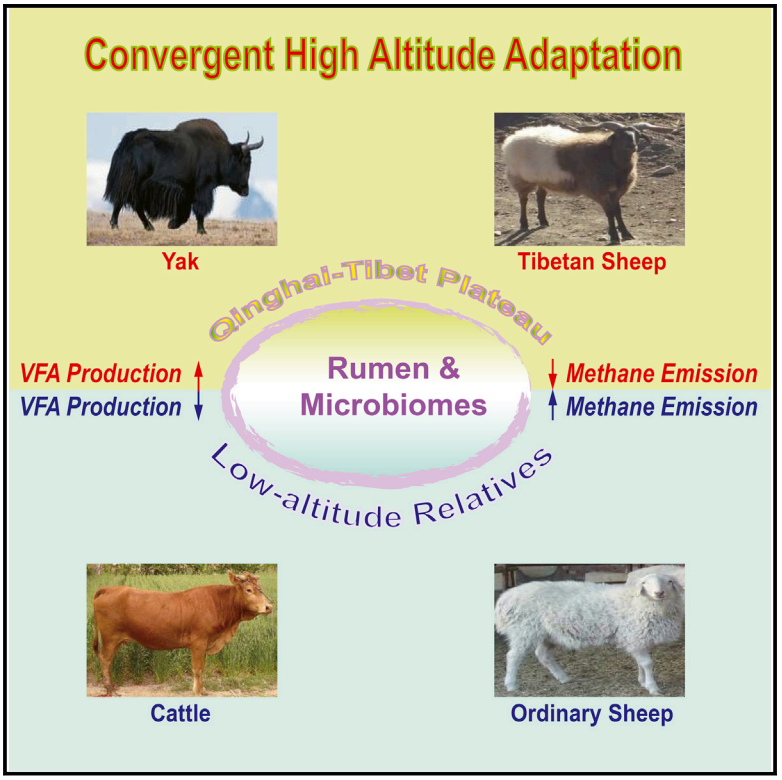
Report

Current Biology

Convergent Evolution of Rumen Microbiomes in High-Altitude Mammals

Graphical Abstract



Highlights

- High-altitude adaptation produces convergent phenotypes of rumen metabolism
- Microbial community structures and compositions contribute to convergent adaptation
- Results show convergence of microbial genes in VFAs and methane-yielding pathways
- Microbiomes co-evolve with host genomes for extremely environmental adaptation

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In Brief

Zhang et al. observe both low-methane emissions and high-VFA production in high-altitude mammals. Using ultra-deep metagenomic sequencing, the authors show co-enrichment in VFA-yielding pathways of rumen microbial genes in high-altitude species, suggesting convergent evolution of rumen microbiomes for their hosts' energy harvesting persistence.

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Convergent Evolution of Rumen Microbiomes in High-Altitude Mammals

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SUMMARY

Studies of genetic adaptation, a central focus of evolutionary biology, most often focus on the host's genome and only rarely on its co-evolved microbiome. The Qinghai-Tibetan Plateau (QTP) offers one of the most extreme environments for the survival of human and other mammalian species. Yaks (*Bos grunniens*) and Tibetan sheep (T-sheep) (*Ovis aries*) have adaptations for living in this harsh high-altitude environment, where nomadic Tibetan people keep them primarily for food and livelihood [1]. Adaptive evolution affects energy-metabolism-related genes in a way that helps these ruminants live at high altitude [2, 3]. Herein, we report convergent evolution of rumen microbiomes for energy harvesting persistence in two typical high-altitude ruminants, yaks and T-sheep. Both ruminants yield significantly lower levels of methane and higher yields of volatile fatty acids (VFAs) than their low-altitude relatives, cattle (*Bos taurus*) and ordinary sheep (*Ovis aries*). Ultra-deep metagenomic sequencing reveals significant enrichment in VFA-yielding pathways of rumen microbial genes in high-altitude ruminants, whereas methanogenesis pathways show enrichment in the cattle metagenome. Analyses of RNA transcriptomes reveal significant upregulation in 36 genes associated with VFA transport and absorption in the ruminal epithelium of high-altitude ruminants. Our study provides novel insights into the contributions of microbiomes to adaptive evolution in mammals and sheds light on the biological control of greenhouse gas emissions from livestock enteric fermentation.

