ARTICLE II

# PCR-DGGE and RT-PCR-DGGE show diversity and short-term temporal stability in the *Clostridium coccoides* ***Eubacterium rectale*** **group in the human intestinal microbiota**

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# PCR DGGE and RT-PCR DGGE show diversity and short-term temporal stability in the *Clostridium coccoides–Eubacterium rectale* group in the human intestinal microbiota

Johanna Maukonen[1], Jaana Mättö[1], Reetta Satokari[1], Hans Söderlund[1], Tiina Mattila-Sandholm[2] & Maria Saarela[1]

[1]VTT Technical Research Centre of Finland, Espoo, Finland; and [2]Valio Ltd, Helsinki, Finland

**Correspondence:** Johanna Maukonen, VTT Technical Research Centre of Finland, PO Box 1000, Tietotie 2, Espoo, FIN-02044 VTT, Finland. Tel.: +358 20 722 7183; fax: +358 20 722 7170; e-mail: johanna.maukonen@vtt.fi

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## Abstract

As the *Clostridium coccoides–Eubacterium rectale* (Erec; clostridial phylogenetic cluster XIVa) group is one of the major groups of the human intestinal microbiota, DNA- and RNA-based population analysis techniques (denaturing gradient gel electrophoresis; DGGE) were developed and applied to assess the diversity and temporal stability (6 months–2 years) of this faecal clostridial microbiota in 12 healthy adults. The stability of the Erec group was compared with the stability of the predominant bacterial microbiota, which was also assessed with PCR-DGGE. In addition, the Erec group was quantified with a hybridization-based method. According to our results, the Erec group was diverse in each subject, but interindividual uniqueness was not as clear as that of the predominant bacteria. The Erec group was found to be temporally as stable as the predominant bacteria. Over 200 clones obtained from two samples proved the developed method to be specific. However, the amount of bacteria belonging to the Erec group was not related to the diversity of that same bacterial group. In conclusion, the newly developed DGGE method proved to be a valuable and specific tool for the direct assessment of the stability of the Erec group, demonstrating diversity in addition to short-term stability in most of the subjects studied.

## Introduction

Clostridia are phylogenetically and metabolically highly diverse bacteria, which together with their close relatives constitute a predominant fraction of the faecal microbiota of human adults (Wilson & Blitchington, 1996; Franks *et al*., 1998; Zoetendal *et al*., 1998; Suau *et al*., 1999). The genus *Clostridium* currently contains 146 species. Clostridia and phylogenetically related bacteria are divided into 19 distinct clusters (Collins *et al*., 1994), and most of the clusters contain species from genera other than *Clostridium*, for example *Eubacterium*, *Ruminococcus*, *Coprococcus*, *Peptostreptococcus*, and *Faecalibacterium*, which are also commonly found in the colonic microbiota (e.g. Suau *et al*., 1999). The 16S rRNA gene-based phylogeny of clostridia is still incomplete, and several prevalent species within these genera have been reclassified as they have been studied more closely (e.g. Wade *et al*., 1999). Studies on the characterization of faecal microbiota of healthy humans by 16S rRNA gene sequencing have revealed abundantly, clones of uncultured bacteria that are distributed in the clostridial clusters (Suau *et al*., 1999; Eckburg *et al*., 2005). The predominant intestinal clostridia belong mainly to clusters XIVa [*Clostridium coccoides–Eubacterium rectale* (Erec) group] and IV (*Clostridium leptum* group) (Franks *et al*., 1998; Suau *et al*., 1999; Sghir *et al*., 2000; Eckburg *et al*., 2005), whereas most of the medically important clostridia belong to cluster I (Stackebrandt *et al*., 1999). Many clostridia are cultivable, but their culture-based quantification is impractical owing to the lack of good subgroup-selective media (with the exception of some clinically important species). Hence, culture-independent techniques have great practical relevance in the study of clostridia (Satokari *et al*., 2005).

Clostridia contain saccharolytic and/or proteolytic species and are able to metabolize a wide variety of substrates (Cato *et al*., 1986). In the colon they are likely to have an important role in the fermentation and putrefaction of food-derived substances, resulting in various metabolites such as fatty acids and gases (Konstantinov *et al*., 2005). Short-chain fatty acids formed by microbial fermentation have an important effect on colonic health. Butyrate, in

particular, has an important role in the metabolism and normal development of colonic epithelial cells and it has been implicated in protection against cancer and ulcerative colitis (Cummings & MacFarlane, 1997). Barcenilla *et al.* (2000) showed that 80% of the butyrate-producing isolates of human faecal origin belonged to the clostridial phylogenetic cluster XIVa. On the other hand, *Clostridium bolteae*, which also belongs to the clostridial cluster XIVa, is postulated to have a role in childhood autism (Song *et al.*, 2004).

The aim of the present study was to develop both DNA- and RNA-based denaturing gradient gel electrophoresis (DGGE) methods using 16S rRNA and its corresponding gene for the detection of the diversity and temporal stability of the Erec group (clostridial phylogenetic cluster XIVa) from human faeces. In addition, the proportion of the Erec group in the total microbiota was evaluated with a recently developed multiplexed and quantitative hybridization-based technique (Satokari *et al.*, 2005). Furthermore, the diversity and temporal stability of the predominant bacterial microbiota were studied with both DNA- and RNA-based DGGE to assess the possible correlation between the temporal stability of the *E. rectale–C. coccoides* group and the predominant microbiota.

## Materials and methods

### Bacterial strains

The bacterial reference strains used for the optimization and validation of PCR and PCR-DGGE of the Erec group (phylogenetic cluster XIVa; Collins *et al.*, 1994) are described in Table 1. The reference strains were grown on media recommended by the culture collections providing the strains.

