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Hydrogenotrophic microbiota distinguish native Africans from African and European Americans

Gerardo M. Nava^{1,6,†}, Franck Carbonero^{2,†}, Junhai Ou³, Ann C. Benefiel², Stephen J. O’Keefe³, and H. Rex Gaskins^{1,2,4,5,*}

¹Division of Nutritional Sciences, University of Illinois, Urbana, IL

²Department of Animal Sciences, University of Illinois, Urbana, IL

³Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA

⁴Department of Pathobiology, University of Illinois, Urbana, IL

⁵Institute for Genomic Biology, University of Illinois, Urbana, IL

Summary

Reduced susceptibility to sporadic colorectal cancer in native Africans (NA) is correlated with low consumption of animal products and greater microbial production of colonic methane. In this context, two hydrogenotrophic microbial groups are of interest, methanogenic Archaea (MA) utilizing H₂ to produce methane and sulfate-reducing bacteria (SRB) generating hydrogen sulfide, which has been linked with chronic inflammatory disorders of the colon. In the present study, stool samples from NA, consuming a diet high in resistant starch and low in animal products, and from African Americans (AA) and European Americans (EA), both consuming a typical Western diet, were examined for genetic diversity and structure of Archaea, MA and SRB communities. In general, a greater proportion of NA than AA and EA harbored the full range of targeted hydrogenotrophic groups. Terminal restriction fragment length polymorphism analysis of 16S rRNA genes and specific functional genes, combined with multivariate statistical analyses, revealed that NA harbored more diverse and different Archaea and MA populations than AA and EA. Also, NA harbored significantly distinct SRB populations compared to AA and EA. Taken together, these data are consistent with diet selecting for distinct hydrogenotrophic microbiota.

Keywords

Hydrogenotrophs; methanogenic Archaea; sulfate-reducing bacteria; TRFLP

Introduction

In the human colon, the main products of microbial fermentation include short chain fatty acids (SCFA; acetate, propionate and butyrate) and gases (CO₂ and H₂). The H₂ produced

*Corresponding Author: Laboratory of Mucosal Biology, 1207 W. Gregory Drive, University of Illinois at Urbana-Champaign, Urbana, IL 61801, Phone: 217-244-3165, Fax: 217-333-8286, hgaskins@illinois.edu.

⁶Present Address: Division of Immunobiology, Washington University School of Medicine, St. Louis, MO

[†]The first two authors contributed equally to this work.

during this metabolic process is either excreted or used *in situ* by hydrogenotrophic microbes, including methanogenic Archaea (MA), sulfate-reducing bacteria (SRB), and reductive acetogens (Smith and Bryant, 1979; Strocchi et al., 1994; Bernalier et al., 1996). Hydrogenotrophic organisms are typically present at much lower densities than are fermentative bacteria (Eckburg et al., 2005). However, in the absence of H₂-consuming organisms, the H₂ partial pressure rapidly reaches a level that thermodynamically inhibits further fermentation (Schink, 1997; Adams et al., 2006). In humans, methane and hydrogen sulfide production have been recognized as the main pathways for H₂ disposal in the colon, and a variety of intestinal factors such as colonic pH, sulfate availability, digesta transit time, and microbial composition may influence which of these pathways predominates (Gibson et al., 1990; Strocchi et al., 1991; Christl et al., 1992; El Oufir et al., 1996; Nakamura et al., 2010).

For example, it has long been recognized that NA excrete more methane in their breath than do individuals in westernized populations (O'Keefe et al., 1999). For example, Segal et al (1988) reported that 84% of rural black Africans excreted breath methane while only 52% of European subjects were methane excreters. Also, it was found that 70% of stool samples from European subjects exhibited sulfate reduction activity and the remaining 30% produced methane. The opposite trend was observed in stool samples from rural black South Africans, where only 15% of the samples contained measurable hydrogen sulfide and the remaining 85% generated methane (Gibson et al., 1988). A similar trend was observed in a study by Christl and coworkers, who reported that approximately 50% of healthy human adults from European and North American populations and 90% of rural black Africans were predominantly methane excreters (Christl et al., 1995). Correspondingly, native Africans (NA) are rarely diagnosed with colorectal cancer (CRC) (O'Keefe et al., 2007). In Western countries, however, sporadic CRC is the second leading cause of cancer death (Horner et al., 2009), and compared to other U.S. racial and ethnic groups, African Americans (AA) have the highest incidence and mortality from sporadic CRC (Horner et al., 2009; Altekruse et al., 2010).

