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ORIGINAL ARTICLE

Abundance and diversity of mucosa-associated hydrogenotrophic microbes in the healthy human colon

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Hydrogenotrophic microbiota have a significant impact on colonic health; however, little is known about their diversity and ecology *in situ*. Here, molecular-based methods and multivariate analyses were used to examine the abundance and diversity of mucosa-associated hydrogenotrophic microbes in 90 biopsies collected from right colon, left colon and rectum of 25 healthy subjects. Functional genes of all three hydrogenotrophic groups were detected in at least one colonic region of all subjects. Methanogenic archaea (MA) constituted approximately one half of the hydrogenotrophic microbiota in each colonic region. Sulfate-reducing bacteria (SRB) were more abundant than acetogens in right colon, while acetogens were more abundant than SRB in left colon and rectum. MA genotypes exhibited low diversity, whereas SRB genotypes were diverse and generally similar across the three regions within subject but significantly variable among subjects. Multivariate cluster analysis defined subject-specific patterns for the diversity of SRB genotypes; however, neither subject- nor region-specific clusters were observed for the abundance of hydrogenotrophic functional genes. Sequence analyses of functional gene clones revealed that mucosa-associated SRB were phylogenetically related to *Desulfovibrio piger*, *Desulfovibrio desulfuricans* and *Bilophila wadsworthia*; whereas MA were related to *Methanobrevibacter* spp., *Mb. smithii* and the order Methanomicrobiales. Together these data demonstrate for the first time that the human colonic mucosa is persistently colonized by all three groups of hydrogenotrophic microbes, which exhibit segmental and interindividual variation in abundance and diversity.

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Introduction

The human intestinal ecosystem is colonized by a vast number of functionally diverse mutualistic microbes, many of which are abundant and dominant in taxonomic surveys obtained by high-throughput sequencing technologies (Turnbaugh *et al.*, 2009). Less abundant taxa are often undetected by such studies; however, abundance does not necessarily reflect metabolic significance. For example, disposal of H_2 gas is fundamental for maintaining efficient microbial fermentation processes, but the microbial groups responsible for this function are present in low abundance (Nakamura *et al.*, 2010). These hydrogenotrophic microbes include acetogens, methanogenic archaea (MA) and sulfate-reducing bacteria (SRB) (Smith and Bryant, 1979; Christl *et al.*, 1992; Strocchi *et al.*, 1994; Bernalier *et al.*, 1996). There is considerable evidence implicating SRB and their end product hydrogen sulfide (H_2S) in the pathogenesis of chronic inflammatory disorders of the colon (Roediger *et al.*, 1997; Levine *et al.*, 1998). In addition, H_2S is a potent genotoxin (Attene-Ramos *et al.*, 2006), and thus may be linked to sporadic colorectal cancer. Although methane has generally been considered an inert colonic gas, it may

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influence energy homeostasis of the host through the impact of methanogenesis on the metabolic efficiency of fermentation (Samuel *et al.*, 2007). Furthermore, there is increasing evidence for a link between colonic methane and motility disorders of the intestine, including irritable bowel syndrome (Sahakian *et al.*, 2010).

The colonic microbiota has most often been characterized from stool samples, which do not accurately reflect mucosa-associated microbiota (Zoetendal *et al.*, 2002; Eckburg *et al.*, 2005). Consequently, relatively little is known about the diversity and ecology of mucosa-associated hydrogenotrophic microbes for any mammalian species. Two molecular-based studies demonstrate that proximal colonic (Zinkevich and Beech, 2000) and rectal (Fite *et al.*, 2004) mucosae are persistently colonized by SRB. Relatively limited MA diversity has been detected, with only *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* cultured from stool (Miller *et al.*, 1982; Miller and Wolin, 1985) and also predominant in molecular surveys (Eckburg *et al.*, 2005; Zhang *et al.*, 2009). More recently, Oxley *et al.* (2010) reported the presence of *Mb. smithii*, *Mb. arboriphilus* and *Msp. stadtmanae* 16S rRNA gene sequences in sigmoid colon mucosa.

The present study characterized the abundance and diversity of mucosa-associated hydrogenotrophic microbes in biopsies from three colonic regions taken from healthy subjects. Ribosomal 16S rRNA or functional genes of acetogens, MA and SRB were analyzed by quantitative PCR (qPCR) and molecular fingerprinting. Phylogenetic affiliations of mucosal SRB and MA were further investigated by clone library analyses. Replicate biopsies were also studied to evaluate the extent of microanatomical variation in hydrogenotrophic microbiota.

Materials and methods

Sample collection and preparation

Colonic biopsies were obtained from 20 healthy subjects (12 women and 8 men) with an age range of 47–64 years undergoing routine colonoscopy at Carle Foundation Hospital (Urbana, IL, USA). None of the recruited subjects had taken antibiotics for at least 8 weeks before sample collection. The biopsies were collected during colonoscopy following standard bowel cleansing methods, snap frozen in liquid nitrogen and stored at −80 °C until DNA extraction was performed. Sixty biopsies were collected from right and left colon and rectum. A duplicate biopsy from each location was collected for confirmation of normal tissue by the Carle Pathology Services Laboratory (Urbana, IL, USA). Also, replicate colonic biopsies <1 cm apart were obtained from right colon, left colon and rectum of five additional healthy subjects. Procedures related to collection and use of tissue from human subjects, including informed consent of participants, were reviewed and approved by the Institutional Review Boards of the University of Illinois at Urbana-Champaign and Carle Foundation Hospital. Demographic information including age, gender, race and endoscopic findings is summarized in Supplementary Table S1.

DNA extraction and PCR amplification

Genomic DNA was extracted from biopsies using a commercial kit (QIAamp DNA Stool Mini Kit; Qiagen, Valencia, CA, USA) following Zoetendal *et al.* (2006). Mechanical cell disruption was not used to avoid excess extraction of eukaryotic DNA, which can interfere with PCR amplification of 16S rRNA genes (Huys *et al.*, 2008; Bakke *et al.*, 2011). The absence of mechanical cell disruption did not affect either the quantity or the quality of microbial DNA isolated as verified by direct comparison of the two approaches with a subset of biopsies (Carbonero *et al.*, in preparation). PCR amplification was performed with primers targeting genes specified in Supplementary Table S2 in 25 μl reactions containing 2.5 μl of 10× buffer, 2.5 μl of BSA (0.1 mg ml^{-1}), 2 μl of 2.5 mM dNTP, 1 μl of each primer (25 μM), 2 μl (0.75 units) of hot start Taq polymerase (Takara, Shiga, Japan), 1 μl of DNA template, and brought to volume with PCR-grade water. Amplifications were performed by initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 94 °C for 1 min, primer annealing at the specific temperature (Table 2) for 1 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min.

