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S.I. Traore, S. Khelaifia, N. Armstrong, J.C. Lagier, Didier Raoult

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Sory Ibrahima TRAORE[1], Saber KHELAIFIA[1], Nicholas ARMSTRONG[1], Jean Christophe LAGIER[1], Didier RAOULT[1*]

1. Aix Marseille Univ, IRD, APHM, MEPHI, IHU-Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13385, Marseille cedex 05, France

*Corresponding author: Prof. Didier RAOULT, Aix Marseille Univ, IRD, APHM, MEPHI, IHU-Méditerranée Infection 19-21 Bd Jean Moulin 13005 Marseille, France. Tel: +33 (0)4 13 73 24 01 fax: + 33 (0) 13 73 24 02.
Email: didier.raoult@gmail.com

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Abstract

Objective

Methanogenic archaea are considered as extremely oxygen-sensitive organisms and their culture is fastidious, requiring specific equipment. We report here the conditions allowing the cultivation of *M. smithii* in an anaerobic chamber without hydrogen addition.

Methods

We first enriched the stool sample in an anaerobic liquid medium, and then, to cultivate *M. smithii* with *B. thetaiotaomicron* and other hydrogen producing bacteria as, on solid medium in an anaerobic chamber, we divided the agar plats in two compartments and we seeded each strain on each compartment. Methane production was assessed by gas chromatographic and the growing colonies were authenticated by MALDI-TOF MS.

Results

We successfully cultured *Methanobrevibacter smithii* from a liquid culture medium inoculated with a stool collected from a healthy donor under an anaerobic chamber. The isolation in pure culture was then a success on agar medium by performing a co-culture with *Bacteroides thetaiotaomicron.* We have also successfully tested the co-cultivation of *M. smithii* with other known hydrogen-producing bacteria. Gas chromatographic tests showed that these strains produced hydrogen in different amounts and agar colonies of methanogens were obtained by co-culture with these bacteria and methane production was detected.

Conclusions

In this work, we propose a new approach to isolate and cultivate new strains of *M. smithii* by using a co-culture based technique that can facilitate and make available the isolation of new methanogenic archaea strains in clinical microbiology laboratories.

Introduction

The cultivation of methanogens is considered difficult and has required until recently the presence of highly specific equipment; both a source of hydrogen and the evacuation of oxygen (1). The absence of oxygen and the presence of hydrogen are required for the culture of methanogens. Recently, we have been able to show that anaerobes are aero-intolerant by culturing them in the presence of antioxidants that allowed culturing most of the anaerobic bacteria (2). The cultivation of some of these anaerobic bacteria generates hydrogen, especially for *Bacteroides,* as previously described (3). This particularity allowed us to develop a culture system for methanogens using a double chamber comprising a medium containing antioxidants, allowing the culture of anaerobes (4). An antibiotic cocktail was added to the agar medium for growing *Methanobrevibacter* to inhibit methanogen-associated bacteria in multi-microbial specimens such as stool specimens (4). This work was the subject of a publication that reported a series of new methanogen isolates (4). We performed in our laboratory the analysis of gut microbiota by culturomics using anaerobic host (5). We were recently surprised to discover by chance that one of the colonies identified on agar by MALDI-TOF MS (Matrix-Assisted Laser Desorption-Ionization Time-Of-Flight Mass Spectrometry) was *Methanobrevibacter smithii,* without having taken special conditions for its cultivation. This surprising discovery led us to believe that in this anaerobic chamber, which also contained cultures of anaerobic bacteria, the presence of hydrogen had been sufficient to allow the cultivation of *M. smithii*. In this work, we report the conditions that could allow the cultivation of *M. smithii* in an ordinary anaerobic chamber without the introduction of hydrogen.

Materials and Methods

Ethics and sample collection

A stool specimen was collected from a healthy 26-year-old Malian female living in France for the last three months. Prior to collection, the donor had given his informed consent and the study was validated by the Ethics Committee of the IHU Méditerranée Infection under number 2016-011. The fecal specimen was collected in a recipient including a small anaerobic generator bag GENbag anaer (bioMérieux) immediately introduced to eliminate oxygen. Thirty minutes after the sampling, the stool sample was transported to the anaerobic chamber where we proceeded to its culture.