The structure of colonic hydrogenotrophic microbiota may explain some of the differences in predisposition to sporadic CRC. Hydrogen sulfide, produced by SRB, has been linked in clinical studies with chronic inflammatory disorders of the colon (Roediger et al., 1997; Levine et al., 1998) and is also a potent genotoxin (Attene-Ramos et al., 2006). Evidence for resource competition between SRB and MA has been shown in natural environments (Purdy et al., 2003), and a similar competition has been suggested to occur in the colon (Pochart et al., 1992; Dore et al., 1995), although this hypothesis remains controversial (Leclerc et al., 1980). A Western diet is characterized by a higher intake of dietary proteins (meat, milk and eggs) that provide sulfate and sulfite to colonic bacteria. An increase in stool sulfide has been demonstrated in subjects fed a diet high in meat compared to a vegetarian diet (Magee et al., 2000). Thus, it can be hypothesized that differences in SRB and MA communities may be related to the low incidence of CRC in NA as a result of their apparent propensity for methanogenic rather than sulfidogenic H₂ disposal. Little is known about the effects of diet or the host's genetic background on the composition of the intestinal microbiota in

general, and the structure of hydrogenotroph populations have been overlooked in recent microbiome studies.

The aim of the present study was to characterize the diversity of SRB and MA populations in stool samples from healthy NA, AA and EA and, thereby, examine possible correlations between hydrogenotrophic microbiota and diet.

Results and discussion

Amplification of Archaea, MA and SRB 16S rRNA and functional genes

The samples evaluated for hydrogenotrophic microbes in the present work were collected in a study that compared dietary, metabolic and physiological parameters in NA recruited from the rural Limpopo Province of South Africa consuming a diet low in animal products versus AA and EA consuming a typical Western diet (O'Keefe et al., 2007). In that study, clear differences were observed in dietary intakes and breath methane concentrations in NA relative to the other two groups. Specifically, NA consumed significantly fewer calories and less total and animal protein, total and saturated fat, and cholesterol than the AA or EA groups. Fasting breath methane was significantly higher in NA than in AA or EA subjects. In contrast, breath hydrogen was highest in AA. Thus, methanogens were expected to be more ubiquitous among NA subjects.

The proportion of stool samples positive for gene amplification in NA, AA and EA were 17/19, 15/18 and 14/17 for Archaea 16S rRNA gene and 17/19, 16/18 and 12/17 for *mcrA*, respectively. Intriguingly, Archaea 16S rRNA gene amplification did not always correlate with the detection of *mcrA* gene sequences. Two EA subjects were positive only for Archaea 16S rRNA gene, and one AA and one NA was positive for *mcrA* but not the Archaea 16S rRNA gene. We suspect these differences reflect amplification biases relating to the relative low abundance of MA in stool and potential DNA degradation or modification, but that presumably all subjects were colonized by MA. Based on data collected from mucosal biopsies of the colon from twenty-five subjects undergoing a screening colonoscopy, we have previously confirmed the prevalence of MA in the colonic mucosa of American (mostly EA) subjects, despite difficulties with detection in stool (Nava et al, 2011). The observation that almost all NA were colonized by MA, in contrast with other groups for which a significant number of subjects were negative is possibly linked to differences in methane excretion, although quantitative data would be needed to directly confirm (or refute) this hypothesis. This would indirectly confirm greater microbial fermentation in NA driven by a diet high in resistant starch.

The SRB functional gene *dsrA* was detected in 19/19 NA, 15/18 AA and 16/17 EA samples, whereas the *Desulfovibrio* 16S rRNA gene was detected in all stool samples from NA and AA and 16 of 17 from the EA group. This observation confirms other reports on the relative prevalence of SRB in the human colon (Pitcher et al., 2000; Zinkevich and Beech, 2000; Fite et al., 2004; Scanlan et al., 2009; Nava et al., 2011).

Diversity and distribution of Archaea 16S rRNA gene and *mcrA* TRFs among subjects

Archaea and *mcrA* profiles were moderately diverse (compared to the SRB TRF profiles) in stool from the three groups (Table 1). Twelve Archaea TRF were identified with BfaI, and nine with Hpy188I. Twenty-four *mcrA* TRF were identified with Hpy188III, and six with DdeI. Figure 1 shows the relative abundance of TRF from the most diverse TRFLP profiles among subjects of the three groups. In the most diverse profile (BfaI), EA harbored one specific Archaea and seven specific *mcrA* TRF, AA had two specific *mcrA* TRF and NA harbored one specific *mcrA* TRF.

A higher diversity of Archaea TRF was observed among NA subjects relative to EA and AA (Table 1), but interindividual variability was also lowest for NA. The greatest interindividual variation was detected in EA with members of this group harboring the greatest number of singletons. For statistical analyses, TRF that were present in less than two different subjects among one or more groups were excluded. With both enzymes, three representative TRF were ubiquitous among subjects and groups, and thus constituted a “core profile.” One additional specific TRF was detected only in NA subjects (10/15, MANOVA $P < 0.05$).