All PCR steps included negative and positive controls (DNA extracted from pure cultures). Amplicons were checked on agarose gel electrophoresis using ethidium bromide fluorescent dye. Triplicate PCR amplifications were performed for each sample, pooled PCR products were gel purified using the QIAquick gel extraction kit (Qiagen).

qPCR analysis of hydrogenotrophic functional genes and SRB genera

Real-time qPCR was performed on all colonic DNA extracts (20 subjects + replicated biopsies from the five additional subjects; A–E, Supplementary Table S1) using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Both primers ACSF1/ACSR1 (Gagen *et al.*, 2010) and FTHFSf/FTHFSr (Leaphart and Lovell, 2001) targeting functional genes *acs* and *fhs* of acetogens were used (Supplementary Table S2). Primers ME1/ME2 (Hales *et al.*, 1996) and DSR1fdeg/DSR4rdeg (Leloup *et al.*, 2007) were used for *mcrA* and *dsrA* genes, respectively (Supplementary Table S2). Primer pairs targeting 16S rRNA genes of *Desulfobacter*, *Desulfobulbus*, *Desulfotomaculum*/*Desulfosporosinus* and *Desulfovibrio* were used to quantify SRB genera (Daly *et al.*, 2000; Supplementary Table S2). Samples were run on a 7900HT Fast Real-Time

PCR System (Applied Biosystems) using a dissociation curve. Standard curves were determined simultaneously using plasmids containing *dsrA*, *mcrA* and *fhs* or diluted PCR products from reference strains for acs and the 16S rRNA genes.

Nested PCR amplification of Desulfovibrio and Archaea 16S rRNA genes

Biopsy DNA was used as a template for PCR amplification using universal primers GM3f/GM4r targeting the 16S rRNA gene of the domain Bacteria (Muyzer *et al.*, 1995), which was then subjected to a second round of amplification (610 bp) using 16S rRNA gene primers DSV230f/DSV838r specific for *Desulfovibrio* spp. (Daly *et al.*, 2000).

For nested PCR of Archaea, the first amplification was carried out using universal primers that target the 16S rRNA gene of the Archaea domain (Hallam *et al.*, 2003), and products were then subjected to a second round of amplification using an internal set of specific Archaea 16S rRNA gene primers (Grosskopf *et al.*, 1998).

PCR amplification of dissimilatory sulfite reductase and methyl-coenzyme M reductase genes

For nested PCR of *dsrA*, DNA extracted from biopsy tissues samples was used as a template for direct PCR amplification using primers DSR1F (Wagner *et al.*, 1998) and DSRAB-R-b (Schmalenberger *et al.*, 2007). Subsequently, these PCR amplicons were subjected to a second round of amplification using internal-specific primers 1FI (Dhillon *et al.*, 2003) and DSR10R (Barneah *et al.*, 2007).

For nested PCR of *mcrA*, direct PCR amplification was carried out using primers ME1 (Hales *et al.*, 1996) and MCRr (Springer *et al.*, 1995). Direct PCR products were then subjected to a second round of amplification using an internal set of specific *mcrA* gene primers ME2 (Hales *et al.*, 1996) and MCRrb (Springer *et al.*, 1995).

Terminal restriction fragment length polymorphism analysis

Genomic diversity of 16S rRNA (*Desulfovibrio,* Archaea) and functional genes (*dsrA* and *mcrA*) was examined in the initial set of 20 subjects (1–20, Table 1) by terminal restriction fragment length polymorphism (T-RFLP; Liu *et al.*, 1997). Forward primers for nested amplification of each target gene were 5′-end labeled with 6-carboxyfluorescein (6-FAM; Integrated DNA Technologies, Coralville, IA, USA). For each microbial gene target, T-RFLP analyses were performed using two independent restriction enzymes. T-RFLP analysis of *Desulfovibrio* spp. 16S rRNA genes was carried out using 10 μl aliquots (3.75 ng of DNA per μl) of the amplicons produced by nested PCR. These DNA products were cleaved for 6 h in a water bath at 37 °C with 2.5 units of restriction endonuclease following the manufacturer recommendations (NE Biolabs, Ipswich, MA, USA). *Hpy*CH4IV and *Scr*FI endonucleases were used for digestion of *Desulfovibrio spp.* 16S rRNA gene amplicons. T-RFLP analyses of Archaea 16S rRNA, *dsrA* and *mcrA* were performed as described above with the following exceptions: For the analysis of Archaea 16S rRNA genes, 10 μl aliquots (7.5 ng of DNA per μl) of nested PCR amplicons were digested with *Bfa*I and *Hpy*188I endonucleases (NE Biolabs). For the analysis of *dsrA*, 10 μl aliquots (6.0 ng of DNA per μl) of nested PCR amplicons were digested with *Sau*96I and *Bst*UI endonucleases (NE Biolabs). For *mcrA*, 10 μl aliquots (6.0 ng of DNA per μl) of nested PCR amplicons were digested with *Dde*I and *Hpy*188III endonucleases (NE Biolabs).

DNA fragment analysis was performed on the ABI Prism 3730xl Analyzer using GeneScan Liz600 marker (Applied Biosystems) as a size standard.

Table 1 Mean abundance of hydrogenotrophic functional genes[a]

	THydro	acs	fhs	mcrA	dsrAB
RC	4.36E+05	9.79E+04 (22.5)	6.62E+04 (15.2)	2.19E+05 (50.2)	1.35E+05 (30.9)
LC	2.46E+05	9.51E+04 (38.7)	6.41E+04 (26.0)	1.17E+05 (47.4)	4.99E+04 (20.3)
Re	2.82E+05	1.06E+05 (37.6)	5.85E+04 (20.8)	1.75E+05 (62.0)	2.50E+04 (8.9)

Abbreviations: LC, left colon; RC, right colon; Re, rectum; THydro, sum of four genes (% of THydro).
[a]Without outliers two times greater than s.d.

Table 2 Mean abundance of SRB genera

	dsrAB	Desulfobacter	Desulfobulbus	Desulfotomaculum	Desulfovibrio
RC	5.50E+07 (2.03E+06)	2.82E+04	5.55E+04	3.13E+03	1.94E+06
LC	5.77E+06 (6.08E+05)	3.71E+04	5.12E+04	3.42E+03	5.16E+05
Re	3.35E+07 (6.68E+05)	2.66E+04	2.86E+05	2.21E+03	3.53E+05

Abbreviations: LC, left colon; RC, right colon; Re, rectum (sum of SRB genera 16S rDNA signal); SRB, sulfate-reducing bacteria.

Terminal restriction fragment (TRF) profiles in each sample were produced using GeneMapper software (version 3.7; Applied Biosystems). For TRF profiles of 16S rRNA genes, terminal fragments smaller than 50 bp and lower than 100 arbitrary fluorescence units were considered background noise and manually removed from the TRF profile in each sample. For TRF profiles of *dsrA* and *mcrA*, terminal fragments smaller than 10 bp were manually removed from the TRF profile in each sample. Background noise found in the T-RFLP analysis of 16S rRNA gene amplicons produced from DNA extracted from pure cultures (positive controls) was also removed from the TRF profiles in each sample.

