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Characterization of bacterial population of raw milk from bovine mastitis by culture-independent PCR-DGGE method

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Abstract:

Culture-independent PCR-DGGE fingerprinting was used to reveal the bacterial composition and diversity associated with raw milk of mastitis cows from Hokkaido, Japan for the first time. All the mastitis milk samples were diagnosed as solely infection by Coliforms using the classical microbiological method based on on-farm culturing. Our results revealed that the bovine mastitis-associated bacteria were host-specific because community structure varied between each sample. *Klebsiella pseudomoniae*, *Lactococcus lactis* *Staphylococcus aureus* and *Escherichia sp* were found to be the widely distributed species. Furthermore, more than one mastitis-causing pathogen was found to be present in some mastitis samples. These pathogens may not only act as etiology agents but also play a role in disrupting the natural microbial ecology in mastitis bovine. This finding highlights the limitation of the traditional identification and characterization strategy. Therefore, it is suggested that the methodology applied in this study might be a valuable addition to mastitis control and prevention.

Key words: Bovine mastitis; PCR-DGGE; raw milk; Microbial population,

1. Introduction

Bovine mastitis is an infection of mammary gland which exists on every dairy farm. This world-wide problem has caused the dairy industry to lose 100 million dollars annually in Hokkaido, one of Japan's milk production areas [1]. These losses are mainly due to reduced milk production and quality, discarded milk and also medication of diseased cow. Mastitis can be caused by over 150 different contagious or environmental microorganisms which can be classified into five groups: gram-positive cocci, gram-negative bacteria (coliforms), *Corynebacterium*, *Mycoplasma* and others, which include *Nocardia*, *Prototheca*, and yeast [2].

Over the years, many studies have dealt with the diagnosis of bovine mastitis, most of which were frequently made on the sole basis of clinical signs or indirect measurements. e.g. somatic cell count (SCC) [3] like the golden standard California Mastitis Test (CMT) and electrical conductivity (EC) measurement of the milk using a hand-held meter [4]. However, none of them have revealed etiological agent to provide information on disease prevention, treatment and control, therefore classical microbiological methods have been introduced as routine identification method [5] but they are tedious and allow only a partial succession of pathogens or bacterial microflora, as its inherent bias is that only 1% of all microorganisms are able to grow fairly rapid in pure culture [6], what's more, some bacteria can not be readily differentiated by current biochemical tests, e.g. *Streptococcus uberis* and *Streptococcus*

parauberis [7].

Culture-independent molecular techniques may provide a more accurate scheme. Previous surveys have been mainly based on PCR by using specific primers for specific genes in a given bacterial species, which makes them biased to limited common pathogens, e.g. *Staphylococci* [8] and *Streptococci* [9]. Furthermore, the detection of the total predominant bacterial population at a time still cannot be realized by these surveys. Denaturing gradient gel electrophoresis (DGGE) [10], which separates amplified partial 16S rDNA fragments of each bacteria based on differences in the GC content and distribution in each fragment, has been developed and widely applied to evaluate the microbial diversity of several environments [11, 12, 13]. The ability of DGGE not only to provide a direct visual image of the bacterial diversity in the sample but also to allow recovery of DNA sequence information from gel bands [10], has proven it to be a very reliable method for studying the variation of dominant bacteria and for characterization of complex microbial populations [14].

In this study, we employed PCR-DGGE approach to characterize the microbial population in raw milk from cows suffering mastitis. In addition, the detected pathogens were also compared with those obtained from classical microbiological method. To our knowledge, this is the first study applying molecular techniques to contribute to an extended knowledge of etiological agent for bovine mastitis.

2. Materials and methods

2.1 Milk sampling and etiological agent identification by classical microbiological method

Raw milk samples were kindly provided by Rakuno Gakuen University in Hokkaido. Four of them (M1-M4) were aseptically collected from cows afflicted with mastitis with different severity, while sample H1 was from a healthy cow. Table 1 gives detailed information about these samples and the corresponding cows. All samples were kept on ice until their transport to the lab, and then they were stored at -20 ˚C until further processing.

