. Hydrog. Energy 42, 21697–21703 (2017).

33. Scholz-Muramatsu, H., Neumann, A., Messmer, M., Moore, E. & Diekert, G. Isolation and characterization of Dehalosporillum multivorans gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. Arch. Microbiol. 163, 48–56 (1995).

34. Kodama, Y., Ha, L. & Watanabe, K. Sulfurospirillum cavolei, a tetrachloroethene-utilizing, strictly anaerobic bacterium isolated from an underground crude oil storage cavity. Int. J. Syst. Evol. Microbiol. 57, 827–831 (2007).

35. Aziz, R. K. et al. The RAST Server: rapid annotations using subsystems technology. BMC Genom. 9, 73 (2008).

36. Goris, T. et al. The complete genome of the tetrachloroethene-respiring Epsilonproteobacterium Sulfurospirillum halorespans. J. Bacteriol. 255, 33–36 (2017).

37. Evans, J. D. & Martin, S. A. Cloning of the L-lactate dehydrogenase gene from the ruminal bacterium Selenomas ruminantium HD4. Curr. Microbiol. 44, 155–160 (2002).

38. Jiang, T. et al. A bacterial multidomain NAD-independent L-lactate dehydrogenase utilizes flavin adenine dinucleotide and Fe-S clusters as cofactors and quinone as an electron acceptor for L-lactate oxidation. J. Bacteriol. 199, 600342-17 (2017).

39. Fuller, J. R. et al. Identification of a lactate-quinone oxidoreductase in Staphylococcus aureus that is essential for virulence. Front. Cell. Infect. Microbiol. 1, 9 (2011).

40. Whitman, W. B., Akanwanda, E. & Wolfe, R. S. Nutrition and carbon metabolism of Methanococcus voltae. J. Bacteriol. 149, 852–863 (1982).

41. Campbell, B. J., Engel, A. S., Porter, M. L. & Takai, K. The versatile epsilonproteobacteria: key players in sulphidic habitats. Nat. Rev. Microbiol. 4, 458–468 (2006).

42. Miroschnichenko, M. et al. Caminibacter profundus sp nov., a novel thermophile of Nautiliales ord. nov within the class ‘Epsilonproteobacteria’, isolated from a deep-sea hydrothermal vent. Int. J. Syst. Evol. Microbiol. 54, 41–45 (2004).

43. John, M. et al. Retentive memory of bacteria: long-term regulation of dehalorespiration in Sulfurospirillum multivorans. J. Bacteriol. 191, 1650–1655 (2009).

44. Romão, M. J. et al. Crystal structure of the xanthine oxidase-related aldehyde oxidoreductase from D. gigas. Science 270, 1170–1176 (1995).

45. Peters, B. et al. Deletion of pyruvate decarboxylase by a new method for efficient markerless gene deletions in Gluconobacter oxydans. Appl. Microbiol. Biotechnol. 97, 2521–2530 (2013).

46. Srikhanta, Y. N., Atack, J. M., Beacham, I. R. & Jennings, M. P. Distinct physiological roles for the two L-asparaginase isozymes of Esherichia coli. Biochem. Biophys. Res. Commun. 436, 362–365 (2013).

47. Kyritsis, P., Hatfeld, O. M., Link, T. A. & Moisils, J. M. The two [4Fe-4S] clusters in Chromatium vinosum ferredoxin have largely different reduction potentials. Structural origin and functional consequences. J. Biol. Chem. 273, 15404–15411 (1998).

48. McCormick, P. M. et al. Engineering the respiratory membrane-bound hydrogenase of the hyperthermophilic archaeon Pyrococcus furiosus and characterization of the catalytically active cytoplasmic subcomplex. Protein Eng. Des. Sel. 28, 11–18 (2015).

49. Lamont, C. M. et al. Expanding the substrates for a bacterial hydrogenylase reaction. Microbiology 163, 649–653 (2017).

50. Hedderich, R. Energy-converting [NiFe] hydrogenases from archaea and extremophiles: ancestors of complex I. J. Bioenerg. Biomembr. 36, 65–75 (2004).

51. Mateus, B. C., Batista, A. P., Duarte, A. M. Pereira, M. M. A. Missing link between complex I and group 4 membrane-bound [NiFe] hydrogenases. Biochim. Biophys. Acta 1827, 198–209 (2013).

52. Thomas, M. T. et al. Two respiratory enzyme systems in Campylobacter jejuni NCTC 11688 contribute to growth on L-lactate. Environ. Microbiol. 13, 48–61 (2011).

53. Pinchuk, G. E. et al. Genomic reconstruction of Shewanella oneidensis MR-1 metabolism reveals a previously unrecognized machinery for lactate utilization. Proc. Natl. Acad. Sci. USA 106, 2874–2879 (2009).

54. Chai, Y., Kolter, R. & Losick, R. A widely conserved gene cluster required for lactate utilization in Bacillus subtilis and its involvement in biofilm formation. J. Bacteriol. 191, 2423–2430 (2009).

55. Zhang, Y. et al. Coexistence of two d-lactate-utilizing systems in Pseudomonas putida KT2440. Environ. Microbiol Rep. 8, 699–707 (2016).

56. Liu, X., Zhao, S., Rensing, C. & Zhou, S. Syntrophic growth with direct interspecies electron transfer between pili-free Geobacter species. Isme J. 12, 2142–2151 (2018).

57. Pribadian, S. et al. Shewanella oneidensis MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components. Proc. Natl. Acad. Sci. USA 111, 12883–12888 (2014).

58. Ueki, T. et al. Geobacter strains expressing poorly conductive Pili reveal constraints on direct interspecies electron transfer mechanisms. MBio 9, e01273-18 (2018).

59. Kim, Y. J. et al. Formate-driven growth coupled with H(2) production. Nature 467, 352–355 (2010).

60. Bradford, M. A. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254 (1976).

61. Wieczorek, S. et al. DAPAR & ProStaR: software to perform statistical analyses in quantitative discovery proteomics. Bioinformatics 33, 135–136 (2017).

Acknowledgements
This work was funded by the German Research Council (DFG)—Jena School for Microbial Communication (JSMC) and Research Unit FOR1530. We would like to gratefully acknowledge Susanne Linde (University Hospital Jena, Center for Electron Microscopy) for the field emission-scanning electron microscopic analysis. The presented work included the use of analytical facilities of the Center for Chemical Microscopy (ProVIS) at the Helmholtz Centre for Environmental Research (UFZ Leipzig). ProVIS is funded by the European Regional Development Funds (EFRE—Europe funds Saxony) and the Helmholtz Association. The authors would like to thank Benjamin Scheer (UFZ Leipzig) for invaluable assistance in the lab with mass spectrometry and Dominique Türkow(University Hospital Jena, Center for Electron Microscopy) for helpful with statistical analysis of proteome data.

Author contributions
S.K. performed the wet lab work, S.K. and T.G. planned experiments, T.G. initiated the study, T.G. and G.D. supervised the study, I.A. performed the mass spectrometric study, T.G. and G.D. supervised the study, L.A. performed the mass spectrometric analysis, S.K., T.G., and G.D. analyzed and discussed the data, M.W. was responsible for electron microscopy, S.K. and T.G. drafted the manuscript, all authors revised, read and approved this manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-07342-3.

Competing interests: The authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.