Curr Microbiol
DOI 10.1007/s00284-015-0787-9

Diversity of Human-Associated *Methanobrevibacter smithii* Isolates Revealed by Multispacer Sequence Typing

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Received: 15 October 2014 / Accepted: 9 January 2015
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Abstract *Methanobrevibacter smithii* is the main archaea in human, detoxifying molecular hydrogen resulting from anaerobic bacteria fermentations into gaseous methane. Its identification relies on gene sequencing, but no method is available to discriminate among genetic variants of *M. smithii*. Here, we developed a multispacer sequence typing (MST) for genotyping the genetic variants of *M. smithii*. Four intergenic spacers recovered from the *M. smithii* reference genome were PCR amplified and sequenced in three *M. smithii* reference strains and in a collection of 22 *M. smithii* isolates from the oral cavity in two individuals and the gut of 10 additional individuals. Sequencing yielded 216 genetic polymorphisms including 89 single nucleotide polymorphisms (41.2 %), 83 insertions (38.4 %), and 44 deletions (20.4 %). Combining these genetic polymorphisms yielded 15 genotypes with an index of discrimination of 0.942 (confidence interval 0.9–0.984; $P < 0.05$). Five *M. smithii* isolates made from the oral cavity yielded five different genotypes; seven gut isolates yielded nine different genotypes; genotypes MST5 and MST6 were found both in the oral cavity and the gut. Multiple genotypes were identified in some individuals at the same anatomical site. MST is a sequencing-based method which discriminates several genetic variants within *M. smithii*. Individuals may harbor several contemporary genetic variants of *M. smithii* in the oral cavity and gut. MST will allow studying population dynamics of *M. smithii* and tracing its circulation between individuals and their environment.

Electronic supplementary material The online version of this article (doi:10.1007/s00284-015-0787-9) contains supplementary material, which is available to authorized users.

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Introduction

Methanobrevibacter smithii is the main human-associated methanogenic archaea, recovered from the vaginal [2], oral, and digestive tract microbiota [9, 16]. We previously showed that *M. smithii* was an almost constant inhabitant of the digestive tract, being detected in up to 97.5 % of individuals [9, 16]. In the digestive tract, this archaea is thought to detoxify molecular hydrogen from fermentation by anaerobic communities, into gaseous methane [25]. Its potential role in pathology remains controversial. Indeed, *M. smithii* has been associated with weight gain and obesity [1, 24]; colonic diseases include ulcerative colitis, Crohn's disease, and colonic cancer [12, 26, 29]. However, these observations warrant confirmation.

M. smithii is a fastidious organism, which requires culture in strict anaerobic atmosphere comprising of up to

Published online: 24 February 2015

80 % hydrogen [9, 16]. Therefore, its diagnosis mainly relies on PCR-based detection of specific sequences, chiefly derived from the 16S rRNA and the *mcrA* gene sequences [17, 23, 31]. Sequencing these genes directly into clinical gut specimens suggested that *M. smithii* comprised several phylotypes [23]. Yet, these data were obscured by difficulties in interpreting sequences directly derived from a complex microbiota and by a lack of acknowledged definition of species versus intraspecies genetic variants of methanogens, based on 16S rRNA and *mcr*A gene sequences.

In order to further explore the diversity of *M. smithii*, we developed multispacer sequence typing (MST), a sequencing-based method combining several intergenic spacer sequences to fingerprint organisms [18], to genotype a collection of *M. smithii* isolates. MST had been initially developed for *Yersinia pestis* [8], and was further applied to genotype several fastidious human pathogens, including *Rickettsia conorii* [32], *Coxiella burnetii* [21], *Bartonella henselae* [19], *Borrelia* spp. [10] and mycobacteria including *Mycobacterium avium* [4], *Mycobacterium abscessus* [28] and *Mycobacterium tuberculosis* [6, 7].

