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Full Length Research Paper

Taxonomical analysis of the suspended bacterial fraction in the dromedary rumen fluid

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An analysis of the dominant microbial taxa present in suspension within the rumen fluid from slaughtered one-humped camel (*Camelus dromedarius*) in Algeria was carried out using culture-independent molecular techniques. The rumen fluid of freshly eviscerated animals was sampled by a syringe and filtered through 0.22 µm filters in sterile conditions. Lyophilized filters were subsequently used as starting material for bacterial lysis and total DNA extraction procedures using DNA purification kits and suitably adapted protocols. The gene corresponding to the small subunit of ribosomal RNA (16S rDNA) was PCR-amplified from the bulk of DNA using eubacterial primers, and the pool of amplicons was ligated to plasmids and cloned in *Escherichia coli*, generating a clone bank of several hundred individuals representative of the rumen bacterial community. A preliminary analysis of 86 clones, sorted by amplified ribosomal DNA restriction analysis (ARDRA), and sequenced by Applied Biosystems automated sequencing using fluorescent terminators yielded the following results. The most abundant amplicon belonged to the *Pseudomonas* genus encompassing over 65% of the clones. *Pseudomonas lutea* appeared the most frequent homology hit in a BLAST GenBank comparison. The remaining flora featured taxa include (in order of deceasing abundance): *Synechococcus* sp., *Moraxella osloensis*, *Sphingomonas* sp., *Diaphorobacter nitroreducens*, *Acinetobacter* sp., *Ruminococcus albus*, *Propionibacterium acnes* and *Comamonas* sp. The data constitute the baseline for a comparison of the results with those that will be obtained by further metagenomic approaches to compare the fluid associated bacterial community with those attached to the solid particulate fraction.

Key words: *Camelus dromedarius*, dromedary camel, rumen fluid, rumen bacteria.

INTRODUCTION

The rumen is a complex environment, hosting a vast array of microorganisms including bacteria, protozoa and fungi with remarkable physiological attitudes. The extent of such microbial diversity has been often pointed out as exemplar also in terms of morphological variation, leading to the choice of a ruminal fluid community as subject for the cover picture of the ninth edition of the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Most studies on the composition of microbial communities refer to cattle rumen, for which a robust body of literature is available (Russell, 2002). The bovine rumen microbial ecosystem was the object of reviews (Kamra, 2005). Methods of study have in the recent past exploited the power of culture-independent techniques such as PCR amplification of 16S rDNA determinants in order to define community identities and structure (Whitford et al., 1998; Tajima et al., 1999; Ramsak et al., 2000; Edwards et al., 2004). The fiber-adherent microbiome in the bovine rumen was the object of recent metagenomic studies (Brulc et al., 2009) while an extensive metagenomic analysis in fistulated cow was recently published (Hess et al., 2011).

A much less explored field is the rumen of other herbivores such as camel, in which microbial investigations have so far been limited to enzymatic assays (Mohamed et al., 2000a, b, 2002) or to the selective isolation of streptococcal species (Ghali et al., 2004). Among the old world camels, *Camelus dromedarius*, known as

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one-humped or Arabian (dromedary) camel, represents an important man-managed animal in north African economy. Its remarkable resistance in arid environments along with its feeding habits, including highly fibrous plants (Haddi et al., 2009), call attention to the potential of its associated microflora, whose degradative capabilities could be exploited in biotechnological applications. The catabolic properties of the camel rumen biota are envisaged to be of particular use in the recycling process of agricultural waste (Bourghoud et al., 2009), among which tomato peels are an example of primary importance due to their large occurrence as industrial byproduct. In this paper, we undertook the characterization of the ruminal prokaryotic community of the Arabian camel rumen by means of a PCR-based approach targeting the 16S gene, followed by cloning in *Escherichia coli*, screening the bank by ARDRA and sequencing the representative cases. We purposely wanted to focus on that portion of rumen bacteria which is carried in suspension in the liquid part of the rumen content, distinguishing it from the bulky food residues on which another large fraction of attached bacteria are commonly associated.

