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An rRNA-based analysis for evaluating the effect of heat stress on the rumen microbial composition of Holstein heifers

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ABSTRACT

We performed a set of heifer feeding trials to investigate the effect of heat and humidity stresses on the rumen bacterial molecular diversity of Holstein heifers (Tajima K, Nonaka I, Higuchi K, Takusari N, Kurihara M, Takenaka A, *et al.* Anaerobe 2007;13:57–64). To further characterize the response of the microbial community to the physiological changes caused by the stresses, we evaluated changes in the ruminal bacterial community composition in the same trials by applying an RNA-based method (sequence-specific small-subunit (SSU) rRNA cleavage method), which was optimized for a comprehensive description of the predominant bacterial groups inhabiting the rumen. Four Holstein heifers were kept at three temperatures (20 °C, 28 °C, 33 °C) in a climatic chamber for two weeks each, and rumen fluid samples were obtained on the last day of each temperature experiment. For quantitative detection, we applied a set of 15 oligonucleotide probes, including those targeting taxa comprised of uncultured rumen bacteria (URB) belonging to phylum *Firmicutes*, to the RNAs extracted from the fluid samples. The relative populations of the *Clostridium coccoides–Eubacterium rectale* group, and the genus *Streptococcus* increased, and that of the genus *Fibrobacter* decreased in response to increasing temperature both in the first (nine months old, 80% relative humidity) and second (15 months old, 60% relative humidity) experiments. In addition, the population of a defined URB group was higher at 33 °C than at 20 °C in the second trial, whereas one of the other URB groups showed a decreasing trend with the temperature rise. These results indicate that the exposure to heat affects the population levels of specific bacterial groups in the ruminal microbial community.

1. Introduction

Ruminant animals are recognized to be among the least tolerant to heat stress, which is caused by a combination of environmental factors (ambient temperatures, relative humidity, solar radiation, air movement) [1,2]. The physiological responses of cattle to heat stress have been well described [3,4], and include increased body temperature [5], increased respiratory rate [6], decreased feed intake [5,7], and increased water intake [8]. These responses have detrimental effects on milk production, reproduction, and health in lactating dairy cows [9–11]. Although a number of studies have been conducted to characterize and alleviate the effects, little information has been available for rumen microbial community, which plays a critical role in the digestion of ingesta in the rumen, in relation to these physiological changes caused by heat stress.

We previously assessed the effect of temperature and humidity on the rumen bacterial diversity of Holstein heifers [12]. In the feeding trial, the ruminal microbial composition, determined by 16S rRNA gene cloning, was significantly changed at elevated environmental temperatures and humidity, along with decreases in dry matter intake, body weight gain and an increase in the digestibility of the feed with rising temperature. However, since the previous experiment lacked quantitative data on the respective bacterial groups, it is still uncertain how the microbial composition changed along with the change in the environmental conditions. We therefore aimed to further investigate how the rumen microbial community responds to heat-stress. For this purpose, we monitored the change in the ruminal bacterial community at the group

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level by analyzing the total RNA solutions extracted from rumen fluid in the same trials as described in the previous report [12]. We did this to determine the change in the activity of respective community members in the rumen in response to the change in environmental temperatures and humidity. The RNase H method that we have developed [13] uses a scissor probe set, covering dominant members of bacteria present in ruminal ecosystems [14] and this was applied for the analysis. To extend the detection coverage of the probe set, we added five scissor probes, including three probes that target clusters mainly comprising uncultured strains belonging to phylum *Firmicutes* retrieved from ruminal samples.

2. Materials and methods

2.1. Heifer feeding trials

The present analysis was performed on specimens collected from a previously described heat-stress trial [12]. Briefly, four rumen-fistulated healthy Holstein dairy cows kept at the National Institute of Livestocks and Grassland Science, Tsukuba, Japan were housed in a climatic chamber with individual tie stalls. Ambient temperature and relative humidity were computer-controlled and monitored continuously. The cows were fed with a sufficient amount of a mixed ration diet composed of 50% hay and 50% concentrate twice a day (09:30 and 17:30) for *ad libitum* consumption and had free access to tap water. The same four cows were subjected to two separate experiments with an interval of about six months between the experiments. The average age and body weight of the cows were 9 months, 250 kg in experiment 1 and 15 months, 430 kg in experiment 2. The cows were kept in the experimental chamber for six weeks: the first two weeks were spent under the reference conditions (20 °C), the next two weeks were spent under a mid-hot condition (28 °C), and the last two weeks were spent under a hot condition (33 °C). Relative humidity was set at 80% in experiment 1 and 60% in experiment 2. The experimental design of this study did not allow us to accurately evaluate the effect of heifer age or relative humidity. In the previous report, three trials were performed but we could obtain samples for this analysis only from the latter two trials. Therefore, experiments 1 and 2 in the present report correspond to experiments 2 and 3 in the previous report, respectively.