Culture procedure

One gram of stool specimen was suspended in 2 mL of phosphate buffered saline (PBS) (Life Technologies, Carlsbad, CA, USA) and inoculated in an anaerobic blood culture bottle (Becton Dickinson, Pont de Claix, France) supplemented with 5% of a mircofiltered (0.2µm) rumen. After 72 hours of incubation at 37 ° C inside the anaerobic chamber, we carried out 10 decreasing serial dilutions using PBS, then 50µL were seeded on 5% sheep blood-enriched Columbia agar (bioMérieux, Marcy l'Etoile, France) (COS). Agar plates were then incubated at 37 ° C inside of the anaerobic chamber (Don Whitley Scientific, France). After 4 days of incubation, the growing colonies were subcultured onto sterile agar plates for purification. The COS agar plate was divided into 8 equal parts and each isolated colony was subcultured onto one part of the agar (Figure 1) and then incubated at 37 ° C for 96 hours.

Co-culture of *Methanobrevibacter smithii* and *Bacteroides thetaiotaomicron*

In order to co-cultivate *M. smithii* with *B. thetaiotaomicron* on solid medium, we divided the agar plats in two compartments, then we seeded *M. smithii* on one compartment and *B. thetaiotaomicron* on the other. The agar plates were then incubated for 72 hours at 37 ° C in a sealed plastic bag with an anaerobiosis generator GENbag anaer (bioMérieux). After 72 hours

of incubation, the gas atmosphere was analyzed by gas chromatography (Figure 1) and the growing colonies were identified by Maldi TOF-MS as previously described (5).

We also co-cultured *M. smithii* with *B. thetaiotaomicron* in liquid medium; an anaerobic blood culture flask containing *M. smithii* and *B. thetaiotaomicron* was prepared and incubated at 37 ° C for 5 days. Then, we proceeded to the measurement of hydrogen and methane contained in the blood culture flasks by mass spectrometry. The anaerobic blood culture flask inoculated with *M. smithii* alone was introduced as negative control.

Co-culture of *M. smithii* with hydrogen-producing bacteria

The co-culture of *M. smithii* with hydrogen-producing bacteria was reported in the anaerobic chamber on 5% sheep blood-enriched Columbia agar medium with the following bacterial species: *Bacteroides fragilis, Bacteroides vulgatus, Parabacteroides distasonis, Marseillibacter massiliensis*, *Allisonella histaminiformansas* and *Desulfovibrio piger*. The agar plates, after seeding the strains, were sealed in a plastic bag with anaerobic generator GENbag anaer (bioMérieux) and incubated at 37 ° C for 72 hours.

MALDI-TOF MS identification

MALDI-TOF MS (Matrix-Assisted Laser Desorption-Ionization Time-Of-Flight Mass Spectrometry) identification was adapted for isolated colonies as previously described (6). Isolated colonies were deposited in duplicate on a MALDI-TOF target for further analysis. Then, a Matrix solution enabled the ionization and desorption of the archaeal or bacterial colonies were deposited on it. A score above 1.9 was considered as proper identification (7).

Hydrogen and methane measurements

Hydrogen and methane were measured using a Clarus 580 gas chromatography system (Perkin Elmer, Villebon-sur-Yvette, France). 100 µL of gas was sampled from Blood culture bottle or airtight bags with a gastight syringe, then directly injected (split 10 mL/min) onto a Shincarbon ST 80/100 micropacked column (2 m x 0.53 mm; Restek, Lisses, France). Injector

and oven were maintained at 110 and 70 ° C respectively. Gases were eluted using either Helium or Argon depending on the compound detected by a Thermal Conductivity Detector (TCD) maintained at 100 ° C. Hydrogen was detected in the negative polarity at 40 mA using Argon as elution/reference gas, whereas methane was detected in the positive polarity at 160 mA using Helium. Data recording and processing was performed using Totalchrom 6.3.2 (Perkin Elmer). A standard gas mixture containing 25 % of hydrogen, nitrogen, methane and carbon dioxyde (Air Products, Allentown, USA) was used to estimate the amounts of gases.

Results

Isolation procedure

Here, a co-culture based procedure was used to isolate and cultivate *M. smithii* from the stool specimen of a 26-year-old healthy Malian female.

The anaerobic Blood culture bottle inoculated by the stool specimen was methane-positive after 72 hours of incubation at 37 ° C. Then, after serial dilutions, colonies appeared on the agar medium after four days of incubation at 37 ° C in the anaerobic chamber. The growing colonies subcultured onto sterile agar plates were strewn with numerous microcolonies that could not be distinguished from each other with the naked eye (Figure 1). The purification step (Figure 1) allowed the isolation of one archaeal species and different bacterial species including: *Methanobrevibacter smithii, Allisonella histaminiformans, Marseillibacter massiliensis, Bacteroides fragilis, Bacteroides vulgatus, Parabacteroides distasonis* and *Desulfovibrio piger*. These bacteria all grew on the same plate as *M. smithii* come from the same sample.

