Lait 81 (2001) 263–280

Evolution, biodiversity, taxonomy

Preliminary experiments for ARDRA validation on flora associated with intestinal mucosa

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Abstract — The human intestinal tract harbours a complex microbial ecosystem which plays a key role in nutrition and health. Although this microbiota has been studied in great detail by culture techniques, microscopic counts on human faeces suggest that 60 to 80% of the observable bacteria cannot be cultivated. Almost all studies used faecal samples, and there is little data on bacteriology of mucosa-associated bacteria of the intestinal tract. We examined the procedure to extract DNA from intestinal tissue in sufficient quantity and quality for PCR amplification. Intestinal mucosa from germ-free and conventional rats was studied. Intestinal samples were washed to remove non-adherent bacteria, and the mucosa scraped off with a sterile scalpel. Microorganisms and cells were lysed by three enzymatic treatments for total community genomic DNA isolation. Released DNA was purified by spin-column and tissue had to be stored for 24 h in a reduced transport media for PCR amplification. Universal and bacterial oligonucleotides complementary to conserved regions in the 16S rDNA of 180 organisms were evaluated, before being used for PCR amplification. All these primers contain mismatches with some organisms which may influence PCR amplification. The 16S rDNA PCR with universal primers hybridises with a mitochondrial gene of rat, producing a 550 bp fragment which contaminates 16S rDNA fragments. Fifteen cycles of amplifications were performed to preserve the biodiversity of the sample. Theoretical 16S rDNA fragments with universal primers were calculated by a restriction fragment length analysis termed ARDRA (amplified rDNA restriction analysis). Three enzymes were used: *Hae*III, *Rsa*I and *Taq*I. Clustering was performed after separate restriction analysis with these enzymes. An example of this method was carried out after PCR amplification of the 16S rDNA fragment of five strains isolated from a jejunal sample by microbiology techniques. Further investigations on a larger number of strains will permit us to validate more precisely this method.

ARDRA / intestinal mucosa / ecosystem

Résumé — Évaluation de la flore associée à la muqueuse intestinale par la méthode ARDRA : essais préliminaires. L'écosystème digestif humain comporte une flore complexe, pouvant avoir un effet positif sur la santé. Les travaux réalisés à ce jour portent essentiellement sur la flore cultivable

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d'échantillons fécaux et peu d'essais concernent la microflore associée à la muqueuse intestinale. Afin de définir les conditions expérimentales optimales d'extraction et de purification de l'ADN pour l'amplification par PCR, des échantillons de muqueuse intestinale provenant de rats, axéniques ou non, ont été utilisés. Les échantillons doivent alors être conservés dans un milieu de transport pré-réduit (RTF), avant lavage et dilacération. L'ADN génomique est obtenu après 3 lyses enzymatiques, puis purifié sur colonne gel filtration. La spécificité des oligonucléotides universels et bactériens complémentaires aux régions conservées de l'ADNr a été contrôlée pour 180 organismes. Ces amorces contiennent des mésappariements pour certains micro-organismes. L'amplification avec des amorces universelles révèle une hybridation avec un gène mitochondrial des cellules de rat. Afin de conserver la biodiversité des échantillons, seulement 15 cycles d'amplification sont réalisés. Les fragments amplifiés sont digérés par 3 enzymes : *Hae*III, *Rsa*I, *Taq*I. Un essai préliminaire de validation de cette méthode d'analyse des profils de restriction d'amplification d'ADNr a été réalisé vis-à-vis de souches isolées de jéjunum de rat. Un plus grand nombre de souches devra être analysé ultérieurement pour valider plus précisément cette méthode.

ARDRA / muqueuse intestinale / écosystème

1. INTRODUCTION

Bacteria of commensal intestinal flora possess a range of important metabolic and immunological properties, and it seems likely that those in the immediate vicinity of the mucosal surface will be particularly relevant to some of these functions [17]. In contrast to numerous studies on faecal bacteria [10, 12, 24, 26, 31], there is little published data on the qualitative composition of mucosa intestinal tract-associated flora [13, 21, 23]. Most qualitative studies are on the human colon and faeces by conventional microbiological methods. One of the limitations in using them is that only easily cultivable and non-damaged organisms are counted. Bacteria that need interactions with the host or other microorganisms, or which require unknown growth conditions, will not be selected in this way. Estimates of cultivability of bacteria in the gastrointestinal tract vary from 10 to 50% [15, 34].

However, culture-independent techniques involving total-community genomic DNA or extraction followed by amplification of 16S rRNA encoding DNA (rDNA) by PCR have revealed immense phylogenetic diversity within a naturally occurring community [32]. The 16S rRNAs are valuable phylogenetic marker molecules for microorganisms because they are universally distributed and constant in function and because different positions of their sequences change at very different rates [36].

This makes the 16S rRNA gene suitable for many analytical methods, like sequence analysis, ribotyping, restriction fragment length polymorphism (RFLP) analysis, and hybridisation with oligonucleotide probes [1, 16], all useful for the identification and typing of microorganisms. Of all these methods, RFLP analysis of PCR generated rDNA fragments named ARDRA (amplified rDNA restriction analysis) was used by many authors [9, 18, 30, 35, 36, 38].

The intention of this study was the improvement of ARDRA methods for analysis of a large number of 16S rDNA clones from intestinal samples collected on rats. Experiments included optimisation of all critical steps: DNA extraction, purification and PCR amplification.

2. MATERIALS AND METHODS

2.1. Bacterial strains

Escherichia coli (CIP54127), *Escherichia coli* TG1, *Bifidobacterium bifidum* (wild strain from laboratory collection) and

Candida albicans (ATCC 2091) strains were used in this study. *E. coli* strains were cultured under aerobic conditions at 37 °C in Luria-Bertani broth or on solid Luria-Bertani medium (Difco Laboratories, Detroit, MI, USA). *Bifidobacterium bifidum* was cultured on De Man, Rogosa & Sharpe medium (MRS) (Difco) anaerobically at 37 °C. *Candida albicans* was cultured under aerobic conditions at 30 °C on Sabouraud medium (Difco). After 24 h incubation, cells were harvested by centrifugation (10 min at 18 000 *g* at 4 °C) for the extraction of total nucleic acids.

2.2. Preparation of faecal and intestinal specimens

Fresh faeces were collected from healthy humans or animals. A 1 g (wet weight) faeces specimen was added to 4 mL of 0.9% (w/v) sterile NaCl aqueous solution and mixed by inverting and vortexing the tube from 5 to 10 min.

Germ-free rats or conventional rats were fasted for 24 h before being killed. Animals were then killed by cervical dislocation. The abdomen was opened under sterile conditions and aliquots of duodenum, jejunum and ileum were collected. The specimens were gently rinsed with sterile saline solution and were scraped with a scalpel. The scrapings were suspended in sterile saline solution and vortexed for 5 min for microbiological analysis.