Statistical analyses of microbial profiles

TRF profiles were aligned on the basis of TRF lengths and individual peak areas using the moving average algorithm included in the T-Align software (Smith *et al.*, 2005), resulting in the generation of data sets of aligned TRFs. Profiles obtained with two independent restriction analyses (endonucleases) were concatenated to form a collective data set, and the resulting output files were used in multivariate statistical analyses using an inferential statistic analysis (non-parametric multivariate ANOVA) for the quantitative assessment of microbial profiles and the use of a non-metric technique, non-metric multidimensional scaling (NMDS) analysis. The results of the non-metric multidimensional scaling ordinations were corroborated by a linear model, principal coordinate analysis, and by a non-parametric clustering technique, multivariate cluster analysis (MCA). For analyses of presence–absence of TRFs, the Kulczynski index was used, whereas analyses of relative abundance-based data were carried out using the Morisita index (Anderson *et al.*, 2011). Interindividual variability within the three colonic regions was estimated using pairwise similarities between right colon, left colon and rectum using those indices. All multivariate statistical analyses were performed with the PAST software package (Hammer *et al.*, 2001). In addition, ANOVA and Fisher's Protected Least Significant Difference test were used to compare intergroup variability among right colon, left colon and rectum. These analyses were carried out using SAS software (Statview, Version 5.0.1; SAS Institute, Cary, NC, USA).

Phylogenetic analysis of dissimilatory sulfite reductase and methyl-coenzyme M reductase genes

Biopsy samples with the highest diversity of *dsrA* or *mcrA* genes (as determined by T-RFLP) were selected, and genomic DNA from these samples was used as a template for nested PCR (without primers 5′-end labeled with 6-FAM). Purified PCR products from the colonic biopsies were pooled and cloned using the TOPO-TA kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer recommendations. Transformants were propagated in LB medium overnight and used for plasmid DNA extraction using the QIAprep Spin MiniPrep Kit (Qiagen). Sequencing using the M13F (−21) and M13R (−48) primers was performed with an ABI 3730XL capillary sequencer (Applied Biosystems). Chromatogram checks, trimming and assembling of sequences were performed with Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA).

Consensus nucleotide sequences were used for BLAST analysis (Altschul *et al.*, 1997) against the non-redundant nucleotide database from GenBank. The 20 best hits (E-value $<$3E-120) for each clone sequence were retrieved and used for phylogenetic analyses. Nucleotide sequences were aligned using ClustalW. Poorly aligned regions (5′- and 3′-flanking segments) were manually removed, and high quality alignments were used for phylogenetic tree reconstructions using the maximum parsimony method. The statistical significance of branch order was estimated by the generation of 500 replications of bootstrap re-sampling of the originally aligned nucleotide sequences. These phylogenetic analyses were conducted with MEGA4 software (Tamura *et al.*, 2007).

Results

Quantification of acs, fhs, mcrA *and* dsrAB *in right colon, left colon and rectal mucosa*

Estimates of the abundance of *acs*, *fhs*, *mcrA* and *dsrAB* gene copies per gram of colonic tissue are shown in Figure 1. Functional genes of all three hydrogenotrophic groups were detected in all colonic regions from all subjects with the exception of *mcrA*. The acetogenic genes *acs* and *fhs* were detected in all biopsies with gene copy numbers ranging from 1.8×10^3 to 8.8×10^6 and from 9.8×10^3 to 3.8×10^7 per gram tissue, respectively. All subjects also harbored significant *mcrA* gene copy numbers in at least one colonic region ranging from 3.0×10^2 to 4.5×10^9. *DsrAB* gene copy numbers ranging from 1.8×10^2 to 1.4×10^9 per gram were detected for all biopsies from all subjects. The *dsrAB* and *mcrA* genes exhibited the greatest variation in abundance among subjects, whereas the abundance of *acs* and *fhs* gene copies was generally in the same order of magnitude (10^4–10^5) across colonic regions for all subjects. Overall, the functional gene data indicate that MA constituted approximately one half of the hydrogenotrophic microbiota in each colonic region (Table 1). SRB were more abundant than acetogens in right colon, while acetogens were more abundant than SRB in left colon and rectum. Multivariate statistical analysis of the combined abundance of *acs*, *fhs, mcrA* and *dsrAB* copy numbers via the Morisita index did not reveal coherent clustering among the 60 biopsies by colonic region or subject (Supplementary Figure S1).

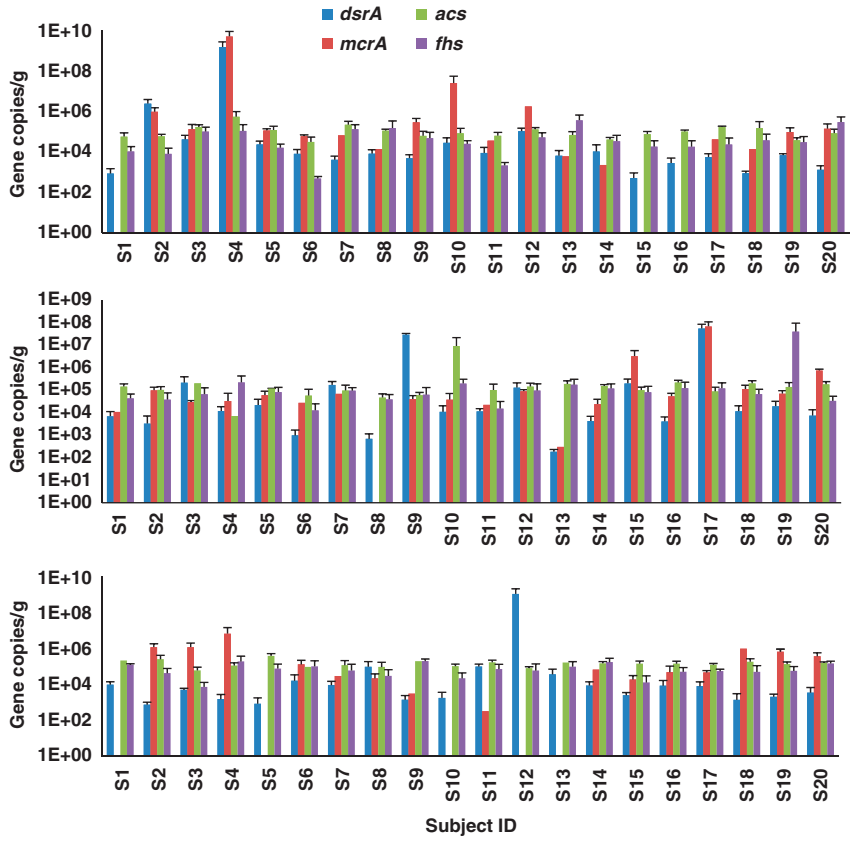

Figure 1 Quantification of *acs*, *fhs*, *mcrA* and *dsrAB* in right colon, left colon and rectal mucosa. Estimates of the abundance of *acs*, *fhs*, *mcrA* and *dsrAB* gene copies per gram of colonic tissue were determined by real-time PCR with a PCR product or plasmid dilution standard curve of the corresponding functional gene. Error bars indicate the s.e.m. of three technical replicates. MCA of the abundance of the four functional genes is presented in Supplementary Figure S1.

Quantification of SRB genera in right colon, left colon and rectal mucosa

Estimates of the abundance of *Desulfobacter*, *Desulfobulbus*, *Desulfotomaculum*/*Desulfosporosinus* and *Desulfovibrio* 16S rRNA gene copies per gram of colonic tissue are shown in Figure 2. *Desulfobacter* and *Desulfobulbus* were detected in all 60, *Desulfovibrio* in 56/60 and *Desulfotomaculum* in 42/60 biopsies. *Desulfotomaculum* was the only SRB genus not detected in all subjects, being absent in three (S1, S4 and S12). When present, the abundance of *Desulfovibrio* 16S gene copy numbers was always greatest (10^4–10^7) followed by *Desulfobacter* and *Desulfobulbus* (10^3–10^6) and finally *Desulfotomaculum* (10^2–10^4). The rectum harbored the least diversity in SRB genera (Figure 2). The total signal for the four genera was roughly 10 times lower than the total *dsrAB* signal (Table 2). On average, *Desulfovibrio* was the most abundant in the right colon and *Desulfobulbus* in the rectum (Table 2). Regional differences were not observed for the abundance of *Desulfobacter* and *Desulfotomaculum*.