### Human faecal samples

The subject group consisted of three male and nine female subjects who were 34–63 years of age. Faecal samples were obtained on two occasions 6 months apart (0 and 6 months; subjects 5–12). For a longer-term stability study, samples were obtained on four occasions (0, 3, 6, and 24 months; subjects 1–4). The main recruiting criterion was a good (normal) intestinal balance [absence of repeating and/or persisting gastrointestinal (GI) symptoms]. The exclusion criteria were regular GI-tract symptoms, lactose intolerance, celiac disease, and antimicrobial therapy during the 2 months immediately prior to each sampling point. The subjects defaecated into a plastic container, which was then made anaerobic with gas generators (Anaerocult A mini, Merck, Darmstadt, Germany) placed on the lid of the container. The samples were transported to the laboratory and processed in an anaerobic workstation (Don Whitley Scientific Ltd, Shipley, UK) within 0–4 h of the defaecation. The samples were maintained at $-70\,^{\circ}C$ until analysed.

The study was approved by the ethical committee of VTT Technical Research Centre of Finland, Espoo, Finland.

### DNA extraction

DNA was extracted from reference strains and 300 mg of faecal material using a FastDNA Spin Kit for Soil (QBIOgene, Carlsbad, CA) according to the manufacturer's instructions but with one modification: the bacterial cells were broken with a Fast Prep instrument (Bio 101 Savant, Holbrook, NY) at $6.0\,m\,s^{-1}$ for 60 s from one (reference strains) to three (some clostridial reference strains and human faecal samples) times. The isolated DNA was stored at $-20\,^{\circ}C$ until examined.

### RNA extraction and biotinylation

RNA was extracted from reference strains and faeces as previously described by Satokari *et al.* (2005) and further purified using the clean-up protocol of the RNeasy mini kit (Qiagen, Hilden, Germany). RNA concentration and purity were determined spectrophotometrically ($A_{260\,nm}/A_{280\,nm}$; BioPhotometer 6131, Eppendorf, Hamburg, Germany), and the integrity of RNA was evaluated with agarose gel electrophoresis in which 23S and 16S RNA were observed.

Purified RNA used for quantitative analysis (TRAC; see below) was biotinylated using Photoprobe® Biotin (Vector Laboratories, CA). Biotinylation was performed by exposing the reaction to long-wave UV light (365 nm) for 30 min, and the subsequent purification of the RNA from free biotin was performed according to the manufacturer's instructions and as described by Satokari *et al.* (2005).

### PCR and reverse transcription PCR of the Erec group

Twenty-four primer combinations, four $MgCl_2$ concentrations, BSA and formamide additions, 11 annealing temperatures, and three cycle numbers were tested in preliminary experiments. The partial 16S rRNA gene of the *C. coccoides–E.rectale* group was subsequently PCR-amplified for DGGE using primer pairs Ccoc-f and Ccoc-r+GC in addition to Ccoc-f+GC and Ccoc-r (Table 2). Optimized PCR amplifications were performed in a total volume of 50 μL containing 1 μL of appropriately diluted template DNA, 0.4 μM of both primers, 0.2 mM dNTP, and 1.25 units of *Taq* polymerase (Invitrogen, Carlsbad, CA) in a reaction buffer with 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 2 mM $MgCl_2$. The PCR program consisted of initial denaturing at 94 °C for 5 min, followed by 30 cycles of denaturing at 94 °C for 45 s, primer annealing at 54 °C for 30 s, and elongation at 72 °C for 60 s, and a final extension at 72 °C for 30 min. PCR products of the expected size were obtained with both primer pairs. Primers Ccoc-f and Ccoc-r+GC

**Table 1.** Bacteria used in the validation of *Clostridium coccoides–Eubacterium rectale*-specific PCR and the results of the PCR specificity tests

| Clostridial cluster* | Species | Strain† | PCR result‡ |
|---|---|---|---|
| XIV | *Anaerostipes caccae* | VTT E-052773 (DSM 14662) | + |
| XIV | *Clostridium bolteae* | VTT E-052776 (DSM 15670) | + |
| XIV | *Clostridium clostridioforme* | VTT E-052777 (DSM 933) | + |
| XIV | *Clostridium coccoides* | VTT E-052778 (DSM 935) | + |
| XIV | *Clostridium indolis* | VTT E-042445 (DSM 755) | + |
| XIV | *Clostridium symbiosum* | VTT E-981051 (DSM 934) | + |
| XIV | *Dorea longicatena* | VTT E-052788 (DSM 13814) | + |
| XIV | *Eubacterium eligens* | VTT E-052844 (DSM 33769) | +§ |
| XIV | *Eubacterium hallii* | VTT E-052783 (DSM 3353) | + |
| XIV | *Eubacterium ramulus* | VTT E-052783 (DSM 15684) | + |
| XIV | *Lachnospira multipara* | VTT E-052784 (DSM 3073) | + |
| XIV | *Roseburia intestinalis* | VTT E-052785 (DSM 14610) | + |
| XIV | *Ruminococcus productus* | VTT E-052786 (DSM 2950) | +¶ |
| I | *Clostridium acetobutylicum* | VTT E-93498 | – |
| I | *Clostridium butyricum* | VTT E-97426 (DSM 10702) | – |
| I | *Clostridium perfringens* | VTT E-98861 (ATCC 13124) | – |
| II | *Clostridium histolyticum* | VTT E-052779 (DSM 2158) | – |
| IV | *Clostridium leptum* | VTT E-021850 (DSM 753) | – |
| IX | *Megasphera elsdenii* | VTT E-84221 (DSM 20460) | – |
| IX | *Pectinatus cerevisiiphilis* | VTT E-79103 (ATCC 29359) | – |
| IX | *Selenomonas lacticifex* | VTT E-90407 (DSM 20757) | – |
| XI | *Clostridium lituseburense* | VTT E-021853 (DSM 797) | – |
| XIX | *Fusobacterium necrophorum* | VTT E-001739 (ATCC 25286) | – |
| XIX | *Fusobacterium nucleatum* ssp. *nucleatum* | VTT E-052770 (RHI 4184) | – |
| | *Atopobium parvulum* | VTT E-052774 (DSM 20469) | – |
| | *Bacteroides fragilis* | VTT E-022248 (DSM 2151) | – |
| | *Bifidobacterium longum* | VTT E-96664 (DSM 20219) | – |
| | *Collinsella aerofaciens* | VTT E-052787 (DSM 3979) | – |
| | *Desulfovibrio desulfuricans* ssp. *desulfuricans* | VTT E-95573 (DSM 642) | – |
| | *Eggerthella lenta* | VTT E-001735 (ATCC 25559) | – |
| | *Enterococcus faecalis* | VTT E-93203 (DSM 20478) | – |
| | *Enterococcus faecium* | VTT E-93204 (DSM 20477) | – |
| | *Escherichia coli* | VTT E-94564 (DSM 30083) | – |
| | *Lactobacillus salivarius* | VTT E-97853 (DSM 20555) | – |
| | *Klebsiella terrigena* | VTT E-96696 | – |
| | *Pediococcus acidilactici* | VTT E-93493 (DSM 20284) | – |
| | *Prevotella melaninogenica* | VTT E-052771 (ATCC 25845) | – |
| | *Veillonella parvula* | VTT E-001737 (ATCC 10790) | – |