For molecular analysis, non-scraped specimens were stored at 4 °C in reduced transport fluid: RTF (3.8 mmol·L^{-1} Na_2CO_3, 1 mmol·L^{-1} EDTA, 1.3 mmol·L^{-1} DTT, 4.3 mmol·L^{-1} K_2HPO_4, 15.4 mmol·L^{-1} NaCl, 3.3 mmol·L^{-1} KH_2PO_4, 6.8 mmol·L^{-1} $(NH_4)_2SO_4$, 1.5 mmol·L^{-1} $MgSO_4 \cdot 7H_2O$, 0.001% resazurin) [28] for 24 h.

2.3. Microbiological analysis

Serial dilution within the range of 1 to 10^{-4} in sterile saline solution of the samples (faeces and scraping) were plated on solid media including Trypcase soy (BioMerieux, Capronne, France**)**, Columbia supplemented with 5% sheep blood (v/v) (BioMerieux) and De Man Rogosa & Sharpe (BioMerieux). The plates were placed in a GENbox anaer system (BioMerieux) for an anaerobic incubation at 37 °C, or were incubated aerobic, for 15 d. Colonies picked from countable plates were selected for the Gram reaction, morphology and biochemical tests (oxidase, catalase) [5]. Some of them were tested for further identification using the API Kit system (BioMerieux). The identification of all strains was confirmed by 16S rDNA analysis.

2.4. Molecular analysis

2.4.1. Isolation of total DNA

Extraction method was used with all samples (faeces, intestinal tissues). Each intestinal sample was washed two times with saline solution to eliminate the transport media and scraped as described above.

One mL of scrapings was centrifuged at 13 000 *g* for 10 min, the pellet was suspended in 150 µL of sucrose lysis buffer (20% [w/v] sucrose, 20 mmol·L^{-1} EDTA, 100 mmol·L^{-1} Tris-HCl; pH 8.0). Mechanical lysis was performed. One mL of suspension was placed into a 2 mL vial containing 2 g of 0.1 mm zirconia/silica beads. The tube was placed for 0.5 to 2 min at 3 000 rpm on a minibead beater (Biospec Corp., Bartlesville, Okla., USA). The lysat was obtained by centrifugation at 16 000 *g* for 2 min. For enzymatic lysis, lysozyme 4 mg·mL^{-1} and 50 units of achromopeptidase (Sigma Aldrich, Saint Quentin Fallavier, France) were added. Incubation was then carried out at 37 °C for 3 h. The preparation was washed with saline solution. The pellet was suspended in 150 µL of $MgSO_4$-$7H_20$, 0.1 mol·L^{-1} Tris-HCl pH 7.2, 0.01 mol·L^{-1} β-mercaptoethanol with 100 units of lyticase solution (Sigma) and was incubat-ed at 30 °C for an additional time of 3 h. After washing with water, the

pellet was suspended in 150 µL of sucrose lysis buffer (20% [w/v] sucrose, 20 mmol·L^{-1} EDTA, 100 mmol·L^{-1} Tris-HCl; pH 8.0) and 7.5 µL of 20% (w/v) sodium dodecyl sulfate and the suspension was incubated at 65 °C for 20 min and 150 µL of phenol-chloroform (1:1) were then added. After centrifugation at 13 000 *g* for 10 min, the aqueous phase was taken. The genomic DNA was allowed to precipitate at –20 °C for 8 h after addition of 350 µL of ice-cold ethanol and DNA was harvested by centrifugation at 13 000 *g* for 20 min. The genomic DNA pellet was purified on a spin column of polyacrylamide gel (Bio-gel P-10 medium, Bio-Rad, Ivry-sur-Seine, France). Finally, DNA was eluted with 50 µL of water.

The DNA concentration (200 ng·µL^{-1}) and its integrity (size >21 kb) were estimated by agarose gel electrophoresis (with 1.0% [w/v] agarose-1X Tris-acetate buffer (0.04 mol·L^{-1} Tris-acetate, 0.002 mol·L^{-1} EDTA).

DNA was visualised by transillumination with UV light after staining the gels with ethidium bromide.

2.4.2. PCR amplification, DNA cloning procedure

Table I lists sets primers used in this study. PCR amplification of 16S rDNA fragments was carried out in an Eppendorf Minicycler (MJ Research, Incline Village, Nevada, USA). Reaction tubes contained 200 ng (1 µL) of DNA extract, 2 U of Taq DNA polymerase, 1X Taq DNA pol buffer, 200 µmol·L^{-1} of each deoxyribonucleotide triphosphate, and 0.44 µmol·L^{-1} of each primer in a final volume of 50 µL. Initial DNA denaturation and enzyme activation steps were performed at 94 °C for 3 min, followed by 15 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min, and elongation at 72 °C for 1 min. PCR products were purified and concentrated using a spin column of polyacrylamide gel. Three reaction mixtures were pooled in order to increase the DNA yield, and amplicons were eluted with 50 µL of water. Their concentration (22 ng·µL^{-1}) and size were estimated by electrophoresis as previously described. The purified products were cleaved at the restriction endonuclease sites *Not*I within the linkers (Tab. I). The pGEM-5Z vector (Promega Corp., Madison, USA) was dephosphorylated by the method of Sambrook et al. [25].

DNA was ligated with T4 DNA ligase as specified by the manufacturer. The ligation mixtures were used to transform competent *Escherichia coli* TG1 cells by electroporation (Gene pulser II Bio-Rad 25 µF, 2.5 kV). Ampicillin-resistant transformants were selected on Luria-Bertani agar containing ampicillin (100 µg·mL^{-1}), 24 µg·mL^{-1} IPTG (isopropyl-β-D-thio-galactopyranoside) and 25.6 µg·mL^{-1} X-Gal (5-bromo-4-chloro-3 indolyl-β-D-galactopyranoside).

2.4.3. Plasmid extraction and digestion

DNA preparations were made with a QIAprep spin plasmid kit (Qiagen, S.A. Hilden, Germany) as specified by the manufacturer. Plasmids were eluted with 50 µL of water, and the products were stored at –80 °C. Plasmid sizes and concentrations were checked by 1% (w/v) agarose gel electrophoresis (Seakem GTG agarose**,** Tebu S.A., Le Perray-en-Yvelines, France). The clones with an insert were digested with the restriction endonucleases *Not*I + *Hae*III, *Not*I + *Rsa*I, *Not*I + *Taq*I. Digests contained 0.2 to 1.0 µg of DNA in a total volume of 20 µL. The digestions were carried out as recommended by the manufacturer, with appropriate restriction buffers at the recommended temperature for 3 h. DNA fragments were resolved in 2% (w/v) agarose gels (Metaphor agarose, Tebu) with Tris-acetate buffer (0.04 mol·L^{-1} Tris-acetate, 0.002 mol·L^{-1} EDTA). Molecular size of the DNA fragments was estimated by comparison with 123 bp DNA ladder (Life Technologies, Paisley, UK) and compared with patterns of different families.

Table I. Oligonucleotides corresponding to conserved regions of 16S rDNA genes used for PCR amplification.

