



Next-generation sequencing analysis of bacterial flora in bovine protothecal mastitic milk and feces

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ABSTRACT. The aim of the present study was to evaluate the bacterial flora in the udder and intestinal environments in cows with and without protothecal mastitis. We used next-generation sequencing (NGS) analysis to identify 16S rRNA genes from bacterial flora present in 13 milk and 13 fecal samples from protothecal mastitic and healthy dairy cows in the Aichi region of Japan. Sequences associated with 5 species (*Calothrix desertica*, *Corynebacterium simulans*, *Corynebacterium striatum*, *Empedobacter falsenii*, and *Rothia endophytica*) showed the highest prevalence in samples of milk and feces from animals with protothecal mastitis. This range of species differed from those detected in the milk and feces from healthy cows.

KEY WORDS: bacterial flora, bovine protothecal mastitis, next-generation sequencing, *Prototheca zopfii*

The genus *Prototheca* consists of achlorophyllic algae that are ubiquitous in cow-barn environments. *Prototheca zopfii* and *P. blaschkeae* are associated with bovine mastitis, a disease that causes a reduction in milk production and secretion of thin watery milk containing white flakes [6–13]. Most cases of bovine protothecal mastitis are chronic and subclinical infections; no effective treatment is known. However, neither the infection mechanism nor a route of infection leading to mastitis have been defined, precluding prevention of this disease. Biochemical and serological tests have previously been used to characterize *P. zopfii* into a minimum of two genotypes, genotype 1 and genotype 2 [11, 12]. Cases of bovine mastitis in Germany, Italy, Japan, Portugal and Poland were almost all as attributed to infection by *P. zopfii* genotype 2, suggesting that this genotype is the main causative agent of bovine protothecal mastitis [1, 2, 6, 7, 9–12].

In a previous study, we evaluated the prevalence of *P. zopfii* genotype 2 in fecal samples from Japanese dairies with or without a history of protothecal mastitis in 2017. *P. zopfii* genotype 2 was detected in 23 of 60 (38%) fecal samples, but only in a herd with a history of protothecal mastitis [5]. Jagielski *et al.* also reported that *P. zopfii* genotype 2 was most frequently isolated from stools and rectums of cows in Poland [2]. These results suggested that the occurrence of bovine protothecal mastitis is related to the intestinal flora and that the source of infection is feces. However, we did not demonstrate clear exchange of intestinal bacterial flora between protothecal mastitic cows and healthy cows. Moreover, we could not account for the observed strong growth of *P. zopfii* genotype 2 in the intestinal environment.

Recent studies have used the metagenomics of bacterial 16S rRNA genes to investigate bacterial DNA diversity in milk samples from mastitic and healthy dairy cows; the results were used to compare the bacterial flora in the udder environment between infected and uninfected animals. Notably, we have employed metagenomic next-generation sequencing (NGS) to identify the 16S rRNA genes of bacterial flora in milk samples from protothecal mastitic dairy cows in the Kushiro area of Japan [3]. That study indicated that changes in bacterial flora are typical in animals with protothecal mastitis [3].

The aim of the present study was to evaluate the bacterial flora in the udder and intestinal environments in cows with and without protothecal mastitis. We used NGS analysis to identify 16S rRNA genes from bacterial flora present in milk and feces samples from cows with and without protothecal mastitis.

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Table 1. Isolation and identification of *Prototheca zopfii* from milk and fecal samples recovered from protothecal mastitic animals (Cows number 14 to 57) and healthy animals (Cows number 1 to 11)

Cow's no.	Cow	Positive milk samples ^{a)}	Positive feces samples ^{b)}
1	healthy	-	1
2	healthy	-	2
3	healthy	-	1
4	healthy	-	2
5	healthy	-	2
7	healthy	-	1
8	healthy	-	1
10	healthy	-	2
11	healthy	-	2
14	mastitis	+	2
47	mastitis	+	2
56	mastitis	+	2
57	mastitis	+	2

a) Positive isolation of *P. zopfii* genotype 2 in milk. b) Positive isolation of *P. zopfii* genotype 1 or 2 in fecal samples.

MATERIALS AND METHODS

Milk samples

A total of 13 milk samples and 13 feces samples were subjected to metagenomic NGS of bacterial 16S rRNA genes. Milk and feces samples (derived from rectums), which were collected in 2018, were obtained by the joint milking of 4 teats from each animal; all were Holstein cows that were housed at a single dairy with a history of protothecal mastitis and located in the Aichi region of Japan [5]. Four clinical strains of *P. zopfii* genotype 2 were isolated from 4 milk and feces samples from 4 distinct cases of protothecal mastitis; the other 9 milk and feces samples were obtained from 9 normal (non-mastitic) animals (Table 1).