*Clostridial phylogenetic cluster number (Collins *et al.*, 1994).
†DSM, www.dsmz.de; ATCC, www.lgcpromochem-atcc.com; VTT, www.inf.vtt.fi/pdf/tiedotteet/1999/T1980.pdf.
‡+, positive PCR result; –, negative PCR result. Each positive strain produced one strong band in DGGE unless otherwise stated.
§Three strong bands in the DGGE profile, one band in agarose gel after PCR.
¶Two strong bands in the DGGE profile, one band in agarose gel after PCR.

were used for the reverse transcription PCR (RT-PCR) (Table 2). RT-PCR was performed with the Qiagen® One-Step RT-PCR Kit (Qiagen) according to the manufacturer's instructions. The PCR program consisted of reverse transcription performed at 50 °C for 30 min, followed by denaturation at 95 °C for 15 min. Thereafter the RT-PCR program was similar to the PCR-program: 30 cycles of denaturing at 94 °C for 45 s, primer annealing at 54 °C for 30 s and elongation at 72 °C for 60 s, and a final extension at 72 °C for 30 min.

## PCR and RT-PCR of the predominant microbiota

The partial 16S rRNA gene was PCR-amplified using primers U968-f+GC and U1401-r (Table 2) as described by Mättö *et al.* (2005). RT-PCR was performed with the Qiagen® OneStep RT-PCR Kit according to the manufacturer's instructions. Primers used for the RT-PCR were the same as those used for the PCR (Table 2). The PCR program consisted of reverse transcription performed at 50 °C for 30 min, followed by denaturation at 95 °C for 15 min.

**Table 2.** Probes and primers used in the present study

| Target group | Short name | Probe/primer | Use | Sequence (5′ → 3′) | Reference or source |
|---|---|---|---|---|---|
| *Clostridium coccoides–Eubacterium rectale* group* | Erec group | Erec482-5A† | TRAC | GCTTCTTAGTCARGTACCG AAAAA | Franks *et al*. (1998) and Satokari *et al*. (2005) |
| *Clostridium coccoides–Eubacterium rectale* group‡ | Erec group | Ccoc-f | PCR-DGGE | AAATGACGGTACCTGACTAA | Matsuki *et al*. (2002) |
| *Clostridium coccoides–Eubacterium rectale* group‡ | Erec group | Ccoc-f+GC | PCR-DGGE | CGCCCGGGGCGCGCCCCG GGCGGGGCGGGGGCA CGGGGGGAAATGACGG TACCTGACTAA | This study |
| *Clostridium coccoides–Eubacterium rectale* group‡ | Erec group | Ccoc-r | PCR-DGGE | CTTTGAGTTTCATTCTTGCGAA | Matsuki *et al*. (2002) |
| *Clostridium coccoides–Eubacterium rectale* group‡ | Erec group | Ccoc-r+GC | PCR-DGGE | CGCCCGGGGCGCGCCCCGGG CGGGGCGGGGGCACGGGGGG CTTTGAGTTTCATTCTTGCGAA | This study |
| Bacteria | | Bact338-IIA§ | TRAC | GCTGCCTCCCGTAGGAGTIIA | Amann *et al*. (1990) and Satokari *et al*. (2005) |
| Bacteria¶ | | U968-f+GC | PCR-DGGE | CGCCCGGGGCGCGCCCCGGG CGGGGCGGGGGCACGGGGGG AACGCGAAGAACCTTA | Nübel *et al*. (1996) |
| Bacteria¶ | | U1401-r | PCR-DGGE | CGGTGTGTACAAGACCC | Nübel *et al*. (1996) |
| Sequencing | | T7 | Seq. | TAATACGACTCACTATAGG | Promega |
| Sequencing | | SP6 | Seq. | GATTTAGGTGACACTATAG | Promega |

*Clostridial phylogenetic clusters XIVa and XIVb (Collins *et al.*, 1994).
†The Erec482 (Franks *et al.*, 1998) probe was tailed with an additional AAAAA sequence (Erec482-5A) in the 3′-end for size distinction purposes.
‡Clostridial phylogenetic cluster XIVa (Collins *et al.*, 1994).
§The Bact338 (Amann *et al.*, 1990) probe was tailed with an additional IIA sequence (Bact338-IIA, where I is inosine) in the 3′-end for size distinction purposes.
¶Partial 16S rRNA gene ($V_6$–$V_8$ hypervariable region).

Thereafter the RT-PCR program was similar to the PCR program: 35 cycles of denaturing at 94 °C for 30 s, primer annealing at 50 °C for 20 s and elongation at 72 °C for 40 s, and a final extension at 72 °C for 10 min.

## Specific quantification of 16S rRNA gene

Multiplexed quantification of clostridial 16S rRNA gene was performed with the TRAC technique (transcript analysis with the aid of affinity capture) according to Satokari *et al*. (2005). Specific 16S rRNA gene-targetted probes for different groups of clostridia (phylogenetic clusters I and II, IV, XI and XIVa and b; Collins *et al*., 1994) and a universal bacterial probe were used in multiplexed hybridization (Table 2; however, only phylogenetic cluster XIVa and b is discussed in this study).