Materials and Methods

M. smithii Isolates

Reference *M. smithii* ATCC 35061 (DSM 861), *M. smithii* DSM 2374, and *M. smithii* DSM 2375 strains were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). A collection of 22 *M. smithii* clinical isolates was made from ten stools specimens collected in ten individuals and from two oral cavity specimens collected in two different individuals, as previously described [16]. All participants had signed the written informed consent formally approved by the ethics committee of Institut Fédératif de Recherche 48, Marseille, France. The isolates were further subcultured into Hungate tubes (Dutscher, Issy-les-Moulineaux, France) prepared as described [9, 16]. Briefly, 4.5 mL of SAB-medium incorporating 1 % agar were prepared anaerobically and maintained at 50 °C. A 100-µL volume of each substrate (Na_2S: 2 %. $NaHCO_3$: 10 %) and vitamin solution were injected in five tubes. One tube was inoculated with 500 µL of the enrichment culture of *M. smithii*, and then a serial dilution from 10^{-1} to at least 10^{-5} was made in additional tubes. Inoculated agar tubes were rolled in ice water to solidify the agar in a thin layer and were then incubated at 37 °C under a H_2/CO_2 (80–20 %) atmosphere at a 2-bar pressure. Growth of any methanogen was monitored by methane production as previously described [9, 16]. After 4- to 8-week incubation, all colonies were picked from the fifth dilution tube using a sterile Pasteur pipette drawn out to a fine capillary, and 90° bend to a terminal 4 mm. The pipette was inserted into a tube, and the desired colony was drawn up into it, and transferred to a sterile screw-cap Eppendorf tube containing 250 µL of sterile phosphate buffer saline (PBS) for molecular analyses.

DNA Extraction and Identification

To extract DNA, 0.3 g of acid-washed beads (≤106 mm, Sigma, Saint-Quentin Fallavier, France) was added in each tube; the suspension was shaken to achieve mechanical lysis in a FastPrep BIO 101 apparatus (Qbiogene, Strasbourg, France) at level 6.5 (full speed) for 2 min. The supernatant was incubated overnight at 56 °C with 180 µL of T1 buffer and 25 µL of proteinase K (20 mg/mL) from the NucleoSpin® Tissue Mini Kit (Macherey–Nagel, Hoerdt, France). After a second cycle of mechanical lysis as above, the supernatant was incubated for 10 min at 100 °C. Total DNA was then extracted using the NucleoSpin® Tissue Mini Kit, according to the manufacturer's recommendations. Extracted DNA was eluted with 100 µL of elution buffer and stored at −20 °C until used. Extraction of 250 µL of sterile water was introduced in each series of DNA extraction as negative control. Isolates were identified as *M. smithii* after PCR amplification and sequencing of the 16S rRNA gene using the broad-range archaeal primers SDArch0333aS15 (5′-TCCAGGCCCTACGGG-3′) and SDArch0958aA19 (5′-YCCGGCGTTGAMTC CAATT-3′) as previously described [17, 31] and PCR amplification and sequencing of the *mcrA* gene using primers *mcrA*-F (5′-GGTGGTGTMGGATTCACACARTA YGCWACAGC-3′) and *mcrA*-R (5′-TTCATTGCRTA GTTWGGRTAGTT-3′) as previously described [20].

Selection of Spacers for MST

The sequences of 154 intergenic spacers were extracted from the reference *M. smithii* ATCC 35061 genome (GenBank accession CP000678.1) using the home-made Perl script which enables to pick non-coding sequences. Spacer sequences retrieved from *M. smithii* ATCC 35061 genome were compared with homologous sequences in *M. smithii* DSM 2374 and *M. smithii* DSM 2375 genome scaffolds (NCBI accession numbers 260590157 and 2101 37298, respectively) using Difseq software in EMBOSS. The NCBI Blast software was used to visualize differences between homologous spacer sequences. A total of 10 spacers were further analyzed on the basis of the following criteria: (i) sequence length of ≤700 bp so that experimental sequences would be in the sequencing range of capillary sequencers and (ii) a difference between homologous sequences of *M. smithii* ATCC, *M. smithii* DSM

2374, and *M. smithii* DSM 2375 would be ≥3 bp. For each one of the 10 selected spacers, a specific PCR primer pair was designed within the genes flanking both extremities of selected spacer using Primer3 and the primer quest software (Table 1) and tested in silico for their specificity using BLAST software (http://www.ncbi.nlm.nih.gov). The PCR conditions were optimized by incorporating DNA extracted from *M. smithii* ATCC 35061, *M. smithii* DSM 2374, and *M. smithii* DSM 2375.

