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ORIGINAL ARTICLE

Methanobrevibacter smithii Is the Predominant Methanogen in Patients with Constipation-Predominant IBS and Methane on Breath

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Abstract
Purpose Among irritable bowel syndrome (IBS) patients, breath methane producers overwhelmingly have constipation predominance (C-IBS). Although the most common methanogen in humans is *Methanobrevibacter smithii*, incidence and type of methanogenic bacteria in C-IBS patients are unknown.
Methods By use of a questionnaire and lactulose breath testing, subjects with Rome II C-IBS and methane (>3 ppm) were selected ($n = 9$). The control group included subjects with IBS who had no breath methane ($n = 10$). Presence of bacterial DNA was assessed in a stool sample of each subject by quantitative-PCR using universal 16S rDNA primer. *M. smithii* was quantified by use of a specific rpoB gene primer.
Results *M. smithii* was detected in both methane and non-methane subjects. However, counts and relative proportion of *M. smithii* were significantly higher for methane-positive than for methane-negative subjects ($1.8 \times 10^7 \pm 3.0 \times 10^7$ vs $3.2 \times 10^5 \pm 7.6 \times 10^5$ copies/g wet stool, $P < 0.001$; and 7.1 ± 6.3 % vs 0.24 ± 0.47 %, $P = 0.02$ respectively). The minimum threshold of *M. smithii* resulting in positive lactulose breath testing for methane was 4.2×10^5 copies/g wet stool or 1.2 % of total stool bacteria. Finally, area-under-curve for breath methane correlated significantly with both absolute quantity and percentage of *M. smithii* in stool ($R = 0.76$; $P < 0.001$ and $R = 0.77$; $P < 0.001$ respectively).
Conclusions *M. smithii* is the predominant methanogen in C-IBS patients with methane on breath testing. The number and proportion of *M. smithii* in stool correlate well with amount of breath methane.

Keywords Constipation IBS · Methane · Methanogens

Introduction

There is growing evidence from indirect breath testing that subjects with IBS have altered intestinal microbiota [1, 2]; two large-scale culture studies have recently revealed elevation of small bowel coliform counts compared with healthy controls [3, 4]. Although breath testing is challenging, a clear finding from this technique is that IBS subjects with positive breath methane more often have a constipation phenotype [5–7], as verified by meta-analysis of nine studies involving a total of 1,277 patients [8]. Moreover, the amount of methane produced, as determined by area under the curve, correlates well with subjective and objective severity of constipation [9].

Methanogens are important constituents of gut microbiota that colonize the human intestinal tract. These organisms are not bacteria but archaea, and generate methane by utilizing hydrogen and carbon dioxide (from syntrophic hydrogen-producing bacteria) [10]. Several decades ago, by use of enrichment cultures, Miller and Wolin isolated methanogens morphologically and physiologically similar to *Methanobrevibacter smithii* from fecal specimens from nine adults with methane production. When examined by immunological methods, these isolates were very closely related to *M. smithii* and unrelated or

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poorly related to other members of the Methanobacteriacaea family [11]. By use of the same morphological and immunological techniques, Weaver et al. detected *M. smithii* in tap water enema samples of 70 % of their subjects before sigmoidoscopy. A small subset of these patients who underwent breath analysis needed at least 2×10^8 methanogens/g dry weight of stool to have detectable breath methane of >6 parts per million (ppm) [12]. However, these studies have not examined subjects with IBS, and have not been replicated by use of molecular techniques, for example PCR.

In this study, we examine the importance of *Methanobrevibacter smithii* as a determinant of methane production in the breath of humans by use of quantitative-polymerase chain reaction (PCR) from stool from IBS patients with and without detectable methane on breath testing.

Methods

Patient Inclusion and Exclusion Criteria

The study was approved by our institutional review board, and informed consent was obtained from all participants. Consecutive Rome II positive IBS subjects aged 18–65 years who presented for lactulose breath testing were eligible for the study. Patients were excluded if they had any of the following: a history of abdominal surgery, for example bowel resection (except cholecystectomy or appendectomy), known intestinal disorder, for example inflammatory bowel disease, abdominal adhesions, perirectal or intestinal fistula, unstable thyroid disease, diabetes, cancer, HIV, pregnancy, use of medications known to affect intestinal motility, for example narcotics, imodium, and tegaserod, or antibiotic use within the past month.