Before transportation, on-farm milking culturing was performed to clarify the major and minor mastitis pathogens existing in these samples [5]. Briefly, the fresh milk was plated onto selective agar including Blood agar TKT agar and MacConkey agar. To further identify organisms, Gram Stain and other tests like morphology, catalase, oxidase and coagulase assays were used if it is necessary. All the four mastitis milk were diagnosed as only Coliforms infected samples, while no bacterium has been identified in H1.

2.2 Direct DNA extraction from raw milk sample and PCR-DGGE

0.5 ml of raw milk sample was mixed with an equal volume of 20% ethanol solution to reduce the interference caused by some components (e.g. lipid) in milk, and the mixtures were centrifuged at 3000×g for 5min, then the bacterial DNA was extracted from pellets using a DNA extraction kit (ISOFECAL, Nippon Gene Co., Ltd, Tokyo, Japan) according to the manufacturer's instructions. Primers 341f with GC-clamp and 907r (Table 2) were used to amplify V3-V5 regions of the bacterial 16S rRNA [15, 16]. Amplification was performed by

using a modified touch-down PCR program [10] and Ampdirect® Plus PCR buffer system (Shimadzu Co., Ltd, Kyoto, Japan). PCR products of approximately 606bp size were confirmed by visualization after electrophoresis through a 2% (w/v) agarose gel and then stored at -20 ˚C.

DGGE analysis of amplicons was performed as described by Sangtian Yan et al. [17] using a DCode universal mutation detection system (Bio-Rad, USA). Polyacrylamide gels (16cm×16cm× 1mm) consist of 6% (v/v) polyacrylamide (37.5:1, acrylamid/bisacrylamide) in 1×TAE buffer with a linear 30% to 70% denaturing gradient (100% denaturant contains 7 M urea and 40% formaide). Electrophoresis was performed at 60 ℃ for 14 h at 110 V. The gel was stained with SYBR Gold (Invitrogen; diluted 1:10000 in 1×TAE) and photographed.

2.3 Sequencing of DGGE bands

Predominant Bands were excised from community DGGE gels and purified by using the freeze and thaw method as described previously [17]. 2 μl of purified DNA was used as template and reamplified using primers 341 and 907 (Table 2) under the same PCR conditions described above. PCR amplicons were purified and ligated into the pGEM-T cloning vector (Promega Co., Ltd, Madion, USA). Ligated DNA was then transformed into *E.coli.* XL-1 Blue competent cells. The recombinant white colonies were screened for inserts of the correct size using primers pGEM-T seq+ and pGEM-T seq- (Table 2). Amplicons of the correct size were then subjected to Restriction Fragment Length Polymorphism (RFLP) analysis by using

MspI and RsaI. One representative clone was chosen from each restriction digestion pattern group within each excised bands, and plasmid was extracted from these representative clones by QIAprep. Spin Miniprep. Kit (Qiagen Sceience, Maryland, USA), and then sequenced by Takara Bio (Takara Bio Co. Ltd, Tokyo, Japan). In order to compare the migration position of the inserted region in the representative clones with their original community profile, the sequenced plasmids were used as templates and the insert regions were amplified using GC-341f and 907r primers, and then analyzed by DGGE using the procedure described.

2.4 Phylogenetic analysis

Closest known relative species of the sequence data were determined by BLAST searches of both the NCBI Genbank database (http://www.ncbi.nlm.nih.gov/) and SEQENCE_MATCH by Ribosomal Database Project [18]. The multiple alignment and phylogenetic tree was made with CLUSTALX 2.0 by using neighborhood-joining method replicated 1000 times. Phylip 3.67 was used for the assessment of the phylogenetic tree. The sequences obtained in this study were deposited in GenBank under accession no. XXXXX to XXXXX

3. Results:

DNA was extracted from each raw milk sample and variable regions 3-5 of the 16S rRNA from samples M1-4 were amplified successfully. The community DGGE fingerprints of amplified partial 16S rDNA associated with these five raw milk samples was shown in Fig.1. For M1-4, the DGGE fingerprints exhibited good banding patterns. Among them, two samples displayed a relatively simple profile with five or less predominant bands although some faint bands were also observed (Fig.1 sample M3 and M4), and others displayed more than 8 predominant bands. However, the community patterns from different samples seemed to be host-specific. Amplification of 16S rDNA from healthy milk failed compared to amplification from mastitis milk samples using the GC-clamped primers [GC-clamped primers appear to reduce PCR efficiency].

