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Diverse hydrogen production and consumption pathways influence methane production in ruminants

Chris Greening [1] · Renae Geier[2] · Cecilia Wang[3] · Laura C. Woods[1] · Sergio E. Morales[3] · Michael J. McDonald[1] · Rowena Rushton-Green[3] · Xochitl C. Morgan[3] · Satoshi Koike[4] · Sinead C. Leahy[5] · William J. Kelly[6] · Isaac Cann[2] · Graeme T. Attwood[5] · Gregory M. Cook[3] · Roderick I. Mackie[2]

Abstract

Farmed ruminants are the largest source of anthropogenic methane emissions globally. The methanogenic archaea responsible for these emissions use molecular hydrogen (H_2), produced during bacterial and eukaryotic carbohydrate fermentation, as their primary energy source. In this work, we used comparative genomic, metatranscriptomic and co-culture-based approaches to gain a system-wide understanding of the organisms and pathways responsible for ruminal H_2 metabolism. Two-thirds of sequenced rumen bacterial and archaeal genomes encode enzymes that catalyse H_2 production or consumption, including 26 distinct hydrogenase subgroups. Metatranscriptomic analysis confirmed that these hydrogenases are differentially expressed in sheep rumen. Electron-bifurcating [FeFe]-hydrogenases from carbohydrate-fermenting Clostridia (e.g., *Ruminococcus*) accounted for half of all hydrogenase transcripts. Various H_2 uptake pathways were also expressed, including methanogenesis (*Methanobrevibacter*), fumarate and nitrite reduction (*Selenomonas*), and acetogenesis (*Blautia*). Whereas methanogenesis-related transcripts predominated in high methane yield sheep, alternative uptake pathways were significantly upregulated in low methane yield sheep. Complementing these findings, we observed significant differential expression and activity of the hydrogenases of the hydrogenogenic cellulose fermenter *Ruminococcus albus* and the hydrogenotrophic fumarate reducer *Wolinella succinogenes* in co-culture compared with pure culture. We conclude that H_2 metabolism is a more complex and widespread trait among rumen microorganisms than previously recognised. There is evidence that alternative hydrogenotrophs, including acetogenic and respiratory bacteria, can prosper in the rumen and effectively compete with methanogens for H_2. These findings may help to inform ongoing strategies to mitigate methane emissions by increasing flux through alternative H_2 uptake pathways, including through animal selection, dietary supplementation and methanogenesis inhibitors.

Introduction

Methane production by livestock accounts for over 5% of global greenhouse gas emissions annually [1]. These emissions mostly originate from the activity of methanogens within ruminants, which generate methane as an obligate

These authors contributed equally: Chris Greening and Renae Geier

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✉ Chris Greening
chris.greening@monash.edu

✉ Roderick I. Mackie
r-mackie@illinois.edu

1   School of Biological Sciences, Monash University, Clayton, VIC 3800, Australia

2   Department of Animal Sciences and Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL

61801, USA

3   Department of Microbiology and Immunology, University of Otago, Dunedin 9016, New Zealand

4   Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan

5   Grasslands Research Centre, AgResearch Ltd., Palmerston North 4410, New Zealand

6   Donvis Ltd., Palmerston North 4410, New Zealand

end-product of their energy metabolism [2]. Several lineages of methanogenic archaea are core members of the microbiome of the ruminant foregut [3–5]. Of these, hydrogenotrophic methanogens are dominant in terms of both methane emissions and community composition [6, 7], with global surveys indicating that *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium* comprise 74% of the rumen methanogen community [5]. These organisms use molecular hydrogen (H_2) to reduce carbon dioxide (CO_2) to methane through the Wolfe cycle of methanogenesis [8, 9]. Rumen methanogens have also been identified that use formate, acetate, methyl compounds and ethanol as substrates, but usually do so in conjunction with H_2 [5, 10–12]. Given their major contribution to greenhouse gas emissions, multiple programs are underway to mitigate ruminant methane production [13, 14]. To date, most strategies have focused on direct inhibition of methanogens using chemical compounds or vaccines [15–18]. A promising alternative strategy is to modulate the supply of substrates to methanogens, such as H_2, for example, through dietary or probiotic interventions [14, 19, 20]. To achieve this, while maintaining health and productivity of the host animal, requires an understanding of the processes that mediate substrate supply to methanogens within the rumen.

H_2, the main substrate supporting ruminal methanogenesis, is primarily produced through fermentation processes [6]. Various carbohydrate fermentation pathways lead to the production of H_2 as an end-product, together with volatile fatty acids and CO_2 [21–23]. This process is supported by hydrogenases, which reoxidise cofactors reduced during carbohydrate fermentation and dispose of the derived electrons by producing H_2. Although it is unclear which rumen microorganisms mediate H_2 production in situ, a range of isolates have been shown to produce H_2 in vitro [24–28]. For example, the model rumen bacterium *R. albus* 7 reoxidises the reduced ferredoxin and NADH formed during glucose fermentation by using two different [FeFe]-hydrogenases depending on environmental conditions [29]. In addition, it is well-established that some rumen fungi and ciliates produce H_2 via hydrogenosomes [30, 31]. A debated source is the nitrogenase reaction, which produces H_2 while fixing N_2; whereas numerous rumen microorganisms encode putative nitrogenases [21], in situ data indicate that N_2 fixation occurs at negligible rates in the rumen [32, 33]. A large proportion of the H_2 produced by hydrogenogenic fermenters is directly transferred to hydrogenotrophic methanogens, in an ecological process known as interspecies hydrogen transfer [25, 34]. Particularly remarkable are the endosymbiotic and ectosymbiotic associations of methanogens, such as *M. ruminantium*, with rumen ciliates [35–37]. In addition to providing a continual substrate supply for methanogens, such symbioses benefit fermenters by maintaining H_2 at sufficiently low concentrations for fermentation to remain thermodynamically favourable [38].

Various hydrogenotrophic bacteria are thought to compete with methanogens for the rumen H_2 supply. Most attention has focused on acetogens, which mediate conversion of H_2/CO_2 to acetate using [FeFe]-hydrogenases [39]. Several genera of acetogens have been isolated from the rumen, including *Eubacterium* [40], *Blautia* [41] and *Acetitomaculum* [42]. However, molecular surveys indicate their abundance is generally lower than hydrogenotrophic methanogens [43–45]. This is thought to reflect that methanogens outcompete acetogens owing to the higher free energy yield of their metabolic processes, as well as their higher affinity for H_2. The dissolved H_2 concentration fluctuates in the rumen depending on diet, time of feeding and rumen turnover rates, but is generally at concentrations between 400 and 3400 nM [46]; these concentrations are typically always above the threshold concentrations required for methanogens (< 75 nM) but often below those of acetogens (< 700 nM) [47]. Despite this, it has been proposed that stimulation of acetogens may be an effective strategy for methane mitigation in methanogen-inhibited scenarios [14, 20, 48, 49]. Various microorganisms have also been isolated from cows and sheep that support anaerobic hydrogenotrophic respiration, including dissimilatory sulfate reduction (e.g., *Desulfovibrio desulfuricans*) [50, 51], fumarate and nitrate reduction (e.g., *Selenomonas ruminantium*, *Wolinella succinogenes*) [52–59] and trimethylamine *N*-oxide reduction (e.g., *Denitrobacterium detoxificans*) [60]. The first described and most comprehensively studied of these hydrogen oxidisers is *W. succinogenes*, which mediates interspecies hydrogen transfer with *R. albus* [25]. In all cases, respiratory electron transfer via membrane-bound [NiFe]-hydrogenases and terminal reductases generates a proton-motive force that supports oxidative phosphorylation [61]. It is generally assumed that these pathways are minor ones and are limited by the availability of oxidants. Promisingly, it has been observed that dietary supplementation with fumarate, sulfate, or nitrate can significantly reduce methane production in cattle, likely by stimulating alternative pathways of H_2 consumption [62, 63].