After removal of singletons, seven representative Archaea 16S rRNA gene TRF were selected with BfaI and Hpy188I, and seven representative *mcrA* TRF were selected with Hpy188III and three with DdeI. With both enzymes, two representative TRF were ubiquitous among subjects and groups. One additional specific TRF was observed only in NA subjects (11/17). Overall, comparable diversity in *mcrA* TRF was observed among the three groups (Table 1). The discordance between Archaea and *mcrA* TRF numbers indicate either the potential presence of *mrtA*-harboring methanogens such as *Msp. stadmaneeae* (Fricke et al., 2006) or non-methanogenic Archaea, which were documented recently with 16S rRNA gene rRNA sequences phylogenetically related to the Crenarchaeota phylum (Rieu-Lesme et al., 2005) and to *Halorubrum alimentarium*, *H. saccharovorum*, *H. koreense* strain and *Halococcus morrhuae* amplified from stool of Korean subjects (Nam et al., 2008).

Multivariate cluster analysis derived from the Kulczynski similarity index and the non-parametric MANOVA ($P < 0.05$) of Archaea populations revealed that NA differ significantly from AA, and that AA and EA harbor comparable Archaea populations (Figure 2A). Principal component analysis confirmed that slight differences exist in TRF content between NA, AA and EA. Similar statistical outcomes were observed for *mcrA* genes (Figure 2B). Thus, all subjects appear to share a core group of 2–3 Archaea/MA, with NA distinguished by the presence of an additional archaeal/MA genotype. While primer specificity may have contributed to the differential amplification efficacy between the two gene targets, consistent detection of additional genotypes in NA with the two primer sets supports the hypothesis that diet or environmental factors shape the composition of archaeal/MA communities. Native Africans were more homogeneous in Archaea 16S rRNA gene and *mcrA* TRF content as a group as illustrated by the NA clusters containing a majority of NA subjects (Figure 2A), whereas AA and EA were more dispersed among the clusters. Although individual diet intake data were not available for this study, the greater heterogeneity in archaeal/MA genotypes in AA and EA subjects may reflect a greater range of food choices in the Western diet.

Diversity and distribution of *dsrA* and *Desulfovibrio* 16S rRNA gene TRF among subjects

The *dsrA* and *Desulfovibrio* 16S rRNA gene TRF were substantially more diverse than Archaea and *mcrA* TRF (Table 1). Among all subjects, 50 *dsrA* TRF were identified with BstUI, and 23 with Sau96I (Figure 3A). Forty-three *Desulfovibrio* 16S rRNA gene TRF were identified with ScrFI and 32 with HpyCh4IV (Figure 3B).

A higher diversity of *dsrA* TRF was observed among NA subjects relative to EA and AA, whereas the three groups harbored similar diversity in *Desulfovibrio* TRF (Table 1). Overall, the extent of interindividual variation in *dsrA* and *Desulfovibrio* genotypes was also substantially greater than for archaeal/MA genotypes. For statistical analyses, TRF that were present in less than two different subjects among one or more groups were excluded. Thus, 16 representative *dsrA* TRF were selected with BstUI and 10 with Sau96I. Twenty-three representative *Desulfovibrio* 16S rRNA gene TRF were selected with ScrFI and 12 with HpyCh4IV.

Multivariate cluster analysis derived from the Kulczynski similarity index and the non-parametric MANOVA of *dsrA* TRF revealed that NA differ significantly from EA, whereas *dsrA* TRF content was indistinguishable for AA and EA (Figure 4A). Principal component analysis confirmed that subtle differences exist in TRF content between NA, AA and EA. For *Desulfovibrio* 16S rRNA gene TRF, similar statistical outcomes were observed (Figure 4B). Native Africans were more homogeneous in *dsrA* and *Desulfovibrio* 16S rRNA gene TRF content as a group as illustrated by the NA clusters containing a majority of NA subjects and only a few AA and EA, whereas AA and EA were more dispersed among the clusters. Thus, both hydrogenotrophic groups appear to be different in NA compared to AA and EA, again possibly reflective of the marked differences in diet or environment among the groups.