MATERIALS AND METHODS

Extraction and processing of ruminal fluid

The one humped camels (*C. dromedarius*) used for this research, originate from the arid natural pastures of the region south of Biskra (Algeria) and feed exclusively on coriaceous wild weeds such as those belonging to the genera *Atriplex*, *Salsola*, *Sueda*, *Limoniastrum* (Haddi et al., 2003). Camels were sampled immediately after slaughtering in the municipal abattoir in Constantine, Algeria. 60 ml of ruminal fluid content were taken by sterile plastic syringes from eviscerated animals. The fluid was filtered through 0.22 µm sterile filters which were stored at -18°C and subsequently lyophilized.

Filter treatment and DNA extraction

The Power Soil™ DNA isolation kit (MoBio, Laboratories, Inc., Solana Beach, CA, USA) was used for the extraction procedure. The circular nitrocellulose filters were cut into small pieces with a sterile scalpel into empty Petri dishes. Fragments from each half filter were introduced into a power bead tube from the above kit and processed according to the manufacturer's instructions; with the exception of the horizontal shaking step that was extended from 10 min to 1 h.

16S Ribosomal rDNA amplification

2 µl of the lysate (or of its 1/10 and 1/100 dilutions) containing the total DNA from filters was treated in a PE 480 Perkin Elmer Thermal Cycler using the two 16S rDNA-targeted universal bacterial primers 63F 5'CAGGCCTAACACATGCAAGTC) (Marchesi et al., 1998) and 1389R (5'ACGGGCGGTGTGTACAAG) (Osborn et al., 2000) in a 50 µl reaction volume, using the following program: initial denaturation at 94°C for 2 min; 35 cycles at 94°C for 80 s, 54°C for 1 min, 72°C for 90 s and a final extension at 72°C for 5 min. The PCR reaction mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 µM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 200 nM of each primer and 1 U *Taq* DNA polymerase, recombinant (Amersham Biosciences). Aliquotes of the amplicons were checked by loading on a 1.5% agarose gel, run electrophoretically for 3 h at 110 V. The ethidium bromide-stained gel (0.3 µg/ml) was visualized over a UV transilluminator and photographed by a Kodak DC290 digital camera.

Gene library construction in *E.coli*

30 µl from the PCR-amplified reaction were purified using the QIAquick PCR purification kit (Qiagen Inc., Chatworth, CA) and amplicons cloned in plasmid pGEM®-T (Promega, Madison, WI) by an overnight ligation at 10°C, and 1.8 to 2 µl were electroporated (2.5 kV, 200 Ω, 25 µF) into 80 µl of electrocompetent *E. coli* XL-1Blue-MRF' in a Gene Pulser chamber (BioRad Inc. Hercules CA, USA). Upon adding 800 µl of LB medium (Luria-Bertani: tryptone 10.0 g/L; yeast extract 5.0 g/L; NaCl 5.0 g/L) and incubating for 1 h; 100 µl aliquotes were plated on LB agar (15 g/L) supplemented with X-Gal (40 µg/ml), IPTG (40 µg/ml) and ampicillin (50 µg/ml). Plates were incubated overnight at 37°C.

ARDRA analysis

To perform amplified ribosomal DNA restriction analysis (ARDRA), 200 recombinant white colonies were streaked, purified on fresh plates and lysed by resuspending a small loopful in 50 µl of lysing buffer (NaOH 0.05 M, sodium dodecyl sulphate 0.25%) in 1.5 polypropylene conical tubes, vortexed for 1 min and heated at 94°C for 15 min. After 10 min of centrifugation at 16.100 x rcf, 10 µl of supernatant was taken and diluted with 90 µl of sterile distilled water. 2 µl of such solution was used for a PCR amplification of the cloned insert using the primer pair GEM-T1: 5'GCAGGCGG CCGCACTAGTGAT (21 bp) and GEM-T2: 5'CCGCCATGGCC GCGGGAT (18 bp).