Name	Sequence (5'– 3')	Position[a]	Reference
Universal			
1	AGCMGCCGCGGTAATWC[b]	F520	Giovannoni et al.[d] [11]
2	ACGGGCGGTGTGTRC[b]	R1392	Olsen et al. [20]
3	**TAATAGCGGCCGC**AGCMGCCGCGGTAATWC[b,c]		
4	**ATAATGCGGCCGC**ACGGCGGTGTGTRC[b,c]		
Bacteria			
1	AGAGTTTGATCCTGGCTCAG	F8	Delong [6]
2	AAGGAGGTGATCCAGCC	R1523	Weisburg et al. [33]
3	**ATAATGCGGCCGC**AGAGTTTGATCCTGGCTCAG[c]		
4	**TAATAGCGGCCGC**AAGGAGGTGATCCAGCC[c]		

[a] The position corresponds to the primer 5' end, using *Escherichia coli* 16S rDNA as reference [3]; F and R correspond to the forward and reverse primers, respectively.
[b] M, an A/C nucleotide degeneracy; W, an A/T nucleotide degeneracy; R, an A/G nucleotide degeneracy.
[c] Containing tails (in boldface type) with restriction site *Not*I (underlined sequence).
[d] This sequence has one nucleotide more.

2.4.4. Sequencing and sequence analysis

The pGEM-5Z vector containing an interesting insert was sequenced directly by Taq cycle sequencing using fluorescence-based chain termination chemistry (Perkin Elmer/Applied Biosystems, Foster City, California, USA) and an automatic DNA sequencer (Perkin Elmer /Applied Biosystems, model 373 A). Sequence analysis was conducted using programs included in the GCG (Genetics Computer Group, Madison WI, USA) package. The insert sequence was compared with the whole set of known nucleic sequences of GENE/EMBL sequence databases, using standard algorithms of biosequence analysis, such as the FastA and the BLAST commands.

2.4.5. Reference rDNA sequences used in cluster analysis

Some references of *Bacteria*, *Archaebacteria*, and Eukaryotes rDNA sequences are listed in Table II with their GenBank accession numbers. In addition, references of *Bacteria*, *Archaebacteria*, and Eukaryotes used in this analysis are reported below.

Acetobacterium carbinolicum (X96956), *Acetobacterium malicum* (X96957), *Acetobacterium paludosum* (X96958), *Acetobacterium wieringae* (X96955), *Acetobacterium woodii* (X96954), *Aeromonas bestiarum* (X60406), *Aeromonas encheleia* (AJ224309), *Aeromonas hydrophila* (X60404), *Aeromonas popoffii* (AJ224308), *Aeromonas salmonicida* (X60405), *Butyribacterium methylotrophicum* (AF064241), *Chromatium minutissimum* (Y12369), *Chromatium purpuratum* (AJ224439), *Desulfovibrio desulfuricans* (AF192154), *Eubacterium callanderi* (X96961), *Eubacterium limosum* (AF064242, U67159), *Halobacterium* sp. (AB009624), *Halococcus saccharolyticus* (ABOO4876), *Lactobacillus jonhsonii* (AJ002515), *Lactobacillus manihotivorans* (AF000163, AF000162), *Lactobacillus mucosae* (AF126738), *Lactobacillus salivarius* (AF089108), *Methanococcus voltae* (M59290), *Methanospirillum hungatei* (M60880), *Mycoplasma capricolum* (U26048), *Nuphar japonica* (D85295), *Paenibacillus lentimorbus* (AF071861), *Paenibacillus popilliae* (AF071860), *Paracoccus carotinifaciens* (AB006899), *Paracoccus marcusii* (Y12703), *Pediococcus inopinutus* (AJ271383), *Pseudomonas azotoformans* (D84009), *Pseudomonas fragi* (D84014), *Pseudomonas taetrolens* (D84027), *Ralstonia gilardii* (AF076645), *Ralstonia paucula* (AF085226), *Saccharomyces cerevisiae* (J01353, Z75578), *Staphylococcus aureus* (Y15856), *Staphylococcus capitis* (AB009937), *Staphylococcus delphini* (AB009938), *Staphylococcus hominis* (X66101), *Staphylococcus succinus* (AF004220), *Staphylococcus vitulus* (AB009946), *Streptomyces sampsonii* (D63871), *Streptomyces scabies* (Y15493), *Streptomyces tendae* (D63873), *Streptomyces thermocarboxydovorans* (U94487), *Sulfolobus solfataricus* (X03235), *Sulfolobus thuringiensis* (X90485), *Zea mays* (AF168884).

3. RESULTS AND DISCUSSION

3.1. Development of DNA extraction procedure from intestinal tissue

Lysis of microbial cells from complex microbiota marks a critical step in a PCR mediated approach. Insufficient or preferential disruption of cells will most likely bias the view of the composition of microbial diversity as DNA, which is not released from the cells, will not contribute to the final analysis of diversity. Our priorities were to lyse the maximal number of cells, thereby releasing all of the genomic DNA, and to purify it to produce templates that could be used in a PCR.

Lysis of microorganism cells was typically performed in two ways: chemical disruption (using compounds and conditions such as SDS plus heat, NaOH, proteinase K

Table II. Sequence alignment of 16S rDNA primers with target sequence for organisms included in this study (only mismatches are represented).

Primer and organism	Primer or target sequence[a]						
Universal 1	5' AGC	MGC	CGC	GGT	AAT	WC	3'
Chlorobium phaeobacteroides	– – –	– – –	– – –	– – –	G– –	– –	
Chlorobium limicola	– – –	– – –	– – –	– – –	G– –	– –	
Micrococcus varians	– – –	– – –	– – –	– – –	G– –	– –	
Propionibacterium[b]	– – –	– – –	– – –	– – –	G– –	– –	
Methanococcus thermolithotrophicus	– – –	– – –	– – –	– – –	– – C	– –	
Methanococcus voltae	– – –	– – –	– – –	– – –	– – C	– –	
Methanococcus maripaludis	– – –	– – –	– – –	– – –	– – C	– –	
Spirochaeta africana	– – –	– – –	– – –	– – –	– – C	– –	
Methanosarcina barkeri	– – –	– – –	– – –	– – –	– – C	– –	
Methanobacterium bryantii	– C –	– – –	– – –	– – –	– – C	– –	
Bifidobacterium thermophilum	– – –	– – –	– – –	– – –	– – –	– A	
Mycoplasma gallisepticum	– – –	– – T	– – –	– – –	– – –	– –	
Universal 2	5' ACG	GGC	GGT	GTG	TRC	3'	
Bifidobacterium breve	– – –	T – –	– – –	– – –	– – –	– –	
Corynebacterium jeikeium	– – –	– – A	– – –	– – –	– – –	– –	
Bacterial 1	5' AGA	GTT	TGA	TCC	TGG	CTC	A 3'
Acinetobacter calcoaceticus	– – –	– – –	– – –	– – A	– – –	– – –	–
Bifidobacterium thermophilum	– – –	– – –	– – –	– – A	– – –	– – –	–
Bifidobacterium inopinatum	– – –	– – –	– – –	– – A	– – –	– – –	–
Butyribacterium methylotrophicum	– – –	– – –	– – –	– – A	– – –	– – –	–
Escherichia coli	– – –	– – –	– – –	– – A	– – –	– – –	–
Lactobacillus manihotivorans	– – –	– – –	– – –	– – A	– – –	– – –	–
Aeromonas[b]	– – –	– – –	– – –	– – A	– – –	– – –	–
Colwellia psyoerythrae	– – –	– – –	– – –	– – A	– – –	– – –	–
Eubacterium limosum	– – –	– – –	– – –	– – A	– – –	– – –	–
Corynebacterium jeikeium	– – –	– – –	– – –	C – –	– – –	– – –	–
Chromatium minutissimum	– – –	– – –	– – –	– – –	– – –	– – G	–
Halobacteroides lacunaris	– – –	– – –	– – –	– – –	– – –	– C T	–
Halorubrum trapanicum	– – –	– – –	– – –	– – –	– – C	– C G	–
Methanobacterium formicicum	– T C	C – –	– – –	– – –	– – G	G – –	–
Methanobacterium thermophilum	– – –	– – –	– – –	– – –	– – G	G – –	–
Bacterial 2	5' AAG	GAG	GTG	ATC	CAG	CC3'	
Sarcina ventriculi	– G A	– – –	– – –	– – –	– – –	– –	
Escherichia coli	– – –	– – –	– – –	– – –	– – A	– –	
Mycoplasma capricolum	– – –	– – –	– – –	– – –	– – T	– –	
Eubacterium limosum	– – –	– – –	– – –	– A –	– – C	– –	
Acholeplasma laidlawii	– – –	– – –	– – –	– – –	– – T	– –	
Spiroplasma citri	– – –	– – –	– – –	– – –	– – T	– –	
Mycoplasma gallisepticum	– – –	– – –	– – –	– – –	– – C	– –	
Clostridium innocuum	– – –	– – –	– – –	– – –	– – T	– –	