We postulate that mitigating methane emissions, while maintaining animal productivity, depends on understanding and controlling H_2 utilisation by methanogens. This requires a system-wide perspective of the schemes for production and concomitant utilisation of H_2 in the rumen. To facilitate this, we determined which organisms and enzymes are primarily responsible for H_2 production and consumption in rumen. First, we screened genome, metagenome and metatranscriptome data sets [21, 64, 65] to identify microbial genera, metabolic pathways and hydrogenase classes [66, 67] that mediate H_2 metabolism. We demonstrate that ruminants harbour a diverse community of

hydrogenogenic fermenters and hydrogenotrophic methanogens, acetogens and sulfate, fumarate and nitrate reducers. Second, we used the model system of the H_2-producing carbohydrate fermenter *R. albus* 7 and the H_2-utilising fumarate-reducing syntrophic partner *Wolinella succinogenes* DSM 1740 [25, 54, 55, 68] to gain a deeper mechanistic understanding of how and why ruminant bacteria regulate H_2 metabolism. We observed significant differences in the growth, transcriptome and metabolite profiles of these bacteria in co-culture compared with pure culture. Finally, we compared gene expression profiles associated with H_2 metabolism between low versus high methane yield sheep [64]. It was recently proposed, on the basis of community structure analysis, that fewer H_2-producing bacteria inhabit low methane yield sheep [69]. In this work, we provide evidence for an alternative explanation: H_2 uptake through non-methanogenic pathways accounts for these differences. Whereas the enzymes mediating fermentative H_2 production are expressed at similar levels, those supporting H_2 uptake through acetogenesis, fumarate reduction and nitrate ammonification pathways are highly upregulated in low methane yield sheep. In turn, these findings support that strategies to promote alternative H_2 uptake pathways, including through dietary modulation, may significantly reduce methane emissions.

Materials and methods

Comparative genomic analysis

The protein sequences of the 501 genomes of cultured rumen bacteria (410 from Hungate Collection [21], 91 from other sources) were retrieved from the Joint Genome Institute (JGI) genome portal. These sequences were then screened against local protein databases for the catalytic subunits of the three classes of hydrogenases (NiFe-hydrogenases, FeFe-hydrogenases, Fe-hydrogenases), nitrogenases (NifH), methyl-CoM reductases (McrA), acetyl-CoA synthases (AcsB), adenylylsulfate reductases (AprA), dissimilatory sulfite reductases (DsrA), alternative sulfite reductases (AsrA), fumarate reductases (FrdA), dissimilatory nitrate reductases (NarG), periplasmic nitrate reductases (NapA), ammonia-forming nitrite reductases (NrfA), DMSO/TMAO reductases (DmsA) and cytochrome *bd* oxidases (CydA). Hydrogenases were screened using the HydDB data set [66, 67], targeted searches were used to screen six protein families (AprA, AsrA, NarG, NapA, NrfA, DmsA, CydA) and comprehensive custom databases were constructed to screen five other protein families (NifH, McrA, AcsB, DsrA, FrdA) based on their total reported genetic diversity [70–74]. A custom Python script

incorporating the Biopython package [75] was designed to produce and parse BLAST results (https://github.com/woodlaur189/get_flanks_blast/releases). This script was used to batch-submit the protein sequences of the 501 downloaded genomes as queries for BLAST searches against the local databases. Specifically, hits were initially called for alignments with an e-value threshold of 1e-50 and the resultant XML files were parsed. Alignments producing hits were further filtered for those with coverage values exceeding 90% and percent identity values of 30–70%, depending on the target, and hits were subsequently manually curated. Table S1 and S2 provide the FASTA protein sequences, alignment details and distribution summaries of the filtered hits. For hydrogenases, the protein sequences flanking the hydrogenase large subunits were also retrieved; these sequences were used to classify group A [FeFe]-hydrogenases into subtypes (A1–A4), as previously described [66], and retrieve diaphorase sequences (HydB) associated with the A3 subtype. Partial [FeFe]-hydrogenase protein sequences from six incompletely sequenced rumen ciliates and fungi genomes were retrieved through targeted blastP searches [76] in NCBI.

Metagenomic and metatranscriptomic analysis

We analysed previously published data sets of 20 paired metagenomes and metatranscriptomes of sheep rumen contents [64]. All profiles were derived from the rumen contents of age-matched, pelleted lucerne-fed rams that were collected 4 h after morning feeding and subject to paired-end sequencing on the HiSeq2000 platform [64]. The samples were taken from 10 rams at two different sampling dates based on their measured methane yields [64, 77]; four rams were consistently low yield, four were consistently high yield, and two others switched in methane yield between the sampling dates (Table S3). The metagenome and metatranscriptome data sets analysed are accessible at the NCBI Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra) accession numbers SRA075938, and SRX1079958—SRX1079985 under bioproject number PRJNA202380. Each metagenome and metatranscriptome was subsampled to an equal depth of five million reads using seqtk (https://github.com/lh3/seqtk) seeded with parameter -s100. Subsampled data sets were then screened in DIAMOND (default settings, one maximum target sequence per query) [78] using the protein sequences retrieved from the 507 rumen microbial genomes (NiFe-hydrogenases, FeFe-hydrogenases, Fe-hydrogenases, HydB, NifH, McrA, AcsB, AprA, DsrA, AsrA, FrdA, NarG, NapA, NrfA, DmsA, CydA). Results were then filtered (length of amino acid > 40 residues, sequence identity > 65%). Subgroup classification and taxonomic assignment of the hydrogenase reads was based on their closest match

to the hydrogenase data set derived from the 507 genomes at either 65 or 85% identity. The full pipeline used is provided on GitHub (https://github.com/Cecilia-Wang/Diamond_line_reader_pipeline). The hits obtained are shown in Table S4 and S5. The number of reads with the rumen-specific hydrogenase data set (15,464 metagenome hits, 40,485 metatranscriptome hits) exceeded those obtained by screening with the generic data set from HydDB [67] (12,599 metagenome reads, 31,155 metatranscriptome reads), verifying that the rumen data set comprehensively captures hydrogenase diversity. For each data set, read count was normalised to account for the average length of each gene using the following formula: Normalised Read Count = Actual Read Count × (1000/average gene length). Independent two-group Wilcoxon rank-sum tests were used to determine whether there were significant differences in the targets analysed between low and high methane yield sheep. Separate analyses were performed based on gene abundance, transcript abundance and RNA/DNA ratio.

Bacterial growth conditions

The bovine rumen isolates *R. albus* 7 [68] and *Wolinella succinogenes* DSM 1740 [54] were cultured anaerobically at 37 °C in modified Balch medium [79] (Table S6). Precultures were grown in Balch tubes (18 × 150 mm; Chemglass Life Sciences, Vineland, NJ) containing 20% v/v culture medium and sealed with butyl rubber stoppers crimped with aluminium caps. Cultures were grown in Pyrex side-arm flanks (Corning Inc., Corning, NY) containing 118 mL modified Balch medium. Two pre-cultures were grown before final inoculation, and all inoculum transfers were 5% (v/v). The headspace consisted of 20% CO_2 and 80% N_2 for *R. albus* pure cultures and the co-cultures, and 20% CO_2 and 80% H_2 for *W. succinogenes* pure cultures. Cultures were periodically sampled at 0, 3, 5, 7, 9 and 11 h for metabolite analysis and bacterial quantification. Each culture was also sampled at 0 and 24 h to analyse H_2 mixing ratios. Culture samples were immediately centrifuged ($16,000 \times g$, 10 min) in a bench-top centrifuge (Eppendorf, Hamburg, Germany). For metabolite analysis, the supernatant was collected and further centrifuged ($16,000 \times g$, 10 min) before high-performance liquid chromatography analysis.

Bacterial quantification

Quantitative PCR was used to quantify the copy numbers of *R. albus* and *W. succinogenes* under pure culture and co-culture. DNA was extracted from each pellet using the Fungal/Bacterial DNA MiniPrep kit according to the manufacturer's instructions (Zymo Research, Irvine, CA). Primers were designed to specifically amplify Rumal_2867 (*R. albus*

glucokinase gene; FW: CTGGGATTCCTGAACTTTCC; RV: ATGCATACTGCGTTAG) and WS0498 (*W. succinogenes flgL* gene; FW: CAGACTATACCGATGCAACTAC; RV: GAGCGGAGGAGATCTTTAATC). For quantification, amplicons of each gene were cloned into pGEM-T Easy vectors (Promega, Madison, WI). Vector inserts were sequenced to confirm proper insertion. Standard curves extended from 10^2 to 10^{10} copies mL^{-1}. DNA was amplified with iTaq Universal SYBR Green (Bio-Rad) on a LightCycler 480 (Roche Holding AG, Basel, Switzerland). The amplification conditions were as follows: initial denaturation for 5 min at 95 °C, 40 amplification cycles (15 s at 95 °C, 20 s at 60 °C, 10 s at 68 °C) and final extension for 5 min at 68 °C. Fluorescence was read during the extension phase of each cycle. The linear regression of the standard curve resulted in an $R^2 > 0.99$, efficiencies were between 97.8–98.5%, and primers were confirmed to not cross-react. Two-tailed tests were done on sample data using SAS Studio (SAS Institute Inc., Cary, NC) to compare growth rates between single and co-cultures. All populations were normally and independently distributed with equal variances.