Collection of Breath and Stool Samples

All patients were first asked to complete a bowel symptom questionnaire to determine the degree of constipation relative to diarrhea on the basis of C-D VAS scoring as previously validated [13]. Subjects then underwent lactulose breath testing (LBT). As part of the LBT, subjects were asked to ingest 10 g oral lactulose in solution (Pharmaceutical Associates, Greenville, SC, USA) after a baseline breath sample. Lactulose is a polysaccharide that is not digested by humans, but can be utilized by enteric flora. Repeat breath samples were then obtained every 15 min after lactulose ingestion until 180 min, and levels of methane and hydrogen were analyzed by gas chromatography (Quintron Instrument Company, Milwaukee, WI, USA). A positive methane breath test was defined as a breath methane level $\geq$3 ppm as previously reported [5, 13]. By use of the questionnaire and breath-test results, subjects who had methane on breath analysis and constipation-predominant IBS were selected. The control group included those with any form of IBS who did not test positive for methane on breath testing. After completion of breath testing, all subjects were provided with a stool container and instructions on how to collect a stool sample. Patients returned the stool sample and it was fresh frozen within 24 h of collection.

Stool PCR Testing

From each stool sample, bacterial DNA was extracted by use of the QIAamp PCR kit (Qiagen, Hilden, Germany). PCR (Eppendorf mastercycler gradient) with previously published universal 16S rDNA primer was used to detect the presence of total bacteria in stool. Quantitative-PCR was performed on the same stool samples using the rpoB gene primer specific for *M. smithii* only (Table 1). In addition, quantitative PCR was also conducted to determine total bacteria count by using universal primers (Table 1).

Quantitative PCR was performed with the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using optical-grade 96-well plates. Duplicate samples were routinely used for determination of DNA by real-time PCR. The PCR reaction was performed in a total volume of 20 µl using the iQ SYBR Green Supermix (Bio-Rad Laboratories), containing 300 nM each of the universal forward and reverse primers. The reaction conditions were set at 95 °C for 3 min followed by 40 cycles at 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 30 s, then 95 °C for 10 s. Data analysis made use of CFX Manager software supplied by Bio-Rad. To generate standard curves for total bacteria, the Ct values were plotted relative to the template DNA extracted from corresponding serial tenfold dilution of cultures of *Escherichia coli* strain

Table 1 Various PCR primers used to detect bacterial DNA in stool

Organism	Target	Primers (5′–3′)	Amplicon size (bp)
Universal	16S rDNA	TCCTACGGGAGGCAGCAGT	466
		GGACTACCAGGGTATCTAATCCTGTT	
M. Smithii	rpoB	AAGGGATTTGCACCCAACAC	70
		GACCACAGTTAGGACCCTCTGG	

ATCC 25922. *Escherichia coli* strain ATCC 25922 was previously grown in TB growth medium (MO BIO Laboratories, Carlsbad, CA, USA) to a concentration of 10^8 CFU then plated on LB (ISC BioExpress, Kaysville, UT, USA) agar plates to verify colony counts. The 10^8 CFU *Escherichia coli* solution was subjected to DNA extraction by use of a Qiaamp DNA mini kit (Qiagen). The extracted DNA was used to create tenfold dilutions and establish a standard curve. Similarly, calibration curves for *M. Smithii* were made by aliquoting tenfold dilutions of 10^8 CFU *M. Smithii* liquid culture. Concentration was determined by measuring optical density at 600 nm.

Statistical Analysis

The Mann–Whitney *U* test was used for non-parametric data, and Student's *t* test was used for normally distributed data. The quantity of *M. smithii* was compared with the amount of methane on breath testing by use of Spearman rank correlation. Comparison of breath test parts per million between hydrogen and methane utilized Pearson regression analysis. In addition, *M. smithii* was determined as a percentage of the combined total bacteria and *M. smithii* count, and this percentage was also compared with breath test status, methane levels, and degree of constipation. All tests were two-tailed and statistical significance was defined as $P < 0.05$.