[a] Obtained from EMBL database. M, an A/C nucleotide degeneracy; W an A/T nucleotide degeneracy; R, an A/G nucleotide degeneracy.
[b] All tested species belonging to this genus have the same mismatches.

or peptidase) or physical disruption (bead mill homogenisation, French press, boiling, microwave treatment, freeze-thaw cycle or sonication).

Nucleic acid extraction by mechanical procedures from faecal samples has been shown to be effective in disrupting bacterial cells from a variety of ecosystems [9, 34, 38]. In our system, host and microorganism DNA were included in extracted DNA from the biopsy. Therefore, quality and quantity of released DNA was difficult to estimate. So first experiments were performed on pure cultures of *Escherichia coli*, *Bifidobacterium bifidum*, and *Candida albicans*. A mechanical procedure (Bead mill homogenisation) was first tested for DNA extraction from the three pure cultures. In this condition, a smear on agarose 1% electrophoresis gel was seen after migration of all released DNA. Whatever the bead beating time, DNA was always degraded when cells were broken (Fig. 1A). Since further treatment affected the DNA integrity, enzymatic procedure was tested in other experiments. Three enzymatic treatments were applied in this procedure: lysozyme, achromopeptidase and lyticase. The lysozyme and the achromopeptidase were used for lysis of Gram-negative and Gram-positive bacteria such as *Bifidobacteria* [2]. The lyticase was used for formation of spheroplast of *Candida albicans* [14, 22]. This enzymatic procedure was effective in the disrupting of bacterial and eukaryotic cells. Observations of Gram-stained samples prepared before and after the cell lysis procedure indicated that over 90% of tested microorganisms were lysed. Therefore, integrity and quality of released DNA was preserved as confirmed by agarose gel electrophoresis (Fig. 1B). Lysis treatment for Gram-positive bacteria and eukaryotic cells did not lead to highly fragmented nucleic acids. Furthermore, our results show that

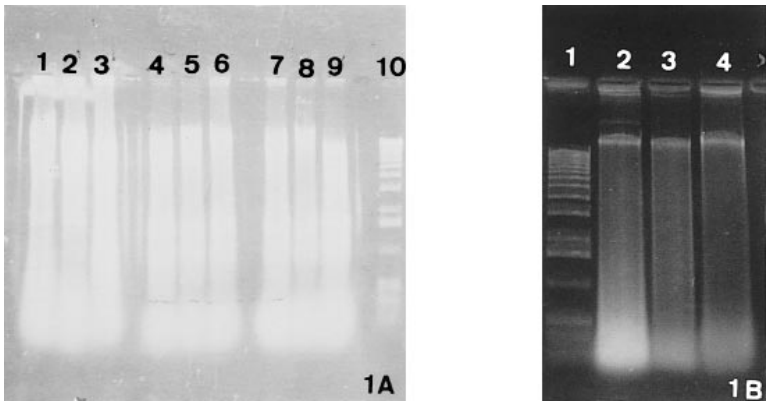

Figure 1. Gel electrophoresis data from the development of lysis procedure for pure cultures or for intestinal samples. One kb DNA ladders (Gibco BRL, Burlington, Ontario, Canada) are included on 1% agarose gels. (**A**) Effect of time for bead beating on extractable DNA from pure cultures. Lanes 1 to 3: DNA extract from *E. coli* after 0.5, 1, 2 min of bead beating respectively, lanes 4 to 6: DNA extract from *Bifidobacterium bifidum* after 0.5, 1, 2 min of bead beating respectively, lanes 7 to 9: DNA extract from *Candida albicans* after 0.5, 1, 2 min of bead beating respectively, lanes 10: DNA ladders. Note the degradation of DNA from *E. coli*, *Bifidobacterium bifidum* and *Candida albicans* whatever the time for bead beating. (**B**) Released DNA by enzymatic lysis from intestinal tissues of rats. Lane 1: DNA ladders, lane 2: DNA extract from duodenal sample, lane 3: DNA extract from jejunal sample, lane 4: DNA extract from ileal sample.

Gram-positive and Gram-negative bacteria can be detected in the same proportions. Finally, enzymatic procedure was chosen rather than mechanical procedure for the cellular lysis.

However, DNA at this stage was still unusable for molecular studies. It was not cut with restriction enzyme. In addition, 16S rDNA was not amplified by PCR. DNA solutions were passed through a spin column of polyacrylamide gel (Bio-gel P10-medium) which removed most of the impurities from them. After this purification, PCR amplification and restriction were possible. Therefore, all these preliminary results were applied for DNA extraction from intestinal tissue. However, our experiments demonstrated that an additional step was necessary for PCR amplification with DNA from the tissue. Direct analysis of the intestinal samples for enzymatic lysis did not enable the detection of nucleic acids, indicating that lysis or detachment of adherent bacteria are limited in this condition. 16S rDNA fragments were amplified only if the sample was stored at 4 °C in reduced transport fluid for 24 h. This transport media was usually used for oral microbiological samples [28]. It was a non-nutritious media which has been shown to be effective in the survival of anaerobic and microaerophilic bacteria (*Prevotella intermedia*, *Actinobacillus*, *Porphyromonas*) [7, 29]. The composition of media containing the intestinal sample at 4 °C appeared to avoid the growth of microorganisms using the sample as a nutriment, despite growth inhibitors in RTF. Our results indicated that the medium, the temperature and the time of pre-incubation are determinant.

Suboptimal binding of primer will result in less efficient amplification of the respective DNA. Especially domain-specific or universal primers must have uniform hybridisation efficiency to guarantee the amplification of all target 16S rDNAs. The specificity of all primers used in this study was so tested.