Liquid and gas metabolite analysis

The concentrations of acetate, ethanol, fumarate, succinate and formate in the culture supernatants were analysed using an Ultra-Fast Liquid Chromatograph (UFLC; Shimadzu, Kyoto, Japan). The UFLC consisted of a DGU-20A5 degasser, a SIL-20ACHT autosampler, an LC-20AT solvent delivery unit, an RID-10A refractive index detector, a CBM-20A system controller, and a CTO-20AC column oven. The mobile phase was 5 mM H_2SO_4 passed through an Aminex HPX-87H ion exclusion column (Bio-Rad, Hercules, CA) at a flow rate of 0.4 mL min^{-1}, 25 °C. Percentage mixing ratios of H_2 were measured using a gas chromatograph (GC; Gow-Mac Series 580 Thermal Conductivity Gas Chromatograph, Gow-Mac Instrument Co., Bethlehem, PA). Samples were withdrawn directly from the culture tube in a gas-tight syringe and 0.5 mL was injected into GC for analysis using N_2 as the carrier gas. The flow rate was 60 mL min^{-1}, the detector was set to 80 °C, the injector was set to 80 °C and the oven was set to 75 °C. For both liquid and gas analyses, peak retention times and peak area were compared to standards of known concentration.

RNA extraction and sequencing

Each pure culture and co-culture used for transcriptome analysis was grown in duplicate in Balch tubes. Growth was monitored until the cultures were in mid-exponential phase; the change in OD$_{600}$ at this phase was 0.14 for *W. succinogenes*, 0.20 for *R. albus* and 0.35 for the co-culture. At mid-exponential phase, 5 mL cultures were harvested by

centrifugation ($13,000 \times g$, $4\,°C$). Cell pellets were resuspended in $400\,\mu L$ fresh lysis buffer ($5\,\text{mM}$ EDTA, 0.5% SDS, $25\,\text{mM}$ lysozyme, $250\,U\,mL^{-1}$ mutanolysin and $150\,\mu g\,mL^{-1}$ proteinase K in $25\,\text{mM}$ sodium phosphate buffer, pH 7.0) and incubated under oxic conditions for 30 min at $55\,°C$ with periodic vortexing. RNA was subsequently extracted under oxic conditions using an RNeasy Mini Kit following the manufacturer's protocol, including all optional steps (Qiagen, Hilden, Germany), and eluted with $50\,\mu L$ ultra-pure DEPC-treated water (Invitrogen, Carlsbad, CA). RNA quantity, quality and integrity were confirmed by Qubit Fluorometry (Invitrogen, Carlsbad, CA), Nanodrop UV-Vis Spectrophotometry (Thermo Fisher Scientific, model 2300c), and agarose gel electrophoresis respectively. Bacterial rRNA was removed from $1\,\mu g$ of total RNA with the MicrobExpress Kit (Life Technologies, Carlsbad, CA). Libraries were prepared on the enriched mRNA fraction using the Stranded RNA Sample Prep Kit (Illumina, San Diego, CA). The barcoded libraries were pooled in equimolar concentration and sequenced on one lane for 101 cycles on a HiSeq2000 using a TruSeq SBS Sequencing Kit (Version 3). Fastq files were generated and demultiplexed with the bc12fastq Conversion Software (Illumina, version 1.8.4). The RNA-seq data were analysed using CLC Genomics Workbench version 5.5.1 (CLC Bio, Cambridge, MA). RNA-seq reads were mapped onto the reference genome sequences of *R. albus* 7 [80] and *Wolinella succinogenes* DSM 1740 [81] (Table S7 & S8). The RNA-seq output files were analysed for statistical significance as described [82] and q values were generated using the q value package in R [83]. Predicted subsystems and functions were downloaded and aligned to the RNA-seq transcriptional data using the RAST Server [84].

Results

H₂ metabolism is a common and diverse trait among rumen bacteria, archaea and eukaryotes

We searched the 501 reference genome sequences of rumen bacteria and archaea [21] for genes encoding the catalytic subunits of H_2-consuming and H_2-producing enzymes (Table S1 & S2). Of these, 65% encoded the capacity to metabolise H_2 via [FeFe]-hydrogenases (42%), [NiFe]-hydrogenases (31%), [Fe]-hydrogenases (2.4%) and/or nitrogenases (23%). This suggests that H_2 metabolism is a widespread trait among rumen microorganisms. We also identified multiple partial sequences of group A1 [FeFe]-hydrogenases in the incomplete genomes of six rumen fungi and ciliates. This is consistent with the known ability of these microorganisms to produce H_2 during cellulose fermentation [31]. The 329 hydrogenase- and nitrogenase-positive genomes spanned 108 genera, 26 orders, 18 classes and 11 phyla (Fig. 1; Figure S1; Table S1 & S2).

We then classified the hydrogenases identified into subgroups. To do so, we used the phylogeny-based, functionally-predictive classification scheme of HydDB [67], which has been used to understand H_2 metabolism in a range of organisms and ecosystems [85–89]. In total, 273 strains encoded hydrogenases from classes that primarily evolve H_2 under physiological conditions (Table S2). These include group A1 and B [FeFe]-hydrogenases and group 4e [NiFe]-hydrogenases that couple ferredoxin oxidation to H_2 production in anaerobic bacteria [90–92]. However, the most widespread hydrogenases are the group A3 [FeFe]-hydrogenases, which were encoded in 43 genera, among them well-characterised carbohydrate fermenters such as *Ruminococcus*, *Lachnoclostridium* and *Bacteroides*. These hydrogenases form heterotrimeric complexes, together with diaphorase subunits, that mediate the recently-discovered process of electron-confurcation: coupling co-oxidation of NADH and ferredoxin produced during fermentative carbon degradation to production of H_2 [29, 93]. This reversible complex can also support hydrogenotrophic acetogenesis [94]. By retrieving the genes immediately upstream and downstream, we verified that the diaphorase subunits (HydB) of this complex were co-encoded with the retrieved hydrogenase subunits (Fig. 1; Table S2).