Results

Baseline Characteristics

A total of nine patients (C-IBS with positive methane breath analysis) and ten controls (IBS with no breath methane) met the inclusion criteria. Most of the subjects in each group were females (8 of 9 in the methane group and 8 of 10 among the non-methane controls). The average age was no different between the two groups (43.8 ± 8.7 years for methane positive vs. 41.9 ± 9.9 years for methane negative subjects). The validated symptom C–D score (range of score from −100 to +100) was 51.1 ± 37.8 mm for the C-IBS with methane group which was greater than −1.0 ± 35.1 mm for non-methane subjects ($P < 0.01$) indicating significant constipation in methane-positive subjects relative to diarrhea. There was no difference in bloating or abdominal pain severity between the groups (Table 2).

Table 2 Baseline characteristics of the study population

	Methane positive ($n = 9$)	Methane negative ($n = 10$)	
Age (years)	43.8 ± 8.7	41.9 ± 9.9	$P > 0.05$
Gender (% females)	88 %	80 %	$P > 0.05$
C–D score[a]	51.1 ± 37.8	−1.0 ± 35.1	$P < 0.01$

[a] Validated Constipation–Diarrhea scoring based on bowel symptom questionnaire [13]

PCR Results from Stool

On q-PCR, *M. smithii* samples were not interpretable because of poor sample for two methane and one hydrogen-producing subjects leaving seven methane and nine non-methane-producing subjects eligible for analysis. In q-PCR for total bacterial counts, six samples were not interpretable leaving 13 (six breath methane positive and seven breath methane negative) for analysis. In determining the percentage of *M. smithii*, there were 12 samples for which both *M. smithii* and bacterial levels were measured.

Examining *M. smithii* first, *M. smithii* was detected in both methane producers and non-methane subjects. However, the presence of *M. smithii* was significantly higher for breath methane-positive subjects ($1.8 \times 10^7 \pm 3.0 \times 10^7$ copies per g wet stool) than for those with negative breath methane ($3.2 \times 10^5 \pm 7.6 \times 10^5$ copies per g wet stool) ($P < 0.001$). On the basis of these findings, the minimum threshold of *M. smithii* resulting in positive lactulose breath testing for methane was deemed to be 4.2×10^5 copies per g wet stool (Fig. 1).

To further evaluate this relationship, the ratio of *M. smithii* to combined total bacteria and *M. smithii* was expressed as a percentage. For non-methane producers, the percentage *M. smithii* was 0.24 ± 0.47 % and for methane-producing subjects it was 7.1 ± 6.3 % ($P = 0.02$) (Fig. 2). On the basis of percentage counts, *M. smithii* greater than 1.2 % was always indicative of positive breath methane.

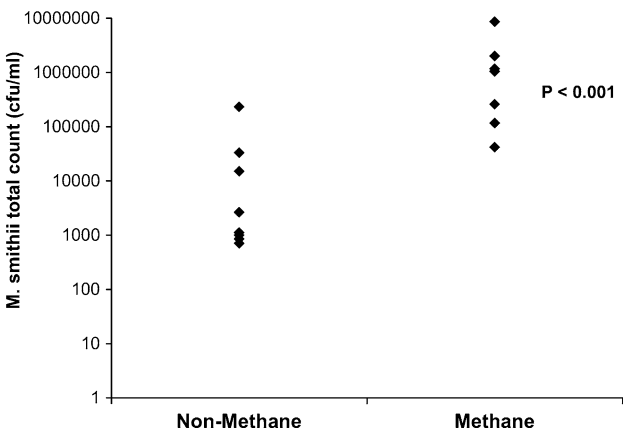

Fig. 1 *M. smithii* counts in methane and non-methane producers in stool

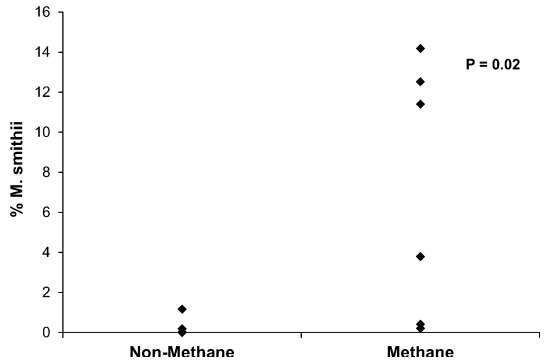

Fig. 2 Percent *M. smithii* relative to prokaryotic bacteria as a determinant of detection of methane on breath