The reaction solution contained 1x buffer, 200 µM dNTP and 200 nM of each of the primers, which flank the vector border sequences. Inserts were amplified in a Bio Rad ICycler170-8740 using the following program: 94°C/2'; (94°C/80"; 60°C/30"; 72°C/60") x 35; 72°C/5'. The product of each reaction was analyzed by 1.2% agarose gel electrophoresis. For the ARDRA analysis, 5 µl of amplified reaction was digested using 10 U of the *Hin*fI and *Hap*II endonucleases (Amersham Biosciences) in a final volume of 20 µl for a minimum of 2 h at 37°C. Fragments were analyzed by 2% agarose gel electrophoresis in 0.5% Tris Borate EDTA buffer, at a constant voltage of 100 V. Gel images were acquired in digital format using an EDAS 290 Image Capturing System (Kodak, Rochester, NY). Profiles were sorted and compared using the GelComparII® software (Applied Maths, Sint-Martens-Latem, Belgium).

DNA sequencing and bioinformatics

1 µl of the amplicon resulting from the earlier described PCR amplification was mixed with 1 µl containing 6.4 pmol of the earlier described forward primer GEM-T1 in a 0.2 ml polypropylene tube and then dried by incubating the tube open for 15 min at 65°C in an I-Cycler thermal cycler. The template and primer mix was directly used for di-deoxy-cycle DNA sequencing with fluorescent terminators (Big Dye, Perkin-Elmer/Applied Biosystems, Foster City CA) and run in an Applied Biosystems ABI Prism 3730XL automated DNA sequencer. Chromatograms were analyzed by Chromas 2.23 software (Technelysium Pty Ltd, Tewantin Australia). Sequences were fed into the BLAST window of the NCBI on line

Table 1. [a]Taxa displaying the highest level of identity with the sequences of the camel rumen clones upon BLAST analysis in NCBI and [b]level of sequence identity with known database record; values above 97% are considered indicative of species identity.

Inferred identity[a]	% Homology[b]	GenBank code of subject	Number of cases
Pseudomonas lutea	99	AY364537	29
Pseudomonas sp.	99	AY574283	27
Synechococcus sp.	97	AF330247	8
Diaphorobacter nitroreducens	99	AB076856	7
Sphingomonas sp.	99	AY646154	5
Moraxella osloensis	99	AY545637	5
Acinetobacter junii	99	AF417863	2
Ruminococcus albus	99	AF104833	1
Propionibacterium acnes	99	AY642051	1
Comamonas testosteroni	94	AY291591	1

platform (http://www.ncbi.nlm.nih.gov/) and the top homologies against available sequences in worldwide databases were obtained.

RESULTS AND DISCUSSION

The membrane obtained upon filtration of the camel ruminal fluid was processed by adapting a kit originally designed for DNA extraction from soil. Such choice was deemed necessary following previous experience with similar materials, resulting in a difficult amplification due to inhibitors inherent to the plant residual tannins and/or to other unidentified ruminal compounds. Such problems are also discussed in cattle rumen literature (Krause et al., 2001). The kit of choice represents an evolution from its original version in which additional steps and different reagents address the problem of DNA extraction from difficult matrices, including highly organic ones. The extracted lysate required subsequent trials, among which was dilutions from 10 to 100 fold in order to achieve the satisfactory PCR amplification. A suitable amount of the amplicon mix was cloned in the appropriate plasmid vector. Electroporation of *E.coli* yielded a clone bank featuring several hundred recombinant clones (white colonies). As preliminary analysis, 86 clones were investigated. Upon reamplifiyng the inserts and digesting with the restriction endonucleases, a number of operational taxonomical units (OTU) could be sorted by computer-assisted comparison. Representatives of each group were picked and their DNA was sequenced. The resulting community composition is summarized in Table 1 and a phylogenetic dendrogram is shown in Figure 1.