RESULTS AND DISCUSSION

Low-Methane and High-VFA Production in High-Altitude Ruminants

We hypothesized that adaptive evolution occurs in cohabiting microbiomes of high-altitude mammals, especially in ruminants, because they extend host metabolic repertoires [4–9]. For instance, volatile fatty acids (VFAs; primarily acetic, propionic, and butyric acids) supplied by rumen microbes lead to energy gain [10]. By contrast, methane, a byproduct of rumen fermentation by methanogenic archaea, causes energy loss [11]. To test this hypothesis, we first measured methane emissions and VFA production, both of which are largely produced by rumen microbes. Using controlled in vitro gas production experiments [12] with the same amount of oat hay (*Avena sativa*) as the fermentation substrate, we compared methane emissions and VFA production from yaks and cattle grazing on the same meadow at 3,000 m altitude 48 hr after in vitro incubation (study 1). Results show that yaks produce significantly ($p = 0.002$, t test) less methane than cattle (Figures 1A and 1B). Conversely, yaks produce significantly ($p < 0.01$, t test) more total VFAs (TVFAs) than cattle, especially for acetate ($p < 0.01$), propionate ($p < 0.05$), and butyrate ($p < 0.001$) (Figure 1C). Independent in vitro experiments involving 72 hr incubation validate these observations (study 2). After 12 hr incubation, cattle produce significantly ($p < 0.001$, t test) more methane than yaks, although the increase in rate of methane emission slows after 48 hr (Figure S1A). Similarly, yaks produce significantly ($p < 0.001$, t test) more VFAs after 72 hr incubation than cattle (Figure S1B). These results coincide with previous in vivo observations [13, 14]. Compared with other similar studies from different cattle strains, yaks produce less methane and more TVFAs (Figure S1C), suggesting that an observable production of both low methane and high VFAs has been fixed in yaks after they split from the common ancestor of cattle.

To determine whether observations for the yak are typical of high-altitude ruminants, we repeated 48 hr in vitro experiments using Tibetan sheep (T-sheep) at 3,000 m and ordinary sheep