In addition, multiple organisms encoded hydrogenases and terminal reductases known to support hydrogenotrophic growth (Fig. 1). All 21 methanogen genomes surveyed harboured [NiFe]-hydrogenases together with the signature gene of methanogenesis (*mcrA*) (Fig. 1; Table S2). These include 14 *Methanobrevibacter* strains, which encoded a complete set of enzymes for mediating hydrogenotrophic methanogenesis through the Wolfe cycle [8], including the [Fe]-hydrogenase and the groups 3a, 3c, 4h and 4i [NiFe]-hydrogenases. Seven genomes encoded both [FeFe]-hydrogenases (A2, A3) and the marker gene for acetogenesis (*acsB*) (Table S2), including known hydrogenotrophic acetogens *Blautia schinkii* [41] and *Acetitomaculum ruminis* [42]. Several subgroups of the group 1 [NiFe]-hydrogenases, all membrane-bound enzymes known to support hydrogenotrophic respiration [66, 95], were also detected. Most notably, various *Selenomonas*, *Mitsuokella* and *Wolinella* strains encoded such hydrogenases together with the signature genes for fumarate reduction (*frdA*) and nitrate ammonification (*narG*, *napA*, *nrfA*). As anticipated, the group 1b [NiFe]-hydrogenase and *dsrA* gene characteristic of hydrogenotrophic sulfate reduction were also encoded in the three genomes of ruminal *Desulfovibrio* isolates (Fig. 1; Table S2).
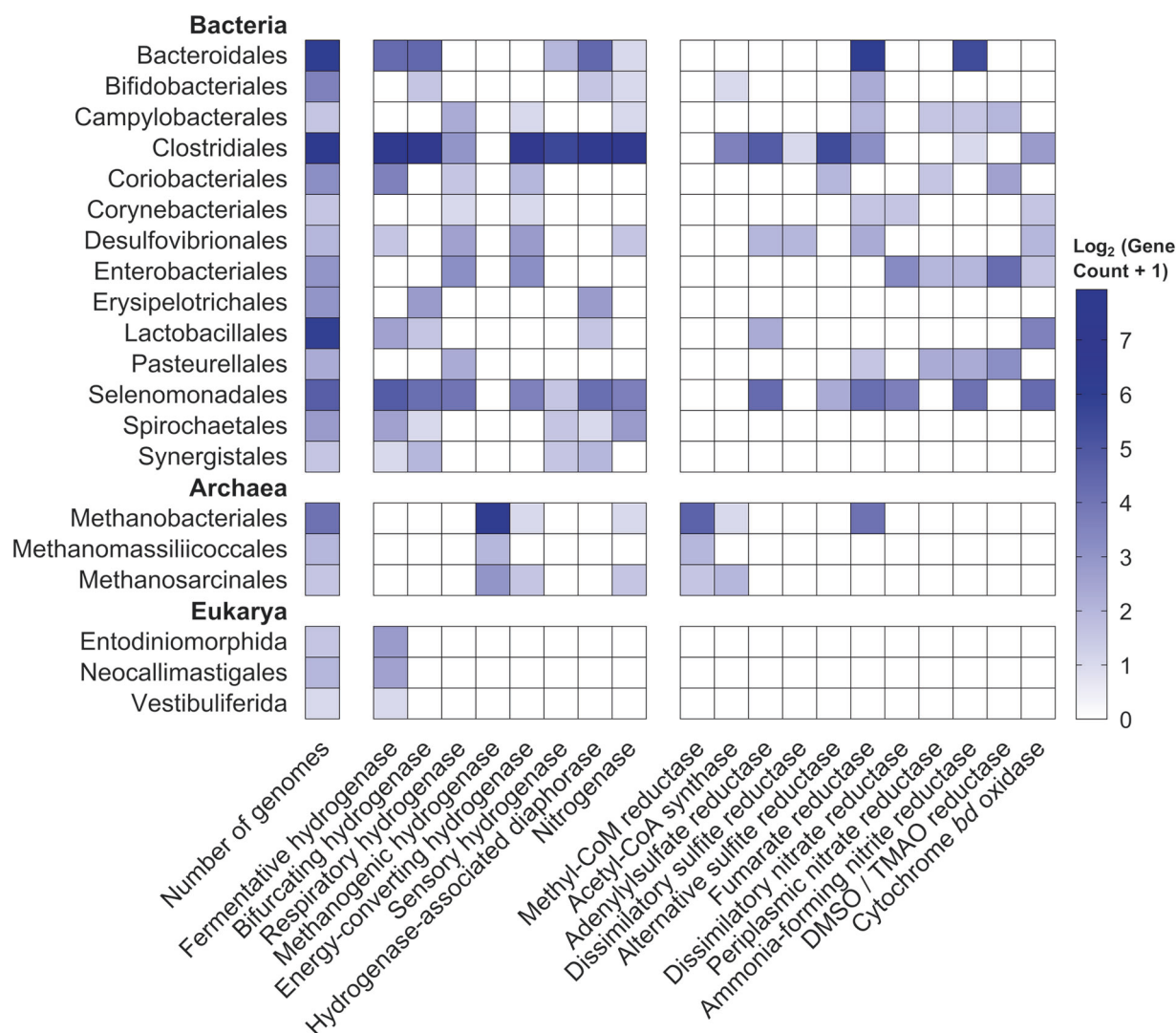
Fig. 1 Heatmap showing distribution of enzymes mediating H_2 production and H_2 consumption in orders of rumen microorganisms. Results are shown based on screens of the 501 genomes of cultured rumen bacteria and archaea (410 from the Hungate collection plus 91 other genomes). Partial hydrogenase sequences were also retrieved and classified from four rumen ciliates and two rumen fungi. The left-hand side of the heatmap shows the distribution of the catalytic subunits of enzymes that catalyse H_2 oxidation and production. These are divided into fermentative hydrogenases (H_2-producing; group A1, A2, B FeFe-hydrogenases), bifurcating hydrogenases (bidirectional; group A3, A4 FeFe-hydrogenases), respiratory hydrogenases (H_2-uptake; group 1b, 1c, 1d, 1f, 1i, 2d NiFe-hydrogenases), methanogenic hydrogenases (H_2-uptake; group 1k, 3a, 3c, 4h, 4i NiFe-hydrogenases, Fe-hydrogenases), energy-converting hydrogenases (bidirectional; group 4a, 4c, 4e, 4f, 4g NiFe-hydrogenases), sensory hydrogenases (group C FeFe-

hydrogenases) and nitrogenases (H_2-producing; NifH). The right-hand side shows the distribution of the catalytic subunits of key reductases in H_2 consumption pathways. They are genes for methanogenesis (McrA, methyl-CoM reductase), acetogenesis (AcsB, acetyl-CoA synthase), sulfate reduction (DsrA, dissimilatory sulfite reductase; AprA, adenylylsulfate reductase; AsrA, alternative sulfite reductase), fumarate reduction (FrdA, fumarate reductase), nitrate ammonification (NarG, dissimilatory nitrate reductase; NapA, periplasmic nitrate reductase; NrfA, ammonia-forming nitrite reductase), dimethyl sulfoxide and trimethylamine *N*-oxide reduction (DmsA, DMSO and TMAO reductase) and aerobic respiration (CydA, cytochrome *bd* oxidase). Only hydrogenase-encoding orders are shown. Table S2 shows the distribution of these enzymes by genome, Figure S1 depicts hydrogenase subgroup distribution by class, and Table S1 lists the FASTA sequences of the retrieved reads

H_2 is mainly produced in sheep by clostridial electron-bifurcating [FeFe]-hydrogenases and consumed by [NiFe]-hydrogenases of methanogens and selenomonads

We then investigated the relative abundance and expression levels of the retrieved hydrogenases in rumen communities.

To do so, we used 20 pairs of metagenomes and metatranscriptomes that were previously sequenced from the rumen contents of age- and diet-matched farmed sheep [64] (Table S3). Screening these data sets with hydrogenases retrieved from the rumen microbial reference genomes yielded 15,464 metagenome hits (0.015% of all reads) and 40,485 metatranscriptome hits (0.040%) (Table S4). Across

the metagenomes, the dominant hydrogenase reads originated from eleven subgroups (A1, A2, A3, B, 3a, 3c, 4e, 4g, 4h, 4i, Fe) (Fig. 2a & S2a) and three taxonomic orders (Clostridiales, Methanobacteriales, Selenomonadales) (Fig. 2c & S3a); this is concordant with the hydrogenase content in the genomes of the dominant community members [64, 65] (Table S2). Metatranscriptome analysis indicated these genes were differentially expressed: whereas A3, 1d, 3a, 3c and 4g genes were highly expressed (RNA/DNA expression ratio > 4), others were expressed at moderate (A1, A2, Fe; ratio 1.5–2.5) or low levels (B, 4e, 4h, 4i; ratio < 1.5) (Figure S2 & S3; Table S1). Though putative nitrogenase genes (nifH) were detected, expression ratios were low (av. 0.45), suggesting nitrogen fixation is not a significant H_2 source in sheep (Figure S4). This is consistent

with previous reports that nitrogenase activity in ovine rumen contents is low [33, 96].