All cases of bovine protothecal mastitis in this study were chronic and subclinical, and were detected as positive results in culture tests of milk samples [1, 9]. Isolation and molecular typing procedures were as described in our previous studies [9, 13].

PCR amplification of bacterial 16S rRNA genes

PCR amplification of bacterial 16S rRNA genes and metagenomic sequence procedures were as described in our previous studies [3]. Briefly, bacterial genomic DNA was isolated from milk using a MORA-EXTRACT kit (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions.

The V3-4 region of bacterial 16S rRNA genes were PCR amplified from each sample using a composite pair of primers containing unique 17- or 21-base adapters, which were used to tag the PCR products from the respective samples (Illumina, Inc., San Diego, CA:U.S.A.) [4]. The forward primer was 5'-**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG**-3', where the bold sequence was the forward primer's overhanging adapter, and the italicized sequence was the universal broadly conserved bacterial primer S-D-Bact-0341-b-S-17 (in which N corresponds to any nucleotide, and W corresponds to A or T). The reverse primer was 5'-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTACHVGGGTATCTAATCC**-3', where the bold sequence was the reverse primer's overhanging adapter, and the italicized sequence was the broad-range bacterial primer S-D-Bact-0907-a-A-20 (in which H corresponds to A, C, or T; and V corresponds to A, G, or C).

The primers S-D-Bact-0341-b-S-17 and S-D-Bact-0907-a-A-20 amplified approximately 460-bp fragments of the bacterial 16S rRNA genes (Illumina).

Genomic DNA samples (100 ng/sample) were subjected to amplification by PCR in a volume of 30 μ l, using a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 mM each deoxynucleoside triphosphate, 1.0 unit of *Taq* polymerase (Takara Bio, Kyoto, Japan), and 50 μ mol each of a pair of primers. Amplification was carried out over 35 cycles, each consisting of template denaturation (1 min, 94°C), primer annealing (1 min, 63°C), and polymerization (2 min, 72°C). PCR products were detected by electrophoresis of an aliquot on a 2% agarose gel followed by staining with ethidium bromide and visualization under UV light.

The 16S metagenomic sequence

PCR amplicons were purified with NucleoSpin® Gel and PCR Clean-up kits (Takara Bio). DNA concentration and purity were evaluated by optical density using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific K.K., Kanagawa, Yokohama, Japan), and the final concentrations of the samples were adjusted to 4 nmol per 5 μ l.

The 16S metagenomic sequence library preparation was performed using the NEBNext Ultra II DNA library prep kit (New

Milk samples		healthy cows
protothecal mastitis		
<i>Calothrix desertica</i>	<i>Corynebacterium argentoratense</i>	<i>Atopostipes suicloacalis</i>
<i>Corynebacterium simulans</i>	<i>Corynebacterium casei</i>	<i>Alkalibacterium psychrotolerans</i>
<i>Corynebacterium striatum</i>	<i>Corynebacterium efficiens</i>	<u><i>Bacteroides plebeius</i></u>
<i>Empedobacter falsenii</i>	<i>Corynebacterium glycinipfilum</i>	<i>Brachybacterium faecium</i>
<i>Rothia endophytica</i>	<i>Corynebacterium kroppenstedtii</i>	<i>Brevibacterium senegalense</i>
	<i>Halothiobacillus neapolitanus</i>	<i>Brevundimonas abyssalis</i>
	<i>Romboutsia ilealis</i>	<u><i>Clostridium ghonii</i></u>
	<i>Sphingomonas sanxanigenens</i>	<u><i>Corynebacterium bovis</i></u>
	<i>Staphylococcus pasteurii</i>	<i>Corynebacterium maris</i>
	<i>Staphylococcus sciuri</i>	<i>Enterococcus gallinarum</i>
		<u><i>Eubacterium tenue</i></u>
		<i>Facklamia ignava</i>
		<i>Facklamia tabacinasalis</i>
		<i>Fastidiosipilla sanguinis</i>
		<u><i>Intestinimonas butyriciproducens</i></u>
		<i>Micrococcus luteus</i>
		<i>Methylobacterium extorquens</i>
		<i>Oligella ureolytica</i>
		<i>Propionibacterium acnes</i>
		<i>Sphingomonas roseiflava</i>
		<i>Staphylococcus saprophyticus</i>
		<i>Weissella koreensis</i>

Fig. 1. The most prevalent (top 10 species in each sample) sequences in milk samples derived from protothecal mastitic and healthy cows. Underlining indicates species shared between milk and feces. The box indicates sequences classified as microbial species from protothecal mastitis samples. The dashed line box indicates sequences classified as microbial species from healthy cow's samples.