Co-culture of *Methanobrevibacter smithii* and *Bacteroides thetaiotaomicron*

Here, *B. thetaiotaomicron*, was used as hydrogen generator required for the growth of *M. smithii* as previously described (4). After 72 hours of incubation, colonies grew on the two agar plates compartments. Analysis of the gas atmosphere by gas chromatography revealed

the presence of methane, characteristic of methanogenic archaea growth (Figure 1). Identification by MALDI-TOF MS then confirmed the authenticity of the two cultivated microorganisms. *M. smithii* colonies were about 0.5 µm in diameter and ocher (Figure 1). Methane was detected in the blood culture bottle when *M. smithii* was co-cultured with *B. thetaiotaomicron*. The quantity of detected methane increased if microfiltred rumen was added (data not shown). No methane was detected when *M. smithii* was cultivated alone in a blood culture bottle.

Co-culture of *M. smithii* with hydrogen-producing bacteria

In order to determine which strain was able to induce the growth of *M. smithii*, each of these strains (*Bacteroides fragilis, Bacteroides vulgatus, Parabacteroides distasonis, Desulfovibrio piger Marseillibacter massiliensis* and *Allisonella histaminiformans)* was subcultured on agar plate with *M. smithii* using the same method as the co-culture with *B. thetaiotaomicron*. Then, the methane and hydrogen production was measured by gas chromatography and the authenticity of the growing colonies was confirmed by MALDI-TOF MS. Growth was observed after three days of incubation at 37 ° C when *M. smithii* was co-cultivated with *B. fragilis, B. vulgatus, P. distasonis* and *D. piger.* Methane was detected by gas chromatography, and represented a production of approximately 50 to 100 ppm (Figure 2). No growth was observed when *M. smithii* was co-cultured with *M. massiliensis* and *A. histaminiformans*.

Discussion

Here, we successfully cultivated *M. smithii* in liquid enrichment and then in pure culture on solid medium without the need for an external supply of hydrogen. This is due to the effectiveness of a culture technique based on a co-culture carried out between a hydrogen-consuming methanogenic strain (*M. smithii*) and a hydrogen-producing bacterium (*B. thetaiotaomicron*). *M. smithii* is known to colonize 97.4% of the human population (8). After

an expected culture of *M. smithii* on agar medium without any addition of hydrogen, we successfully cultivated this methanogenic archaea in a liquid medium by culture enrichment of stool specimen collected from a healthy donor, to which we added *B. thetaiotaomicron* as hydrogen-generating bacterium. This enrichment was methane-positive after three days of incubation, indicating the growth of methanogenic archaea (9). The isolation in pure culture was then successfully obtained on agar medium still in co-culture with *B. thetaiotaomicron.* Subsequently, we were able to subculture this isolate in co-culture with other known hydrogen-producing bacteria isolated at the same time and on the same agar plate as *M. smithii*. We then tested the effect of each of these strains on the growth of *M. smithii* by performing co-culture on solid medium. Growth of *M. smithii* was observed with each of these strains and gas chromatography measurements showed that these strains produced hydrogen. This gas was detected in different amounts ranging from 0 to 75 ppm (Figure 3). Viability and growth were confirmed by observing colony formation on solid medium and methane production was measured gas chromatography.

Culture-based techniques for the identification of methanogenic archaea mainly use the culture technique in Hungate tubes developed by Hungate et al 1969 (2). This technique remains fastidious and not accessible to some clinical microbiology laboratories that do not have the necessary equipment for its application despite evidence of presence of archaea in pathologies such as brain abscesses, paravertebral muscle abscess or periodontis as recently demonstrated (10-12). Recently, we have developed in our laboratory a new culture technique in a double chamber, where methanogenic archaea are co-cultured with *Bacteroides thetaiotaomicron* which produces the hydrogen necessary for their growth (4). This technique subsequently allowed us to isolate a large number of *M. smithii* strains from different samples collected from different human mucosae (oral cavity, gut and vagina). In this work, and thanks to the acquisition of a new anaerobic chamber in our laboratory, we propose a new

190 approach to isolate and cultivate *M. smithii* isolates. The simplicity of this technique and the
191 few means necessary to put it into practice make the cultivation of methanogenic archaea
192 within the reach of all clinical microbiology laboratories that do not have specific equipment
193 enabling them to study methanogenic archaea.