PCR for MST

PCRs were carried out in a PTC-200 automated thermal cycler (MJ Research, Waltham, USA) in a 25-µL PCR mixture containing 5 µL of 10× buffer (Qiagen, Courtaboeuf, France); 0.2 µM of each primer (10 pM; Eurogentec, Seraing, Belgium); 200 µM (each) of dATP, dCTP, dGTP, and dTTP; 1.5 mM of MgCl2; 1.25 U of HotStarTaq polymerase (Qiagen); 13 µL of Dnase/Rnase free distilled water (Gibco, Cergy Pontoise, France); and 5 µL of DNA template. An initial 15-min denaturation at 95 °C was followed by 40 cycles of 30-s denaturation at 95 °C, 45-s annealing at the appropriate Tm (57 or 60 °C), and 60-s extension at 72 °C. Amplification was completed by 5-min holding at 72 °C to allow complete extension of the PCR products. Negative controls consisting of PCR mixture without DNA template were included in each PCR run. PCR products were purified using the PCR filter plate Millipore NucleoFast 96 PCR kit as recommended by the manufacturer (Macherey–Nagel).

Sequencing reactions were carried out using the BigDye Terminator, version 1.1, cycle sequencing kit DNA according to the manufacturer's instructions (Applied Biosystems, Foster City, USA). All PCR products were sequenced in both directions using the same primers as used for PCRs in a 2720 Thermal Cycler (Applied Biosystems) with an initial 1-min denaturation step at 96 °C, followed by 25 cycles of 10-s denaturation at 96 °C, 20-s annealing at 50 °C, and 4-min extension at 60 °C. Sequencing products were purified using the MultiScreen 96-well plates Millipore (Merck, Molsheim, France), containing 5 % of Sephadex G-50 (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France), and sequences were analyzed on an ABI PRISM 3130× Genetic Analyzer (Applied Biosystem). The sequences were edited using the ChromasPro software (version 1.42; Technelysium Pty Ltd), aligned using Clustal W (MEGA 5 software), and compared with the *M. smithii* ATCC35061, *M. smithii* DSM 2374 and *M. smithii* DSM 2375 homologous sequence spacers using the online BLAST program of NCBI. Stability of spacer sequences was experimentally ensured on a subset of 10 *M. smithii* isolates S40-1, S40-2, D63-1, D63-2, S36-2, S36-3, D27-1, D27-2, D27-3, and S-1 by two additional runs of PCR sequencing after one and two subcultures.

Sequence Analysis

For each intergenic spacer, a spacer type (ST) was defined as a sequence exhibiting unique genetic polymorphism (SNPs and indels). MST genotypes were defined as a unique combination of four spacer types. The discrimination power was calculated using the Hunter-Gaston index/Simpson's index of diversity [15], and the 95 % confidence interval was determined using the online tool Darwin.phyloviz.net/ComparingPartitions [14]. Multiple alignments of sequences were carried out using the online tool clustalW2 of EMBL-EBI and MultAlin version 5.4.1 [5] to show the stability of the alignments when using different alignment tools. For each tool, the default parameters were selected.

Table 1 Characteristics of intergenic spacers and primers used in this study

Intergenic spacer	Position	Position in *M. smithii* strain ATCC 35061 genome	Primers	Tm (°C)	Size (bp)*
1	1647–2123	exoribonuclease VII, large subunit, XseA	F: AATTATTGATTGCACTGATTGAGTAA	57	477
		Integrase-recombinase protein	R: AGATACTCATCCAAATATCCTGAAAG		
2	1667928–1668282	Hypothetical protein	F: GATTGGTGTATTGAAAGAGGATATTT	57	355
		Arylsulfatase regulator, AslB	R: ATTATGGATTTGATGATGAAACCA		
3	134368–134821	Exonuclease	F: TGACAATATCGCTTCCAACAAATA	60	469
		Hypothetical protein	R: TTTTAGCTTCATTATTGCAACCTT		
4	237842–238465	Hypothetical protein	F: ATCCTTTTCTTCACCCACACC	60	625
		Transcription regulator	R: TTCAAAAGTTCGTCATCTTTTTGTG		