Comparing *M. smithii* and Breath Methane and Hydrogen Levels on Breath Test

The amount of breath methane produced as determined by 180 min AUC correlated significantly with the quantity of *M. smithii* in stool ($R = 0.76$, $P < 0.001$) (Fig. 3). Although total bacterial counts did not correlate with methane on breath testing, the percentage of *M. smithii* was highly correlated with the level of methane on breath test ($R = 0.77$, $P = 0.001$) (Fig. 4).

In contrast with methane, when breath hydrogen was compared with quantities of *M. smithii*, total prokaryotic bacteria, and the percentage of *M. smithii*, no trend was seen. However, there was expected hydrogen utilization by methane as suggested by an inverse correlation between breath methane AUC and hydrogen AUC ($R = -0.61$, $P = 0.005$) (Fig. 5).

Constipation Symptoms, *M. smithii*, and Total Bacterial Count

Using the previously validated score examining constipation relative to diarrhea (C–D), we examined whether *M. smithii* and total bacterial levels were predictive of constipation severity. Neither absolute *M. smithii* (Fig. 6) ($R = 0.43$, $P = 0.1$) nor percentage *M. smithii* (Fig. 7) ($R = 0.47$, $P = 0.12$) quite reached significance in a comparison with the severity of constipation by C–D. Also, there was no correlation between absolute *M. smithii* and percentage *M. smithii* and severity of abdominal pain or bloating.

In the case of total bacterial counts, there was no association with C–D score and no association with bloating. Although, there was an inverse correlation between bacterial levels and abdominal pain VAS scores ($R = -0.51$), it did not reach statistical significance ($P = 0.07$) (Fig. 8).

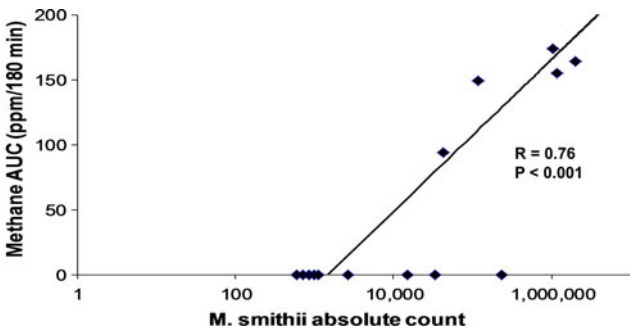

Fig. 3 Correlation between *M. smithii* and breath methane AUC

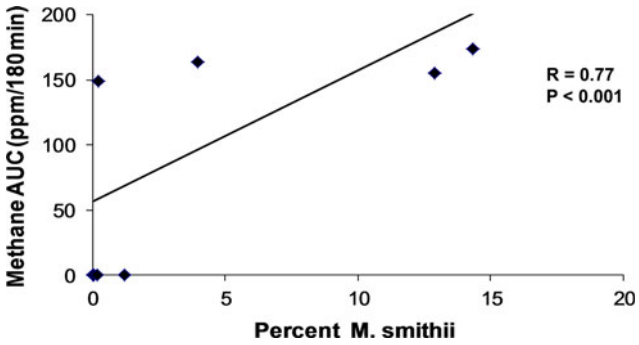

Fig. 4 Correlation between percentage *M. smithii* to total prokaryotes and breath methane AUC