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200 **Conflict of Interest**

201 There is no conflict of interest

202

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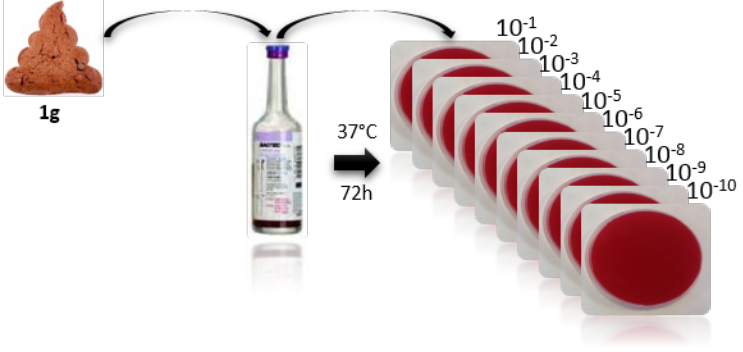
Figures legends

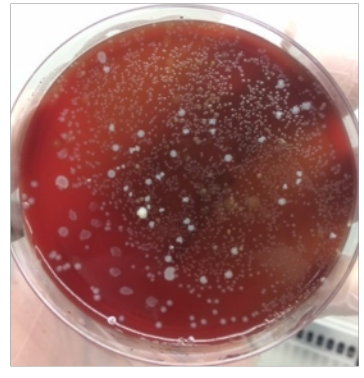
Figure 1: Illustration of the different steps of the isolation procedure and the cultivation of *M. smithii* by co-culturing with *B. thetaiotaomicron.*

Figure 2: Methane measurements by gas chromatography. Each plot represents the gas phase from the co-culture of *M. smithii* with: A, *P. distasonis*; B, *B. fragilis*; C, *B. vulgates*; D, *D. piger*.

Figure 3: Hydrogen measurements by gas chromatography. Each plot represents the gas phase from the co-culture of *M. smithii* with: A, *P. distasonis*; B, *B. fragilis*; C, *B. vulgates*; D, *D. piger*.



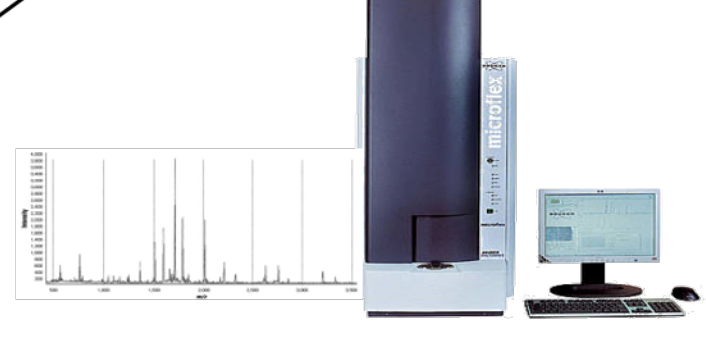
Incubation for
4 days



37°C
96h

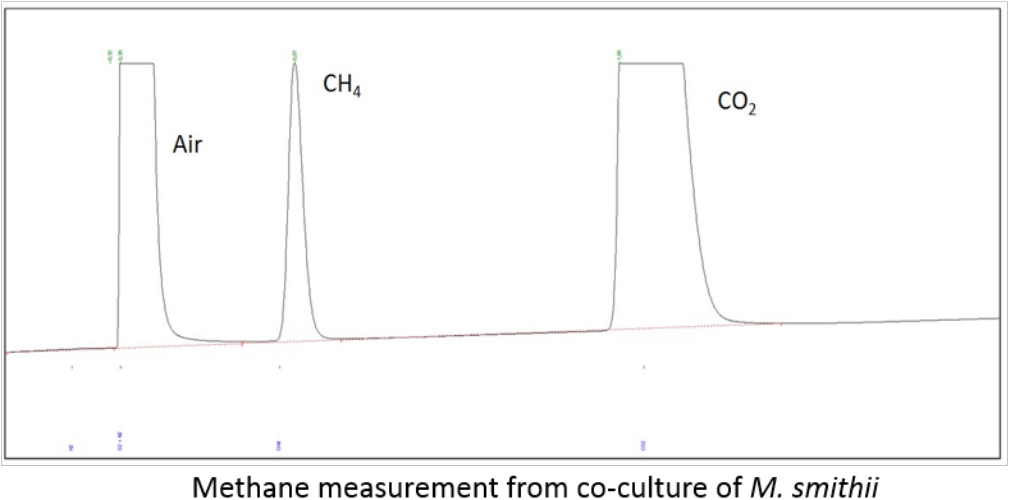
Colonies purification

MALDI-TOF (Bruker)