There are no available literature studies addressing the camel rumen yet, as this study constitutes to our knowledge the first example of its kind. However, in order to compare data with the cow situation, one should consider that marked zoological differences occur between bovines and camels, as the former belong to the suborder Ruminantia, the latter, along with llamas and allies, are included in the suborder Tylopoda which have no omasum. This anatomical difference brings about physiological consequences as an omasum is filled with muscular laminae and its short and sturdy papillae take care of mechanical aspects of the digestion by grinding the dry and rough plant materials that typically fills its cavity. In the absence of such a compartment, its functions are in part to be taken over by the rumen itself, in which a more thorough digestion of the plant tissues is expected compared to the cattle situation. Such difference would also lead to a possible enrichment in plant-associated bacteria within the rumen camel and could concur to explain the abundance of taxa not as commonly observed in bovine-derived libraries. In fact, reports on the bovine situation adopting culture-independent approaches normally list, as most abundant phyla, members of the firmicutes and of the cytophaga-flexibacter-bacteroides group (Edwards et al., 2004; Ozutsumi et al., 2005), while proteobacterial sequences are usually confined within 4 to 5% (Tajima et al., 1999). In this study, it is worth noticing that the two most abundant cases display 99% homology with *Pseudomonas lutea* (29 clones out of 86) and to *Pseudomonas* sp. (27/86). Thus, over 65% of the clones from this rumen fluid fraction pertain to aerobic gamma proteobacteria. As regards possible effects of the slaughterhouse practice that might affect rumen biota, it is important to point out that at the abattoir of choice, the camels are either processed straight upon arrival or, in case of postponed treatment, they are regularly fed until slaughtering, and in no instances are they constrained from feed. This excludes possible alterations of the biota composition due to unhealthy conditions or prolonged fasting practices.

It is also important to point out that the object of the present report is the liquid fraction of the rumen content, separated from the insoluble food residues. Therefore, the clone libraries do represent bacteria not attached to food particles. In this respect, it can be expected that this community does not include the fractions engaged in the active degradation of the substrate to which as a