F forward primer, *R* reverse primer

* Theoretical size of PCR products was determined according to the position of primers within *M. smithii* strain ATCC 35061 genome (accession number NC_ CP000678.1)

Nucleotide Sequences Accession Numbers

All the spacer sequences reported in this paper have been deposited in GenBank (accession no LK054642 to LK054667) and in Mediterranean Infection database at http://www.mediterranee-infection.com/article.php?laref=331&titre=mst-methanobrevibacter-smithii.

Results

Identification of the Clinical Isolates

The 22 isolates made from 12 clinical specimens in this study were firmly identified as *M. smithii* on the basis of a 16S rRNA and *mcrA* genes sequence exhibiting >99 % similarity with the *M. smithii* ATCC 35061 reference genome. The 16S rRNA and *mcrA* gene sequences of two *M. smithii* isolates made from the oral cavity in two unrelated individuals were deposited in the GenBank database under accession numbers LK054635, LK054636, LK054626, and LK054627.

Spacer Sequencing

Of the 154 intergenic spacers detected in the genomic sequence of *M. smithii* strain ATCC 35061 using the perl script program of our laboratory, 22 spacers fulfilled our selection criteria, and 10 spacers were randomly chosen for tentative amplification in *M. smithii* ATCC 35061, *M. smithii* DSM 2374, and *M. smithii* DSM 2375 reference strains. As six of these 10 spacers were not amplified in the three reference strains, four spacers herein referred to as spacers 1–4 were further PCR amplified and sequenced in 22 clinical isolates and in the three reference strains (Table 2). Spacer 1 yielded sequences of 421–477 bp corresponding to five different alleles; spacer 2 yielded sequences of 315–355 bp corresponding to five different alleles; spacer 3 yielded sequences of 415–454 bp corresponding to five different alleles; and spacer 4 yielded sequences of 575–625 bp corresponding to six different alleles (supplementary Figure S1). Altogether, comparing the sequences of the four spacers in *M. smithii* ATCC 35061, *M. smithii* DSM 2374, *M. smithii* DSM 2375, and the 22 clinical isolates yielded 216 genetic polymorphisms

Table 2 MST genotypes of *M. smithii* reference and clinical strains used in this study

Individuals	Sex	Age	Isolates	ST1	ST2	ST3	ST4	MST
Type strain	/	/	ATCC35061	1	1	1	1	1
Type strain	/	/	DSM2374	2	2	2	2	2
Type strain	/	/	DSM2375	3	3	3	3	3
S40	F	74	S40-1	2	4	7	3	4
			S40-2	2	6	6	3	5
D63	M	53	D63-1	2	6	6	3	5
			D63-2	2	2	6	5	6
S36	F	57	S36-2	2	2	6	5	6
			S36-3	2	3	6	5	7
S1	M	76	S1-1	2	2	6	3	8
			S1-2	2	2	6	3	8
S3	M	77	S3-1	2	2	6	3	8
			S3-2	2	2	6	3	8
S5	M	69	S5-1	3	3	2	3	9
S4	M	86	S4-1	3	3	2	3	9
S38	F	73	S38-1	3	3	2	5	10
			S38-2	3	3	2	5	10
S34	M	66	S34-2	3	3	2	5	10
			S34-1	3	3	2	5	10
D27[a]	F	56	D27-3	4	3	4	3	11
			D27-2	4	3	4	4	12
			D27-1	5	5	5	4	13
S2	M	97	S2-1	6	3	2	7	14
S6	F	92	S6-1	6	3	2	7	14
			S6-2	6	3	2	6	15

"S" denotes an individual for whom stool *M. smithii* isolates were studied, "F" denotes a female individual, and "M" an male individual

[a] "D" denotes an individual for whom oral cavity *M. smithii* isolates were studied

including 89 SNPs (41.2 %), 83 insertions (38.4 %), and 44 deletions (20.4 %).