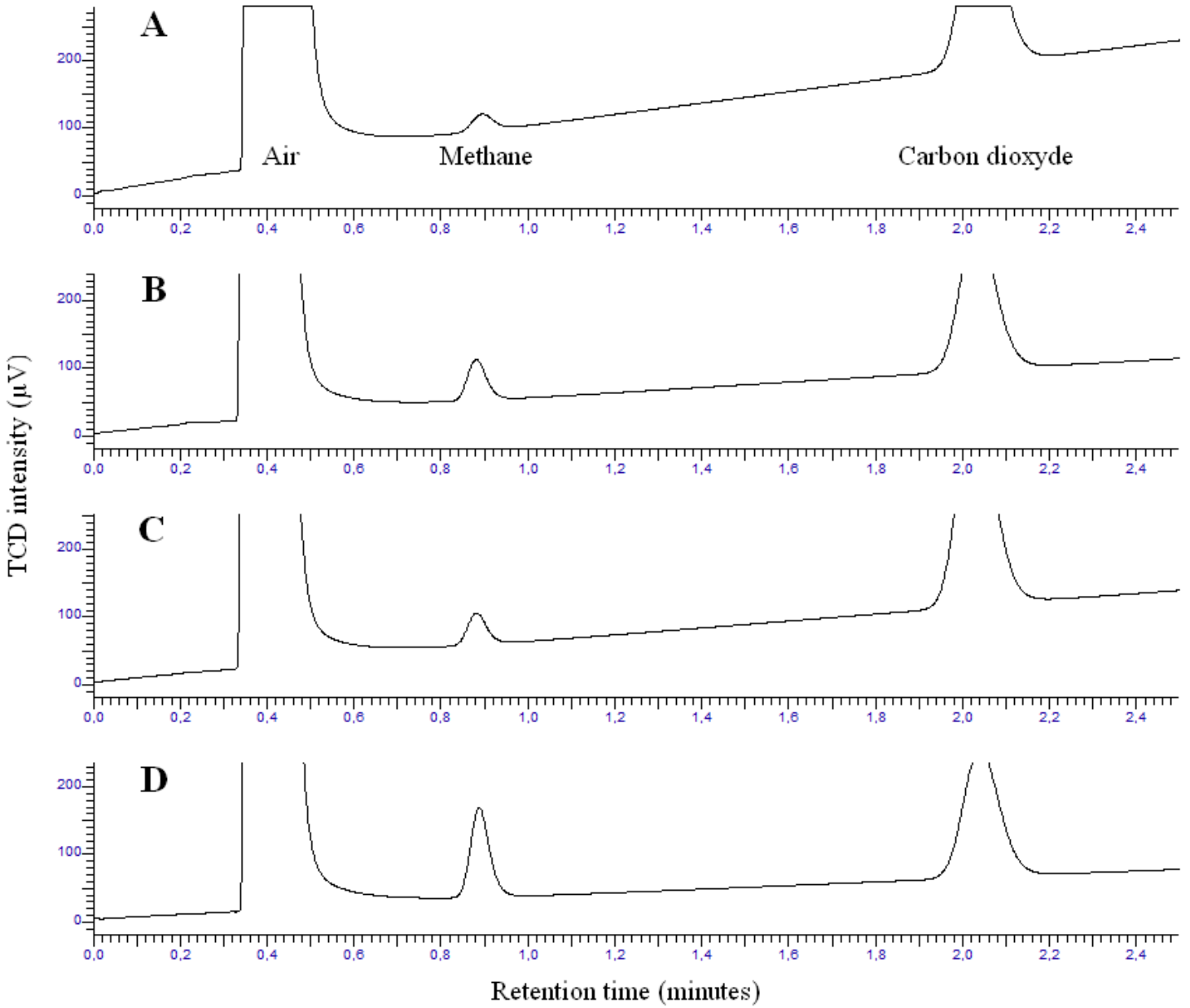


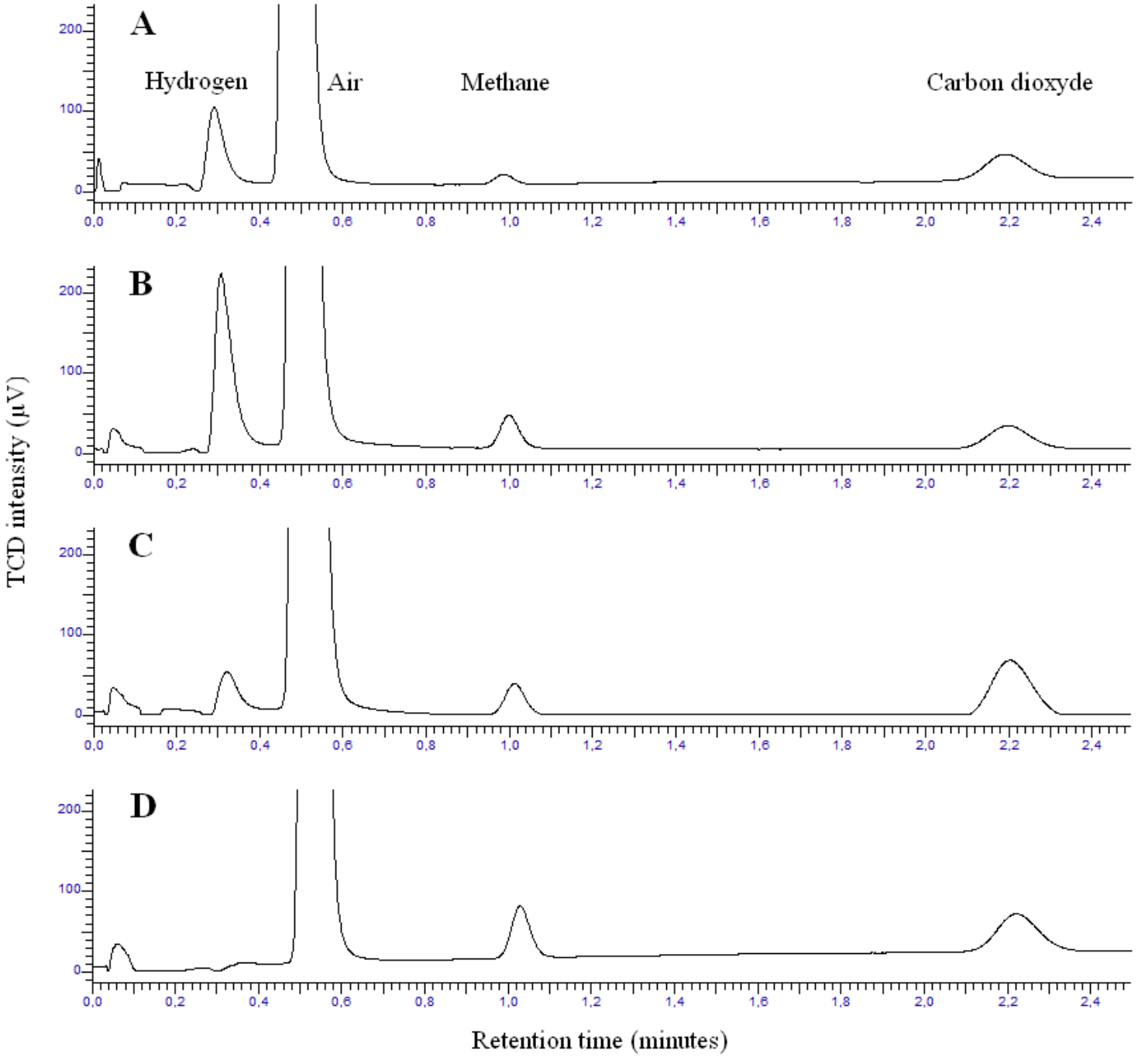
MALDI-TOF MS-based identification

Subculuring *M. smithii*
by co-culture with *B. thetaiotaomicron*



Methane measurement from co-culture of *M. smithii*
and *B. thetaiotaomicron*


A
B
C
D
Air
Methane
Carbon dioxyde
TCD intensity (µV)
Retention time (minutes)
0
100
200
0,0
0,2
0,4
0,6
0,8
1,0
1,2
1,4
1,6
1,8
2,0
2,2
2,4


A
Hydrogen
Air
Methane
Carbon dioxyde
B
C
D
TCD intensity (µV)
Retention time (minutes)