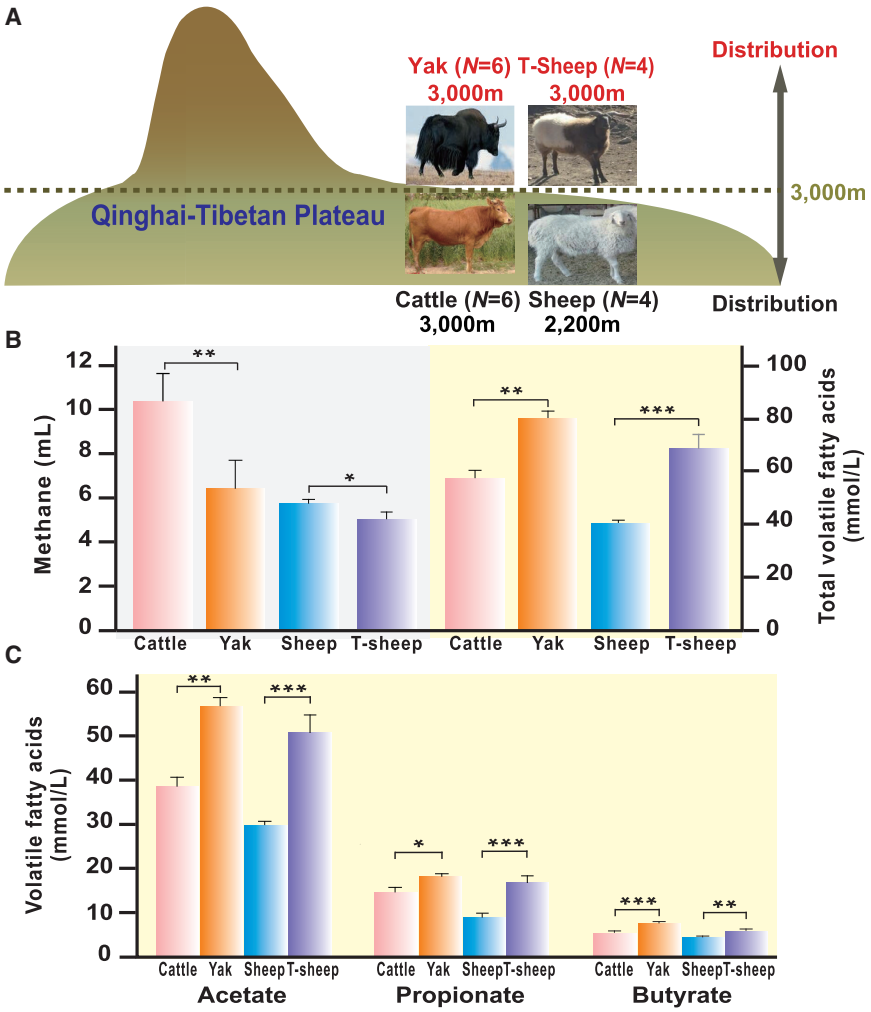

Figure 1. In Vitro Measurements Show Phenotype Differences in Methane Emission and VFA Yield

(A) Ruminant species and their distributions used for all in vitro experiments. The 3,000 m above sea level (a.s.l.) marked by the dashed line denotes the approximate survival threshold of animals that have become evolutionarily adapted to high-altitude environment. Arrows show animals' distributions along the elevation gradient on the QTP. Details are given in the Supplemental Experimental Procedures.

(B) Methane and TVFAs yield 48 hr after in vitro incubation (study 1 and sheep pair).

(C) VFA production 48 hr after in vitro incubation related to (B).

Variables are expressed as the mean ± SE statistic, Student's t test: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. See also Figure S1.

grazing in a similar pasture at 2,200 m altitude. Similarly, T-sheep produce significantly less methane ($p < 0.05$, t test) and more TVFAs ($p < 0.001$, t test) than sheep (Figure 1). These results reveal phenotypic convergence of low-methane and high-VFA production in high-elevation ruminants and indicate that gut microbiomes might play critical roles in high-altitude adaptation.

Structural Convergent Variation of Gut Microbiota in High-Altitude Ruminants

To investigate the structuring of gut microbial communities that contribute to phenotypic convergence, we compare the fecal bacterial communities of seven yaks, six cattle, four T-sheep, four ordinary sheep (sample information is detailed in the Supplemental Experimental Procedures), and the 17 other herbivore species [8]. The 16S rRNA gene commonly serves to characterize the composition and diversity of the microbiota [15]. Principal coordinates analysis (PCoA) clustering analysis based on 16S rRNA gene sequences shows that the structure of the fecal bacteria communities of yaks and T-sheep consistently differs from those of cattle, ordinary sheep, and the other 17 herbivore species [8] (Figure 2A). Moreover, seven individual yaks from four altitude populations clearly cluster together, suggesting that the yak's gut microbiota is highly conserved for inter-species comparisons, despite inter-individual variation.

Deep sequencing with subsampling of 100,000 reads adequately estimates microbial species richness in the rumen samples (Supplemental Experimental Procedures). Analyses of these data (Table S1) indicate that the rumen bacterial (Figure 2B) and archaeal (Figures S2A and S2B) community structure in yaks and T-sheep is distinct from those in cattle and sheep. The microbial communities of high-altitude ruminants show convergence (Figure 2C). This pattern is largely caused by those operational taxonomic units (OTUs) with significant differences in relative abundance between high-altitude ruminants and their low-altitude relatives (Figures S2A and S2B) and might be associated with phenotypic differences in methane yield and VFA accumulation. For example, methylotrophic methanogens, belonging to the class Thermoplasmata, have been associated with reduced methane production [16], and relative abundances are significantly ($p < 0.01$, t test) higher in yaks and T-sheep than in cattle and ordinary sheep (Figure S2C). In contrast, *Methanobrevibacter gottschalkii*, which is associated with high methane production [17], shows higher relative abundance in cattle ($p = 0.063$, t test) and sheep ($p = 0.021$, t test) than in yaks and T-sheep, respectively (Figure S2C). Likewise, yaks and T-sheep have a higher relative abundance of *Prevotella* spp., which can produce propionate or succinate and acetate [18] (Figure S2D). This group may contribute to the abundance of VFAs in high-altitude ruminants. Thus, changes in rumen microbiome structure and composition can contribute to the low-methane and high-VFA phenotypes.