Genotypic diversity of mucosa-associated Desulfovibrio spp.

The nested PCR approach revealed that all 20 of the initial set of subjects harbored mucosa-associated *Desulfovibrio* spp. populations in at least one colonic region. The percentages of biopsy tissues positive for *Desulfovibrio* spp. in right colon, left colon and rectum were 95, 90 and 100, respectively. Thirteen *Desulfovibrio* spp. TRFs were obtained with *Hpy*CH4IV and thirty-four with *Scr*fI. The mean abundance of individual *Desulfovibrio* spp. TRFs with *Scr*fI across the three colonic regions is shown in Figure 3a. Three major TRFs constituted >75% of the total profiles and the three colonic regions generally possessed similar TRF composition. Broadly the *Desulfovibrio* spp. TRFs formed four major groups represented in a minimum of three subjects for each (Figure 3a).

Statistical comparisons by means of ANOVA and Fisher's Protected Least Significant Difference test indicated that right colon harbored significantly more homogenous *Desulfovibrio* spp. TRFs than left colon ($P=0.0059$) based on the Kulczynski index; however, the three colonic regions were shown to harbor comparable variability in *Desulfovibrio* spp. TRFs using the Morisita index ($P>0.05$). Furthermore, pairwise similarities between these colonic sections using both indices revealed that right colon, left colon and rectum possess comparable variability in *Desulfovibrio* spp. TRFs ($P>0.05$). Similar results were obtained using multivariate ANOVA with both

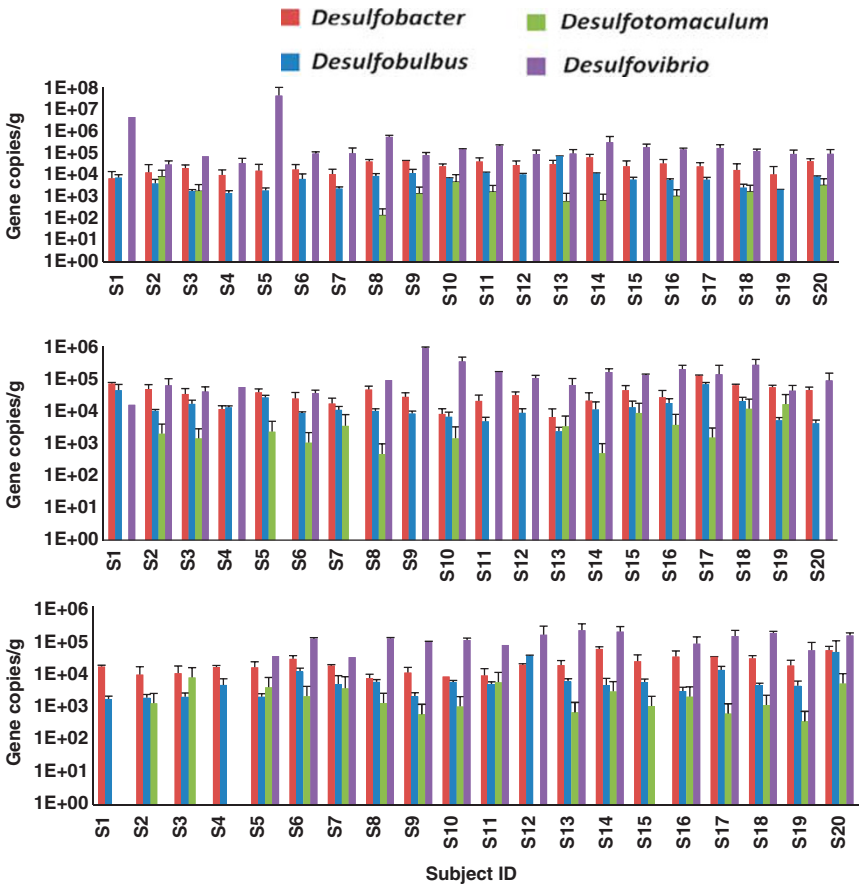

Figure 2 Quantification of SRB genera in right colon, left colon and rectal mucosa. Estimates of the abundance of *Desulfobacter*, *Desulfobulbus*, *Desulfotomaculum*/*Desulfosporosinus* and *Desulfovibrio* 16S rRNA gene copies per gram of colonic tissue were determined by real-time PCR with a PCR product dilution series standard curve of genomic DNA from corresponding type strains. Error bars indicate the s.e.m. of three technical replicates.

indices. MCA confirmed the absence of regional-specific *Desulfovibrio* spp. TRF patterns. However, subject-specific clusters were observed for *Desulfovibrio* spp. TRF patterns (Supplementary Figures S2A and B). These ordination patterns were confirmed by non-metric multidimensional scaling and principal coordinate analysis.

The diversity of mucosa-associated Desulfovibrio 16S rRNA genes was also analyzed in replicate colonic biopsies. Specifically, mucosa-associated *Desulfovibrio* spp. communities clustered (>60% similarity) mainly by subject using presence–absence data (Kulczynski index); only subject E exhibited contrasting TRF profiles (Supplementary Figure S3A). Statistical analysis of *Desulfovibrio* spp. profiles using TRF abundance data (Morisita index) revealed that mucosa-associated *Desulfovibrio* spp. communities clustered (>70% similarity) by subject and colonic site (Supplementary Figure S3B). *Desulfovibrio* spp. profiles from subjects A and B formed a distinct cluster with two exceptions, and right colon and left colon of subject C formed a distinct cluster. Rectal *Desulfovibrio* spp. communities from four subjects clustered together rather than by subject. The replicate samples of only five of fifteen biopsy pairs clustered together, and these matches were not coherent for the two statistical indices (Supplementary Figure S3). This observation is consistent with microheterogeneity in *Desulfovibrio* spp. communities within colonic regions.

Diversity of dsrA *genes of mucosa-associated microbes*
The nested PCR approach revealed that 19 out of 20 subjects harbored mucosa-associated *dsrA* sequences in at least one region of the colon. The percentages of biopsy tissues positive for *dsrA* gene amplification in right colon, left colon and rectum were 90, 80 and 80, respectively. The *dsrA* TRF profiles were less diverse than the *Desulfovibrio* spp. 16S TRFs. Fourteen SRB TRFs were recovered with *Bst*UI and sixteen TRFs with *Sau*96I. Mean abundances of *dsrA* TRFs across the three colonic tissues from *Sau*96I are shown in Figure 3d. Broadly, the *dsrA* TRFs formed one major (14/20 subjects) and two minor groups, which were consistent across colonic regions (Figure 3d).