Accounting for 54% of hydrogenase transcripts detected (Figs. 2b, 3a, S2), group A3 [FeFe]-hydrogenases appear to be the primary catalysts of H_2 production in ruminants. We assigned the retrieved transcripts to taxa based on their closest hits to the rumen genome hydrogenase data set (Table S4). Clostridia accounted for the majority of the hits (Fig. 2d), including *Ruminococcus* (22%), *Saccharofermentans* (9.2%) and *Lachnoclostridium* (7.4%) species known to fermentatively produce H_2 [29, 34, 97] (Figure S5 & S6). Transcripts from the characterised fermentative genera *Bacteroides*, *Butyrivibrio*, *Clostridium* and *Sarcina* were also moderately abundant. A further 21% of group A3 [FeFe]-hydrogenase hits were assigned to three
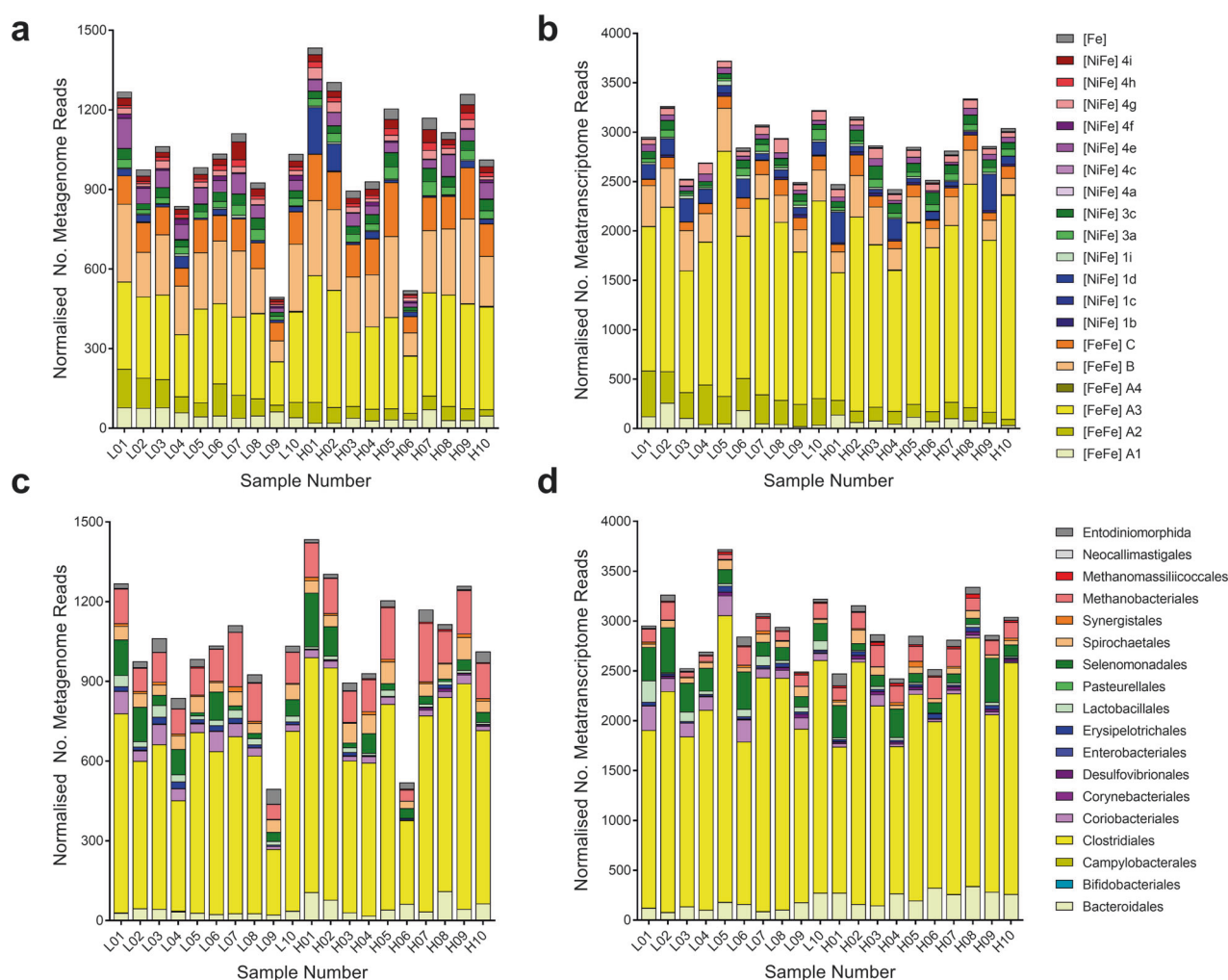

Fig. 2 Hydrogenase content in the metagenomes and metatranscriptomes of the microbial communities within rumen contents of high and low methane yield sheep. Hydrogenase content is shown based on hydrogenase subgroup **a**, **b** and predicted taxonomic affiliation **c**, **d** for metagenome data sets **a**, **c** and metatranscriptome data sets **b**, **d**. Hydrogenase-encoding sequences were retrieved from 20 paired shotgun metagenomes and metatranscriptomes randomly subsampled at five million reads. Reads were classified into hydrogenase subgroups and taxonomically assigned at the order level based on their closest match to the hydrogenases within the genomes screened (Fig. 1). L01 to L10 are data sets for sheep that were low methane yield at time of sampling, H01 to H10 are data sets from sheep that were high methane yield at time of sampling (see Table S3 for full details)
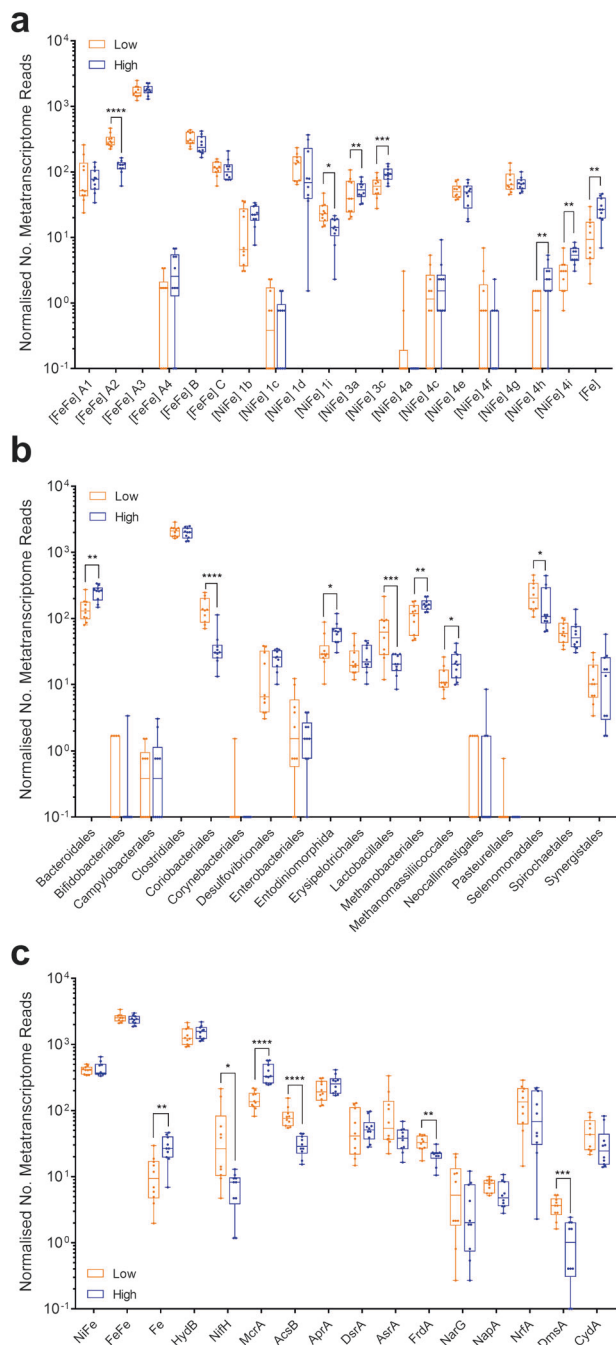
a

b

c

Fig. 3 Comparison of expression levels of H_2 production and H_2 uptake pathways in low and high methane yield sheep. Results are shown for 10 metatranscriptome data sets each from low methane yield sheep (orange) and high methane yield sheep (blue) that were randomly subsampled at five million reads. **a** Normalised count of hydrogenase transcript reads based on hydrogenase subgroup. **b** Normalised count of hydrogenase transcript reads based on predicted taxonomic affiliation. **c** Normalised count of transcript reads of key enzymes involved in H_2 production and H_2 consumption, namely the catalytic subunits of [NiFe]-hydrogenases (NiFe), [FeFe]-hydrogenases (FeFe), [Fe]-hydrogenases (Fe), hydrogenase-associated diaphorases (HydB), nitrogenases (NifH), methyl-CoM reductases (McrA), acetyl-CoA synthases (AcsB), adenylylsulfate reductases (AprA), dissimilatory sulphite reductases (DsrA), alternative sulfite reductases (AsrA), fumarate reductases (FrdA), dissimilatory nitrate reductases (NarG), periplasmic nitrate reductases (NapA), ammonia-forming nitrite reductases (NrfA), DMSO/TMAO reductases (DmsA) and cytochrome *bd* oxidases (CydA) are provided. For FrdA, NrfA and CydA, the numerous reads from non-hydrogenotrophic organisms (e.g., Bacteroidetes) were excluded. Each boxplot shows the 10 datapoints and their range, mean and quartiles. Significance was tested using independent two-group Wilcoxon rank-sum tests (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; full p values in Table S9, S10 and S11). Note the metagenome abundance and RNA/DNA ratio of these genes is shown in Figure S2 (hydrogenase subgroup), Figure S3 (hydrogenase taxonomic affiliation) and Figure S4 (H_2 uptake pathways). A full list of metagenome and metatranscriptome hits is provided for hydrogenases in Table S4 and H_2 uptake pathways in Table S5

these organisms to date. The expression of formate hydrogenlyases and other formate-dependent hydrogenases were extremely low in metatranscriptome data sets (Fig. 3c), indicating formate-dependent H_2 production is not a major pathway in situ.

