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ORIGINAL ARTICLE

Long-term ecological impacts of antibiotic administration on the human intestinal microbiota

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Antibiotic administration is known to cause short-term disturbances in the microbiota of the human gastrointestinal tract, but the potential long-term consequences have not been well studied. The aims of this study were to analyse the long-term impact of a 7-day clindamycin treatment on the faecal microbiota and to simultaneously monitor the ecological stability of the microbiota in a control group as a baseline for reference. Faecal samples from four clindamycin-exposed and four control subjects were collected at nine different time points over 2 years. Using a polyphasic approach, we observed highly significant disturbances in the bacterial community that persisted throughout the sampling period. In particular, a sharp decline in the clonal diversity of *Bacteroides* isolates, as assessed by repetitive sequence-based PCR (rep-PCR) and long-term persistence of highly resistant clones were found as a direct response to the antibiotic exposure. The *Bacteroides* community never returned to its original composition during the study period as assessed using the molecular fingerprinting technique, terminal restriction fragment length polymorphism (T-RFLP). Furthermore, using real-time PCR we found a dramatic and persistent increase in levels of specific resistance genes in DNA extracted from the faeces after clindamycin administration. The temporal variations in the microbiota of the control group were minor compared to the large and persistent shift seen in the exposed group. These results demonstrate that long after the selection pressure from a short antibiotic exposure has been removed, there are still persistent long term impacts on the human intestinal microbiota that remain for up to 2 years post-treatment.
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Introduction

The human gastrointestinal tract is a complex ecosystem, with approximately 10^{11} bacteria per gram faeces (Franks *et al.*, 1998). These bacteria play an important role in health and disease through their involvement in nutrition, pathogenesis and immunology of the host (Gibson and Roberfroid, 1995; Noverr and Huffnagle, 2004; Kelly *et al.*, 2005; Gill *et al.*, 2006). Traditionally, cultivation-based techniques have been used to determine the bacterial composition in faeces. However, molecular techniques based on analysis of 16S rRNA genes directly amplified from DNA extracted from faeces have estimated that less than 25% of the faecal bacterial populations have been cultured to date (Suau *et al.*, 1999; Eckburg *et al.*, 2005). Therefore, we cannot fully understand their roles in the intestinal environment or how the ecological balance of the bacterial community can be disrupted.

It is known that antimicrobial agents not only affect the pathogens to which they are directed but may also impact other members of the intestinal microbiota (Sjölund *et al.*, 2003; De La Cochetiere *et al.*, 2005). Thus they inhibit susceptible organisms and select for resistant ones. A number of different molecular fingerprinting techniques have previously been used to analyse the impact of antibiotics on the faecal microbiota such as denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) (Donskey *et al.*, 2003; Harmoinen *et al.*, 2004), temporal TGGE (TTGE) (De La Cochetiere *et al.*, 2005), terminal restriction fragment length polymorphism (T-RFLP) (Jernberg *et al.*, 2005; Engelbrektson *et al.*, 2006) and denaturing high-performance liquid chromatography (DHPLC) (Goldenberg *et al.*, 2006). These techniques all

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bypass the necessity for cultivation, enabling comparative analyses of community fingerprints and the ability to monitor relative shifts in specific populations. Recently, Gill *et al*. (2006) used metagenomic cloning to increase further our understanding of the composition and function of the gastrointestinal microbiome, that is the collective genome of our microbiota. In addition, real-time PCR can be used for absolute or relative quantification of specific genes of interest in DNA isolated from faecal material (Bartosch *et al*., 2004). Now that there are different molecular tools available it is possible to select appropriate tools to address particular questions and to use a polyphasic approach to assess the composition and function of the gut microbiota.

The impact of antibiotics on *Bacteroides* species is of particular interest because these represent one of the most dominant intestinal bacterial groups and they have important functions in the gut. In the colon the *Bacteroides* species ferment a variety of carbohydrates, and some, such as *B. thetaiotaomicron*, can ferment plant polysaccharides (Salyers, 1984). Some of the species of this genus are opportunistic pathogens, such as *B. fragilis,* and can cause a variety of infections, for example in the blood stream and intra-abdominal area. *B. fragilis* is the most frequently isolated species from clinical samples, followed by *B. thetaiotaomicron*. *Bacteroides* are resistant to a wide range of antitiotics, including animoglycosides, β-lactam antibiotics and tetracycline. Clindamycin has been the drug of choice when treating *Bacteroides* infections as the antibacterial spectrum of this antibiotic covers almost all anaerobic bacteria. Although some *Bacteroides* spp. are suppressed by clindamycin (Sullivan *et al*., 2003), others have been shown to be resistant to clindamycin and several other antibiotics (Hedberg and Nord, 2003; Löfmark *et al*., 2006) and resistance to this antibiotic is increasing (Hedberg and Nord, 2003). Previously, we found that 7 days administration of clindamycin resulted in a dramatic increase in antibiotic resistant *Bacteroides* spp. isolated from faecal samples and resistant strains could still be isolated up to 2 years post treatment. Resistance levels for clindamycin among analysed isolates were approximately 50% in post-exposure samples, compared to 0–1% in samples from untreated subjects (Löfmark *et al*., 2006). Our study therefore demonstrated long-term persistence of antibiotic resistance in the human gut microbiota and suggested that resistant *Bacteroides* strains could serve as a reservoir of antibiotic resistance.