In order to determine which bacterial group each band could be ascribed to, a total of 26 bands were excised from DGGE gels and sequenced, Analysis of the results showed that totally 31 bacteria were identified, most of the sequences had at least 98% similarity to reference strains found in the NCBI database, while one clone was most probably new genera with similarities of less than 95% (Table 3). Among the identified bacteria, nearly half of them were uncultivable, while some of the other bacteria were closely related to bacteria previously described in diary environments. These include some psychrotrophs (e.g. *Flavobacteriaceae and Chryseobacterium*) and LAB (e.g. *Lactococcus*). Psychrotrophs have long been

considered the cause for spoilage of raw milk and processed dairy products, because they are capable of growing at 7 ˚C or less, and some of their enzymes can still be active after pasteurization or other heat treatments thus leading to the degradation of milk components [19]

Each sample displayed a different bacterial composition. Raw milk sample M1 and M2 had the highest bacterial diversity among the tested samples, followed by sample M4, and sample M3. However, at species level, *K. pseudomoniae*, *L. lactis*, *S. aureus* and *Escherichia sp*, was the most widely distributed species, they were present in at least two samples. For, *L. lactis*, we observed that representing band in M2 was much stronger that in M1. Although DGGE is not a quantitative method, the density change of each band can be explained as a consequence of a change in the relative abundance of the same microbes in the microbial community [20], indicating that the concentration of *L.lactis* in M2 might be higher than that in M1. For the other widely distributed bacteria, *K. pseudomoniae* and *S. aureus* have been reported to be common mastitis-causing pathogens, other major pathogens were also found in only one sample including *Enterobacter. Species* (M1), *S. uberis* and *Corynebacterium glutamicum* (M4).

Fig.3 shows the phylogenetic relationships based on the sequence results. 31 bacteria derived from DGGE bands were divided into eight classes: *Bacilli*, *Flavobacteria*, *Unclassified Bacterocidetes, Sphingobacteria, Bacterocidetes, Actinobacteria,*

Alphaproteobacteria and Gammaproteobacteria. It was clear that *Gammaproteobacteria* was most diversified in the four Coliform-infected samples identified by classical microbiological method. However, it was interesting to find that the second most abundant bacteria were the *Bacilli* class which including contagious pathogenic *S. aureus*. Finally, we were not able to identify some minor bands because they could not be excised from the gels (too low intensity) or no amplification product was obtained from the excised fragment.

4. Discussion

The overall knowledge of the consortia community and the role of specific etiology agents of bovine mastitis remain poor, as most studies to date were constrained by the limitations of traditional microbiological techniques. Indeed, some important components may substantially escape the detection, particularly those at low relative abundance.

In the present study, 31 isolates obtained from four mastitis milk samples were identified at species level by a 16S rDNA-based PCR-DGGE approach. These isolates comprised specific bacterial pattern in individual sample, despite the fact that a small fraction of common bacteria were found, this provides the basis that microbial community differs from cow to cow. However, none of bacteria could be identified in healthy milk in the present study; we found the amplification of bacterial 16S rDNA genes from healthy milk was much more difficult than amplification from diseased samples, indicating that the overall bacterial abundance is lower in healthy milk. Increasing the bacterial abundance in healthy milk by concentrating large volumes of sample or improving the extraction procedure might be alternative ways [21], but this paper is intended to gain a better understanding of the microbial diversity and causal agents within mastitis milk. In fact, the relative easiness to amplify DNA from mastitis milk revealed that pathogenic bacteria might play a role in disrupting the natural microbial ecology. This might reinforce the recent studies that prior colonization of some pathogens in the teat canal of cow may have altered the protective properties of the teat canal thus render it more

susceptible to other bacteria's penetration followed by multiplication.[22].