## Cloning of the PCR-amplified products

PCR amplicons generated with the set of Erec-group primers were purified using a Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Two samples were selected based on the TRAC results – one sample containing the highest percentage of Erec-group bacteria and another with a low percentage. DNA purity and yield were estimated by electrophoresis in 1% (w/v) agarose gels. Purified amplification products were ligated into pGEM-T vector system II and transformed into *Escherichia coli* JM109 high-efficiency competent cells (Promega, Madison, WI) according to the manufacturer's instructions. One hundred and thirty randomly picked colonies of ampicillin-resistant transformants from each sample were further subcultured on Luria–Bertani (LB) agar (Atlas, 1997) plates supplemented with ampicillin (100 μg mL$^{-1}$; Sigma, St Louis, MO), X-gal (100 μg mL$^{-1}$; Promega), and IPTG (0.5 mM; Promega) and incubated at 37 °C overnight. A loop full of bacterial mass was transferred into 50 μL of Tris-EDTA and incubated at 95 °C for 15 min to lyse the cells. PCR with pGEM-T-specific primers T7 and SP6 (Table 2) was performed from the lyzed cells to check the size of the inserts. The PCR products of the plasmids containing inserts of the right size were purified with the Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions and sequenced with an ABI PRISM BigDye terminator cycle sequencing kit v.3.1 (Applied Biosystems,

Foster City, CA) according to the manufacturer's instructions using the primer T7. All the sequences were analysed with an ABI PRISM 3100 automated capillary DNA cycle sequencer (Applied Biosystems) and checked and edited with the CHROMAS program (Technelysium Pty Ltd, Helensvale, Australia). CLUSTALW (http://align.genome.jp) was used for the alignment of the sequences and for checking of the sequence similarities. All unequal sequences were thereafter identified through the GenBank database (www.ncbi.nlm.nih.gov) using the BLAST (basic local alignment search tool) algorithm (Altschul *et al*., 1997) and using the 'Classifier' tool of the Ribosomal Database Project (RDP) II (Cole *et al*., 2005). CLUSTALW and DNAMAN 4.1 (Lynnon BioSoft) were used for the creation of operational taxonomic units (OTUs). An OTU, as employed here, consisted of all sequences with less than 2% divergence from 440 aligned homologous sequences (Suau *et al*., 1999). Phylogenetic analysis of OTU subgroups was performed with DNAMAN 4.1. One representative of each OTU was deposited in the GenBank database, and the sequences are available under the accession numbers DQ307759–DQ307802.

### DGGE analysis of 16S rRNA gene fragments

DGGE analysis of predominant bacteria was performed as described by Mättö *et al*. (2005). The primer pair Ccoc-f and Ccoc-r+GC was chosen for the analysis of the samples with DGGE because of specificity and optimal migration. DGGE analysis of the Erec group of clostridia was similar to the DGGE analysis of predominant bacteria. Various denaturing gradients were tested and subsequently a denaturing gradient from 38 to 60% [where 100% is 7 M urea and 40% (v/v) deionized formamide] was chosen because of optimal migration and differentiation. The similarity of the PCR-DGGE profiles of the samples obtained from a single subject at different sampling points was compared in order to evaluate the temporal stability of the predominant and clostridial faecal bacterial populations. The comparison of the profiles was performed by calculating a similarity percentage using BIONUMERICS software version 4.01 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Clustering was performed with Pearson correlation and the unweighted-pair group method (UPGMA). Amplicons with a total surface area of at least 1% were included in the similarity analysis.

After sequence analysis of the clones, all the clones with different sequences were subjected to Erec-PCR-DGGE, after which the migration of each clone was compared with the migration of different amplicons in the original sample.

### Statistical analysis

The mean and standard deviation were calculated for each experiment. Student's *t*-test (two-sample assuming unequal variances) was used for the statistical analysis of the results.

## Results

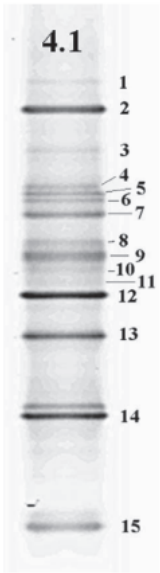
### Optimization and validation of PCR-DGGE and RT-PCR-DGGE of the Erec group

When the specificity of primer pairs Ccoc-f – Ccoc-r+GC and Ccoc-f+GC – Ccoc-r (Table 2) was evaluated with reference strains, the primers gave positive PCR results for all the target bacteria belonging to the clostridial phylogenetic cluster XIVa (Table 1). No false-positive results were obtained (Table 1). In most cases only one strong or one strong and one additive weak band was/were seen in the DGGE profiles of the reference strains. However, the DGGE profiles of *Ruminococcus productus* and *Eubacterium eligens* contained two and three strong bands, respectively (Table 1). Most of the amplicons of the used reference strains migrated differently and could thus be distinguished. However, the amplicons of *Clostridium bolteae*, *Clostridium clostridioforme*, and *C. coccoides* migrated to the same position (data not shown).

Two samples were cloned after Erec-PCR to validate the specificity of the newly developed method. All the sequenced 205 clones belonged to the Erec clostridial cluster XIVa, as expected. The 108 clones of subject 4 fell into 21 OTUs, and the 97 clones of subject 6 into 22 OTUs, when an OTU was defined as a phylogenetic group that consisted of members having over 98% sequence similarity to each other. The DGGE profiles of the cloned samples and the sequence information obtained from the cloning and Erec-DGGE are presented in Figs 1 and 2. OTU 5 (band number 9) of subject 4 was divided into two subgroups: OTUs 5A and 5B, since, according to the BLAST results, OTUs 5A and 5B had 100% similarity to different species (although having over 98% similarity to each other; Fig. 1). Similarly, OTU 21 (band numbers 3, 7, 11, 12, and 13) of subject 6 was divided into subgroups according to phylogenetic analysis and migration in the DGGE gel (Fig. 2). When cloned samples 4.1 and 6.1 were run in the same DGGE gel, several amplicons migrated similarly – for example band number 2 of sample 4.1 migrated similarly to band number 2 of sample 6.1, and both were identified as *Anaerostipes* IE4 (Fig. 1).