Statistical comparisons by means of ANOVA and Fisher's Protected Least Significant Difference test

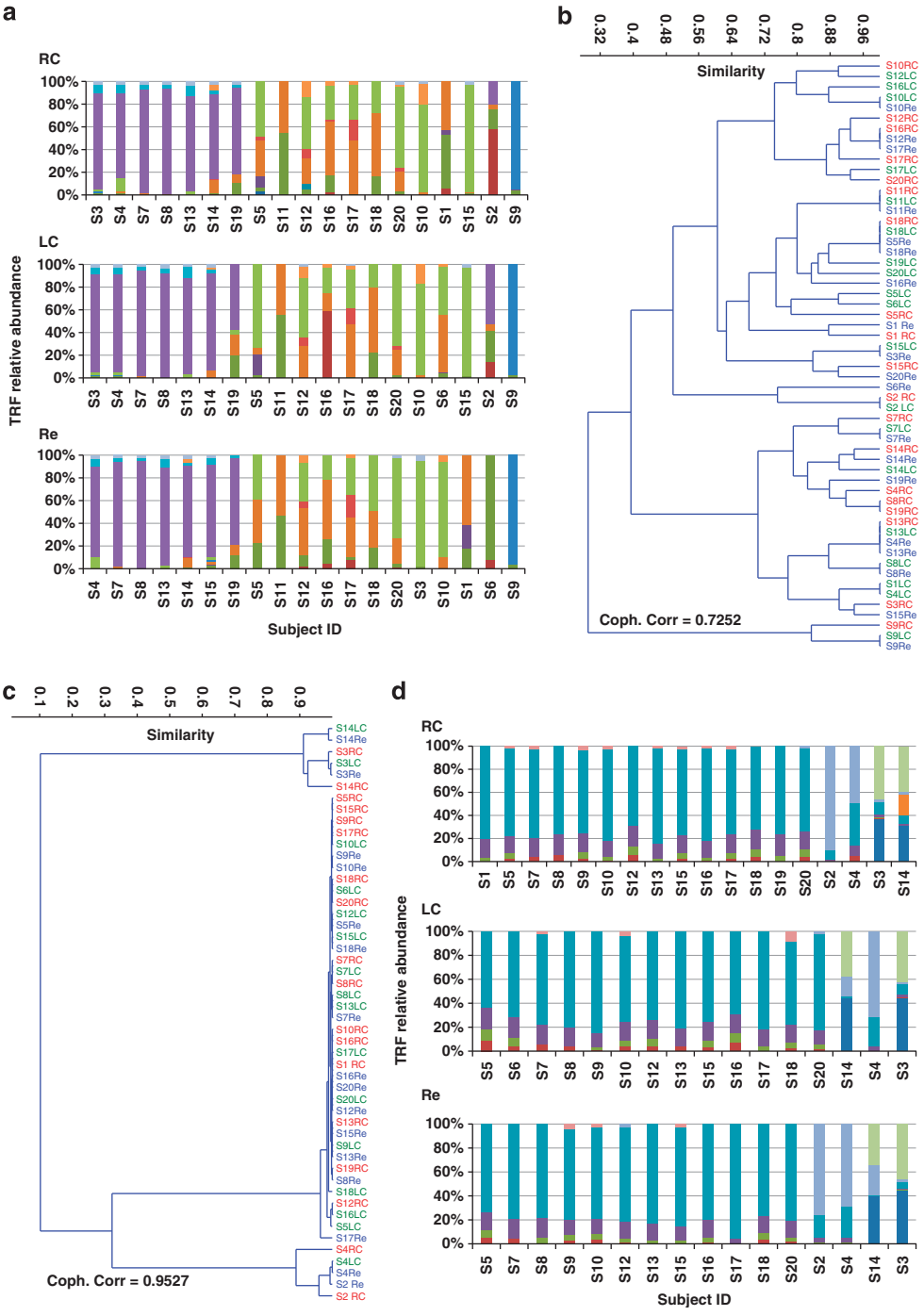

Figure 3 Individual T-RFLP profiles of *Desulfovibrio* 16S rRNA and *dsrA* gene sequences. A nested PCR T-RFLP approach was used to examine diversity in *Desulfovibrio* spp. 16S rRNA genes in mucosal biopsies from right colon (RC), left colon (LC) and rectum (Re). Relative abundance is demonstrated by terminal fragments recovered from the T-RFLP analysis using *Scr*FI endonuclease for *Desulfovibrio* 16S rRNA (**a**). Only TRFs that were present in at least two regions and representing >1% of the total profile signal are presented. Subjects are grouped according to similarities of TRF profiles. MCA of *Desulfovibrio* spp. 16S rRNA gene profiles across RC (red), LC (green) and Re (blue) was performed from the TRF profiles using Kulczynski (**b**) and Morisita (**c**) similarity indices. Cophenetic correlation coefficient (Coph Corr) values are shown. A similar nested PCR T-RFLP approach was used to examine diversity in *dsrA* using *Sau*96I endonuclease (**d**). MCAs of *dsrA* T-RFLP profiles are presented in Supplementary Figure S2.

using the Kulczynski index revealed that right colon is colonized significantly by more homogenous SRB populations than rectum ($P=0.0452$); however, the three colonic regions harbored comparable variability using the Morisita index ($P>0.05$). Furthermore, pairwise similarities between these colonic sites using either index revealed that right colon, left colon and rectum possess comparable variability in

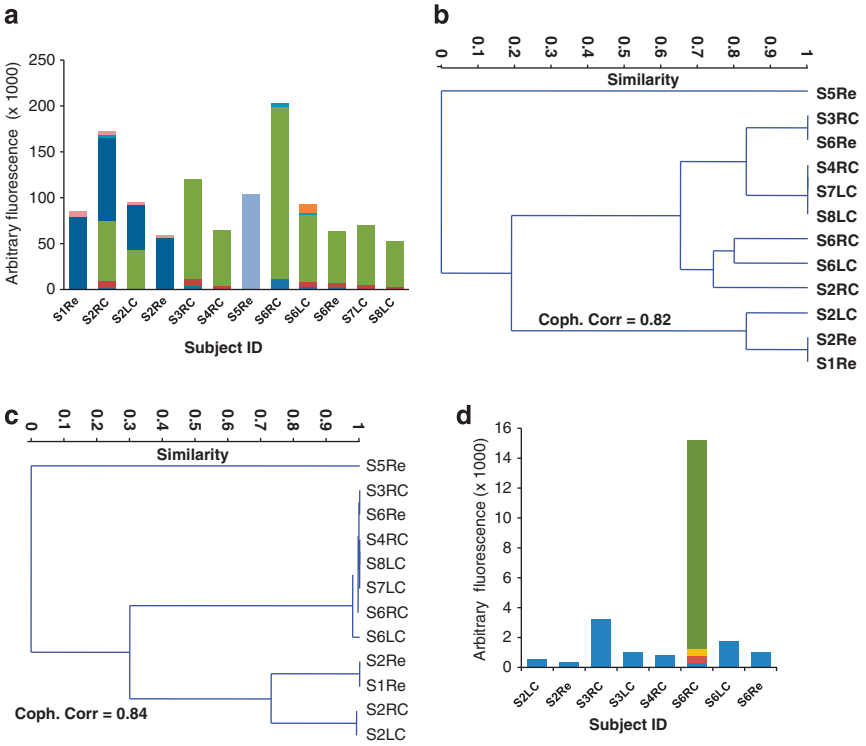

Figure 4 Individual T-RFLP profiles of Archaea 16S rRNA and *mcrA* gene sequences. A nested PCR T-RFLP approach was used to examine diversity in Archaea 16S rRNA and *mcrA* genes in mucosal biopsies from right colon (RC), left colon (LC) and rectum (Re). Relative abundance is demonstrated by terminal fragments recovered from the T-RFLP analysis using *Hpy*188III endonuclease for Archaea 16S rRNA (**a**) and *mcrA* (**d**). Each color represents a terminal fragment and its proportion within each chart represents relative abundance. Regional similarities were estimated by means of MCA across RC (red), LC (green) and Re (blue) using Kulczynski (**b**) and Morisita (**c**) similarity indices.