2.2. The extraction of total RNAs from rumen fluid samples

Rumen fluid samples were obtained anaerobically via fistula on the last day of each temperature condition before the morning feeding. The rumen fluid was sampled from each cow and processed for the extraction of total RNA. Nucleic acid extraction was performed essentially according to Krause et al. [15] and Whitford et al. [16]. The obtained fluid was filtered through two layers of cheesecloth, and then clarified by centrifugation for 5 min at 800 × g at 4 °C, to separate the protozoan cells and residual feed particles from the fluid. The supernatants were subjected to immediate extraction of total RNAs. The prokaryotic cells in the supernatants were disrupted by glass bead beating, and the total RNAs were extracted with phenol equilibrated with a pH 5.1 buffer (10 mM EDTA, 50 mM sodium acetate [pH 5.1]), as previously described [13]. DNAs were removed with RNase-free DNase (Promega). *In vitro* SSU rRNA transcripts were generated from the PCR amplicons of the T7 promoter-conjugated SSU rRNA genes and T7 RNA polymerase by a T7 RiboMAX express kit (Promega, Madison, WI), as previously described [13]. Total RNAs and the SSU rRNA transcripts were stored at −80 °C until they were used for analyses.

2.3. Phylogenetic analysis and probe design

In our previous study [14], we addressed the usefulness of developing probes for unknown bacterial clusters related to the genus *Clostridium* to extend the detection coverage of the ruminal bacterial community. We therefore aimed at determining the phylogenetic positions of uncultured rumen bacteria groups belonging to the genus. For this purpose, a phylogenetic analysis was performed by obtaining 16S rRNA gene sequences of uncultured bacteria and relative bacterial strains from public databases and aligning them with the ARB program [17]. The aligned data were manually corrected based on information about primary and secondary structures. A phylogenetic tree was generated by using the neighbor-joining algorithm implemented in the ARB program to determine the position of respective taxa. The PAUP* program was used for bootstrap analysis of tree topologies (1000 resampling). Three clusters comprising uncultured rumen bacteria (URB) were estimated ('Unknown cluster B', 'Unknown cluster C', and 'Unknown group 2'), which were mentioned in previous reports [18,19], as shown in Fig. 1. A probe for 'Unknown cluster B' (URBI432) and 'Unknown group 2' (URBII611) and a probe for a subcluster, which also comprises URB and was related to *Clostridium viride* (Cvir432), was designed by determining the signature sequences of the group using the ARB program. As we could not determine any signature sequence of 'Unknown cluster C', no probe for the group was designed. Specificity of these newly designed probes was confirmed as described previously [14]. Briefly, for each scissor probe, the 16S rRNAs of the target and non-target reference strains were subjected to a cleavage experiment at various formamide concentrations in the RNase H reaction mixture. Since no cultured strain has been available for the probes URBI432 and URBII611, two clonal sequences (H60N90 and H60N88) were used for *in vitro* transcription of target rRNAs. *C. viride* (DSM 6856) was used for the target strain in the evaluation of the probe Cvir432 (Fig. 1). Strains, the 16S rRNA sequence of which has the least number of mismatches with the target site of the probe sequence, were selected as the non-target reference strain of the respective scissor probe: *Lactobacillus acidophilus* (DSM 20079) and *Ruminooccus albus* (DSM 20455), for the URBI432 probe (4 base mismatches [MM]), *L. acidophilus* (DSM 20079) for the URBII611 probe (1 MM), and *Clostridium thermocellum* (DSM 1237) and *Ruminococcus bromii* (ATCC 2725) for the Cvir432 probe (3 MM). By doing so, we determined a formamide concentration at which the 16S rRNA of the non-target strain was not cleaved at all, while the 16S rRNA of the target strain was sufficiently cleaved. The sequence, formamide concentration, and cleavage coefficient of respective probes were shown in Table 1. At the optimum formamide concentration, non-target rRNAs were not cleaved by any of the probes tested (data not shown).

2.4. RNA quantification based on 16S rRNA cleavage by an oligonucleotide and RNase H

In addition to the probes described above, we used probes for bacterial groups under the same reaction conditions as described in previous papers [13,14] (Table 1). The probes Atop291 and Osc808 were also employed in this study (Table 1). The sequence-specific cleavage of rRNA fragments was performed as described previously [13]. Briefly, 10 μl of RNA solution (100 ng/μl), 5 μl of 15× hybridization buffer (375 mM Tris–HCl [pH 7.5], 15 mM EDTA, 375 mM NaCl), 2 μl of oligonucleotide probe solution (10 pmol/μl), and a defined amount of formamide were mixed to make a hybridization solution, and then diethyl pyrocarbonate-treated water was added to make a final volume of 75 μl. The mixture was subsequently heated at 95 °C for 1 min to unfold the RNA molecules, and