A range of nutritionally and physiologically distinct SRB have been cultured from stool (Gibson et al., 1991; 1993; Willis et al., 1997) with *Desulfovibrio* being the most predominant SRB genus in the human colon (Gibson et al., 1991). Here, the multivariate analyses revealed that differences between NA and EA in the diversity of SRB populations were likely associated with the presence or absence of particular *dsrA* TRF. These analyses suggest that non-*Desulfovibrio* SRB are probably more specific to the three racial groups and, thus, could have been selected primarily by diet. These taxa must be identified and described, as they could significantly influence the rate and production of hydrogen sulfide in the colon. Moreover, the present data indicate the importance of understanding how diet impacts hydrogen metabolism and its potential contribution to colonic health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Intergroup interindividual variation in the diversity and relative abundance of Archaea 16S rRNA gene and *mcrA* TRF

A nested PCR-TRFLP approach was used for the amplification of Archaea 16S rRNA gene and *mcrA* from stool samples from healthy native Africans (NA; n = 19), African Americans (AA; n = 18) and European Americans (EA; n = 17) taken from fresh feces voided during the first defecation of the morning and immediately stored under anaerobic conditions at -80°C until DNA extraction was performed. Genomic DNA was extracted from 200 mg of stool sample using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen, Valencia, CA). Methodological details for the nested PCR-TRFLP analyses are described in Nava et al. (2011). All primer pairs used for nested PCR are listed in Table S1.

Relative abundance of (A) Archaea 16S rRNA gene and (B) *mcrA* TRF in stool samples from NA, AA and EA using BfaI endonuclease for Archaea 16S rRNA gene and Hpy188III endonuclease for *mcrA*. Each color represents a TRF and its relative size within each chart represents relative abundance; the black bars represent the TRF that were represented more often in NA.

Figure 2. Diversity of Archaea 16S rRNA gene and *mcrA* sequences

A nested PCR-TRFLP approach was used for the amplification of Archaea 16S rRNA gene and *mcrA* from stool samples from healthy NA, AA and EA. Sample preparation and data analyses were executed as described in Fig. 1. Differences in the structure and composition of Archaea and *mcrA* sequences between the three groups were examined by multivariate analysis. **(A)** Cluster analysis of Archaea 16S rRNA gene TRF derived from the Kulczynski similarity index (presence-absence data) and **(B)** Cluster analysis of *mcrA* TRF derived from the Kulczynski similarity index. Green symbols represent NA; blue symbols, AA and red symbols, EA. Each symbol designates a single subject. Intergroup differences among NA, AA and EA were estimated by means of non-parametric MANOVA. Differences were considered significant if $P < 0.05$.

Figure 3. Intergroup interindividual variation in the diversity and relative abundance of *dsrA* and *Desulfovibrio* 16S rRNA gene TRF

A nested PCR-TRFLP approach was used to examine diversity in (A) dissimilatory sulfite reductase (*dsrA*) and (B) *Desulfovibrio* spp. 16S rRNA genes in stool samples from NA, AA and EA. Sample preparation and data analyses were executed as described in Fig. 1. Each color represents a TRF and its relative size within each chart represents relative abundance using BstUI endonuclease for *dsrA* and ScaFI endonuclease for *Desulfovibrio* 16S rRNA gene.

Figure 4. Diversity of *dsrA* and *Desulfovibrio* 16S rRNA gene sequences

A nested PCR-TRFLP approach was used for the amplification of *dsrA* and *Desulfovibrio* spp. 16S rRNA genes from stool samples. Sample preparation and data analyses were executed as described in Fig. 1. Differences in the structure and composition of *dsrA* and *Desulfovibrio* spp. 16S rRNA gene sequences between the three groups were examined by multivariate analysis. **(A)** Cluster analysis of *dsrA* TRF derived from the Kulczynski similarity index (presence-absence data) and **(B)** Cluster analysis of *Desulfovibrio* spp. 16S TRF derived from the Kulczynski similarity index. Green symbols represent NA; blue symbols, AA and red symbols, EA. Each symbol designates a single subject. Intergroup differences among NA, AA and EA were estimated by means of non-parametric MANOVA. Differences were considered significant if $P < 0.05$.

Table 1

Diversity indices for the four taxonomic targets *

Group	TRF number				Shannon index				Evenness			
	Arch	<i>merA</i>	<i>dsrA</i>	DSV	Arch	<i>merA</i>	<i>dsrA</i>	DSV	Arch	<i>merA</i>	<i>dsrA</i>	DSV
NA	4.29 ^a	2.82	4.87	5.18	0.91	0.28	0.85 ^a	0.89	0.63	0.53	0.52 ^a	0.53
AA	3.77 ^b	2.63	5.2	4.91	0.85	0.22	0.79 ^a	0.83	0.69	0.56	0.45 ^b	0.57
EA	3.04 ^c	2.67	4.88	5.46	0.73	0.27	0.72 ^b	0.94	0.73	0.57	0.46 ^b	0.58

* Averages for the two restriction enzymes based on non-singleton TRF

Letters indicate significant differences (t-test)