MST Genotyping

Combining the four spacer sequences obtained in 22 isolates and the three reference strains yielded fifteen genotypes named MST 1 to MST 15 (Table 2) with an index of discrimination of 0.942 (confidence interval 0.9–0.984; $P < 0.05$). Using two different alignments tools with default parameters yielded the same results. Although each one of the three reference isolates have yielded one unique MST, the 22 clinical isolates yielded twelve MST including six MST unique to one isolate and six MST with, at least, two isolates which are shared by two different, unrelated individuals. Five *M. smithii* isolates made from the oral cavity yielded five different genotypes in two individuals: individual D63 harbored two genotypes (MST5 and MST6) and individual D27 harbored three genotypes (MST11, MST12, and MST13). Seven gut isolates yielded nine different genotypes. Genotypes MST5 and MST6 were found both in the oral cavity and the gut of unrelated individuals. We further observed that individual S40 harbored two different genotypes MST4 and MST5, individual S36 harbored two different genotypes MST6 and MST7, and individual S6 harbored two different genotypes M14 and M15. Repeated PCR sequencing yielded the same MST genotype for each one of the 10 tested isolates.

Discussion

The intraspecific diversity of the human-associated methanogen *M. smithii* is poorly known. Indeed, only one *M. smithii* complete genome has been published and this genome sequence was derived from an environmental (sewage digester) isolate, not a clinical one [27]; a few studies of *M. smithii* 16S rRNA and *mcr*A genes reported phylotypes which were tentatively attributed to several *M. smithii* lineages, without clear-cut evidence of whether these phylotypes could be in fact attributed to highly related, yet different species [23].

Here, we studied clinical isolates firmly identified as *M. smithii* on the basis of a 16S rRNA gene and a *mcr*A gene sequences exhibiting 99 % similarity with the *M. smithii* ATCC 35061 reference genome currently available in the Genbank database. This is the largest collection of clinical *M. smithii* ever published as the previous one comprised 20 isolates [11]. We then took advantage of the availability of this reference complete genome and two draft genomes (*M. smithii* DSM 2374 and DSM 2375) to analyze intergenic spacers for potential sequence variability. As for Bacteria, the 16S-23S rDNA intergenic spacer is widely used to type bacterial isolates. Indeed, this spacer has been shown to exhibit variability both in copy number and sequence in many bacterial species [3, 13, 22, 30, 32]. Studying the 16S-23S rDNA spacer is not possible in *M. smithii* because in this Archaea, the 16S rRNA gene is separated from the 23S rRNA genes [27]. We therefore developed MST as a sequencing-based method for genotyping *M. smithii.* Combining the sequences of four variable intergenic spacers in a multi-spacer format, we identified 15 different genotypes among 25 *M. smithii* isolates, including 22 clinical isolates from our laboratory and three reference isolates. As MST is a sequencing-based method, unsurprisingly, we observed the robustness and reproducibility of MST data. These data illustrate the capacity of MST to depict intraspecies genetic variability in the Archaea *M. smithii.* Interestingly, we observed that several individuals were harboring several *M. smithii* genotypes either in the oral cavity or the gut. We further observed that oral cavity and the gut could harbor several different genotypes of *M. smithii* with genotypes MST5, MST6 being detected in both anatomical sites, suggesting a lack of specificity of the various *M. smithii* genotypes for one particular anatomical niche.

We propose that MST could be used as a first-line method for genotyping *M. smithii*. This task is becoming of interest in the perspective of the role of this methanogen in the gut physiology, and its potential role in some pathology directly or indirectly connected with gut [12, 26, 29]. In particular, MST could be used to study the dynamics of *M. smithii* populations and to trace inter-individual transmission of *M. smithii* including mother to infant transmission, as well the potential effect of various factors such as antimicrobials and diseases of this major human-associated archaea.

Acknowledgments VN was supported by a Grant of "Mediterranean Infection Institute," Marseille, France. The work was supported by Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, Marseille, France. The authors acknowledge the Ethic Committee of Institut Fédératif de Recherche 48 for reviewing the ethics of the Project.

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