### Proportion, diversity and temporal stability of the *C. coccoides–E. rectale* group from faecal samples of healthy adults

DNA-based PCR-DGGE and RNA-based RT-PCR-DGGE analyses targetted to the *C. coccoides–E. rectale* group showed intraindividual diversity of the faecal microbiota (Fig. 3). In addition, the interindividual profiles were divergent (Figs 3 and 4). After comparison of both DNA-




| Band number | OTU | Blast-result | similarity | clones /OTU | Band number in sample 6.1 |
|---|---|---|---|---|---|
| 1 & 2 [a] | 1 | *Anaerostipes* IE4 | 97-98 % | 13 | 2 |
| 3, 4 [b] | 8 | *Ruminococcus lactaris* | 96 % | 5 | |
| 5 | 21 | *Clostridium hathawayi* | 97 % | 1 | |
| 6 | 11 | *Clostridium nexile* | 99 % | 5 | 5 |
| 7 | 2 | *Eubacterium hallii* | 98 % | 5 | |
| 7 | 7 | *Eubacterium oxidoreducens* | 96 % | 6 | |
| 8 | 3 | *Ruminococcus* sp. CE2 | 96 % | 2 | 7 |
| | | *Ruminococcus schinkii* | 94 % | | |
| 9 | 5A | *Roseburia faecalis* | 99-100 % | 8 | 8 |
| | 5B | *Roseburia intestinalis* | 100 % | 2 | |
| 9 | 6 | *Eubacterium rectale* | 100 % | 1 | 8 |
| 10 | 10 | *Dorea formigerans* | 99 % | 1 | |
| 11 | 14 | *Clostridium* sp. cTPY-17 | 97 % | 8 | |
| | | *Ruminococcus obeum / schinkii / torques / lactaris* | 94-96 % | | |
| 12 | 4 | *Ruminococcus* sp. CE2 | 98-100 % | 12 | 13 |
| | | *R. obeum / R. schinkii* | 94-96 % | | |
| 13 | 18 | *Clostridium clostridioforme* | 98 % | 7 | 14 |
| 14 [c] | 9 | *Ruminococcus lactaris* | 99 % | 1 | 15 |
| 14 [c] | 16 | *Ruminococcus obeum* | 97 % | 13 | |
| 14 [c] | 17 | *Ruminococcus productus* | 95 % | 4 | |
| 15 | 13 | *Ruminococcus gnavus* | 96 % | 4 | |
| n.d. | 12 | *Ruminococcus gnavus* | 99 % | 1 | |
| n.d. | 15 | *Ruminococcus obeum* | 99 % | 1 | |
| n.d. | 19 | *Clostridium amygdalinum / indolis* | 96 % | 1 | |
| n.d. | 20 | *Clostridium hathawayi* | 95 % | 1 | |

[a] 12 clones migrated as band 2 and one clone as band 1
[b] 3 clones migrated as band 3, 1 clone as band 4 and 1 clone a little above band 6
[c] a double band

**Fig. 1.** The cloned and sequenced bands from the *Clostridium coccoides–Eubacterium rectale*-specific PCR-DGGE of the baseline sample of subject 4 (n.d., the clone is not visible in the community profile).

and RNA-based profiles with BioNumerics software – using Pearson correlation for similarity analysis and UPGMA for clustering of the profiles – all samples of a given subject clustered together in most of the cases (nine out of 12 subjects). Only samples of three subjects (subjects 1, 9, and 11) clustered within another subject's cluster (Fig. 4). The DNA-based profiles of 10 out of 12 subjects and the RNA-based profiles of eight out of 12 subjects were stable or rather stable in the 6-month follow-up study (Fig. 4). When DNA- and RNA-based profiles from the same time point of a given subject were compared, nine DNA vs. RNA profiles out of 12 from baseline and 10 out of 12 from the 6-month sampling point were similar (data not shown). The long-term study (2-year period) showed that the DNA-based profiles of subjects 3 and 1 were unstable or rather unstable, respectively, and the profiles of subjects 2 and 4 contained minor differences (Figs 3 and 5).

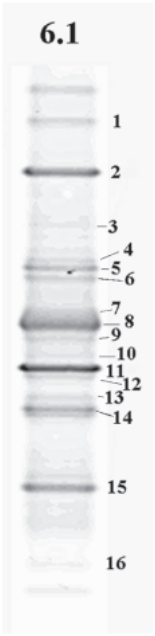
The *C. coccoides–E. rectale* group comprised an average of 45% (range 18–71%) of the total bacteria from human faecal samples as detected with the hybridization-based TRAC technique (Table 3). There were clear differences in intraindividual temporal stability (change in proportions between the two sampling points; Table 3).

### Stability and diversity of the predominant bacteria from faecal samples of healthy adults

Both DNA- and RNA-based DGGE analyses targetted to the predominant bacterial population showed considerable intraindividual diversity as well as uniqueness of the faecal microbiota. The DNA- and RNA-based profiles in the follow-up study (6 months) were stable or rather stable in nine out of 12 subjects (data not shown), and in the DNA-based long-term study (2 years) in two out of four subjects (Fig. 5). When DNA- and RNA-based profiles from the same time point of a given subject were compared, the temporal intraindividual similarity values of RT-PCR-DGGE and PCR-DGGE profiles were significantly higher than the intraindividual similarity values between DNA- and RNA-based DGGE profiles ($P < 0.05$) (data not shown). However, since each individual had unique PCR-DGGE and RT-PCR-DGGE profiles, the intraindividual RNA- and DNA-based DGGE profiles resembled each other more than the interindividual RNA-based profiles (data not shown).

