academicJournals

Vol. 7(49), pp. 5546-5549, 11 December, 2013
DOI: 10.5897/AJMR2013.2356
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Study of the distribution of *Megasphaera micronuciformis* in oral cavities of the Japanese by species-specific polymerase chain reaction (PCR) assay

Ran Murayama, Shinko Abe, Yuji Hasegawa, Taku Inoue, Kenji Tanaka, Yukiko Bando, Kazumasa Tonooka, Naoshi Fujimoto, Masaharu Suzuki and Akihiro Ohnishi*

Department of Fermentation Science, Faculty of Applied Bio-Science, Tokyo University of Agriculture, 1-1 Sakuragaoka 1-chome, Setagaya-ku, Tokyo 156-8502, Japan.

Accepted 6 November, 2013

***Megasphaera micronuciformis* is an anaerobic microbe isolated from humans. However, since the microbe is strictly anaerobic, its cultivation requires complicated facilities, making detection costly. For rapid, inexpensive detection and identification of *M. micronuciformis* in the clinical setting, a new technique is necessary. This study aimed to develop a species-specific PCR primer set for the detection of *M. micronuciformis*. A ribosomal DNA-specific PCR primer Mm2F was designed for *M. micronuciformis*. Analytical specificity data showed that the PCR primer set Mm2F/Mega-X produced amplicons from *M. micronuciformis* but not from the other species tested, including 4 *Megasphaera* species and representative related species. Of the 52 oral samples from Japanese subjects evaluated in our study, 71% were positive for *M. micronuciformis*, suggesting the likelihood that *M. micronuciformis* is widely distributed in the oral cavity of the Japanese population.**

Key words: *Megasphaera micronuciformis*, specific polymerase chain reaction (PCR), Mm2F, oral bacteria.

INTRODUCTION

Megasphaera micronuciformis is a Gram-negative, anaerobic microorganism that was first isolated from a liver abscess and pus sample in adult women (Marchandin et al., 2003). It belongs to the phylum Firmicutes and genus *Megasphaera,* which includes *Megasphaera cerevisiae*, *Megasphaera elsdenii*, *Megasphaera paucivorans* and *Megasphaera sueciensis* (Haikara and Helander, 2006; Vos et al., 2009; Ohnishi et al., 2010). Although *Megasphaera* species are commonly found in contaminated foods and the rumen, *M. micronuciformis* has been detected only in clinical specimens, including vaginal and oral samples (Marchandin et al., 2003; Oakley et al., 2008; Preza et al., 2009). Despite *M. micronuciformis* being associated with oral and vaginal infections, few studies have investigated their main habitat and behavior. Since its culture is difficult (Kumar et al., 2005), the abovementioned characteristics must be investigated using culture-independent methodologies such as cloning, microarray and pyro sequencing (Riggio et al., 2008; Preza et al., 2009; Guss et al., 2010).

However, these methodologies require special devices and are very expensive or time consuming. Although several species-specific detection methods for other *Megasphaera* species are available, no such methods have been developed for *M. micronuciformis* detection (Satokari et al., 1998; IIJIMA et al., 2008; Juvonen et al., 2008). Since an effective tool for *M. micronuciformis* detection is necessary, we aimed to develop a polymerase

*Corresponding author. E-mail: a1ohnish@nodai.ac.jp. Tel: +81-3-5477-2387. Fax: +81-3-5477-2287.

chain reaction (PCR) primer set for use in rapid *M. micronuciformis* detection.

MATERIALS AND METHODS

Bacterial strains

All strains for this study (Table 1) were obtained from a culture collection of prokaryotes and handled in an anaerobic glove box (ANX-1; Hirasawa; Japan) with an N_2-CO_2-H_2 atmosphere (85:10:5, v/v/v), and were cultivated using the recommended medium (DSMZ medium 104) and conditions (Ohnishi et al., 2011b).

Primer design

Sequences were obtained from the National Center for Biotechnology Information website (http://www.ncbi.nlm. nih.gov/). The 16S rRNA gene sequences of *M. micronuciformis* were aligned with each other and with sequences of 13 closely related species (Table 1), using Clustal X (version 1.83; www-igbmc.u-strasbg.fr) (Thompson et al., 1997). Thereafter, a search for *M. micronuciformis*-specific primer-binding sites was performed. The specificities of the potential primer sequences were tested *in silico* by using the basic local alignment search tool (BLAST; www.ncbi.nlm.nih.gov/BLAST/), allowing us to design a specific primer for *M. micronuciformis*; this primer was termed Mm2F, 5′-CTTGTGGTTAATACCCATAAGAA-3′ (*Escherichia coli* genome, position 401–423). Comparison of the primer sequence against GenBank/EMBL/DDBJ databases showed that the primer was not complementary to DNA from any non-target microbe. The primer sequence was typical of *M. micronuciformis*. Mega-X, a primer specific to genus *Megasphaera*, was used as the reverse primer (Ohnishi et al., 2011b).