3.2. Evaluation of oligonucleotide target sequence (Tab. II)

DNA extraction should reflect quantitative abundance species, however, in PCR amplified 16S rDNA from complex microbiota a mixture of homologous molecules serve as a template. Amplified DNA can also reflect quantitative abundance in species only if the amplification efficiencies are the same for all molecules. This requires several assumptions [27]:

– all molecules are equally accessible to primer hybridisation;

– primer-template hybrids form with equal efficiencies;

– extension efficiency of DNA polymerase is the same for all templates;

– and limitations by substrate exhaustion equivalently affect the extensions of all templates.

These assumptions seem difficult to retain, as universal primers employed for amplification of rDNA often contain degeneracy which may influence the formation of primer-template hybrids.

From the databank, 180 organisms were chosen to represent the three domains of *Bacteria*, *Archaea* and *Eukarya*. One hundred and forty-eight microorganisms were selected to represent the bacterial domain: *Firmicute*, *Proteobacteria alpha*, *beta*, *gamma*, *delta subdivision*, *green sulfur* and *spirochaetale*, 29 organisms were chosen to represent *Archaea* domain: *Methacoccale*, *Methanobacteria*, *Halobacteria*, *Crenarchaeata*, and three phylogenically diverse eukaryotes: *Saccharomyces cerevisiae*, *Nuphar japonica*, and *Zea mays* were selected to represent the *Eukarya*.

In addition to phylogenetic considerations, 16S rDNA sequence availability from the databank was an important consideration for the selection of organisms. Each one of the selected organisms has a nearly complete rDNA sequence available.

The primer Universal 1 consisted of a mixture of four oligomers. The degenerate

sites were present at the fourth base (M = A or C) and at the sixteenth base (W = T or A). These four oligomers hybridised with all *Bacteria* and *Eukarya* organisms included in this study. However one internal T:C mismatch was observed with the *Archaea: Methanobacteria*, some *Methanococcus* and *Spirochaeta*. One A:G mismatch with *Propionibacteria* genus, some *Chlorobium* and *Micrococcus* species was also found.

Eighty-seven percent of bacteria had an A nucleotide at the fourth base of their 16S rDNA sequence, the remainder had a C nucleotide at the corresponding position and belong to *Sulfolobus*. An A nucleotide at the sixteenth base was characteristic of the *Bacteria* and *Archaea* domains, whereas a T nucleotide represents the *Eukarya* domain.

The percentage of 16S tested rDNA sequences which had no, one or two mismatches with this probe was 6%.

All these mismatches may have modified the primer hybridisation with template, and may have influenced the PCR amplification.

The primer Universal 2 consisted of a mixture of two oligomers differing from each other at the fourteenth base. The probe mixture was synthesised by adding an equimolar mixture of A and G during the corresponding synthesis step. The probe perfectly matched the *Eukarya* and *Archaea* domains. However, for the *Bacteria* domain, two strains had one mismatch: *Bifidobacterium breve* and *Corynebacterium jeikeium*. The oligomers with an A perfectly matched the *Bacteria* and *Eukarya*, while the oligomers with a G matched the *Archaea*. Therefore, when the probe hybridised with rDNA from the *Archaea* there was one additional G:C pair, compared with those from the other two domains, however, the percentage of 16S tested rDNA sequences which had no, one or two mismatches with this probe was only 1.5%.

The primer Bacteria 1 was specific to *Bacteria* domain, contained no base degenerate but had a C:A mismatch with some bacteria. One mismatch was present with some species of *Bifidobacteria* and *Lactobacillus*. This specific probe for domain bacteria could also hybridise with one mismatch with an *Archaeabacteria*: *Chromatium minutissimum*. The other *Archaebacteria* contained two or more mismatches. The twelfth base could be changed by nucleotide degeneracy (M = C or A) which may perfectly match with *Bifidobacteria*, *Escherichia coli* and *Lactobacillus*. The percentage of 16S tested rDNA sequences which had no, one or two mismatches with this new probe was only 3%, whereas the percentage was 8% with the non-modified probe.

The primer Bacteria 2 was specific to the bacteria domain. The third base was different for the *Mycoplasma* genus, *Acholeplasma* species, *Clostridium innocum*, and *Escherichia coli*. Two mismatches with *Eubacterium limosum* were observed. The percentage of bacterial 16S rDNA sequence which had no, one or two mismatches with this probe was 4.5%. The two bacterial primers tested had high specificity for bacterial sequences with only minor affinity for archaeal sequences.

Similar analysis by Brunk et al. [4] has been reported. They examined the 16S rRNA sequences in the Ribosomal Database Project (RDP) to evaluate the efficiency and specificity of universal and bacterial probes. They found that the universal probe corresponding to universal primer 1 with M = A and W = A had a perfect match with 71% of 16S rDNA sequence. The other three iterations of this probe sequence, collectively had only 32 additional perfect matches. They reported that using a degenerate probe at either or both positions would add only marginally to hybridisation, however, this analysis was performed with pure cultures and not with a mixture of 16S rDNA sequences. Zheng et al. [37] have analysed the specificity of universal primers; they found that in pure cultures or in mixed

cultures, hybridisation was modified. In their study, Suau et al. [26] have examined human faecal samples with direct analysis of genes encoding 16S rRNA from using bacterial primer 1 for PCR amplification. Since the analysed faecal samples contained genetic material of bifidobacteria based on RNA dot blot hybridisation, the lack of bifidobacterial sequences with PCR analysis could be attributed to mismatches between the sequence of the 5' PCR primer bacterial 1 and bifidobacterial sequences. However, DNA-DNA hybridisation and PCR are two different methods and cannot be compared in the same way.

Mismatch position could modify PCR amplification. Imperfect primer annealing at 3' end could result from more inappropriate hybridisation stringency for PCR amplification than primer annealing at 5' end. In addition, *Lactobacillus*, *Bifidobacteria* and *Escherichia coli* may have been encountered in the intestinal tract, and could be underestimated or absent in our analysis. Mismatches within primers may have influenced PCR, resulting in a wrong evaluation of the diversity from a complex microbiota. Despite this, universal primers were chosen for ARDRA validation because they are internal sequences from 16S rRNA databank sequences and all mismatches can be verified by amplification with bacterial primers. Future experiments will be carried out to determine the impact of mismatch position on PCR amplification.