Functional Metagenomic Profile of the Rumen Microbiome

To gain further insight into the metabolic pathways involved in production of methane and VFAs, we generated 379 Gb of

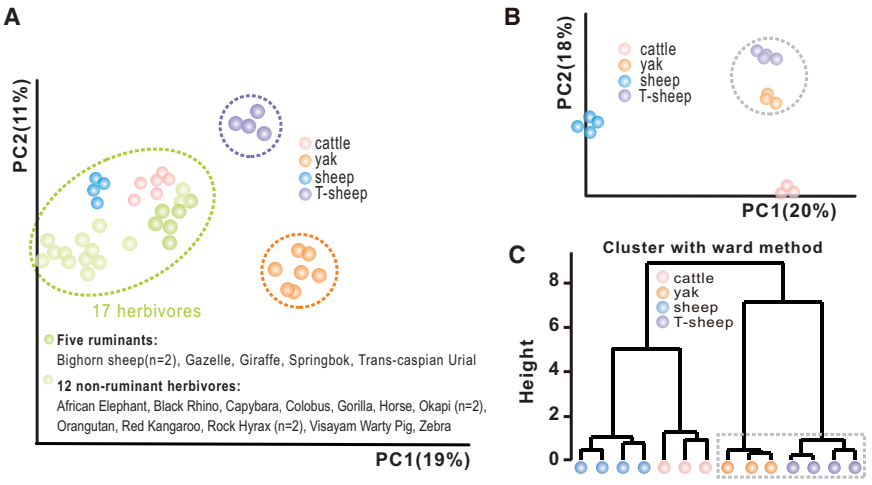

Figure 2. Convergent Variation in the Structure of the Gut Microbial Community of Yaks and T-Sheep

(A) PCoA clustering analysis of fecal bacterial communities of yak, cattle, T-sheep, ordinary sheep, and 17 herbivores [8] via both unweighted UniFrac full tree and binary Jaccard matrices. Sample details are included in the Supplemental Experimental Procedures.

(B) PCoA clustering analysis of rumen bacterial communities via both UniFrac g-full tree and Bray Curtis matrices based on subsampling at 100,000 reads. Three yak and three cattle samples came from study 1.

(C) Hierarchical clustering of rumen bacterial communities based on Pearson's correlation dissimilarity measure corresponding to (B).

See also Figures S2A and S2B for rumen archaea community structures and Figures S2C and S2D for rumen bacteria and archaea compositions. Basic information of rumen microbial 16S rRNA-based analysis is given in Table S1.

metagenomic sequence data (Table S2) from nine rumen microbiomes sampled from three independent yak populations along an elevational gradient on the QTP. We bound these data together to generate a core rumen microbiome representing the yak and then compares it to previous data from cattle (268 Gb sequence data) [4]. Further, we generated 86 Gb and 110 Gb of sequence data from the rumen microbiomes of three T-sheep and four sheep, respectively (Table S2). Ultra-deep sequencing data ensure highly non-redundant metagenomic assemblies (Table S3) that can identify genes involved in rumen metabolism. After applying a series of strict criteria for quality control, we de novo assembled and predicted 4,629,980 (yak), 4,461,287 (cattle), 3,616,639 (T-sheep), and 3,335,612 (sheep) open reading frames (ORFs). The NCBI non-redundant database has annotations for about 77.9% of the predicted ORFs.

Enrichments of gene number in the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) functional categories provide insights into the genetic basis of adaptive phenotypic changes. To avoid a bias caused by differences in sequencing depth among samples, we first identified a core KO dataset shared among the sequenced individuals. We evaluated the functional enrichments of 3,160 KOs shared by yaks and cattle and 2,947 shared by T-sheep and sheep. Analyses identified 716, 275, 313, and 88 significantly enriched KO categories in the metagenomes of yaks, cattle, T-sheep, and sheep, respectively (Data S1a–S1d). The rumen microbiomes of yaks and T-sheep have significantly more energy- and carbohydrate-metabolic-related KO categories than do those of cattle and sheep (Figure 3A; $p < 0.05$–0.0001, Fisher's exact test). This result indicates that rumen microbiomes may have co-evolved with host genomes via an expansion of gene families related to energy metabolism [2, 3]. Among the KOs, high-altitude ruminants show enrichment in the carbon fixation pathways of prokaryotes, which is consistent with the highly efficient formation of VFA [10, 19] and fatty acid [20] biosynthesis. For example, significant enrichment via six enzymes occurs in the carbon fixation pathway of yaks and T-sheep; the enzymes contribute to the production of acetate, propionate, and butyrate (Figures 4). Moreover, high-altitude ruminants show convergent enrichment of the essential enzyme acetyl-CoA carboxylase, which regulates the metabolism of fatty acids by catalyzing the committed and rate-limiting step in fatty acid biosynthesis [20]. This directly contributes to rumen energy by converting VFAs to medium-or-long chain fatty acids. In contrast, cattle show enrichment in methanogenesis, including the CO_2/H_2 pathway and the methylotrophic pathway [17] (Figures 4 and S3), which is consistent with the high-methane-yielding phenotype.