Polymerase chain reaction

The PCR was set up in a 50-µl reaction volume containing 25 µl of GoTaq Hot Start Green Master Mix (Promega), 1 µM of each primer set (Mm2F/Mega-X for *M. micronuciformis* -specific detection, or 20F/1540R for all bacteria (Ohnishi et al., 2011a), and 1 µl of DNA solution. PCR was performed using the PTC-200 DNA Engine Thermal Cycler (Bio-Rad). The amplification profile was 94°C for 2.5 min followed by 30 cycles of 15 s at 94°C, 30 s at annealing temperature, and 30 s at 72°C. The last extension step was for 7 min at 72°C. The annealing temperature is 58°C for *M. micronuciformis* -specific PCR using the primer set Mm2F/Mega-X, and 55°C for other bacteria using the primer set 20F/1540R. A negative control without template DNA was included in all runs. Five microliters of the PCR products was separated on the basis of size by 1.5% agarose gel electrophoresis in 1× TAE buffer and then stained with ethidium bromide and detected under UV light with a transilluminator (AE- 6943V-FX; ATTO). The λ*Hin*dIII digest (Takara) was used as the molecular size marker.

DNA extraction

For DNA extraction, 1 ml of the cell suspension was centrifuged at 10,000 *g* for 5 min at 4°C, and the pellet was resuspended in 1 ml TE buffer. This process was repeated twice for washing. The cells were boiled for 10 min, and the debris was removed by centrifugation at 10,000 *g* for 10 min at 4°C. Thereafter, 1 µl of the supernatant was used for PCR analysis according to a previous study (Ohnishi et al., 2011b).

RESULTS AND DISCUSSION

The universal primer set 20F/1540R was used as the positive control (Ohnishi et al., 2011b). The analytical specificity of the constructed primer set Mm2F/Mega-X was evaluated using PCR. Genomic DNA extracted from strains representing 14 related bacterial species was used as template DNA. A PCR product of suitable size (approximately 1500 bp) was obtained as the positive reaction from all samples by using the universal primer set 20F/1540R. PCR products of approximately 900 bp were obtained using Mm2F/Mega-X and amplified from only *M. micronuciformis* using optimized annealing conditions (58°C). Using these methods, we generated a primer set Mm2F/Mega-X that was specific to *M. micronuciformis* (Table 1).

To test the analytical sensitivity of PCR, the detection limit was defined as the lowest concentration of *M. micronuciformis* genomic DNA that could be detected by PCR. Genomic DNA was obtained using a FastPure DNA Kit (Takara, Japan), according to the manufacturer's instructions. The concentration of extracted DNA was measured by NanoDrop ND-1000 (LMS, Japan). The analytical sensitivity ranged from 100 ng to 0.1 fg using 10-fold dilutions. The primer set Mm2F/Mega-X could detect 100 fg of the genomic DNA of *M. micronuciformis.*

To survey the distribution of *M. micronuciformis*, which is sometimes detected in the human oral cavity, saliva samples from 52 Japanese subjects were obtained. Saliva samples were obtained using a buccal brush attached to a Master Amp Buccal Swab DNA Extraction Kit (Epicentre Technologies, USA), according to the manufacturer's instructions. The DNA yield usually falls within the range of 2 to 8 ng/µl. The brushes were swirled in 1.5-ml tubes containing 0.5 ml MasterAmp Buccal Swab DNA Extraction Solution to remove the adhering bacteria. Each sample was vortex-mixed for 10 s and incubated at 60°C for 30 min, then vortex-mixed for 15 s and incubated at 98°C for 8 min, and again vortex-mixed for 15 s. This process was repeated twice for lysis. The tubes were placed on ice briefly to reduce the temperature, and the cell debris was removed by centrifugation at 10,000 *g* for 10 min at 4°C. Finally, 5 µl of the supernatant of each sample was used for PCR.

When the universal primer set 20F/1540R was used, all the samples showed positive PCR results. When the *M. micronuciformis*-specific primer set Mm2F/Mega-X was used, 37 samples were positive and 15 were negative. Thirty PCR products were selected randomly and sequenced using the primer Mm2F. Sequencing and phylogenetic analysis were performed using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM Model 310 genetic analyzer (Applied Biosystems) (Figure 1). Nucleotide

Table 1. Results of the specificity tests for the *M. micronuciformis*-specific primer set.