SRB populations ($P>0.05$). Similar results were obtained using multivariate ANOVA with both indices. MCA confirmed the absence of regional-specific *dsrA* spp. TRF patterns. However, subject-specific clusters were observed for *dsrA* spp. TRF patterns (Supplementary Figures S2A and B).

Diversity of mucosa-associated Archaea populations

Forty percent of the twenty subjects harbored mucosa-associated Archaea populations in at least one region of the colon. Twenty percent of the subjects were positive for Archaea 16S rRNA gene amplification for all of the three regions. Eight Archaea TRFs were recovered with *Bfa*I and ten with *Hpy*188I. The mean abundances of Archaea TRFs across colonic sites for *Hpy*188I are shown in Figure 4a. There were 10 (*Hpy*188I) or 8 (*Bfa*I) major TRFs and the TRF composition overlapped except for subject 7 left colon. Distinct TRF profiles were observed for the two individuals (2 and 6) that were positive for Archaea for the three colonic sites.

Ordinations derived from the Kulczynski (Figure 4b) and Morisita (Figure 4c) indices revealed two major clusters with the rectal TRF profile of subject 5 being an outlier. The different colonic regions of subjects 2 and 6 formed coherent and distinct clusters corresponding to these two major clusters.

Diversity of methyl-coenzyme M reductase gene of mucosa-associated microbes

In contrast to Archaea 16S rRNA, only 20% of the subjects were positive for *mcrA* amplification in at least one region of the colon. Two *mcrA* TRFs were recovered with *Dde*I and four with *Hpy*188III, and only one biopsy (RC) from subject 6 exhibited that diversity. The mean abundances of *mcrA* TRFs across colonic sites for *Hpy*188III are shown in Figure 4d.

Phylogenetic analysis of dissimilatory sulfite reductase and methyl-coenzyme M reductase genes

Twenty-one *dsrA* gene clones were obtained from the sixty initial colonic biopsies. On average, the recovered mucosa-associated *dsrA* clones were 98.5% homologous to *dsrA* genes archived in GenBank. Phylogenetic tree reconstruction (Figure 5a) using maximum parsimony and statistical significance of branch order (500 replications of bootstrapping) revealed that mucosa-associated SRB were genetically related to *Desulfovibrio piger* (three clones), *Desulfovibrio. desulfuricans* (three clones), uncultured SRB (six clones) and *Bilophila wadsworthia* (nine clones).

Nineteen *mcrA* gene clones were recovered from these colonic biopsies. On average, the mucosa-associated *mcrA* clones were 97.2% homologous to

mcrA genes archived in GenBank. Maximum parsimony phylogenetic tree reconstruction (Figure 5b) and statistical significance of branch order (500 replications of bootstrapping) revealed that the mucosa-associated MA were genetically related to uncultured *Methanobrevibacter* sp. (15 clones), *Methanoculleus chikugoensis* (3 clones) and *Mb. smithii* (1 clone).

The *dsrA* sequences are deposited in GenBank under accession numbers GU180102 through GU180122 and *mcrA* sequences under accession numbers GU180123 through GU180141.

Abundance of mucosa-associated hydrogenotrophic microbiota in replicate colonic biopsies

Estimates of the abundance of *acs*, *fhs, mcrA* and *dsrAB* gene copies per gram of colonic tissue for the thirty replicate biopsies from three colonic sites of five additional subjects are shown in Figure 6a. Consistent with observations from the set of 60 biopsies from 20 subjects, functional gene sequences for each of the hydrogenotrophic groups were present in at least one colonic region for the five additional subjects. Again, MA abundance was the most variable in the set of replicate biopsies ranging from not detected to 10^8 *mcrA* gene copy numbers per gram. Statistical analysis of the combined abundance of *acs*, *fhs, mcrA* and *dsrAB* copy numbers via the Morisita index did not reveal coherent clustering among the replicate biopsies by colonic region or subject (Figure 6b).

Discussion

This study provides the first molecular characterization of low-abundant hydrogenotrophic microbes