Functional Convergence of Rumen Microbiomes in High-Altitude Ruminants

Adaptive divergence at the level of gene sequence may also contribute to the emergence of low-methane-yielding and high-VFA phenotypes. A Ka/Ks ratio (nonsynonymous-to-synonymous substitution rate ratio) of >1 usually indicates adaptive selection [22]. Analyses obtain 16,745 one-to-one orthologs in the yak and cattle metagenomes and 16,722 for T-sheep and sheep. Ka/Ks values are significantly larger than 1, after false discovery rate (FDR) correction, in 5,107 (30.5%) and 6,107 (36.9%) genes, respectively. These appear to be rapidly evolved genes (REGs; Data S1e and S1f). Enriched REGs between yaks and cattle and between T-sheep and sheep overlap significantly in functional categories involved in energy metabolism, including carbohydrate metabolism and amino acid metabolism categories (Figure 3B; $p < 0.05$–0.001, Fisher's exact test). Notably, REGs show enrichment in carbon fixation pathways in prokaryotes (58 enzymes, in pathway ec00720 in KEGG). Specifically, REGs include 9 of 12 enzymes that participate in the formation of VFAs (Figure 4). Thus, both an increase in gene numbers and adaptive sequence change in VFA-formation pathways contribute to the high level of production of VFAs in high-elevation ruminants. The increase in VFA production could greatly inhibit the production of methane by competing for hydrogen with the methane-producing pathway.

Generally, energy harvesting in ruminants usually requires nitrogen balance [23]. Rumen microbiomes of high-altitude ruminants show significant enrichment in amino acid metabolism both in overall gene number and in the number of REGs (Figure 3; $p < 0.05$, Fisher's exact test). It would be interesting to test