The metatranscriptome data sets indicate that multiple H_2 uptake pathways operate in ruminants (Figs. 2 and 3). In agreement with historical paradigms [6], hydrogenotrophic methanogenesis appears to be the largest sink of H_2; methanogens accounted for 5.3% of normalised hydrogenase reads (Fig. 2d) and methyl-CoM reductase (*mcrA*) is the most expressed of the reductases surveyed (Fig. 3c). Consistent with their central roles in the CO_2-reducing pathway of methanogenesis [9], the F_{420}-reducing [NiFe]-hydrogenase (3a) [98] and the heterodisulfide reductase-associated [NiFe]-hydrogenase (3c) [99] of *Methanobrevibacter* species were among the most transcribed of all H_2 uptake enzymes (Fig. 3a and S5). In contrast, the Eha-type (4h), Ehb-type (4i) and [Fe]-hydrogenases were expressed at lower levels (Fig. 3a and S5), reflecting their secondary roles in the physiology of methanogens [100–102]. There was also strong evidence that hydrogenotrophic acetogenesis may be a more significant ruminal H_2 sink than previously recognised. Across the data set, acetyl-CoA synthases (*acsB*; 1135 normalised reads) were expressed at a quarter of the level of methyl-CoM reductases (*mcrA*; 5246 normalised reads) (Fig. 3c). For 74% of the reads, the closest matches were to predicted hydrogenotrophic acetogens isolated from rumen, including

uncharacterised cultured lineages within the Clostridia: Clostridiales R-7, Ruminococcaceae P7 and Lachnospiraceae YSB2008 (Figure S5 & S6). This is compatible with our previous studies showing unclassified microorganisms, especially from R-7 group, are abundant in rumen [21]. H_2-evolving hydrogenases from the A1 and B subgroups were also detected, but their RNA/DNA expression ratios were threefold lower than the A3 hydrogenases. Rumen ciliates such as *Epidinium* dominated A1 reads (Fig. 2d & S5), but it is likely that their abundance in the data sets is underestimated owing to the minimal genome coverage of

Blautia, *Acetitomaculum* and *Oxobacter* (Figure S7 & Table S5). Consistently, genes encoding close homologues of the group A2 and group A3 [FeFe]-hydrogenases from these genera were moderately expressed in the metatranscriptomes (3.7%) (Figure S5). The other *acsB* reads likely originate from acetogens that use other electron donors, such as formate. No *acsB* hits from methanogens were detected.

Surprisingly, however, the most highly expressed H_2 uptake hydrogenase overall is the group 1d [NiFe]-hydrogenase of Selenomonadales (4.1%) (Figs. 3a, b and S5). This enzyme is likely to mediate the long-known capacity of *Selenomonas* species to grow by hydrogenotrophic fumarate reduction and nitrate ammonification [52, 53, 58]. Consistently, fumarate reductases (*frdA*), nitrate reductases (*narG*) and ammonia-forming nitrite reductases (*nrfA*) homologous to those in *S. ruminantium* were expressed in the metatranscriptomes (Fig. 3c). Normalised *nrfA* expression was fivefold higher than *narG*, indicating selenomonads may preferentially use external nitrite, although further studies are required to determine the source of nitrite, this compound is known to accumulate in the rumen depending on nitrate content of feed [103]. Reads corresponding to the group 1b [NiFe]-hydrogenase, periplasmic nitrate reductase (*napA*), *nrfA* and *frdA* from *Wolinella* was also detected, but at low levels (Table S4 & S5; Figure S5). Several other pathways in low abundance in the metagenome were also highly expressed, notably group 1b [NiFe]-hydrogenases and *dsrA* genes from *Desulfovibrio* species, as well as group 1i [NiFe]-hydrogenases from metabolically flexible Coriobacteriia (e.g., *Slackia*, *Denitrobacterium*) (Figure S4 & S5). The expression levels of the group 1b and 1d [NiFe]-hydrogenases, together with the functionally unresolved group 4g [NiFe]-hydrogenases, were the highest of all hydrogenases in data sets (RNA/DNA ratio > 10) (Figure S3). Though these findings need to be validated by activity-based studies in situ, they suggest that respiratory hydrogenotrophs are highly active and quantitatively significant H_2 sinks in the rumen despite often being detected in low abundance [5].

Culture-based studies demonstrate that hydrogenases mediating H_2 production and uptake are differentially regulated in response to hydrogen levels

In order to better understand how rumen bacteria regulate H_2 metabolism, we performed a culture-based study using *R. albus* 7 and *Wolinella succinogenes* DSM 1740. This system was chosen given it is the best-studied example of rumen microorganisms that participate in interspecies hydrogen transfer [25] and is relevant to H_2 transactions in the rumen, given *Ruminococcus* electron-bifurcating hydrogenases are the most abundant in the sheep

metatranscriptome data sets (Figure S5). We compared the growth, transcriptome and extracellular metabolite profiles of these strains in either pure culture or co-culture when grown on modified fumarate-supplemented Balch medium (Table S6). The concentrations of the metabolites consumed and produced by the strains varied between the conditions (Table S1; Figure S8) in a manner consistent with previous reports [24, 25, 29, 54] and the transcriptomic results (Fig. 4). Based on the ratios of the products observed, *R. albus* fermentatively degraded cellobiose to H_2, acetate and ethanol in pure culture (glucose + 3.3 ADP + 3.3 P_i → 2.6 H_2 + 1.3 acetate + 0.7 ethanol + 2 CO_2 + 3.3 ATP [29]) and H_2 and acetate in co-culture (glucose + 4 ADP + 4 P_i → 4 H_2 + 2 acetate + 2 CO_2 + 4 ATP [29]) (Fig. 4a–c). *W. succinogenes* grew by hydrogenotrophic fumarate respiration under both conditions by using exogenously supplied H_2 in pure culture and syntrophically-produced H_2 in co-culture (Fig. 4–f). Hence, *R. albus* channels fermentation through the pathway that yields stoichiometrically more ATP, H_2 and acetate, provided that H_2 concentrations are kept sufficiently low through interspecies hydrogen transfer for this to be thermodynamically favourable (see Zheng et al. [29] for stoichiometric and thermodynamic considerations).

Transcriptome profiling revealed that *R. albus* tightly regulates the expression of its three hydrogenases (Fig. 4a, b). Overall, 133 genes were differentially expressed (fold change > 2, *q* value < 0.05) in co-culture compared with pure culture (Table S7). Of these, the greatest fold change was the 111-fold downregulation of a putative eight-gene cluster encoding the ferredoxin-only hydrogenase (group A1 [FeFe]-hydrogenase), a bifunctional alcohol and aldehyde dehydrogenase, and regulatory elements including a putative sensory hydrogenase (group C [FeFe]-hydrogenase) (Fig. 4a, b). By suppressing expression of these enzymes, *R. albus* can divert carbon flux from ethanol production to the more energetically efficient pathway of acetate production; acetate fermentation produces equimolar levels of NADH and reduced ferredoxin, which can be simultaneously reoxidised by the electron-bifurcating hydrogenase (group A3 [FeFe]-hydrogenase) (Fig. 4c). Glycolysis enzymes and the phosphate acetyltransferase, acetate kinase and electron-bifurcating hydrogenase of the acetate production pathway were expressed at similarly high levels under both conditions (Fig. 4a, b). However, there was a significant increase in the biosynthesis of thiamine pyrophosphate, a cofactor for pyruvate dehydrogenase complex, in co-culture (Fig. 4a).