## Discussion

The aim of this study was to develop both DNA- and RNA-based population analysis techniques (DGGE) for the




| Band number | OTU | Blast-result | similarity | clones /OTU |
|---|---|---|---|---|
| 1 | 11 | *Ruminococcus torques* | 98 % | 1 |
| 2 | 7 | *Anaerostipes* sp. IE4 | 98 % | 4 |
| 3 | 21A | *Ruminococcus* sp. CE2 | 97 % | 1[a] |
| | | *Eubacterium rectale / Roseburia faecalis* | 95 % | |
| 4 | 10 | *Ruminococcus lactaris* | 96 % | 4 |
| 5 | 13 | *Clostridium nexile* | 99 % | 9 |
| 6 | 6 | *Eubacterium hallii* | 98 % | 4 |
| 7 | 21B | *Ruminococcus* sp. CE2 | 98 % | 1[a] |
| | | *Ruminococcus obeum / schinkii* | 96& | |
| 8 | 16 | *Eubacterium rectale* | 99-100% | 15 |
| 8 | 18 | *Roseburia faecalis* | 99-100% | 10 |
| 9 | 14 | *Ruminococcus obeum / schinkii* | 96 % | 4 |
| 10 | 22 | *Eubacterium rectale* | 95 % | 1 |
| 11 | 21C | *Ruminococcus* sp. CE2 | 98 % | 9[a] |
| | | *Ruminococcus obeum / schinkii* | 96 % | |
| 12 | 21D | *Ruminococcus* sp. CE2 | 99 % | 4[a] |
| | | *Ruminococcus obeum / schinkii* | 97 % | |
| 13 | 21E | *Ruminococcus* sp. CE2 | 99 % | 3[a] |
| | | *Ruminococcus obeum / schinkii* | 96 % | |
| 14 | 1 | *Clostridium clostridioforme* | 98 % | 7 |
| 15 | 5 | *Ruminococcus productus / schinkii* | 95 % | 4 |
| 15[b] (& 9) | 4 | *Ruminococcus obeum* | 96 % | 1 |
| 15[b] | 2 | *Clostridium amygdalinum / Cl. indolis / Ruminococcus obeum* | 96 % | 1 |
| 16 | 12 | *Ruminococcus gnavus* | 97 % | 1 |
| n.d. | 3 | *Ruminococcus obeum* | 96 % | 1 |
| n.d. | 8 | *Clostridium hathawayi / Hespellia porcina* | 95 % | 1 |
| n.d. | 9 | *Clostridium amygdalinum / jejuense / algidixylanolyticum* | 95 % | 1 |
| n.d. | 15 | *Ruminococcus obeum* | 96 % | 1 |
| n.d. | 17 | *Eubacterium rectale* | 97 % | 1 |
| n.d. | 19 | *Roseburia faecalis* | 96-97 % | 2 |
| n.d. | 20 | *Eubacterium rectale* | 97 % | 1 |
| n.d. | 21F | *Ruminococcus sp. CE2* | 98 % | 3[a] |
| | | *Ruminococcus obeum / schinkii* | 96 % | |

[a] There were altogether 21 clones in OTU 21
[b] a double band

**Fig. 2.** The cloned and sequenced bands from the *Clostridium coccoides–Eubacterium rectale* group-specific PCR-DGGE of the baseline sample of subject 6 (n.d., the clone is not visible in the community profile).

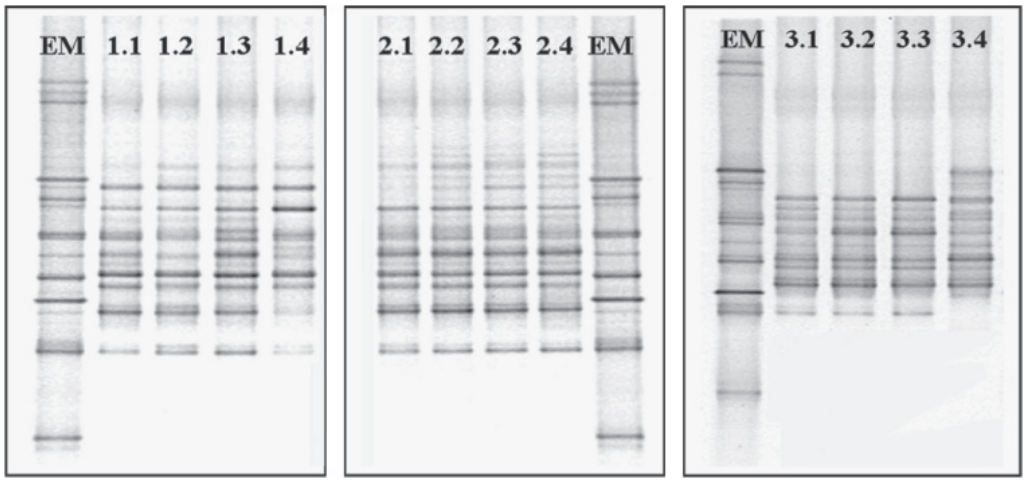



**Fig. 3.** DNA-based long-term stability study from faeces of three healthy adults with *Clostridium coccoides–Eubacterium rectale* group-specific PCR-DGGE. The samples were obtained at time points 0 (1.1, 2.1, 3.1), 3 months (1.2, 2.2, 3.2), 6 months (1.3, 2.3, 3.3), and 2 years (1.4, 2.4, 3.4). EM, Erec marker.