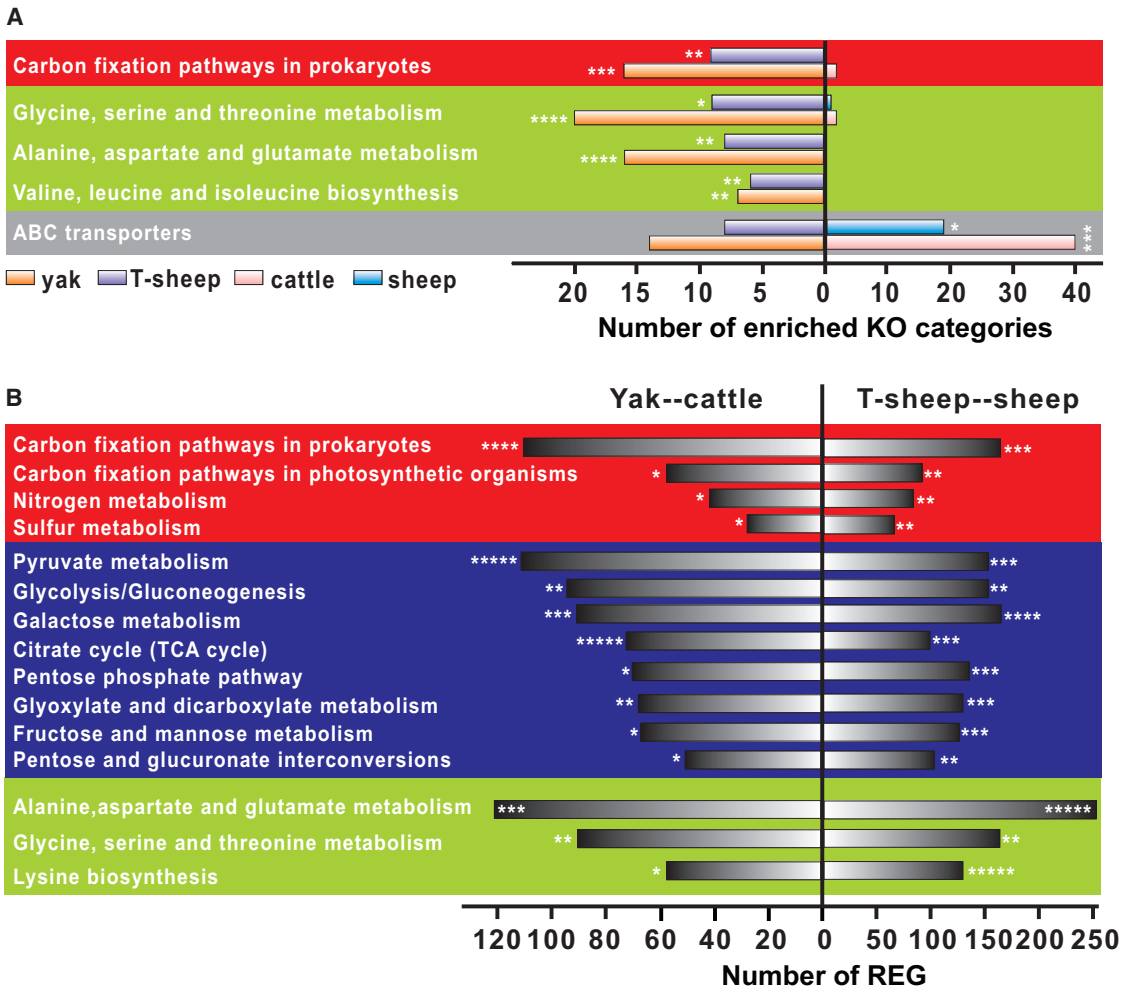

Figure 3. Functional Profiles of Rumen Microbiomes

(A) Significant differences of KEGG Orthology (KO) functional categories between high-altitude ruminants and their low-altitude relatives. See also Data S1a–S1d for enriched KOs. Detailed functional descriptions and gene counting of KO categories are given in Data S1g.

(B) Functional categories with significant enrichment in rapidly evolved genes (REGs).

See also Data S1e and S1f for identified REGs. For REG-enriched categories, detailed functional descriptions and REG counting of KO categories are given in Data S1h. The energy metabolism category is marked by a red box, carbohydrate metabolism by a blue box, amino acid metabolism by a green box, and membrane transport by a gray box. p values from two-sided Fisher's exact test with 10,000 bootstrap replicates are indicated as follows: *****p < 0.00001, ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05. See also Tables S2 and S3 for basic metagenomic data generation.

whether the highly efficient nitrogen metabolism matches the high production of VFAs in the future.

Co-evolution of the Rumen Microbiomes and Their Host Genomes in High-Altitude Ruminants

VFAs formed in the rumen are largely absorbed across the host's ruminal epithelium. Thus, high-altitude ruminants might possess the ability to more efficiently transport and absorb VFAs than their lowland counterparts. Comparative transcriptome analysis of ruminal epithelium show significant ($p < 0.05$, t test) upregulation of 36 genes associated with VFA transport and absorption [24–26] when compared to cattle (Figure S4). This result suggests that high-altitude ruminants also evolved highly efficient VFA transport. Future efforts via evaluation of plasma VFA levels are needed to reinforce the finding associated with VFA transport in high-altitude ruminants. Such analyses may provide valuable insights into how microbiomes and host genetic changes associate with one another.

Conclusions

Taken together, our phenotypic and metagenomic analyses provide important insights into convergent adaptation of yaks and T-sheep to high altitude and highlight the vital role microbial genomes play as "the second genome" of adaptation. Future understanding may be gained by investigations of mechanisms of high efficiency of microbiome in high-altitude mammals in the context of the hosts' physiological conditions (e.g., gut capacity, feed intake, digestion rate, digesta transit, and retention time). In addition, our study of low-methane-producing ruminants at high altitudes and their unique microbiome structures and genes