Species	Strain	Reaction with primer set[a]	
		Mm2F/Mega-X	20F/1540R
Anaeroglobus geminatus	CCUG 44773^T	-	+
Anaerovibrio lipolytica	DSM 3074^T	-	+
Megasphaera cerevisiae	DSM 20462^T	-	+
Megasphaera elsdenii	DSM 20460^T	-	+
Megasphaera micronuciformis	DSM 17226^T	+	+
Megasphaera paucivorans	DSM 16981^T	-	+
Megasphaera sueciensis	DSM 17042^T	-	+
Mitsuokella jalaludinii	DSM 13811^T	-	+
Pectinatus cerevisiiphilus	DSM 20467^T	-	+
Pectinatus frisingensis	DSM 6306^T	-	+
Pectinatus haikarae	DSM 16980^T	-	+
Schwartzia succinivorans	DSM 10502^T	-	+
Selenomonas ruminantium	DSM 2872^T	-	+
Veillonella atypica	DSM 20739^T	-	+

[a]+, Positive reaction; -, negative reaction.

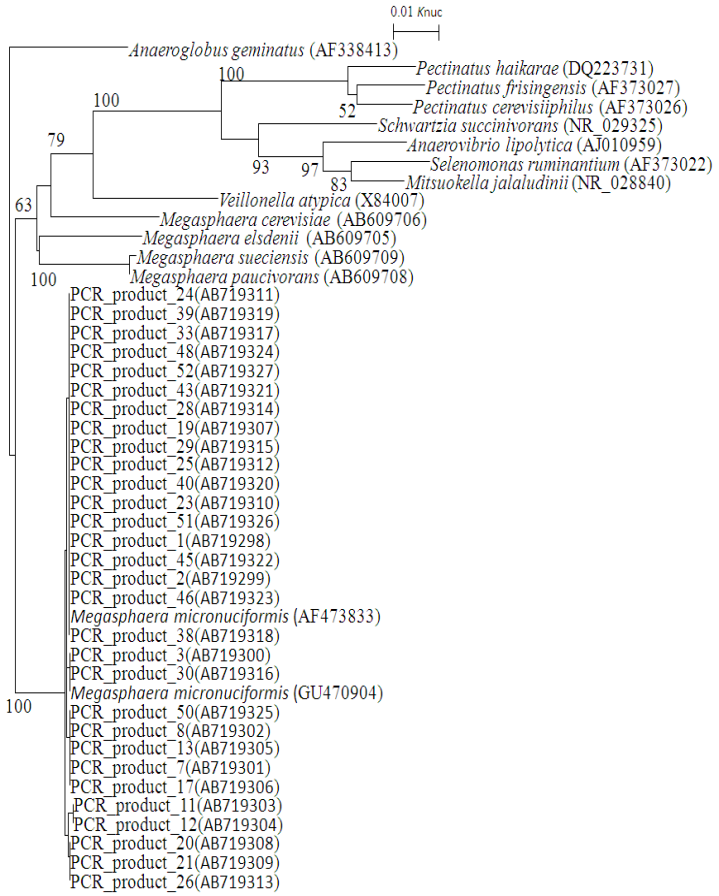

Figure 1. Phylogenetic relationship between the isolates and related bacteria based on 16S DNA sequences. The phylogenetic tree, which was constructed using the neighbor-joining method, is based on the comparison of approximately 600 nucleotides of the 16S rRNA gene. Bootstrap values, expressed as a percentage of 1000 replicates, are shown at the branching points; only values ≥50% are shown.

sequences were compared with known sequences by using BLAST to search for close evolutionary relatives in the GenBank database. The neighbor-joining method (Saitou and Nei, 1987) was used for constructing a phylogenetic tree. http://www.ncbi.nlm.nih.gov) (Altschul et al., 1997).

All the sequenced PCR products were identified as *M. micronuciformis* (similarity, 99-100%; Figure 1). Our results show that the primer set Mm2F/Mega-X is a useful tool for rapid and specific detection of *M. micronuciformis* from saliva samples. Moreover, approximately 71% of healthy Japanese individuals tested positive for *M. micronuciformis* in their saliva; therefore, it is likely that *M. micronuciformis* is widely distributed as an indigenous bacterium in the saliva of Japanese individuals.