In samples M1 and M2, *Lactococcus lactis* had been identified, such strains have been considered commensal species since they are capable of secreting antimicrobial substances and can even be used to prevent infection by mastitis-causing Gram-positive bacteria such as staphylococci [23].We assumed the concentration of *L.lactis* in M2 was higher in M1, actually, M2 was also the sample that no gram-positive pathogens were detected. This led to our hypothesis that the outgrowth of *L. lactis* might have inhibited the growth of some gram-positive pathogens such as staphylococci in sample M2.

Staphylococcus aureus is considered to be a major contagious organism that commonly produces long-lasting infections in bovine mastitis. Interestingly, it was found in two mastitic samples out of four in this study. Actually the detection of this species should always be thought an intramammary infection instead of a contamination in milk sample, and even small numbers of SA are sufficient to cause infection thus should not be overlooked [5]. In contrast, culturing had failed to identify this causative species because only Coliform has been diagnosed as represents of the etiological agent in all four mastitis milk. Other possible etiological agents that had escaped the classical method but were found by PCR-DGGE approach including *K. pneumoniae, S. ubris*, et.al. This finding leads to our suspicion that standard identification techniques might not be as reliable or accurate as needed. A statistical analysis have shown that bacteria may not be able to be isolated from up to half of the milk

samples by culturing [24]. In fact, to understand the mastitis better and to optimize the efficacy of disease treatment, it is important to do epidemiologic study of mastitis for clinicians, with better and accurate information improving the chances of a control and prevention program succeeding. Another interesting observation was that more than one pathogen had been found in some mastitis sample. Similar phenomena have been also reported by the others, Sabry A groups showed that mixed infection by *Klebsiella pneumoniae* and *E.coli* was more prevalent than single infection on both animal and quarter level [25], Hogan JS groups reveled that *Corynebacterium bovis* infection could increase the rate of the secondary infection toward bovine udder by *Streptococcus ubris* or some gram-negative pathogens [26]. All these suggest that the cause of an individual case of mastitis might be quite complicated than previously thought, if it is true, it might be useful to elucidate the reason why current antibiotic therapy toward bovine mastitis is inefficient.

Of the five tested samples, M2 and M4 were collected from the cows which showed the most severe mastitis symptoms. Although the highest bacterial diversity was found in sample M2, it is quite difficult to establish a relationship between the characteristics and composition of bacteria and the severity of mastitis. It is accepted that bacterial, environmental, management and cow factors may affect the occurrence and severity of mastitis. Indeed some reports have indicated that mastitis mainly depends on cow factors as shown by cases where cows were infected by the same species [27].

The PCR-DGGE technique was useful for the identification of a broad range of bacteria in raw milk samples from mastitic cows at a time, but the method also has some limitations. Based on our study, firstly, the co-migration of the partial 16S rDNA fragment amplified from different species occurred in some cases. For example, band M4-4 was assigned to an *Uncultured bacterium* clone or *Shigella flexneri.* Secondly, distinct bands in the gels did not necessarily correspond to different bacteria, e.g. band M2-3, M2-5(I) and M2-6 were all identified as *Lactococcus lactis*. These issues were also observed in other studies [28, 29]. By altering the resolution capability and denaturing condition of DGGE, one can improve the band separation. Other limitations associated with PCR-DGGE method such as potential biases related to extraction of community DNA, the PCR step, and other enzymatic reactions have also been reported repeatedly [30].

5. Conclusions

PCR-DGGE method described here gives an increasingly comprehensive and more precise picture of the bacterial populations associated with bovine mastitis milk. Although more studies are required, our results show every sample had its own unique bacteria profile. The attempts to identify causal agents within mastitis milk also demonstrate that currently used traditional culture techniques are not likely to be the best way. However efforts to improve DGGE technology's resolution are still needed although it is quite mature nowadays. The combination of DGGE with other molecular technologies such as direct cloning analysis of

16S rDNA and fluorescence in situ hybridization may lead to more accurate and robust analysis of the microbial communities.

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Figure legends

Fig.1. DGGE analysis of bacterial composition in raw milk samples collected from mastitis cows. The numbered bands were cut and sequenced.