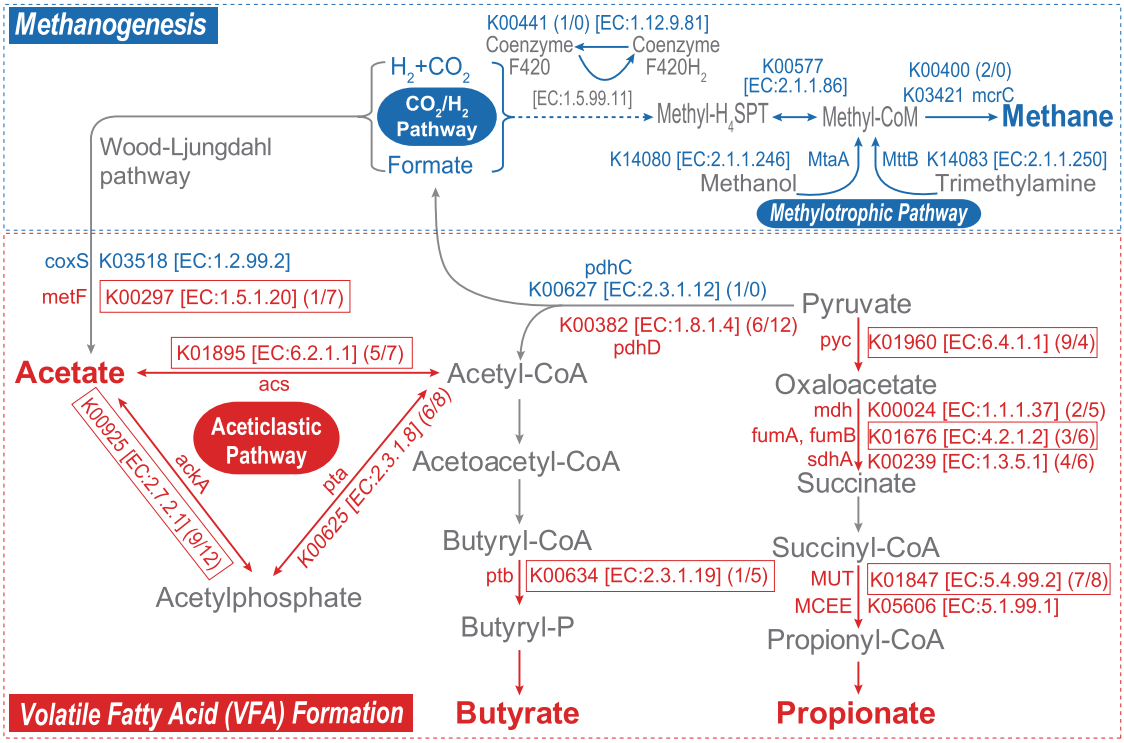

Figure 4. Reconstruction of the Metabolic Pathway Associated with VFA Formation and Methanogenesis in Tibetan Ruminants

All KO numbers, or K numbers, EC numbers, and gene names obtained from KEGG database [21]. See also Figure S3 for a complete view of rumen microbial genes mapped to the predominant CO_2/H_2 pathway. KO categories enriched in yaks are highlighted with red. Boxed KO categories are co-enriched in yaks and T-sheep. KO categories denoted by blue uniquely are enriched in cattle. REG numbers between yaks and cattle and between T-sheep and ordinary sheep are noted in parentheses. Six rapidly evolved enzymes (represented by K00297, K01895, K00925, K01960, K01676, and K01847) were found in the carbon fixation pathways in prokaryotes (detailed given in Data S1g and S1h). See also Tables S2 and S3 for basic metagenomic data generation and Figure S4 for upregulated genes implicated in fatty acid transport and absorption across the ruminal epithelium of yak.

provides promising directions for the biological control of greenhouse gas emissions in other high-methane-producing ruminant species.

EXPERIMENTAL PROCEDURES

Sample Preparation and Experimental Designs

A total of 15 yaks were used in this study, including six from 3,000 m a.s.l., three from 3,500 m a.s.l., three from 4,200 m a.s.l., and three from 4,500 m a.s.l. In addition, we used six cattle from the same Tibetan farm at 3,000 m a.s.l. where six yaks were located. We collected the fresh rumen contents from six yaks and six cattle at 3,000 m a.s.l. for the measurements of methane emissions and VFA production. These samples were divided into two groups for methane and VFA measurements: one group for in vitro experimentation after 48 hr incubation (study 1), and another group for experimentation after 72 hr incubation (study 2). We also repeated the 48 hr time point in vitro experiment by comparing four T-sheep (3,000 m a.s.l.) and four sheep (2,200 m a.s.l.).