3.3. 16S rDNA amplification from intestinal tissue by PCR

Universal and bacterial primers were used for PCR amplification of 16S rDNA fragments from intestinal or faecal samples. PCR with universal primers amplified a fragment from 862 bp for *Paracoccus marcusii* to 913 bp for *Bifidobacterium inopinatum* with an average of 895 bp for all tested 16S rDNAs. PCR amplification with bacterial primers produced a 1 532 bp fragment of 16S rDNA corresponding to the *Escherichia coli* sequence. PCR amplification of a 16S rDNA fragment from biopsy (duodenal, jejunal, ileal and colon mucosa), intestinal content or a faecal sample from germ-free rats was performed. With PCR bacterial primers, any amplification was detectable on agarose electrophoresis gel. Surprisingly, a 550 bp fragment was amplified by PCR using universal primers, only with intestinal samples. This DNA fragment was cloned in PGEM 5Z vector and was sequenced. Comparison of 550 bp sequence with sequences available in the EMBL database revealed 99.7% homology with 3 beta-hydroxysteroid dehydrogenase isomerase type II2 (S63167), a mitochondrial gene of rats. The universal primer 1 hybridised at 100% with this gene, whereas the universal primer 2 had two mismatches. Therefore, the absence of amplification of samples from germ-free rats at the expected size was confirmed with no growth on Trypcase soy, Columbia supplemented with sheep blood and MRS plates incubated in aerobic and anaerobic conditions. PCR amplification from tissue of conventional rats included 550 bp and 890 bp fragments for the universal primers (Fig. 2). An additional step was necessary to purify the 890 bp fragment. PCR amplification with bacterial primers produces a 1 530 bp fragment without additional fragments (Fig. 2), however, this PCR amplification may underestimate microbial diversity. The effect of template concentration on PCR amplification was studied by amplifying biopsy DNA at different dilutions. Dilution to approximately 10 pg of DNA·μL^{-1} did not affect the PCR amplification. This indicated that DNA from tissue was present in abundance, suggesting that very little pieces of intestinal samples are necessary for this experiment. Due to the PCR capacity to amplify small amounts of DNA, organisms occurring in small numbers in a complex microbiota are now detectable.

In addition, to avoid the bias caused by template annealing by PCR amplification

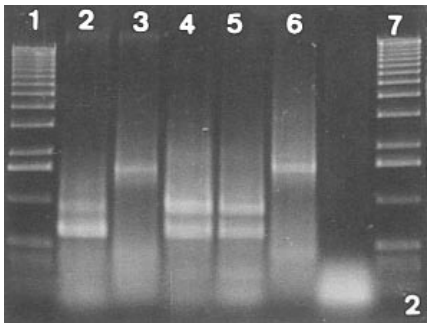

Figure 2. 16S rDNA amplification from intestinal samples with universal and bacterial primers. One kb DNA ladders (Gibco BRL) are included on 1% agarose gels (lanes 1 and 7). PCR amplification of 16S rDNA from the duodenal sample with universal primers 3, 4 (lane 2), with bacterial primers 3, 4 (lane 3), from the jejunal sample with universal primers (lane 4) and from the ileal sample with universal primers (lane 5) and bacterial primers (lane 6). Note the presence of 550 bp fragment with PCR universal primers.

[8, 27], the number of cycles were minimised. Wilson et al. [34] reported that when the number of temperature cycles was minimised, biodiversity was preserved, and there was a good agreement between culturing bacteria and sampling rDNA directly. PCR amplification of 15, 20, 35 cycles were tested with DNA from tissue. Therefore, 15 cycles were chosen for further experiments. The cloning of 16S rDNA was possible only if primers contained a rare-cutting restriction enzyme site such as *Not*I. With a genome containing 50% G + C, the probability of finding a *Not*I (8 bp) cleavage site is one for 65 kb compared to one for 4 kb for a 6 bp site. PCR efficiency was not affected by the presence of a restriction site. Moreover, the risk of losing clones because of an internal restriction site *Not*I using PCR primers was limited. All the 180 organisms' 16S rDNA sequences were tested for the presence of this restriction site and none of these had this site.

Furthermore, the distribution of 16S rDNA clones should approximate with the relative distribution of cells in the tissue. Therefore, a large number of clones should be analysed in a short time. For this, a PCR insert product was amplified with universal or bacterial primers from plasmidic DNA. In this case, no amplification of DNA from *Escherichia coli* TG1 was observed due to this lower concentration of plasmidic DNA. In addition, crude bacterial lysat can be used for PCR 16S amplification.

3.4. ARDRA validation

From the databank, 180 organisms were chosen to represent the three domains of *Bacteria*, *Archaea* and *Eukarya*. Their theoretical ARDRA patterns with universal primers and tetrameric endonuclease *RsaI*, *Taq*I and *Hae*III digestions were calculated. Because all enzymes recognise a tetranucleotide sequence, they statistically cut a given fragment with similar frequencies. Clustering of the library was performed after combining the similar results of ARDRAs. Inside the following genera: *Acetobacteria*, *Aeromonas*, *Halococcus*, *Lactobacillus*, *Paenibacillus*, *Paracoccus*, *Propionibacterium*, *Ralstonia* and *Staphylococcus*, no difference was seen for all the species tested. For species distinction of these bacteria, restriction with another enzyme would be necessary. The 890 bp fragment of 16S rDNA yielded restriction fragments in the size range from 890 bp to 123 bp (lower limit for size evaluation). All the fragments below 123 bp were eliminated from Table III. Obviously, the analysis of separate digestions with three restriction enzymes instead of double digestion gives more precise results. Double digestion produces small bands, which are difficult to detect in the gel. The accuracy of distinction between different 16S DNA fragments by ARDRA depends on the number of restriction enzymes used. This relationship was discussed in detail by Nei and Li [19]. Two strains belonging to the same species may have the same profile, for example

Table III. ARDRA patterns with restriction enzymes *Hae*III, *Rsa*I, *Taq*I. Sizes were expressed in bp.

Genus	Species	Accession number	*Hae*III	*Rsa*I	*Taq*I
Staphylococcus			889	503 373	443 361
Strain 3			**838**	**492 369**	**450 369**
Paenibacillus			885	372 340 146	443 322
Bacillus	*tipchiralis*	AF039408	884	712 146	443 441
Acetobacterium			878	501 272	443 435
Mycoplasma	*capricolum*	U26048	874	366 304 147s	434 354
Acholeplasma	*polakii*	AF031479	873	349 304 144	434 355
Acholeplasma	*palmae*	L33734	872	349 166 144 137	433 355
Acholeplasma	*modicum*	M23933	863	497 304	434 355
Acetobacterium	*bakii*	X96960	786	501 365	443 435
Eubacterium			728 151	501 272	444 435
Entomoplasma	*freundtii*	AFO36954	712 165	864	440 437
Ralstonia			684 198	371 353 146	441 357
Clostridium	*fusiformis*	AF028349	671 215	502 372	360 244 198
Spiroplasma	*citri*	X63781	653 222	367 304 159	439 221 148
Spiroplasma	sp.	AJ006775	652 222	557 304	434 354
Sarcina	*ventriculi*	AF110272	563 319	370 238 145	442 244 196
Acholeplasma	*laidlawii*	M23932	560 316	366 349 145	436 356
Methanogenium	*thermophilum*	M59129	538 355	340 220 176 157	412 165 147
Methanomicrobium	*mobile*	M59142	538 355	340 220 176 157	661 207
Bacillus	*vortex*	AF039409	507 377	712 146	442 442
Methanofollis	*liminatans*	Y16429	503 355	340 220 176 157	721 147
Bifidobacterium	*inopinatum*	ABO29087	482 318	378 375 148	468 445
Propionibacterium	*acnes*	Y12288	481 224 154	358 305 147	362 326
Propionibacterium	*cyclohexanicum*	D82046	477 378	305 206 148 147	358 316
Mycoplasma	*gallisepticum*	M22441	477 223 164	405 368	439 425
Acinetobacter	*radioresistens*	Z93444	476 223 187	514 243 129	444 324
Halococcus	*turkmenicus*	AB004878	470 193 163	260 219 178 157	476 419
Halococcus	*salifodinae*	AB004877	469 193 163	260 219 178 156	635 259
Halobacteroides	*lacunaris*	L37421	461 411	634 244	448 323
Clostridium	*innocuum*	M23732	461 223 171	700 161	377 355
Coryneform			458 379	375 355 147	445 314
Bacillus	*sphaericus*	L38654	458 410	503 371	445 441
Deinococcus	*radiodurans*	Y11332	458 373	502 240	439 360
Micrococcus	*radiodurans*	M21413	458 373	502 355	439 360
Lactobacillus			457 411	373 355 146	444 244 199
Strain 1			**463 408**	**355 327 120**	**420 255 237**
Strain 2			**448 426**	**370 369 136**	**446 246 220**
Bacillus	*pseudomycoides*	AF013121	457 377	373 355 146	443 443
Bacillus	*thuringiensis*	Z84594	457 377	373 355 146	443 443
Pediococcus	*acidilactici*	M58833	457 386	373 278 146	443 244 199
Bacillus	*subtilis*	AF058766	457 409	501 371	441 359
Bacillus	*cereus*	Z84590	456 377	373 354 146	442 443
Strain 5			**468 369**	**369 356 134**	**438 446**
Clostridium	*frigidicarnis*	AF069742	456 410	372 354 146	442 442
Corynebacterium	*jeikeium*	U87823	454 376	372 203 147	439 357