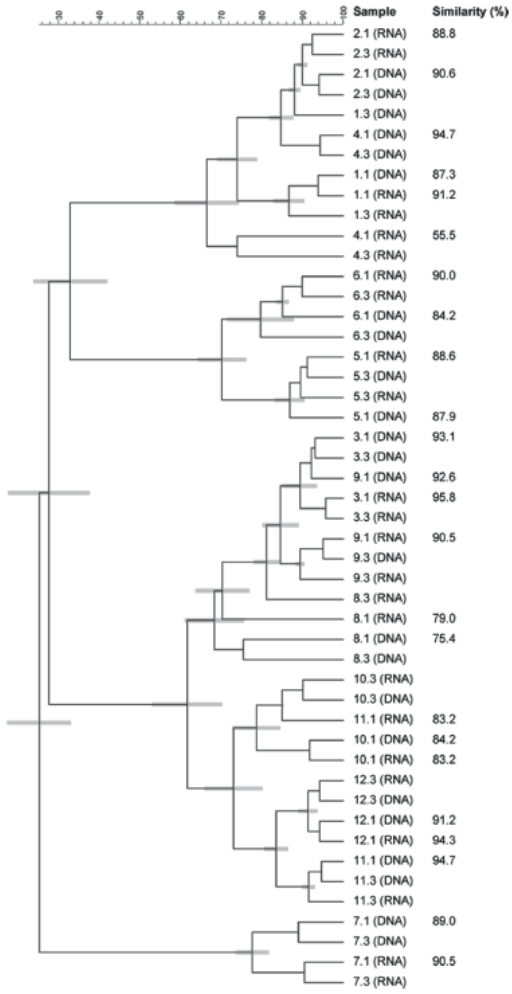



**Fig. 4.** Similarities of the DNA- and RNA-based DGGE profiles of the *Clostridium coccoides–Eubacterium rectale* group of human faeces. The samples were taken on two occasions: baseline (RNA1 and DNA1) and 6 months later (RNA3 and DNA3). The comparison of the profiles was performed by calculating the similarity percentage using BioNumerics software version 4.01. The similarity percentage presented indicates either DNA- or RNA-based similarity between the DGGE profiles of the two sampling points (baseline and 6 months) of the same individual. DNA-based similarity values are represented in the same row as the DNA-based baseline sample, and RNA-based similarity values in the same row as the RNA-based baseline sample. Clustering was performed with Pearson correlation and the UPGMA method. Amplicons with a total surface area of at least 1% were included in the similarity analysis. The grey bars represent the error bars.

detection of diversity and temporal stability of the Erec group (Erec group; clostridial phylogenetic cluster XIVa; Collins *et al.*, 1994) from human intestinal samples. To our knowledge, this is the first study to use DGGE for studying the diversity of the Erec group. The Erec group is one of the major bacterial groups in human faeces (e.g. Wilson & Blitchington, 1996; Franks *et al.*, 1998; Suau *et al.*, 1999; Vaughan *et al.*, 2000), and before this study it was only possible to quantify the number of bacteria belonging to this group with either hybridization-based methods (Franks *et al.*, 1998; Seksik *et al.*, 2003; Satokari *et al.*, 2005) or real-time PCR (Matsuki *et al.*, 2004; Rinttilä *et al.*, 2004; Song *et al.*, 2004). DGGE methods have already been developed for other relevant faecal/GI bacterial groups/species – namely bifidobacteria (Satokari *et al.*, 2001), the *Lactobacillus* group (Walter *et al.*, 2000; Heilig *et al.*, 2002; Vanhoutte *et al.*, 2004), the *Bacteroides fragilis* group (Vanhoutte *et al.*, 2004), and predominant bacteria (e.g. Nübel *et al.*, 1996). Because there is extensive evidence that Erec-group bacteria are abundant in the human intestinal microbiota, we developed a simple method (PCR-DGGE) to assess the diversity of this group, which does not necessitate the building of a clone library from each sample. The new DGGE method was validated with a clone library, which consisted of 205 clones originating from two subjects (one sample per subject). All 205 sequenced clones proved to belong to the clostridial phylogenetic cluster XIVa. All the amplicons seen in the profiles were identified through the clone library, and each DGGE band consisted of either one OTU or a few OTUs that were phylogenetically closely related (although several bacterial species migrated similarly). Therefore the number of amplicons seen in the DGGE profiles correctly reflected the clostridial phylogenetic diversity of the samples. The majority of the sequences retrieved from the 205 clones of the two subjects were different. This has also been shown to be the case in other studies in which extensive numbers of predominant bacterial clones from different individuals were sequenced (Eckburg *et al.*, 2005).

We also studied the number of bacteria belonging to the Erec group with a hybridization-based method (TRAC; Satokari *et al.*, 2005). The average percentage of the Erec group observed in healthy adults ($45 \pm 10\%$) in this study was somewhat higher than what has previously been reported with FISH, namely 11–35% (Franks *et al.*, 1998; Jansen *et al.*, 1999; Sghir *et al.*, 2000; Marteau *et al.*, 2001; Harmsen *et al.*, 2002a, b; Zoetendal *et al.*, 2002; Hold *et al.*, 2003; Rigottier-Gois *et al.*, 2003; Rochet *et al.*, 2004). The proportion of the Erec-group bacteria varied greatly within our study group (18–71%), and it has been reported that, when the human faecal microbiota has been analysed with the 16S rRNA gene library method, bacteria from the Erec group have comprised 10–59% of the total faecal flora (Wilson & Blitchington, 1996; Suau *et al.*, 1999; Hayshi *et al.*, 2002; Hold *et al.*, 2002; Wang *et al.*, 2003). These results are more in accordance with our results. The two samples that were cloned in this study were chosen based on

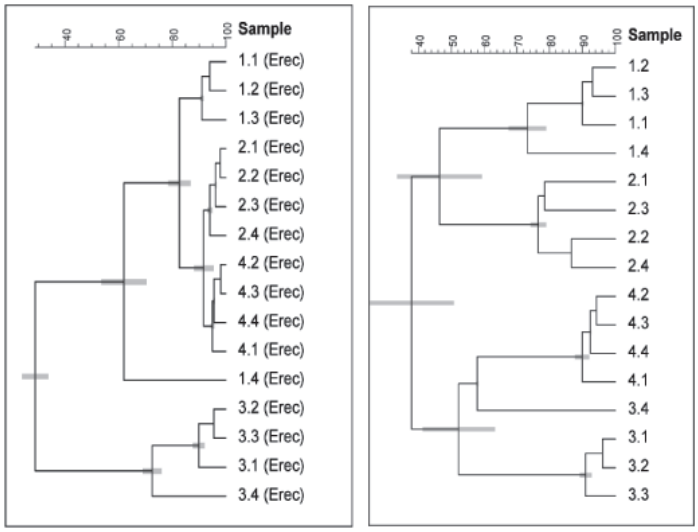



**Fig. 5.** Similarities of the DNA-based DGGE profiles of the long-term (24 months) study. The dendogram on the left is based on the profile similarities of the *Clostridium coccoides–Eubacterium rectale* group, whereas the dendogram on the right is based on the profile similarities of the predominant bacterial microbiota. The comparison of the profiles was performed by calculating the similarity percentage using BioNumerics software version 4.01. Clustering was performed with Pearson correlation and the UPGMA method. Amplicons with a total surface area of at least 1% were included in the similarity analysis. The samples were obtained at time points 0 (1.1, 2.1, 3.1, 4.1), 3 months (1.2, 2.2, 3.2, 4.2), 6 months (1.3, 2.3, 3.3, 4.3), and 2 years (1.4, 2.4, 3.4, 4.4). The grey bars represent the error bars.