The fermentation stoichiometries of *R. albus* 7 measured in pure culture compared with co-culture (Table 1) were the same as we previously reported for the bacterium at high vs low concentrations of H_2 [29]. This suggests that the differences in regulation are primarily determined by H_2
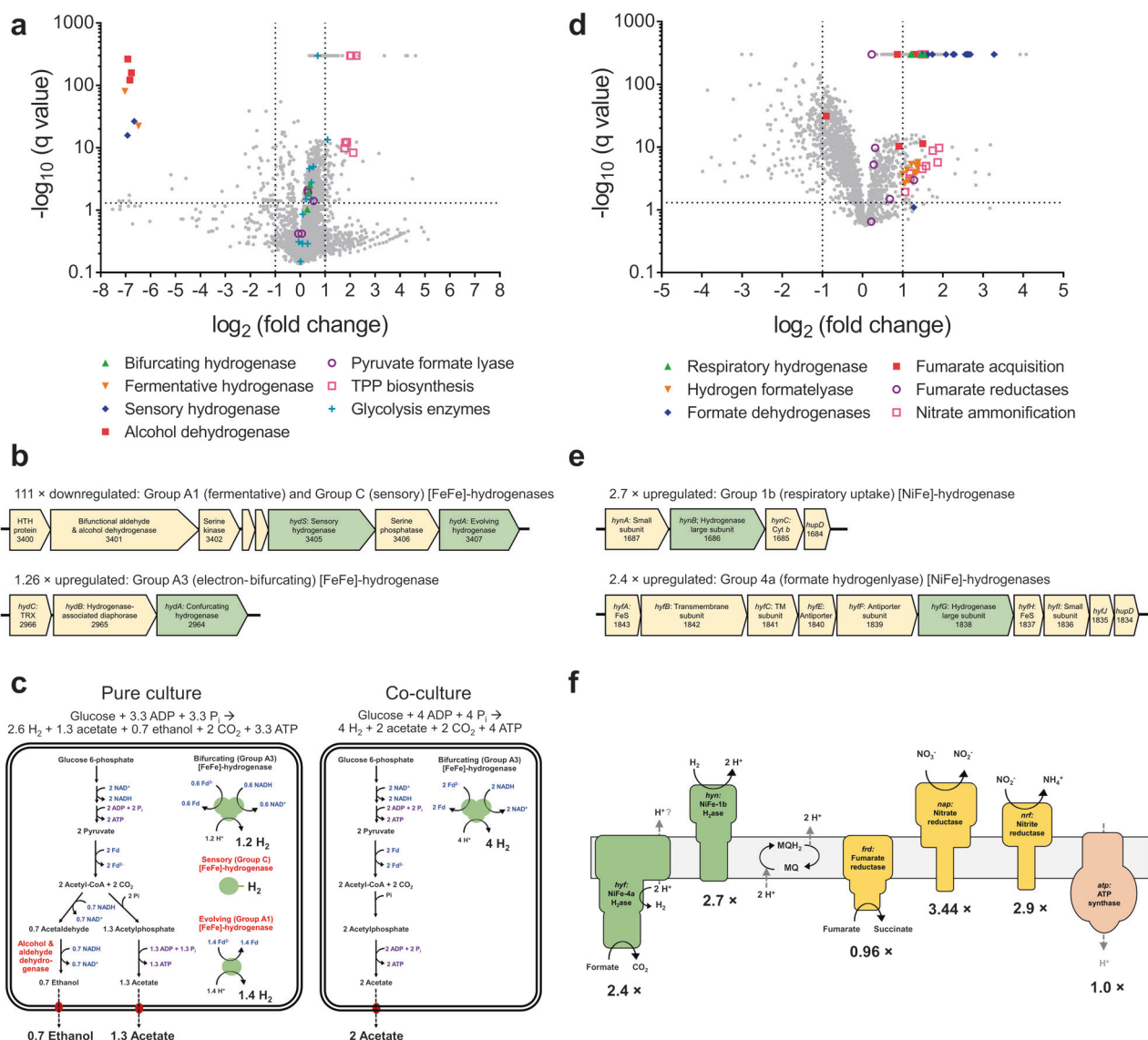
Fig. 4 Comparison of whole genome expression levels of *Ruminococcus albus* and *Wolinella succinogenes* in pure culture and co-culture. Pure cultures and co-cultures of *Ruminococcus albus* 7 **a–c** and *Wolinella succinogenes* DSM 1740 **d–f** were harvested in duplicate during mid-exponential phase and subject to RNA sequencing. **a, d** Volcano plots of the ratio of normalised average transcript abundance for co-cultures over pure cultures. Each gene is represented by a grey dot and key metabolic genes, including hydrogenases, are highlighted as per the legend. The horizontal dotted lines indicate *q* values of 0.05 and the vertical dotted lines indicate twofold changes. **b, d** Predicted operon structure of the

three hydrogenases of *R. albus* and two hydrogenases of *W. succinogenes*. **e** Comparison of dominant fermentation pathways of *R. albus* in pure culture (left) and co-culture (right) based on transcriptome reads and metabolite profiling. Three enzymes with decreased expression in co-culture are in red font. **f** Respiratory chain composition of *W. succinogenes* in pure culture and co-culture based on transcriptome reads. Metabolite profiling indicated that the respiratory hydrogenase and fumarate reductases were active in both conditions. Proton translocation is thought to occur primarily through redox-loop mechanisms. A full list of read counts and expression ratios for each gene is provided in Table 1

levels, rather than by direct interactions with syntrophic partners. This regulation may be achieved through direct sensing of H_2 by the putative sensory group C [FeFe]-hydrogenase co-transcribed with the ferredoxin-only hydrogenase and alcohol dehydrogenase (Fig. 4e). In common with other enzymes of this class [66, 104, 105], this enzyme contains a subunit containing a H-cluster for H_2 binding and a PAS domain for signal transfer, as well as a

putative serine or threonine phosphatase subunit that may modify downstream regulators. Thus, analogous to the well-studied regulatory hydrogenases of aerobic bacteria [106, 107], this enzyme may directly sense H_2 levels and induce expression of the alcohol/aldehyde dehydrogenase and ferredoxin-only hydrogenase when H_2 concentrations are high through a feedback loop. H_2 sensing may be a general mechanism regulating hydrogenase expression in

Table 1 Comparison of growth parameters and metabolite profiles of *Ruminococcus albus* and *Wolinella succinogenes* in pure culture and co-culture

	Ruminococcus albus	*Wolinella succinogenes*	Co-culture
Growth parameters			
Growth yield (OD$_{600}$)	0.79 ± 0.01	0.36 ± 0.01	0.93 ± 0.01
Specific growth rate (h^{-1})	0.58 ± 0.19	0.33 ± 0.06	0.57 ± 0.34 (*Ra*) 0.54 ± 0.11 (*Ws*)
Concentration changes of extracellular metabolites			
Hydrogen (%)	$+5.3$	-78.4	BDL
Fumarate (mM)	-5.5	-46.3	-43.1
Succinate (mM)	$+2.2$	$+54.6$	$+55.4$
Acetate (mM)	$+21.8$	0	$+32.4$
Ethanol (mM)	$+8.7$	0	$+0.3$
Formate (mM)	BDL	BDL	BDL
pH	-0.4	-0.4	-0.6

Growth of pure cultures and co-cultures of *Ruminococcus albus* 7 and *Wolinella succinogenes* DSM 1740 was monitored by qPCR. Values show means ± standard deviations of three biological replicates. Also shown is the change in extracellular pH, percentage hydrogen gas (measured by gas chromatography), and concentrations of fumarate, succinate, acetate, ethanol and formate (measured by ultra-fast liquid chromatography) between 0 and 12 h. Growth media was the same between the three conditions, except 80% H$_2$ was added for *W. succinogenes* growth, whereas no H$_2$ was added for the other conditions. Full liquid metabolite measurements are shown in Figure S8. BDL = below detection limit (1 mM for formate, 0.1% for H$_2$).

ruminants, given group C [FeFe]-hydrogenases are abundant in ruminant genome (Fig. 1), metagenome (Fig. 2a), and metatranscriptome data sets (Fig. 2b and 3a).

The transcriptome results also clarified understanding of hydrogenotrophic fumarate respiration by *W. succinogenes* (Fig. 4d). In both pure culture and co-culture, the group 1b [NiFe]-hydrogenase, fumarate reductase and F$_1$F$_o$-ATPase that mediate this process were expressed at high levels (Table S7; Fig. 4f). A periplasmic asparaginase, aspartate ammonia-lyase and dicarboxylate-binding proteins were also highly expressed; this suggests that the organism can efficiently produce and import additional fumarate from amino acid sources (Table S7). In total, 352 genes were significantly differentially regulated in co-culture (fold change > 2, *q* value < 0.05). The respiratory hydrogenase was among the upregulated genes (Fig. 4e), which may reflect the strain's faster growth rate in co-culture (Table 1). The periplasmic nitrate reductase and ammonia-forming nitrite reductase (Fig. 4f) were also induced, indicating some plasticity in electron acceptor usage, in line with the metatranscriptomic findings. Two formate dehydrogenases and a

formate hydrogenlyase (group 4a [NiFe]-hydrogenase) were highly expressed in co-culture (Fig. 4d, e), indicating the bacterium can potentially use formate transferred from *R. albus* as a supplemental electron donor to H$_2$ (Fig. 4a); consistently, *R. albus* is known to produce through formate pyruvate lyase activity and this enzyme was highly expressed in both pure culture and co-culture (Table S7), though any formate produced was below detection limits (1 mM) (Table 1).