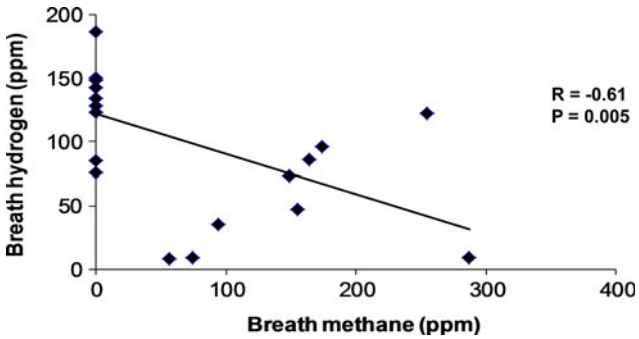

Fig. 5 Correlation between hydrogen and methane in breath AUC

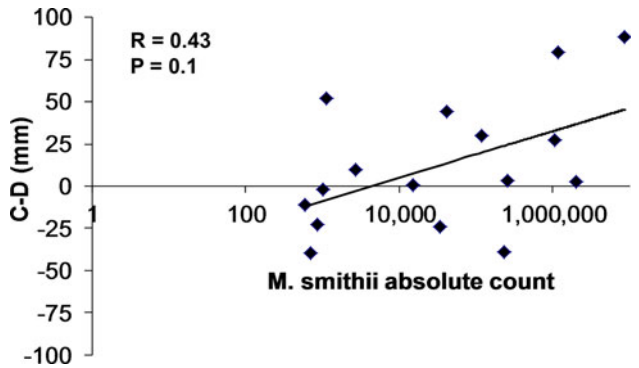

Fig. 6 Relationship between *M. smithii* level and the relative degree of constipation to diarrhea. C–D is a validated measure of the relative degree of constipation to diarrhea. The larger the number the more constipation is relative to diarrhea

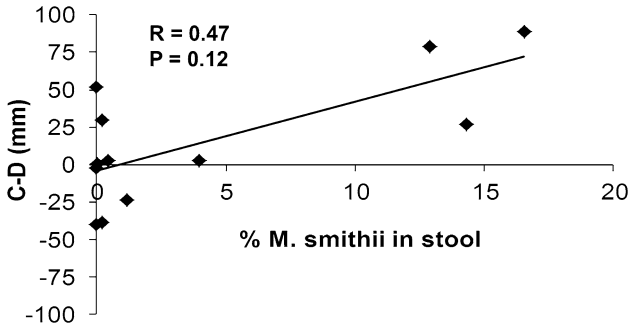

Fig. 7 Comparison of the percent of *M. smithii* to total bacteria in the stool and relative degree of constipation. C–D is a validated measure of the relative degree of constipation to diarrhea. The larger the number the more constipation is relative to diarrhea. The % M. smithii is determined by the amount of *M. smithii* relative to total prokaryotic bacteria

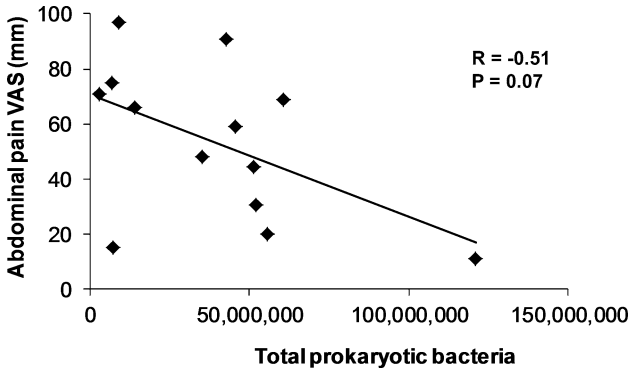

Fig. 8 Correlation between total prokaryote bacteria counts in stool and abdominal pain scores

Discussion

In this study we discovered that *Methanobrevibacter smithii* is likely to be the important methanogen responsible for breath methane in subjects with IBS. Furthermore, *M. smithii* levels and relative proportions in stool correlate with amount of methane produced, suggesting this may be the major methanogen responsible for methane during breath testing in humans. Finally, this is the first study to demonstrate by qPCR that *M. smithii* is important in C-IBS subjects with methane on LBT.