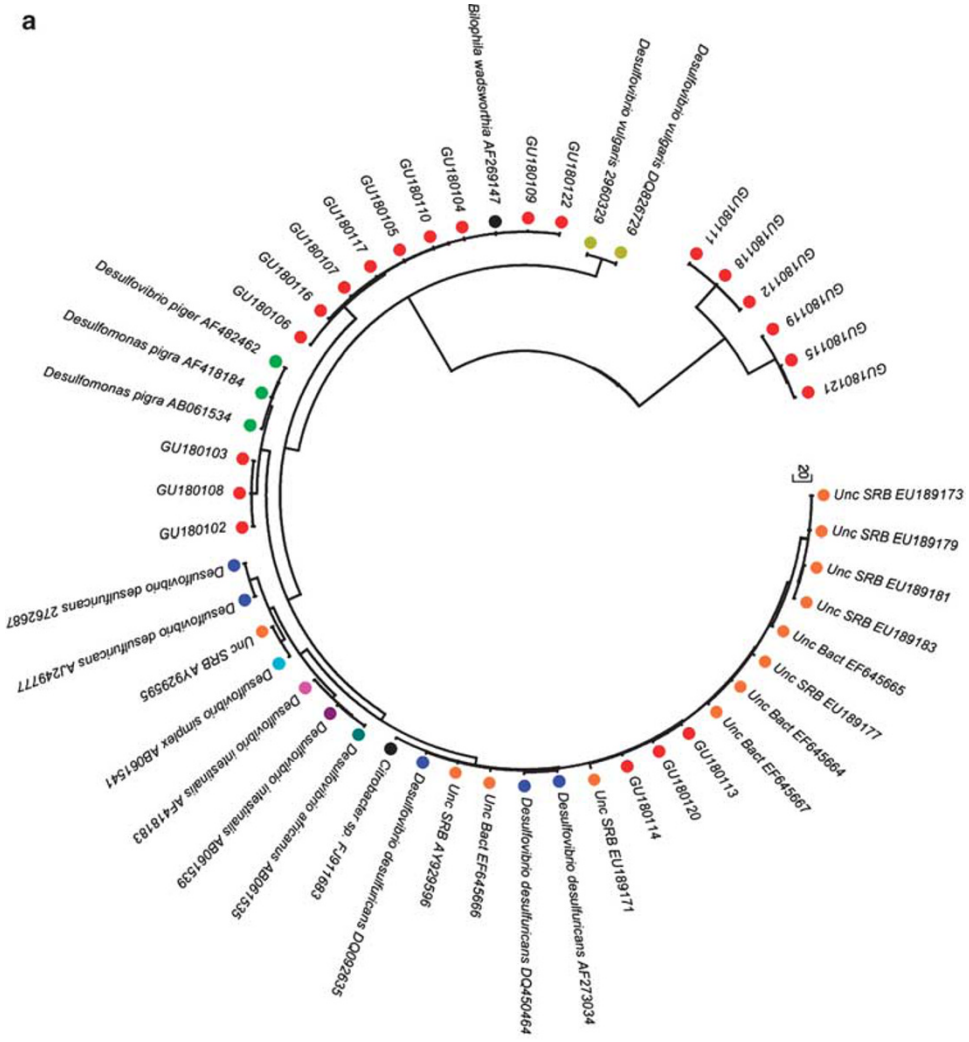

Figure 5 Phylogenetic affiliations of *dsrA* and *mcrA* clone sequences. Twenty-one clones of *dsrA* genes and nineteen clones of *mcrA* genes were recovered from colonic biopsies and sequenced. Phylogenetic tree reconstructions for *dsrA* (**a**) and *mcrA* (**b**) using the maximum parsimony and statistical significance of branch order (500 replications of bootstrapping) are shown. Red symbols represent the clones obtained in the present study. The scale bar represents the number of nucleotide substitutions per site.

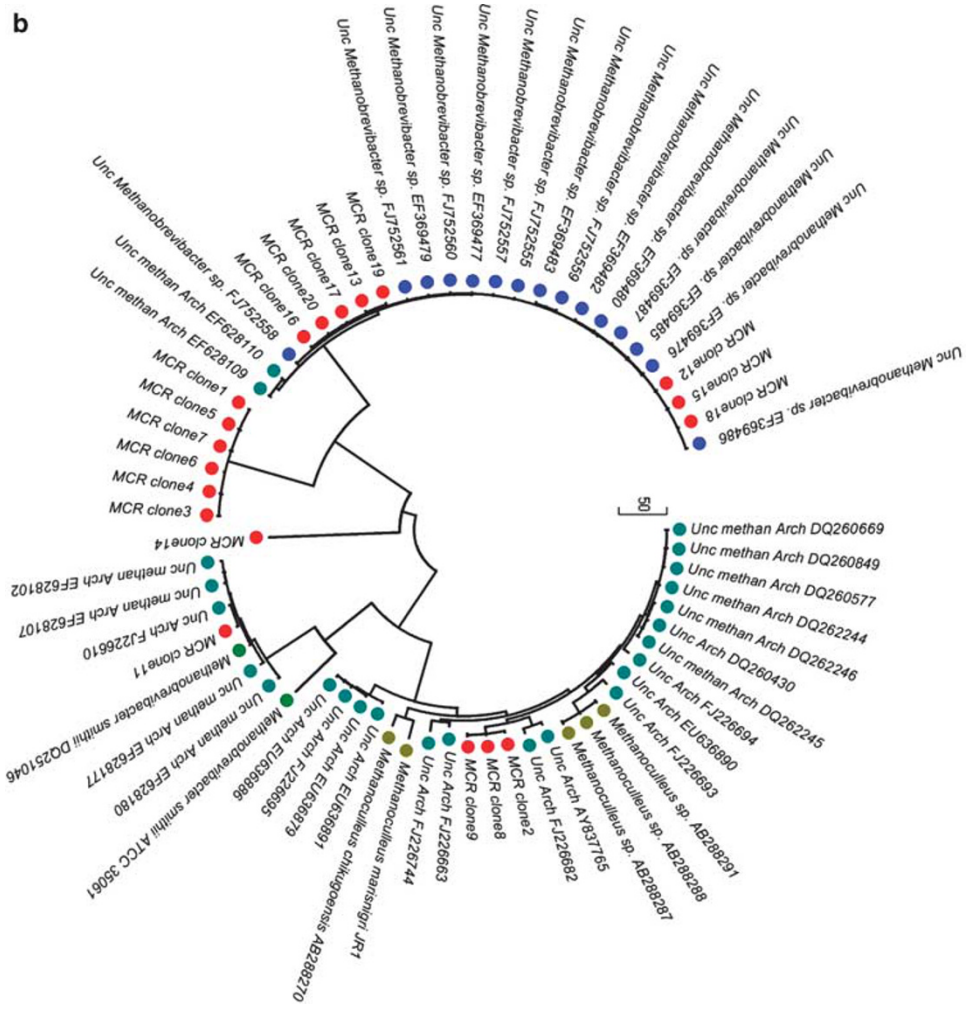

Figure 5 Continued.

that are associated with the colonic mucosa of healthy humans. These data confirm that SRB populations are ubiquitous and found associated with the mucosa throughout the colon. Moreover, these data are the first to demonstrate that mucosa-associated MA and acetogens are also prevalent, with all 25 subjects examined generating a signal for related functional genes in at least one colonic region. In addition, this study confirms that *Desulfovibrio* is generally the most abundant mucosa-associated SRB genus. Consistent with other microbial groups in the colonic ecosystem, both SRB and MA genotypes were highly variable among subjects and without clear region-specific patterns. Statistical analyses supported clustering of particular SRB genotypes by subject but not by colonic region.

Cultivation-based studies have estimated that the number of acetogens ranges from 10^2 to 10^8 CFU per gram human feces (Dore *et al.*, 1995; Bernalier *et al.*, 1996). Acetogenesis is thought to be a relatively less important hydrogenotrophic pathway in the colon because the oxidation of H_2 by methanogenesis or sulfate reduction is thermodynamically more favorable than reductive acetogenesis (Nakamura *et al.*, 2010). However, recent metagenomic data indicated that genes involved in reductive acetogenesis were more abundant than methanogenesis and sulfate respiration genes (Rey *et al.*, 2010). Our data indicate that mucosal acetogens are present in numbers comparable to MA and SRB. Thus, these data stress the need to further define the metabolic contributions of reductive acetogens to hydrogen disposal in the colonic mucosa.

Both the qPCR and nested PCR data indicate persistent colonization of SRB throughout the colon and *Desulfovibrio* spp. in particular. These data are consistent with previous reports using cultivation (Macfarlane *et al.*, 1992) as well as *Desulfovibrio* spp. 16S rRNA (Fite *et al.*, 2004) and functional gene (Zinkevich and Beech, 2000) primers. Unexpectedly, *dsrA* genes were less diverse than *Desulfovibrio* spp. 16S rRNA genes, which might indicate that the *dsrA* primers failed to amplify all of the genetic diversity. In this regard, subsequent efforts to obtain a complete census of mucosal SRB would benefit from the use of more degenerate *dsrA* primers.