Hydrogenotrophic acetogenesis, fumarate reduction and nitrate reduction pathways are significantly upregulated in low methane yield sheep

Finally, we tested whether the abundance and expression of hydrogenases and H$_2$ uptake pathways differed between high and low methane yield sheep. A current leading hypothesis, proposed on the basis of community composition [69], asserts that H$_2$ production levels account for differences in methane yield between sheep. To the contrary, the expression levels of the dominant H$_2$-evolving hydrogenases (e.g., group A3 [FeFe]-hydrogenases) and taxonomic orders (e.g., Clostridiales) were in fact extremely similar between the groups (Fig. 3a, b; Table S9 & S10).

We therefore tested an alternative hypothesis: H$_2$ utilisation through non-methanogenic pathways can reduce methane yield. In line with this hypothesis, the expression levels of the five methanogen hydrogenases and methyl-CoM reductase are significantly reduced in low methane yield sheep (Fig. 3a, c; Table S9 & S11), confirming a strong correlation with methane yield (Table S3). Concurrent increases in the gene expression for two major alternative H$_2$ sinks were detected, namely acetogenesis (*acsB*; *p* < 0.0001) and fumarate reduction (*frdA*; *p* = 0.002) (Fig. 3c; Table S10), concomitant with significant increases in the expression levels of *Blautia* and *Selenomonas* hydrogenases (Figure S5). Expression levels of *nrfA* were also on average 1.8-fold higher in low methane yield sheep, though there was much inter-sample variation in the read count for this gene. Whereas there are more transcripts of *mcrA* than other terminal reductases combined in high methane yield sheep, the transcript levels of *acsB* and *nrfA* together exceed those of *mcrA* in low methane yield sheep. Depending to what extent expression levels predict activity, hydrogenotrophic acetogens and selenomonads may therefore be more active than methanogens in low methane yield sheep and may significantly limit substrate supply for methanogenesis. Two other potential H$_2$ sinks are also upregulated in the low methane yield sheep: the putative group 1i [NiFe]-hydrogenase of Coriobacteriia and, consistent with previous observations [65], the functionally unresolved group A2 [FeFe]-hydrogenase of *Sharpea*, *Olsenella* and *Oribacterium* (Fig. 3a, b, S5).

Discussion

To summarise, H_2 metabolism is a more widespread and complex process in ruminants than previously realised. Together, the genomic, metagenomic and metatranscriptomic surveys suggest that multiple orders of bacteria, archaea and eukaryotes encode and express enzymes mediating H_2 production and consumption in the rumen. We infer that fermentative Clostridia are the main source of H_2 in the rumen, which largely agrees with findings from activity-based and culture-based studies [6, 25, 26, 29], and that a range of methanogenic, acetogenic and respiratory hydrogenotrophs are present. Although this manuscript provides a relatively comprehensive understanding of H_2 transactions in the rumen, it is probable that other uncharacterised organisms and genes also contribute to this process. A more-detailed perspective of which taxa mediate H_2 cycling in the rumen would be possible by profiling the thousands of metagenome-assembled genomes recently recovered from ruminants [23, 108] and also better accounting for the role of the largely unsequenced rumen ciliates and fungi, which to date are underrepresented in genomic data sets. Further studies are also necessary to extend findings to other ruminants, such as bovines, and investigate how H_2 cycling responds to environmental factors such as diet.

One of the most-important findings of this work is that the recently characterised electron-bifurcating hydrogenases appear to primarily mediate ruminal H_2 production. These enzymes are highly upregulated compared with ferredoxin-only hydrogenases in situ and constitute over half of hydrogenase reads in these metatranscriptomes. We provide a rationale for this finding by showing that *R. albus*, a dominant H_2 producer within the rumen, expresses its electron-bifurcating hydrogenase and suppresses its ferredoxin-only hydrogenase when grown syntrophically with *Wolinella succinogenes*. In this condition, H_2 concentrations remain sufficiently low that the fermentation pathway producing higher levels of ATP, H_2 and acetate remains thermodynamically favourable. In the rumen, where tight coupling of hydrogenogenic and hydrogenotrophic processes usually keeps H_2 at sub-micromolar concentrations [46], Clostridia will also preferentially oxidise carbohydrates through higher ATP-yielding pathways and reoxidise the NAD and ferredoxin reduced using the electron-bifurcating hydrogenase [29]. It is likely that the ferredoxin-only hydrogenases are preferentially upregulated during the transient periods where H_2 levels are high, for example immediately after feeding [46]. Based on these findings and previously published results [29, 66, 104, 105], we propose that the hydrogenases and fermentation pathways are differentially regulated as a result of direct H_2 sensing by putative sensory [FeFe]-hydrogenases. A further surprising finding is that uncharacterised lineages within the Clostridia account for a large proportion of the electron-bifurcating hydrogenase reads, emphasising the need for physiological and bacteriological characterisation of these organisms.

The other major finding of this work is that there are multiple highly active H_2 sinks in the rumen. We provide evidence, based on transcript levels of their hydrogenases and terminal reductases, that acetogens (*Blautia*, *Acetitomaculum*), fumarate and nitrate reducers (*Selenomonas*, *Wolinella*) and sulfate reducers (*Desulfovibrio*) are quantitatively significant H_2 sinks in sheep. In support of these findings, our culture-based study confirmed that the enzymes mediating hydrogenotrophic fumarate reduction and potentially nitrate ammonification are highly expressed by *W. succinogenes* in co-culture with *R. albus*. Although alternative H_2 uptake pathways have been previously detected in vitro [41, 42, 50–53, 57, 58], it has generally been assumed that they are quantitatively insignificant compared to hydrogenotrophic methanogenesis [5, 6, 46]. To the contrary, hydrogenase and terminal reductase transcripts from alternative H_2 uptake pathways are more numerous than those of methanogens in low methane yield sheep, and hence these pathways may collectively serve as a larger H_2 sink than methanogenesis under some circumstances. These findings justify activity-based studies to quantify H_2 flux within ruminants between methanogenic and non-methanogenic pathways. There is also evidence of other novel pathways operating in the rumen, mediated by the functionally unresolved group 1i [NiFe]-hydrogenases (*Slackia*, *Denitrobacterium*), group 4g [NiFe]-hydrogenase (*Clostridium*) and group A2 [FeFe]-hydrogenases (*Sharpea*, *Oribacterium*, *Olsenella*).

These findings also provide some insight into the basis of variation in methane emissions between ruminants. Methane yield in ruminants is a quantitative heritable trait [64, 77], to some extent, and there is much interest in developing host or microbiome markers to enable selection of animals with low methane yield traits [109]. Our study compared the abundance and expression of key functional genes in the rumen microbial communities of sheep that differed by an average of 28% in methane yield. In low methane yield sheep, there was significantly decreased expression of methyl-CoM reductase, increased expression of acetyl-CoA synthase and fumarate reductase and multiple changes in hydrogenase profiles. However, we detected few significant differences at the gene level. The similar metagenome profiles, combined with the metatranscriptome profiles of the phenotype-switching sheep, therefore suggest that alternative H_2 uptake pathways are partly inducible. Further studies with larger sample sizes are needed to assess whether transcripts of these functional genes can predict methane yield and, if so, whether they can be adopted

as biomarkers to facilitate animal selection or evaluate methane mitigation strategies. Our findings, in conjunction with other studies [109], indicate that the methyl-CoM reductase and hydrogenases of methanogens may be particularly promising and relevant biomarkers.

The strong correlation between H_2 uptake pathways and methane yield phenotypes nevertheless supports that modulating H_2 metabolism may be an effective methane mitigation strategy. One strategy is to develop inhibitors that redirect electron flux from H_2 production towards volatile fatty acid production. However, given the central role of H_2 metabolism in the physiology and ecology of most rumen microorganisms, this would be challenging to achieve without compromising rumen function and consequently ruminant nutrition. Furthermore, such strategies may have a converse effect on methane production, given lower H_2 concentrations restrict acetogens more than methanogens [46]. Instead, our metatranscriptome analyses support the concept that a more promising approach may be to stimulate alternative H_2 pathways such as fumarate, nitrate and sulfate respiration. One solution may be to supplement animal feeds with electron acceptors, such as fumarate, nitrate, or sulfate, that stimulate the dominant respiratory hydrogenotrophs. Such approaches have shown some promise in mitigating methane production both in vitro [110–112] and in field trials [62, 63, 113, 114]. These strategies may complement methanogenesis inhibitors [16, 17] by facilitating the redirection of H_2 flux from methanogens to other pathways. Although further studies are required, there is also growing evidence that redirecting H_2 flux can favour animal production by enabling recovery of energy that would otherwise be lost through methane emissions [115].

Compliance with ethical standards

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