Thus far, very few cells of *M. micronuciformis* have been detected using culture-independent methodologies. Riggio et al. (2008) detected 2 sequences of *M. micronuciformis* in 553 clones (approximately 0.4%) obtained from 20 subjects with halitosis. Likewise, Fishman (2009) reported that 0.5% of 2,854 clones obtained from plaques and saliva samples of 30 subjects were *M. micronuciformis*. Kumar et al. (2005) reported that 0.6% of 4,500 clones obtained from plaques in 30 subjects were *M. micronuciformis*. It was also shown that the prevalence of the genus *Megasphaera*, including *M. micronuciformis*, was elevated in cases of periodontitis. In addition, sequences sharing a high level of similarity with that of *M. micronuciformis* during BLAST searches had previously been deposited as clones detected during cultivation in independent studies of the human vaginal epithelium microbiota and the skin microbiome, as well as in sputum samples from adult patients with cystic fibrosis, biofilms of extubated endotracheal tubes from intensive care unit patients, skin microbiomes of children with atopic dermatitis, intestinal biopsies from patients with diverse inflammatory bowel diseases and microbiota associated with dental caries (Guss et al., 2010). These data suggest that the association between these diseases and *M. micronuciformis* is an important topic for future study. However, these detection methods used were time consuming and might have low sensitivity for *M. micronuciformis*. *M. micronuciformis*-specific PCR supported rapid detection of *M. micronuciformis* and revealed its widespread distribution in our subjects.

In conclusion, our specific PCR method is useful for the rapid detection and identification of *M. micronuciformis* from oral cavities. This method may offer substantial insights into the distribution of *M. micronuciformis* in clinical specimens.

REFERENCES

Altschul S, Madde T, Schaffer A, Zhang J, Zhang Z, Miller W, Lipman D (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25:3389.

Guss AM, Roeselers G, Newton ILG, Young CR, Klepac-Ceraj V, Lory S, Cavanaugh CM (2010). Phylogenetic and metabolic diversity of bacteria associated with cystic fibrosis. The ISME Journal 5:20-29.

Haikara A, Helander I. (2006). *Pectinatus*, *Megasphaera* and *Zymophilus*. Prokaryotes 4:965-981.

IIJIMA K, Asano S, Suzuki K, Ogat T, Kitagawa Y (2008). Modified multiplex PCR methods for comprehensive detection of *Pectinatus* and beer-spoilage cocci. Biosci. Biotechnol. Biochem. 72:2764-2766.

Juvonen R, Koivula T, Haikara A (2008). Group-specific PCR-RFLP and real-time PCR methods for detection and tentative discrimination of strictly anaerobic beer-spoilage bacteria of the class *Clostridia*. Int. J. Food Microbiol. 125:62-169.

Kumar PS, Griffen AL, Moeschberger ML, Leys EJ (2005). Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. J. Clin. Microbiol. 43:3944.

Marchandin H, Jumas-Bilak E, Gay B, Teyssier C, Jean-Pierre H, de Buochberg MS, Carrière C, Carlier JP (2003). Phylogenetic analysis of some Sporomusa sub-branch members isolated from human clinical specimens: description of *Megasphaera micronuciformis* sp. nov. Int. J. Syst. Evol. Microbiol. 53:547.

Oakley BB, Fiedler TL, Marrazzo JM, Fredricks DN (2008). The diversity of human vaginal bacterial communities and their association with clinically-defined bacterial vaginosis. Appl. Environ. Microbiol. 74(15):4898-4909.

Ohnishi A, Abe S, Nashirozawa S, Shimada S, Fujimoto N, Suzuki M (2011b). Development of a 16S rRNA gene primer and PCR-restriction fragment length polymorphism method for rapid detection of members of the genus *Megasphaera* and species-level identification. Appl. Environ. Microbiol. 77:5533-5535.

Ohnishi A, Bando Y, Fujimoto N, Suzuki M (2010). Development of a simple bio-hydrogen production system through dark fermentation by using unique microflora. Int. J. Hydrog. Energy. 35:8544-8553.

Ohnishi A, Nagano A, Fujimoto N, Suzuki M (2011a). Phylogenetic and physiological characterization of mesophilic and thermophilic bacteria from a sewage sludge composting process in Sapporo, Japan. World J. Microbiol. Biotechnol. 27:333-340.

Preza D, Olsen I, Willumsen T, Grinde B, Paster BJ (2009). Diversity and site-specificity of the oral microflora in the elderly. Eur J Clin Microbiol Infect Dis 28:1033-1040.

Riggio M, Lennon A, Rolph H, Hodge P, Donaldson A, Maxwell A, Bagg J (2008). Molecular identification of bacteria on the tongue dorsum of subjects with and without halitosis. Oral Dis 14:251-258.

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406.

Satokari R, Juvonen R, Mallison K, von Wright A, Haikara A (1998). Detection of beer spoilage bacteria *Megasphaera* and *Pectinatus* by polymerase chain reaction and colorimetric microplate hybridization. Int. J. Food Microbiol. 45:119-127.

Thompson J, Gibson T, Plewniak F, Jeanmougin F, Higgins D (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876.

Vos P, Garrity G, Jones D, Krieg N, Ludwig W, Rainey F et al. (2009). Bergey's manual of systematic bacteriology 2nd edn, vol. 3: The Firmicutes.