Table III. (Continued).

Genus	Species	Accession number	*Hae*III	*Rsa*I	*Taq*I
Clostridium	*cocleatum*	AF028350	453 222 184	497 304	438 355
Corynebacterium	*jeikeium*	U87815	450 377	373 200 147 145	439 353
Corynebacterium	*jeikeium*	U87816	449 377	373 347 143	351 242 196
Halorubrum	*trapanicum*	D14125	429 193 163	260 219 178 157	476 419
Sulfolobus			424 183 136	514 220 156	435 314
Propionibacterium	*acnes*	Y12288	419 224 154	305 272 147	362 326
Acinetobacter	*calcoaceticus*	Z93434	418 223	515 372	445 324
Spirochaeta	*alkalica*	X93927	409 172 145	393 207 146 127	441 360
Spirochaeta	*halophila*	M88722	409 171 145	726 146	441 444
Rhizobium	sp.	XJ96060	409 278 179	872	441 311
Paracoccus			409 279 157	480 371	338 216 139
Desulfovibrio	*gabonensis*	U31080	408 281 180	243 231 161 127	440 363
Chlorobium	*okenii*	AJ223234	408 276 162	499 229 139	355 216 140
Chlorobium	*phaeobacteroides*	Y08104	406 276 162	368 348 149	355 214 140
Haloarcula	*marismortui*	X61689	401 355	259 220 178 157	381 254 169
Streptomyces	*acidiscabies*	D63865	384 224 154	374 212 150 147	366 326
Propionibacterium	*granulosum*	AJ003057	379 319 165	360 306 147	365 326
Propionibacterium	*propionicus*	AJ003058	379 317 165	359 306 147	363 327
Propionibacterium	*lymphophilum*	AJ003056	379 364	353 306 147	358 326
Corynebacterium	*pseudogenitalium*	U87822	379 358	237 200 146 138	442 354
Micrococcus	*varians*	X87754	378 316 146	252 205 154 147	444 363
Pseudomonas	*stutzeri*	AF237677	378 220 171	374 357 146	444 361
Kocuria	*rosea*	X87756	378 313 146	356 252 147	444 360
Arthrobacter	*agilis*	X80748	378 207 146	356 252 147	444 360
Arthrobacter	*uratoxydans*	X83410	378 313 146	356 252 147	444 446
Arthrobacter	*ureafaciens*	X80744	378 146	356 252 147	444 360
Corynebacterium	*xerosis*	AF024653	378 377	355 235 147 139	444 359
Corynebacterium	*glutanicum*	X80629	378 364	374 253 147	444 359
Clostridium	*indolis*	AF028351	378 319 189	502 372	442 360
Pseudomonas	*aeruginosa*	AF237678	377 219 171	356 235 146 138	360 303
Chromatium	*limicola*	Y08102	377 279 145	373 357 146	361 303
Pseudomonas	*synxantha*	D84025	376 220 171	372 357 146	442 361
Pseudomonas	*stutzeri*	Y18006	376 220 171	372 357 146	361 303
Corynebacterium	*genitalium*	U87821	375 354 371	199 146 144	437 351
Methanococcus	*thermolithotrophicus*	DSM2095	369 273	171 354 220 176	156 906
Corynebacterium	*genitalium*	U87824	368 357	364 328 146	421 276
Methanosarcina	*barkeri*	AJO12094	358 217 157 138	260 220 176 157	563 232
Alcaligenes	*faecalis*	AF155147	356 319 198	498 371	441 356
Halorubrum	*trapanicum*	X82168	355 222 175	278 220 177 157	260 255 217
Halobacterium	*sodomense*	X82169	355 248 222	278 220 177 157	260 255 217
Methanobacterium	*bryantii*	AF028688	355 203 152	340 220 176 157	469 326
Methanococcus	*voltae*	U38461	355 267 247	340 220 176 156	892
Methanococcus	*vannielli*	M36507	355 241 171	340 220 176 156	567 325
Spirochaeta	*smaragdinae*	U80597	349 223	722 441	439
Spirochaeta	sp.	U40791	338 280	446 427 447	439
Sulfolobus	*metallicus*	X90479	331 184	736 156	793

Table III. (Continued).