Fresh fecal samples used for 16S rRNA sequencing came from seven yaks (three in study 2 and four randomly selected from nine yaks above 3,000 m a.s.l.), six cattle (study 1 and study 2), four T-sheep, and four sheep. Rumen community analysis was performed using the rumen samples from three yaks (represented the yak species, study 1), three cattle (represented the cattle species, study 1), four T-sheep, and four sheep. Rumen metagenomes were sequenced from the rumen samples from nine yaks representing three populations (above 3,000 m a.s.l.), four T-sheep, and four sheep. Transcriptome sequencing was conducted using ruminal epithelium of nine yaks (above 3,000 m a.s.l.) and three cattle (study 2). All the samples for molecular analysis were kept in the liquid nitrogen until further experimental analyses.

The Supplemental Experimental Procedures provide detailed sample information including location, age, sex, and weight of all experimental animals and detailed procedures on in vitro experimentation, molecular analysis, and data generation.

Metagenomic Analysis of the Rumen Microbiome

Filtered paired-end reads were used to build de novo assemblies using IDBA_UD v1.1.1 [27]. The MetaGeneMark version 2.8 gene prediction tool [28] was used to predict ORFs. Predicted ORFs were functionally annotated using NCBI non-redundant (nr) database and the KO database [21]. For taxonomic annotation, we computed the lowest common ancestor (LCA) of all species in the collected BLAST hits and to determine its taxonomic origin as described in a previous study [29]. Further detailed descriptions, functional enrichment analyses, and evolutionary analyses are provided in the Supplemental Experimental Procedures.

RNA-Seq Data Processing

We filtered out low-quality reads from raw data of nine individual yaks and three individual cattle using fastq_quality_filter tool in FASTX-Toolkit (version 0.0.13) (http://hannonlab.cshl.edu/fastx_toolkit/index.html) with parameters -Q 33 -q 20 -p 80. The quality-filtered reads (95%) were mapped to the mRNA of yaks using Bowtie2 software (version 2.1.0) [30], with a mean overall alignment rate of 58.7% in nine samples, and the RPKM (reads per kilobase of gene per million reads mapped) [31] values of the genes were calculated using eXpress (version 1.3.1) [32] for each sample. Gene expression level was defined determining RPKM as previously described [31]. A total of 15,368 genes were expressed in nine yak samples.

For identification of differentially expressed genes, RNA-Seq-based transcriptome data from the rumen epithelium of four cattle individuals in the

control group from a previous study [33] and three cattle individuals were selected as controls for this study. We found 10,184 expressed orthologous genes between yaks and cattle with best reciprocal blast hits. Raw gene expression data represented by RPKM values were transform into logarithm, $\log_2$(raw + 1), and were normalized with quantiles methods [34] supplied by the affy package in R. Genes were defined as significantly upregulated (or downregulated) if the difference in ratio to the control was at least 2-fold log ratio with Student's t test p value < 0.05, which were further visualized using the heatmap.2 software in R 3.2.0. Finally, we identified 2,176 upregulated genes in yaks, which were further annotated by Gene Ontology (GO) using DAVID bioinformatics resources (version 6.7) [35, 36] to find upregulated genes associated with VFA absorption.

ACCESSION NUMBERS

The accession numbers for all sequences reported in this paper are the Genome Sequence Archive (GSA; http://gsa.big.ac.cn/): PRJCA000114–PRJCA000116. The accession number for RNA-seq data reported is NCBI Short Read Archive (SRA): SRP066581.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, three tables, and one dataset and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.05.012.

AUTHOR CONTRIBUTIONS

P.S. and Z.Z. performed project planning. P.S., F.Z., and R.L. performed project coordination, execution, and facilitation. Z.Z., L.W., X.Z., W.W., J.Z., X.H. and Q.Q. conducted sample collection and preparation. Z.Z., L.W., W.W., and R.L. performed metabolic assays and analyses. Z.Z. and X.Z. performed nucleic acid purification and library construction. F.Z. and J.W. carried out assemblies, gene prediction, and annotation. Z.Z., D.X., J.H., and P.S. processed the 16S rRNA gene sequence data and conducted the metagenomic data analyses. P.S., F.Z., R.L., and Z.Z. prepared the manuscript.

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