England BioLabs, Inc., Ipswich, MA, U.S.A.).

The library of the V3-4 region of bacterial 16S rRNA genes was sequenced using the MiSeq™ System (Illumina).

To facilitate a detailed (species-level) analysis of the sequences, the following steps were followed: 10,000 to 15,000 sequences from each sample were used to create a new FASTAQ sequence file. This file was trimmed to remove the 16S primers and filtered to remove additional sequences of low quality (non specific amplicons for bacterial 16S rRNA genes). The resulting file was uploaded in the OTU (Operational Taxonomic Unit) picking tool (World Fusion US, Inc., San Diego, CA, U.S.A.; <http://www.w-fusionus.com/metagenome-kin>), and then processed by the complete linkage clustering tool (which clustered the aligned sequences into OTUs). Finally, the dereplicate function was used to create one representative sequence for each OTU. Eventually, a new file of representative sequences was created. The homology analysis with the Basic Local Alignment Search Tool (BLAST algorithm) for bacterial 16S was performed by the Metagenom@Kin (World Fusion US, Inc.).

The NGS produced 10,000 to 15,000 sequences in each DNA sample. To facilitate comparison, the sequencing results were grouped according to the culture-based protothecal mastitis diagnosis; sequences derived from the milk samples obtained from healthy cows were grouped separately. The nucleotide sequence identity for bacterial species was 98% to 100% among the sequence samples examined in the present work, including the reference sequences of bacterial 16S in the BLAST database.

RESULTS

The bacterial sequences detected most frequently by NGS of organisms in milk samples obtained from protothecal mastitic and healthy cows are shown in Fig. 1.

Sequences classified by the Metagenom@Kin as 5 species (*Calothrix desertica*, *Corynebacterium simulans*, *Corynebacterium striatum*, *Empedobacter falsenii*, and *Rothia endophytica*) were the most prevalent sequences in each bacterial 16S amplicon sample in the groups of samples characterized by culturing as present in samples from cows with protothecal mastitis (Table 1 and Fig. 1).

At the same time, sequences representing 22 bacterial species (*Atopostipes suicloacalis*, *Alkalibacterium psychrotolerans*, *Bacteroides plebeius*, *Brachybacterium faecium*, *Brevibacterium senegalense*, *Brevundimonas abyssalis*, *Clostridium ghonii*, *Corynebacterium bovis*, *Corynebacterium maris*, *Enterococcus gallinarum*, *Eubacterium tenue*, *Facklamia ignava*, *Facklamia tabacinasalis*, *Fastidiosipilla sanguinis*, *Intestinimonas butyriciproducens*, *Micrococcus luteus*, *Methylobacterium extorquens*, *Oligella ureolytica*, *Propionibacterium acnes*, *Sphingomonas roseiflava*, *Staphylococcus saprophyticus*, and *Weissella koreensis*) were prevalent (included among the top 10 species in a given sample) only in the milk samples derived from healthy cows (Fig. 1). Notably, *C. desertica* was not detected in any of the milk samples derived from healthy cows.

Ten bacterial species (*Corynebacterium argentoratense*, *Corynebacterium casei*, *Corynebacterium efficiens*, *Corynebacterium glycinipfilum*, *Corynebacterium kroppenstedtii*, *Halothiobacillus neapolitanus*, *Romboutsia ilealis*, *Sphingomonas sanxanigenens*, *Staphylococcus pasteurii*, and *Staphylococcus sciuri*) also were prevalent (included among the top 10 species in each sample) in the milk samples derived from protothecal mastitic and healthy cows (Fig. 1).

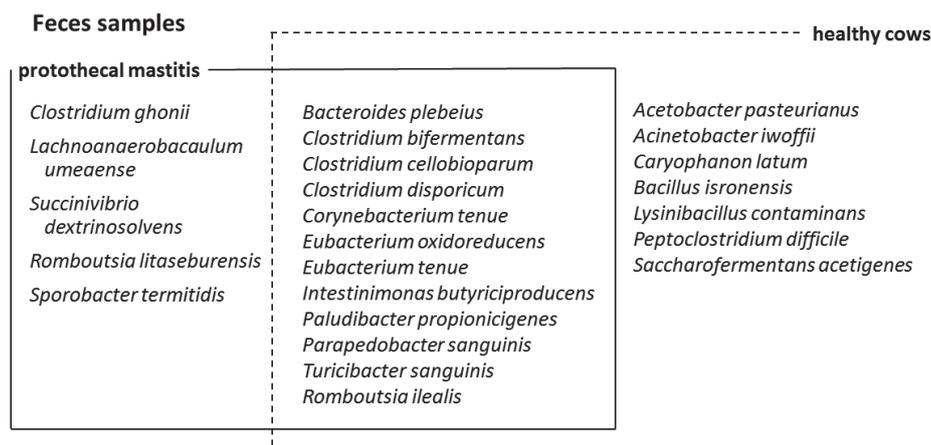


Fig. 2. The most prevalent (top 10 species in each sample) sequences in feces samples derived from protothecal mastitic and healthy cows. The box indicates sequences classified as microbial species from protothecal mastitis samples. The dashed line box indicates sequences classified as microbial species from healthy cow's samples.