**Table 3.** Similarity values of DGGE profiles of human faecal samples obtained at two different time points from 12 healthy subjects (baseline and 6 months later) with primers specific to the *Clostridium coccoides–Eubacterium rectale* (Erec) group of clostridia and with primers that target predominant microbiota in addition to proportions of the Erec group as quantified with the TRAC technique

| | | Mean ± SD | Range |
|---|---|---|---|
| Qualitative DGGE analysis | | | |
| With Erec primers | | | |
| RNA-based similarity (%)* | Baseline vs. 6 months | 85.5 ± 10.6 | 55.5–95.8 |
| DNA-based similarity (%)* | Baseline vs. 6 months | 88.2 ± 5.8 | 75.4–94.7 |
| RNA-based diversity† | Baseline | 13.3 ± 2.3 | 10–16 |
| RNA-based diversity† | 6 months | 13.8 ± 1.7 | 12–17 |
| DNA-based diversity† | Baseline | 14.3 ± 1.6 | 12–17 |
| DNA-based diversity† | 6 months | 14.3 ± 1.9 | 12–18 |
| With universal bacterial primers | | | |
| RNA-based similarity (%)* | Baseline vs. 6 months | 84.2 ± 10.0 | 68.2–92.4 |
| DNA-based similarity (%)* | Baseline vs. 6 months | 83.4 ± 13.4 | 58.8–96.7 |
| RNA-based diversity† | Baseline | 18.5 ± 3.2 | 12–25 |
| RNA-based diversity† | 6 months | 19.0 ± 2.0 | 16–22 |
| DNA-based diversity† | Baseline | 32.9 ± 7.4 | 24–51 |
| DNA-based diversity† | 6 months | 31.5 ± 5.9 | 24–42 |
| Quantitative TRAC analysis | | | |
| Erec (%)‡ | Baseline | 44.7 ± 16.0 | 17.7–71.2 |
| Erec (%)‡ | 6 months | 46.0 ± 10.1 | 35.1–66.9 |
| ΔErec (%)§ | | 9.3 ± 7.9 | 0.2–22.7 |

*Similarity values were counted with BioNumerics 4.01 software using Pearson correlation. Amplicons with a total surface area of at least 1% were included in the similarity analysis.
†Diversity is presented as the number of amplicons detected by the BioNumerics 4.01 software.
‡Percentage of bacteria that hybridized with the Erec482 probe as determined in triplicate.
§Difference in proportions (%) between the two sampling points.

the results obtained from our hybridization studies – one sample contained a high percentage of Erec-group bacteria and the other a low percentage. Our results showed that the amount of bacteria belonging to the Erec group was not related to the diversity of that same bacterial group. One sample (subject 4, baseline sample) containing 71% of Erec-group bacteria had 15 DGGE bands representing 21 OTUs, whereas another sample containing 27% of Erec-group

bacteria (subject 6, baseline sample) had 16 DGGE bands representing 22 OTUs.

The Erec group was found to be temporally as stable or unstable as the predominant bacteria in this study. In the 2-year stability study, the DGGE profiles of two subjects (1 and 3) were unstable/rather unstable, and two subjects (2 and 4) had minor differences with both predominant bacterial DGGE and Erec DGGE. In the shorter follow-up study (6 months) nearly all of the profiles that were unstable/rather unstable with either RNA- or DNA-based (or both) Erec DGGE were also unstable with predominant bacterial DGGE. However, the hybridization-detected proportional stability was not found to correlate with the DGGE-based stability. Five subjects showed instability in the proportion of the Erec group as detected with TRAC, whereas the DGGE profiles of only two of these subjects were found to be rather unstable. This finding further addresses the above-discussed difference between the proportion and diversity of a given bacterial group.

We found considerable intraindividual diversity as well as uniqueness of the predominant faecal bacterial population with both DNA-based (24–51 amplicons per sample) and RNA-based (12–25 amplicons per sample) PCR-DGGE analyses. The same intraindividual diversity was not observed in Erec-group DGGE profiles (12–18 amplicons per DNA sample; 10–17 amplicons per RNA sample), even though this group comprised the majority of the microbiota in some individuals according to the hybridization results. However, since it is possible to detect only those bacteria that constitute over 1% of the total population with DGGE (Muyzer *et al.*, 1993), we were able to detect the majority of the Erec-group bacteria, as can be seen from our clone-library results. The interindividual uniqueness of Erec profiles was not as clear as was the inter-individual uniqueness of the predominant bacterial profiles. Regardless of this, samples of a single subject clustered together in most cases. In addition, the clone libraries of the two subjects (4 and 6) indicated that, even though the DGGE profiles resembled each other and contained several similar OTUs, less than 20% of their sequences were identical.

RNA-based DGGE profiles of the predominant microbiota contained significantly fewer bands than the DNA-based DGGE profiles in this study. The differences were seen in all parts of the denaturing gradient. A clear difference between the DNA- and RNA-derived predominant faecal bacterial populations has also been reported by Tannock *et al.* (2004). However, this was not observed as commonly in our Erec-group DGGE profiles. Most of the intraindividual DNA- and RNA-based profiles were quite similar (10 out of 12 subjects).

In conclusion, the newly developed PCR-DGGE and RT-PCR-DGGE methods for the Erec group proved to be specific and useful in studying the diversity and stability of the human faecal microbiota. The DGGE profiles of all 12 healthy adults studied were diverse. In addition, the developed DGGE method proved to be a valuable tool for the direct assessment of the stability of the Erec group, demonstrating stability in most of the subjects studied.

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