Recent literature suggests involvement of methanogenic gastrointestinal microbiota in the pathophysiology of functional gastrointestinal disorders such as IBS. Specifically, methane gas on LBT is associated with a constipation phenotype [5–7]. Our group has shown that methane is not an inert gas as previously thought; but slows intestinal transit [14]. In an in-vivo study on five dogs, infusing methane through mid-small bowel fistula reduced proximal small bowel motility by an average of 59 % [14]. The presence of breath methane has also been associated with significant slowing of intestinal transit in human studies [8, 15, 16]. Among patients with IBS, it has been confirmed in a multitude of publications that methane on lactulose breath testing is almost universally associated with constipation-predominant disease [5–8]. However, evaluation of stool in such patients to determine the source of methane has never been attempted in IBS.

In this study, we established that *Methanobrevibacter smithii* is present ubiquitously in the stool of IBS patients. However, patients with methane-positive breath test harbor significantly more *M. smithii* than methane negative patients. These patients also have higher proportions of *M. smithii* in their stool relative to other bacteria. The higher the count or relative proportion of *M. smithii* in stool, the greater the degree of breath methane. This implies that stool quantitative PCR is a much more sensitive tool than breath analysis for detection of intestinal methanogens. However, the clinical relevance of such testing remains to be determined, especially for those harboring bacteria unable to generate sufficient breath methane.

Interestingly, methanogens alone may not be problematic. In this study, most subjects had detectable *M. smithii* in their stool. However, the level of *M. smithii* may be the issue. On the basis of this study, methane on the breath seems to be detectable when the level of *M. smithii* exceeds 4.2×10^5 copies per g of wet stool or 1.2 % of total stool bacteria. This is important, because in the original description of methane on breath and constipation IBS, not all constipation-predominant IBS subjects had methane. However, nearly all methane subjects were constipated. Combined, these findings suggest that stool testing by qPCR may identify a threshold for producing constipation that a breath test is not sensitive enough to detect.

In this study, the threshold of *M. smithii* to cause detectable methane on breath analysis was much smaller than that reported earlier by Weaver et al. [12]. This difference is likely to be because of the use of different techniques. In the study by Weaver et al., methanogens were cultured from the stool sample and identification as *M. smithii* was based on morphological and immunological methods. Handling and culture of stool for methanogens can be difficult, because the organisms are anaerobic. Exposure of the stool sample to air might harm the organisms limiting their growth. In q-PCR, handling is not problematic because PCR will detect both viable and non-viable organisms.

Although the diagnostic application of this technique is intuitive, these data may also have therapeutic and clinical significance, because elimination of methanogens by non-absorbable antibiotics can significantly improve gut symptoms [17, 18]. For methane producers with constipation-predominant IBS, neomycin resulted in 44.0 ± 12.3 % versus 5.0 ± 5.1 % improvement in constipation,

compared with placebo, that correlated well with elimination of methane on follow up breath testing [19]. In a retrospective study, combination of rifaximin and neomycin for 10 days resulted in significantly greater reduction in methane (87 %) and constipation symptoms (85 %) than neomycin (33 and 63 %, respectively) or rifaximin (28 and 56 %, respectively) alone [20].

One limitation of this study is that the small number of subjects did not enable better characterization of symptoms as they relate to *M. smithii* total counts and relative proportions. Although breath methane has been correlated well with both subjective and objective severity of constipation in earlier studies, we could not demonstrate significant correlation between *M smithii* and constipation [8, 9]. However, we observed encouraging positive trends of an association between *M. smithii* and constipation. Unfortunately, it did not reach statistical significance as the study was underpowered for this result. Future studies should include collection of a large number of unselected IBS subjects to determine if there is a clear threshold of *M. smithii* in stool that correlates with the C-IBS phenotype.

In conclusion, our results suggest that *M. smithii* is the predominant methanogenic archaeabacteria in the gut of C-IBS patients responsible for methane on breath testing. This is supported by the correlation between *M. smithii* level in stool and methane AUC on breath testing. Further studies are needed to determine if levels of *M. smithii* determine the constipation phenotype of IBS and if this finding predicts response to antibiotic therapy.

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Conflict of interest Dr Mark Pimentel is a consultant for and has grants from Salix Pharmaceuticals. Cedars-Sinai has a licensing agreement with Salix Pharmaceuticals. All other authors have no financial conflicts to disclose.

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