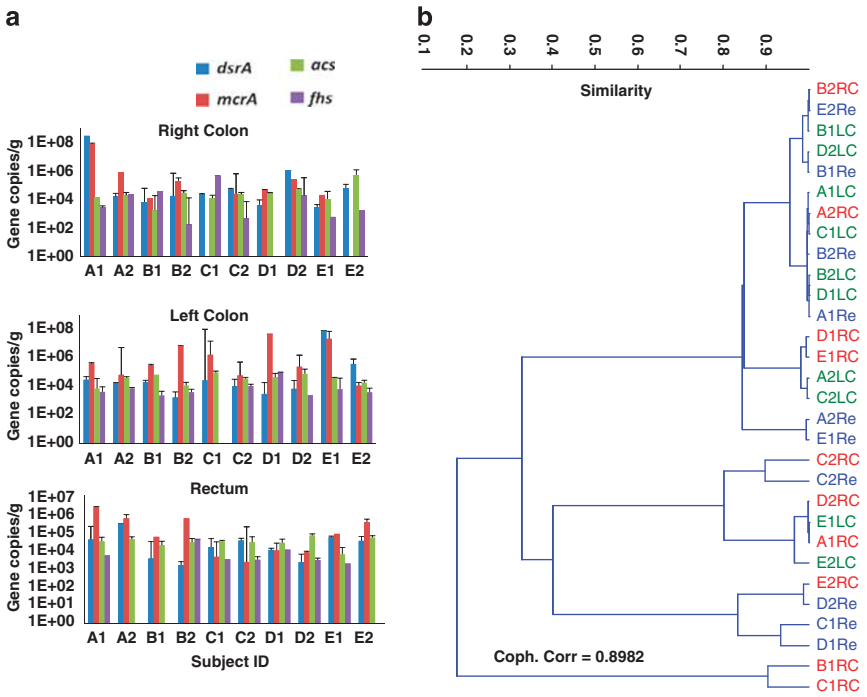

Figure 6 Quantification of *acs*, *fhs*, *mcrA* and *dsrAB* in replicate biopsies from five additional subjects. Estimates of the abundance of *acs*, *fhs*, *mcrA* and *dsrAB* gene copies per gram of colonic tissue were determined by real-time PCR with a corresponding PCR product or plasmid dilution standard curve (**a**). Error bars indicate the s.e.m. of three technical replicates. MCA of *acs*, *fhs*, *mcrA* and *dsrAB* across the RC (red), LC (green) and Re (blue) was performed using the Morisita similarity index (**b**). Cophenetic correlation coefficient (Coph Corr) values are shown.

Heretofore, colonic SRB had been characterized mainly by culture-based studies using stool or colonic contents as inocula (Nakamura *et al.*, 2010). For example, Gibson *et al.* (1993) cultivated SRB from stool of healthy subjects that were classified as *Desulfovibrio* spp., *Desulfobacter* spp., *Desulfobulbus* spp. and *Desulfotomaculum* spp. *Desulfovibrio* spp. are typically considered to be the predominant SRB genus in the human colonic ecosystem based on a limited number of culture-based studies (Loubinoux *et al.*, 2002). The present data fully corroborate previous reports on the predominance of *Desulfovibrio* spp. as well as the presence of other SRB genera including *Desulfobulbus* spp. and *Desulfobacter* spp., which were consistently detected throughout the colon; *Desulfotomaculum*/*Desulfosporosinus* exhibited a more irregular distribution.

Based on the phylogenetic analysis of *dsrA* clones, mucosa-associated SRB populations included *D. piger*, *D. desulfuricans,* uncultured *Desulfovibrio* spp. and *B. wadsworthia*. *B. wadsworthia* is a member of the mutualistic microbiota but has also been linked to gangrenous and perforated appendicitis in humans (Urban *et al.*, 2004). This genus does not reduce sulfate but can use sulfite as a terminal electron acceptor during taurine metabolism in anaerobic respiration (Laue *et al.*, 2001). It is important to acknowledge that the presence of SRB does not necessarily reflect the occurrence of sulfate reduction. Moreover, it is not possible to reach conclusions on the activity of mucosal SRB from the present molecular data. Nonetheless, evidence for persistent colonization of the colonic mucosa with SRB stresses the importance of gaining knowledge on their *in situ* metabolic activities.

To our knowledge, the present data provide the first direct evidence for widespread colonization of the colonic mucosa with MA. A *mcrA* signal was detected in at least one colonic region for all of the 25 subjects examined. In addition, MA were the predominant hydrogenotrophic group throughout the colon. Based on a breath methane concentration of 1 p.p.m. or greater, the percentage of methane producers varies significantly in humans of different ethnic groups, ranging from 34% to 87% (summarized in Levitt *et al.*, 2006). Although breath methane reliably reflects the abundance of colonic methanogens, non-methanogenic subjects, as thereby defined, often harbor a relatively lower number of stool MA (El Oufir *et al.*, 1996). One study, based on a fortuitous measurement of colonic gases, suggested that methane was predominantly produced in the left colon (Bond *et al.*, 1971). Culture-based data consistent with the abundance of MA increasing in a proximal to distal gradient have also been reported (Macfarlane *et al.*, 1992). To a certain extent, our observations concur with this gradient, as *mcrA* abundance increased along the colon to comprise two thirds of the hydrogenotrophic gene content in

the rectum. Archaeal diversity (16S rRNA gene TRFs) was greater than MA diversity (*mcrA* TRFs). This observation indicates significant colonization by non-MA, which is in accordance with previous detection of non-MA from colonic mucosa (Oxley *et al.*, 2010). Alternatively, it is possible that MA harboring the *mrtA* functional gene, such as *Msp. stadtmanae*, were not detected by the nested PCR approach.

Culture- and molecular-based studies to date indicate that *Mb. smithii* is the predominant methanogen in the human colon (Miller *et al.*, 1982; Weaver *et al.*, 1986; Eckburg *et al.*, 2005; Scanlan *et al.*, 2008; Zhang *et al.*, 2009). *Msp. stadtmanae*, also a member of the order Methanobacteriales, was isolated from human stool at a lower abundance (Miller and Wolin, 1985). Phylogenetic analysis of the *mcrA* clones recovered from colonic biopsies revealed that mucosa-associated MA were related to *Mb. smithii,* uncultured *Methanobrevibacter* spp. and one clade belonging to the Methanomicrobiales order and distantly related to the *Methanoculleus* genus. The presence of these sequences confirms that MA lineages other than the two most commonly described are also present in the human colon. Specifically, *mcrA* and 16S rRNA gene sequences related to Methanosarcinales (Scanlan *et al.*, 2008) and to a putative new order related to Thermoplasmatales (Mihajlovski *et al.*, 2008) have been detected in stool.

The abundance of SRB and MA was highly variable among subjects and without clear host or region-specific patterns. On the other hand, both *Desulfovibrio* spp. and *dsrA* TRF profiles generally clustered by subject. Replicate biopsies collected <1 cm apart did not cluster by host or colonic site for the abundance of hydrogenotrophic functional genes. Thus, the composition of hydrogenotrophic microbiota may be individualized, similar to mutualistic microbiota in general; while the abundance of the three groups appears to vary both regionally and in a microanatomical scale. It should be noted that the bowel cleansing preparation may have modified loosely adherent microbiota, which might have led to underestimation of the diversity or abundance of the microbial groups examined. It is not feasible to sample the right or transverse colon of healthy subjects without bowel cleansing; thus, it would be difficult to empirically address these potential biases. Regardless, standardized methodologies were used for the collection and analysis of all biopsy samples validating the relative differences observed.

In summary, the present data from three colonic regions of 25 human subjects provide clear evidence for coexistence of acetogens, SRB and MA, indicating that these three groups are not mutually exclusive in healthy mucosa. These baseline data provide a framework to determine the extent to which dysbiosis in hydrogenotrophic microbiota may be linked to colonic disorders. Moreover, the evidence for microheterogeneity highlights the need to define spatial scales of microbial hydrogenotrophy relevant to disease etiology in the human colon.

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