The bacterial sequences detected most frequently by NGS in feces samples obtained from protothecal mastitic and healthy cows are shown in Fig. 2.

Sequences classified by the NGS as belonging to 5 species (*Clostridium ghonii*, *Lachnoanaerobaculum umeaense*, *Succinivibrio dextrinosolvens*, *Romboutsia litaseburensis*, and *Sporobacter termitidis*) were the most prevalent (included among the top 10 species in each sample) only in the samples characterized from protothecal mastitic feces (Fig. 2). However, *C. desertica* was not detected in any of the samples derived from protothecal mastitic cow feces.

Sequences classified as belonging to 7 species (*Acetobacter pasteurianus*, *Acinetobacter iwoffii*, *Caryophanon latum*, *Bacillus isronensis*, *Lysinibacillus contaminans*, *Peptoclostridium difficile*, and *Saccharofermentans acetigenes*) were prevalent only in feces samples derived from healthy cows (Fig. 2).

Sequences classified as belonging to 12 species (*Bacteroides plebeius*, *Clostridium bifermentans*, *Clostridium cellobioparum*, *Clostridium disporicum*, *Corynebacterium tenue*, *Eubacterium oxidoreducens*, *Eubacterium tenue*, *Intestinimonas butyriciproducens*, *Paludibacter propionigenes*, *Parapedobacter sanguinis*, *Turicibacter sanguinis*, and *Romboutsia ilealis*) also were commonly prevalent in the feces samples derived from protothecal mastitic and healthy cows (Fig. 2).

DISCUSSION

The risk factors for protothecal mastitis are believed to include multiple intramammary treatments, milker hygiene, and milking equipment performance [2]. Based on their history, the protothecal mastitic cows examined in the present work had not been exposed to any of these known risk factors. To improve our understanding of protothecal mastitis while clarifying the mechanism of infection, we surveyed these cows' bacterial flora. Specifically, we used NGS analysis to evaluate the bacterial flora in the udder and intestinal environments in protothecal mastitic and non-mastitic cows.

Notably, *Calothrix desertica* (a cyanobacterium) showed higher prevalence in milk from cows with protothecal mastitis than in that from healthy cows (Fig. 1). In a previous study, we reported the first demonstration that *C. desertica* sequences, which were effectively absent from samples derived from healthy animals, were highly prevalent in samples from protothecal mastitic cows in one dairy, located in the Kushiro area of Japan, that had a history of protothecal mastitis infection. *C. desertica* may inhabit the dairy and natural environments in Japan, but the ecology of this species remains poorly understood. Unfortunately, we could not explain the prevalence of this cyanobacterium in protothecal mastitic milk.

The present work revealed that the profile of bacterial species in milk from cows with protothecal mastitis differed from the profile in milk from healthy cows (Fig. 1). Oikonomou *et al.* reported that differences in bacterial flora are detected between milk samples from cows with bacterial mastitis and those from healthy dairy cows, regardless of the bacterial distribution defined by metagenomic analyses obtained by pyrosequencing of the 16S rRNA [8]. Those results, like those of the present study, indicated that changes in bacterial flora are a common phenomenon in protothecal mastitis.

In the present study, we compared the bacterial flora in feces between protothecal mastitic and healthy cows. Bacterial species with high prevalence in protothecal mastitic milk were not similarly prevalent in the feces from these animals (Figs. 1 and 2). Thus, the bacterial flora in milk from cows with bacterial mastitis might not correlate with the flora in the feces of these animals.

These results suggested that infection by *Prototheca* species can be considered an opportunistic infection that reflects alterations in the udder environment and host immunity in mastitic cows compared to those of healthy cows.

Unfortunately, we could not account for the observed strong growth of *P. zopfii* genotype 2 in the intestinal environment in this study. In subsequent work, we intend to investigate additional protothecal mastitic cows and dairy environments in the other dairy

farms in an attempt to clarify the mechanism of infection and to identify potential preventive tools. Moreover, we will investigate the prevalence mechanism of *C. desertica* in protothecal mastitic milk.

ETHICAL APPROVAL. This study did not use experimental animals.

CONFLICTS OF INTEREST. The authors report no conflicts of interest. The authors alone were responsible for the content and writing of the paper.

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