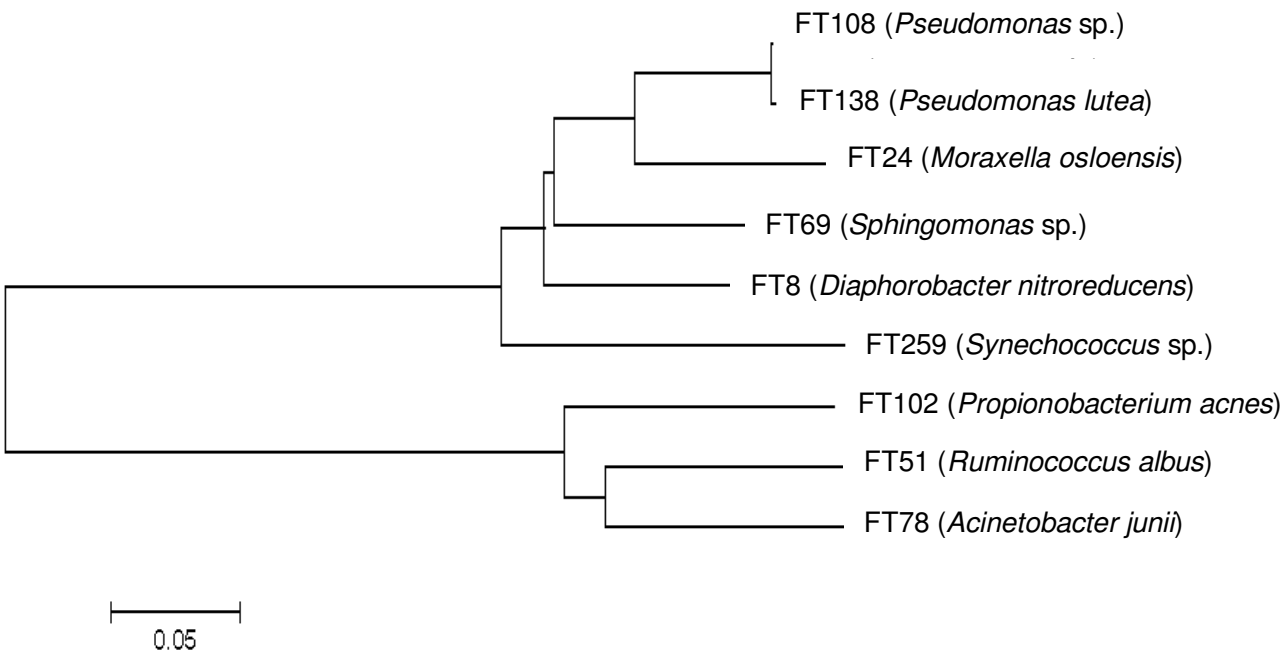

Figure 1. Evolutionary relationships of the nine different taxa found in the sample as resulting from the clustering of the aligned 16S sequences of the clones. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.17709493 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 449 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

conseqence they are tightly bound. It is known that, in order to detach plant-lytic bacteria from rumen food debris, soluble cellulose derivatives (as methyl cellulose) need to be used as scavengers. Conversely, the loosely associated bacteria that will be preferentially found in the fluid can represent biota that can have no active role in the digestion. These could likely include the vast fraction of plant epiphytic microorganisms that are widely introduced as part of the massive amounts of plant leaves ingested by ruminants. Therefore, one could expect the rumen fluid to be enriched in a community pertaining to plant-associated guilds of bacteria. In the present case, the two pseudomonadaceae species sharing high homology with the sequences resulting at the top of the list indeed do pertain to epiphytic habitats. Various *Pseudomonas* species are also reported as plant endophytes (Reiter et al., 2003) and it is known how endophytes could easily reach densities over 10^7 cells per gram of plant tissue dry weight in several plants (Hallmann et al., 1997; Sattelmacher, 2001). Hence, considering the volume of bulk foraged material hosted and processed in the rumen, an abundance of DNA from pseudomonads and other plant-residing bacteria is not surprising. Even though such taxa may not play active roles in the anaerobic ruminal physiology, their residual DNA is likely to show up in rumen libraries. The presence of *Pseudomonas aeruginosa* in the rumen of other livestock is documented by different reports. Kozak and Forsberg (1979) found this aerobic microorganism active in ruminal de-methylation, while Duncan et al. (1999) could isolate and culture live *P. aeruginosa* from sheep rumen. A second fact worth of attention when screening the list of occurrencies found in camel rumen is the presence of different taxa with pathogenic attitude towards animals and humans such as *Moraxella osloensis, Acinetobacter junii* and *Propionibacterium acnes*.

Another reason for which we choose to separate out the rumen solid bolus particles to focus on the particular fraction of bacteria that are free in the fluid, was to deal with a smaller subcommunity, offering chances of a better coverage in a clone-bank based approach. On the other hand, the solid particles of the food residues will supposedly hold a substantially larger fraction of the bacterial rumen diversity as many active degraders would be substrate bound, taking part in enzymatic activities on cellulose, pectin and other insoluble fibers. The array of prokaryotic diversity that pertains to those guilds will be the object of a future study in which we will address such compartments by means of an extensive metagenomic sequencing, in order to verify the identities of such complementary populations. The notions acquired will be useful to plan the digestibility and feed ratios of different plant substrates by camel rumen bacteria in comparison with the bovine counterparts. These studies will benefit *in vitro* techniques apt to measure the kinetics of gas

emission such as the gas production technique (Menke and Steingass, 1988).

In conclusion, we hereby provide a first microbiological investigation of the arabian camel rumen microbial content and open an insight that allows to move towards a more thorough and massive characterization of its microbial contingent. The molecular approach of the microbial genetic diversity in the rumen proves apt to overcome the constraints inherent to cultivation of several ruminal strains. A better knowledge of this unique animal habitat prefigures perspective applications of the available phenotypes to access and seize their yet unexploited high biotechnological potential.

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