Fig.2. Phylogenetic tree of the bacteria retrieved from bands in DGGE profile of each samples. The letter-number combination before the dash designates the tested sample; the number after the dash corresponds to the bands in Fig.1; the Roman numerals distinguish between different species from the same band. The scale bar length of 0.1 denotes the number of amino acid replacements per site validated with 1000 bootstraps. The number on the branches indicates the support proportion of each branch.

Table 1 Information on cows which provided raw milk samples

Cow No.	Calving times	CMT [a]	Condition of collected milk	Udder status	Age of cow
M1	2	+	Clotted	No	42 month
M2	2	+++	Clotted	Red udder	43 month
M3	3	++	Clotted	No	55 month
M4	3	+++	Clotted	Red udder	56 month
H1	3	_	_	No	57 month

[a] California Mastitis Test designation. Negative score denotes healthy cow with average SCC of 88000 cells/ml. Positive score indicates severity of mastitis as follows: +, subclinical mastitis with average SCC of 921000 cells/ml; ++, serious mastitis with average SCC of 2073000 cells/ml; +++, serious mastitis infection with average SCC of 3761000 cells/ml.

Table 2 Oligonucleotide primers used for PCR

Primer	Sequence(5'→3')
GC-341f	(GC clamp) *-CCTACGGGAGGCAGCAG
907r	CCGTCAATTCCTTTGAGTTT
pGEM-T seq+	ATTGGGCCCGACGTCGCATG
pGEM-T seq-	GGAGCTCTCCCATATGGTCG

*GC-clamp: GCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG

Table 3 Sequence and phylogenetic affiliation of the bacteria based on bands in the community DGGE gel of raw milk samples collected from mastitic cows

Band No.	Closest relative in GenBank database	Similarity (%)
Sample M1-1	*Lactococcus lactis isolate D29*	100%
M1-2(I)	*Staphylococcus aureus strain 185060 (VRSA)*	99%
M1-2(II)	*Uncultured Porphyromonadaceae bacterium clone EMP_O3*	97%
M1-3	*Uncultured bacterium clone 11-12*	100%
M1-4	*Uncultured bacterium clone 11-11*	99%
M1-5(I)	*Enterobacter sp. Nj-68*	98%
M1-5(II)	*Uncultured bacterium clone 75-ORF02*	91%
M1-6	*Klebsiella sp. TS34*	99%
M1-7	*Uncultured bacterium clone P4D7-456*	99%
M1-8	*Uncultured bacterium clone P4D7-662*	99%
M1-9	*Escherichia sp. TX3*	99%
Sample M2-1	*Chryseobacterium joostei LMG 18212*	100%
M2-2	*Chryseobacterium joostei isolate H197*	99%
M2-3	*Lactococcus lactis isolate D29*	100%
M2-4	*Uncultured bacterium clone F2X*	99%
M2-5(I)	*Lactococcus lactis isolate D29*	99%
M2-5(II)	*Uncultured bacterium clone P4D7-472*	99%
M2-6	*Lactococcus lactis isolate D29*	100%
M2-7	*Uncultured bacterium clone 13-1*	100%
M2-8(I)	*Raoultella terrigena isolate m30*	99%
M2-8(II)	*Uncultured bacterium clone 13-5*	95%
M2-9(I)	*Klebsiella pneumoniae strain 6.2T*	99%
M2-10	*Uncultured bacterium clone 13-4*	100%
Sample M3-1	*Uncultured bacterium clone 11-8*	99%
M3-2	*Uncultured bacterium clone 11-1*	98%
M3-3(I)	*Klebsiella pneumoniae strain HR11*	98%
M3-3(II)	*Escherichia sp. TX3*	99%

Band No. [a]	Closest relative in GenBank database	Similarity(%)
Sample M4-1	*Streptococcus uberis*	100%
M4-2	*Staphylococcus aureus strain XJTUMS2*	99%
M4-3(I)	*Legionella-like amoebal pathogen 2*	97%
M4-3(II)	*Corynebacterium glutamicum strain CICCHLJ Q91*	99%
M4-3(III)	*Flavobacteriaceae bacterium SM33*	99%
M4-4(I)	*Shigella flexneri strain FBD001shig*	99%
M4-4(II)	*Uncultured bacterium clone HH_aai34c08*	96%
M4-5	*Uncultured bacterium clone ZG7000_SP6.ab1*	100%

[a] The letter-number combination before the dash designates the tested sample; the number after the dash corresponds to the bands in Fig.1; the Roman numerals distinguish between different species from the same band.