Genus	Species	Accession number	*Hae*III	*Rsa*I	*Taq*I
Micrococcus	*lylae*	X80750	319 314 146	357 252 147 122	444 361
Alcaligenes	*faecalis*	D88008	319 198 183 182	499 371	441 357
Bifidobacterium	*thermophilum*	ABO16246	314 224 154	358 251 154 123	454 443
Stomatococcus	*mucilaginosus*	X87758	313 287 146	356 252 147 123	445 360
Bifidobacterium	*bifidum*	S83624	313 224 154 146	356 252 147 474	415
Bifidobacterium	*bifidum*	U25952	313 224 154 146	356 252 147 123	445 444
Bifidobacterium	*breve*	X70973	313 224 154 146	356 252 147 123	444 360
Bifidobacterium	*bifidum*	U25951	313 224 154 146	356 252 147 123	445 444
Dermacoccus	*nishinomiyaensis*	X87757	310 224 154 146	252 204 149 147	444 357
Desulfovibrio	*halophilus*	U48243	282 215 165	345 243 161	439 364
Desulfomonas	*pigra*	AF192152	281 215 192 180	369 344 161	363 306 133
Desulfovibrio	*faifieldensis*	U42221	281 215 192 180	369 344 161	439 363
Acinetobacter	*lwoffi*	AF188302	280 180 154	358 356 146	361 325
Bradyrhizobium	sp.	D86355	280 223 186 179	503 371	441 361
Rhodoplanes	*roseus*	D25313	280 223 186 179	503 229 142 361	308 133
Rhodopseudomonas	*palustris*	D25312	280 224 185 179	503 371	441 361
Blastochloris	*viridis*	D25314	280 223 186 179	503 229 142	361 308
Photobacterium	*profundum*	AB003191	279 215 180	401 372 102	442 361
Photobacterium	*angustum*	D25307	279 215 180	401 234 138	442 361
Acinetobacter	*johnsonii*	AF188300	279 223 180	357 243 146 129	361 324
Colwellia	*psyroerythraea*	ABO11364	279 215 180 161	357 234 146 138	442 361
Photobactérium	*phosphoeum*	Z19107	279 215 180	401 372	442 361
Rhodospirullum	*rubrum*	D30778	279 215 179 160	502 320	444 441
Photobacterium	*histaminum*	D25308	278 180 146	502 372	499 303
Photobacterium	*damselae*	Y18496	278 180 146	502 372	442 303
Chromatium			277 215 181 162	373 356 147	361 303
Spirochaeta	*africana*	X93928	277 154	515 138	443 359
Acinetobacter	*calcoaceticus*	AF15904	277 223 171	372 355 146	359 324
Spirochaeta	*asiatica*	X93926	275 185 154	367 146 138	440 357
Halorubrum	*lacusprofundi*	X82170	248 239 222	340 220 177 157	260 255 217
Methanobacterium	*formicicum*	M36508	247 244	340 220 176 157	893
Methanobacterium	*subterraneum*	X99045	246 242 152	338 220 175 157	890
Methanobacterium	*thermophilum*	X99048	243 193 163	341 220 175 158	469 192 135
Methanococcus	*maripaludis*	AF005049	241 204 171 151	340 220 176 156	892
Bifidobacterium	*animalis*	ABO2987	225 207 146	356 252 147 123	445 360
Streptomyces			224 204 154 146	374 211 150 147	365 326
Bifidobacterium	sp.	X89111	224 207 146	356 251 147 123	443 360
Aeromonas			220 215 171 161	372 357 146	361 303
Isochromatium	*buderi*	AJ224430	215 179	361 314 145	327 170 133
Escherichia	*coli*	JO1859	215 210 180 161	502 372	360 303
Strain 4			**238 238 148 148**	**503 385**	**376 350**
Chromatium	*warmingii*	Y12365	200 180 179 155	371 342 145	356 302
Halobacterium	sp.	AB009624	193 163 158 131	240 178 160 157	476 268 151
Slackia	*exigua*	AF101240	193 151 143	497 371	440 308
Methanococcus	*vulcanus*	AFO51404	191 164 164	340 220 176 156	793

Bifidobacterium bifidum U25951, S83624 or different profile, for example *Corynebacterium jeikeium*.

Therefore, this analysis can give some phylogenetic relationships. If we classify all the restriction profiles by the size with *Hae*III restriction (Tab. III). It can be observed that the closest to the *Staphylococcus* genus was *Bacillus*. It can be correlated to FastA analysis of the 16S *Staphylococcus* sequence which also gives the information that Bacillus have homology with this genus. If close relationships between 16S rDNAs should be analysed, the use of two restriction enzymes is not sufficient. Moreover, the type of restriction enzyme used also influences the accuracy of differentiation. In addition, these three enzymes were complementary for this analysis. For example, the difference between the genus *Lactobacillus* and *Clostridium fridicarnis* was carried out with *Taq*I restriction. Also, genera *Eubacterium* and *Acetobacterium* cannot be discriminated with restriction profiles *Rsa*I and *Taq*I, but restriction with *Hae*III makes it possible. Finally, restriction with *Hae*III and *Taq*I cannot be differentiated between genera *Staphylococcus* and *Paenibacillus*, only *Rsa*I restriction could discriminate these two genera. All these examples confirmed the usage of three enzymes for this analysis. For all ARDRA tested, some organisms could be differentiated at genus level (25%), and to some extent at species level (69%) and 6% at strain level.

Another example of this analysis was ARDRA patterns of five strains isolated from jejunal tissue of the rats. Bacterial strains were identified by traditional microbiology and in parallel by 16S PCR amplification with universal primers. PCR restriction with *Hae*III, *Rsa*I and *Taq*I were performed. Results were included in Table III. The resulting products were separated by gel electrophoresis. Seakem agarose gel electrophoresis (1%) did not resolve the fragments satisfactorily, therefore, Metaphor agarose 2% was used for visualisation of restriction products. Strains 1 and 2 were isolated on MRS medium. Carbohydrate fermentations of these strains were tested using the API 50CH L gallery. The resulting identifications were *Lactobacillus paracasei* for the strain 1 and *Lactobacillus pentosus* for the strain 2. Database comparison of ARDRA patterns indicated that the strain was affiliated to the genus *Lactobacillus*. The strain 3 was isolated from Columbia supplemented with sheep blood medium. Identification (Gram-stained, catalase API gallery) of strain 2 corresponds to *Staphylococcus hominis*. ARDRA patterns of this strain were similar to the S*taphylococcus* genus. Strains 4 and 5 were obtained on Trypcase soy plates. Strains 4 and 5 were identified by microbiology techniques as belonging *Escherichia coli* for strain 4 and *Bacillus cereus* 1 for strain 5. ARDRA patterns of these 5 strains were reported in Table III. The results indicated a correlation between these two methods. Therefore ARDRA patterns with these three enzymes cannot always discriminate at species level. But the genera corresponding to identified strains 1, 2 and 3 have the same pattern for all tested species. The limit resolution of this analysis is between genus and species level. Further investigations on a larger number of strains including cultivable and non-cultivable microorganisms will permit us to validate more precisely this method. Thereby, the ARDRA method may provide the means by which new microorganisms can be discovered.

4. CONCLUSION

Cell lysis, DNA extraction, PCR amplification and cloning process are potential sources of bias in 16S rDNA analysis and specially for ARDRA. Our points of interest are to minimise these biases, in order to preserve microbial diversity from the intestinal sample. We successfully extracted and amplified the 16S rDNA from intestinal samples without cultivation. Grouping of clones

from 16S rDNA libraries by the reported method is a useful tool to reduce the number of clones that have to be further analysed to determine their phylogenetic affiliation and to examine the diversity of intestinal communities. The resolution limit for this analysis appears to be between the genus and the species level. Future experiments will be carried out to validate this method with a large number of strains. Even if complementary analysis (biochemical, phenotypical features, sequencing 16S rRNA…) are necessary for strain identification at genus or species level, this method would permit a rapid diversity evaluation of a complex microbial ecosystem. It is likely that adherence of intestinal bacteria is influenced by factors within the lumen, properties of organisms themselves and local immune response. Many bacteria in the gastrointestinal tract are opportunist pathogens. Therefore, it is important to know which bacteria are in the most intimate association with the mucosa. There are probably several reasons why there is little published data on this important topic. Faeces and the contents of intestinal lumen are readily available for study, whereas there are logistic and ethical difficulties in obtaining fresh biopsy tissue to allow investigation on flora associated with the mucosa. The validation of such a method directly applicable to biopsies may lead, despite its limitations, to the characterisation of the genus closer to the mucosa.

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