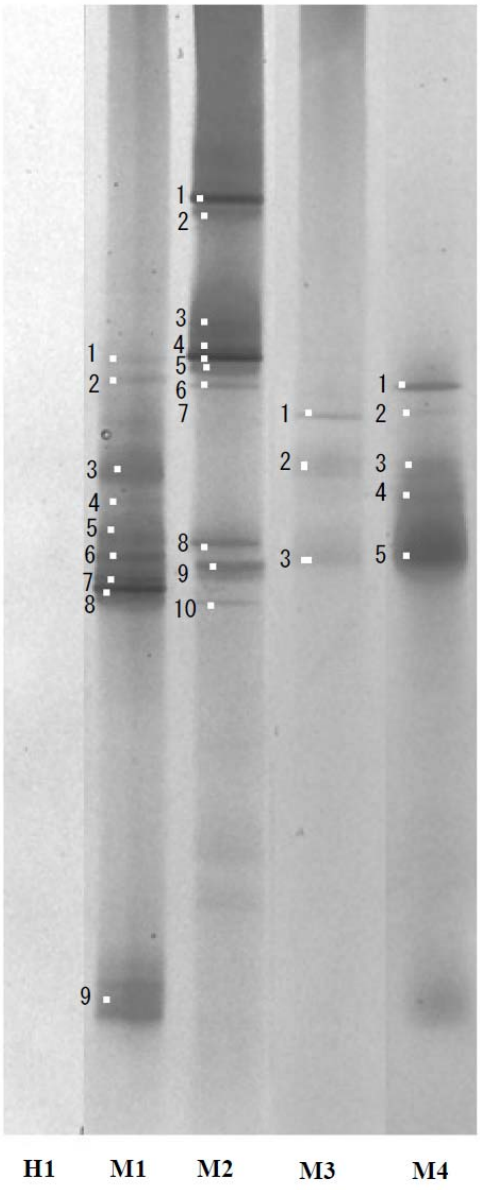

Fig.1

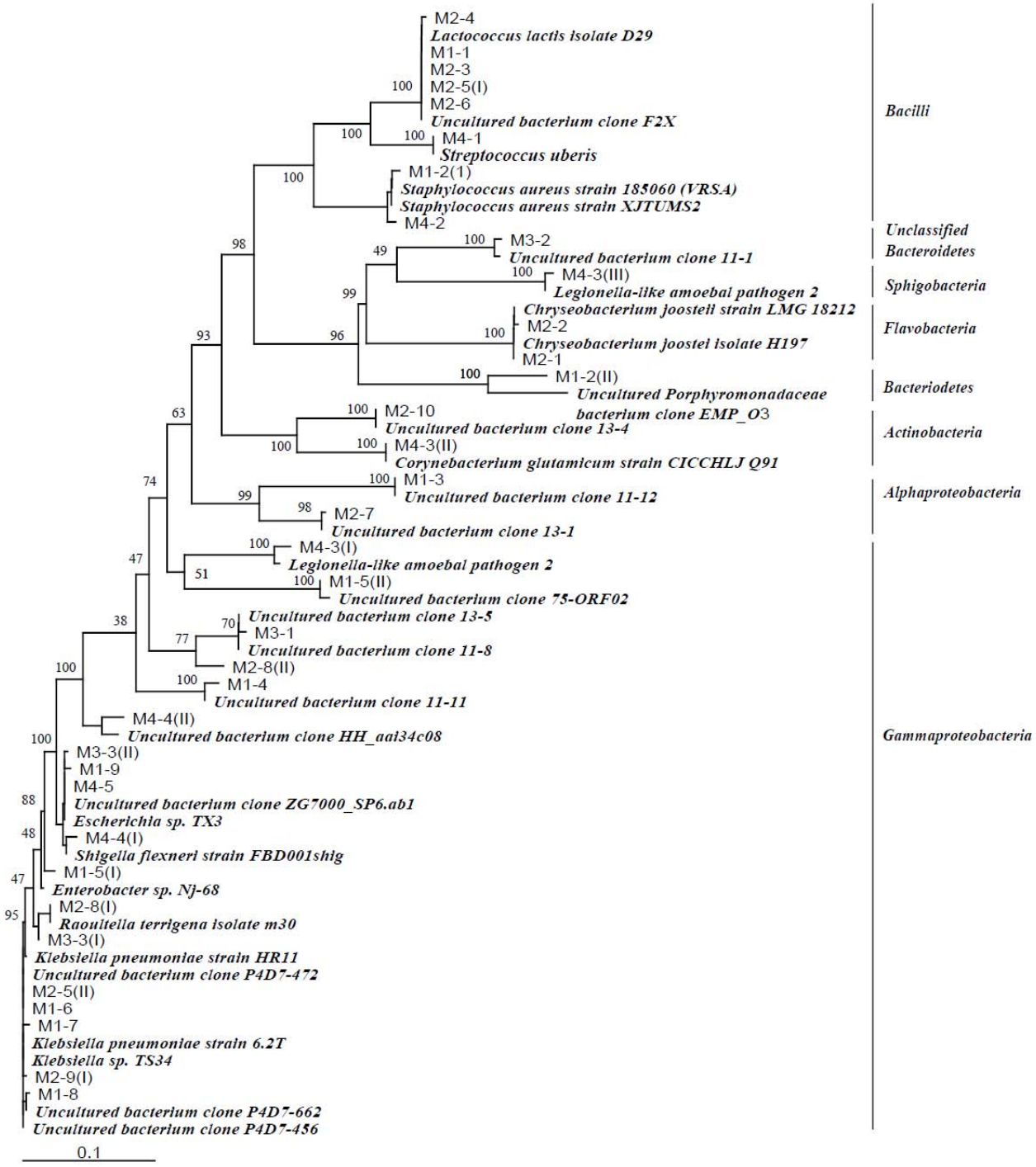
M2-4
Lactococcus lactis isolate D29
M1-1
M2-3
M2-5(I)
M2-6
Uncultured bacterium clone F2X
M4-1
Streptococcus uberis
M1-2(1)
Staphylococcus aureus strain 185060 (VRSA)
Staphylococcus aureus strain XJTUMS2
M4-2
M3-2
Uncultured bacterium clone 11-1
M4-3(III)
Legionella-like amoebal pathogen 2
Chryseobacterium joosteii strain LMG 18212
M2-2
Chryseobacterium joostei isolate H197
M2-1
M1-2(II)
Uncultured Porphyromonadaceae bacterium clone EMP_O3
M2-10
Uncultured bacterium clone 13-4
M4-3(II)
Corynebacterium glutamicum strain CICCHLJ Q91
M1-3
Uncultured bacterium clone 11-12
M2-7
Uncultured bacterium clone 13-1
M4-3(I)
Legionella-like amoebal pathogen 2
M1-5(II)
Uncultured bacterium clone 75-ORF02
Uncultured bacterium clone 13-5
M3-1
Uncultured bacterium clone 11-8
M2-8(II)
M1-4
Uncultured bacterium clone 11-11
M4-4(II)
Uncultured bacterium clone HH_aai34c08
M3-3(II)
M1-9
M4-5
Uncultured bacterium clone ZG7000_SP6.ab1
Escherichia sp. TX3
M4-4(I)
Shigella flexneri strain FBD001shig
M1-5(I)
Enterobacter sp. Nj-68
M2-8(I)
Raoultella terrigena isolate m30
M3-3(I)
Klebsiella pneumoniae strain HR11
Uncultured bacterium clone P4D7-472
M2-5(II)
M1-6
M1-7
Klebsiella pneumoniae strain 6.2T
Klebsiella sp. TS34
M2-9(I)
M1-8
Uncultured bacterium clone P4D7-662
Uncultured bacterium clone P4D7-456
0.1
Bacilli
Unclassified Bacteroidetes
Sphigobacteria
Flavobacteria
Bacteriodetes
Actinobacteria
Alphaproteobacteria
Gammaproteobacteria

Fig.2