In this study, we aimed to investigate the long-term ecological stability of the gut microbiota after clindamycin administration, with a focus on the *Bacteroides* group, using a combination of molecular approaches. One question to address was whether specific *Bacteroides* clones became resistant after clindamycin exposure and if so, whether these were enriched in the community. Therefore, our first aim was to monitor specific clones of *Bacteroides* spp. that were previously isolated from faecal samples (Löfmark *et al*., 2006) in both clindamycin exposed and control groups. Specific *Bacteroides* clones were typed by repetitive sequenced based PCR (rep-PCR) (Moraes *et al*., 2000). The rep-PCR method generates fingerprints of DNA fragments that are size separated and visualized by electrophoresis, enabling high-resolution typing of individual bacterial clones or strains (Versalovic *et al*., 1998).

In addition, our aim was to use culture-independent approaches to monitor the impact of clindamycin on the total bacterial community, and the *Bacteroides* group in particular, in the faecal samples. Community DNA was extracted from faecal samples from both clindamycin-exposed individuals and from a control group over the 2-year study period. T-RFLP was used as a fingerprinting approach to determine the community structure of both dominant members of the gut microbiota and the *Bacteroides* group. Information obtained from the control group was used to determine natural fluctuations in diversity and clonal stability of specific members of the faecal microbiota and these data provided us with a baseline for reference to those subjects in the treated group.

Finally, our aim was to determine the relative changes in the levels of specific resistance genes, *erm*(B), *erm*(F) and *erm*(G), belonging to the *erm* (erythromycin resistance methylases) gene family. The gene product of these genes methylates the ribosomal target for the antibiotic and thereby prevents its binding. These *erm* genes are known to confer resistance to clindamycin and also to macrolides (Roberts, 2004), and they have all previously been detected in *Bacteroides* spp. (Shoemaker *et al*., 2001). Previously, specific *erm* genes were quantified by real-time PCR in clindamycin-resistant *Bacteroides* strains isolated on culture medium from the faecal samples (Löfmark *et al*., 2006) with the finding that resistant strains harboured either *erm*(F) or *erm*(G). Here, we aimed to quantify the relative levels of *erm* genes in DNA extracted directly from the faecal samples by real-time PCR to determine the relative amounts of these genes in the faecal microbiota at large, in both the exposed and control groups.

Materials and methods

Experimental setup and sampling

Details of the experimental set-up and sampling procedure are described in a previous study (Löfmark *et al*., 2006). Briefly, eight healthy subjects were divided into two groups. Four of the subjects, 1–4 (three females and one male, ages 49, 31, 35 and 36 years, respectively) received 150 mg clindamycin capsules (Dalacin; Pharmacia, Stockholm, Sweden) perorally four times a day for 7 days, the recommended dosage in Sweden. As a control group, four

subjects, 5–8 (three females and one male ages 58, 52, 33 and 34 years, respectively), who did not receive any antibiotics during the study period were included. No restriction on diet was made to enable normal variations in the faecal microbiota to be explored. Faecal samples were collected before administration (day 0), on the last day of clindamycin administration (day 7), 2 weeks after administration, as well as after 3, 6, 9, 12, 18 months and 2 years after administration. For the control group sampling was performed at corresponding intervals. The samples were stored at −70°C until analysis. None of the subjects had taken any antibiotics at least 1 year before the study period. The study was approved by the ethics committee of Karolinska Institute, Stockholm, Sweden.

Clonal typing by rep-PCR

Up to 20 *Bacteroides* isolates previously obtained from each faecal sample (Löfmark *et al.*, 2006) were typed to the clonal level by rep-PCR (approximately 1300 total). Primer sequences, REP1R-I and REP2-I and ERIC1R and ERIC2 (Versalovic *et al.*, 1991), were used during PCR amplification (for primer sequences, see Table 1). For isolates that were difficult to type owing to few bands an additional primer was used, BOXA1R (Versalovic *et al.*, 1995). The PCRs were performed with standard PCR recipes and programs according to published protocols (Versalovic *et al.*, 1991, 1995), except for the addition of 2.5 μl dimethylsulphoxide per 50 μl reaction volume. Rep-PCR was performed using 1 μl of each *Bacteroides* colony suspended in sterile water as a template. The PCR products were run on 1.5% agarose gels at 80 V for 3 h followed by ethidium bromide staining. DNA molecular weight marker III (0.12–21.2 kbp) (Roche Diagnostics GmbH, Germany) was included in the gel. Comparisons between obtained fingerprints were carried out by visual inspection and isolates with a banding difference of ⩾3 bands were considered to be different clones. The clone types were designated according to individual and species. For example, subject 1, *B. thetaiotamicron* clone type 1 has the following type designation: 1tI.

T-RFLP

DNA was extracted from triplicate 100 mg faecal samples from each subject using the FastDNA SPIN Kit for Soil (Q-BIOgene, Carlsbad, CA, USA) following the manufacturer's instructions. The 16S rRNA genes in the extracted DNA were amplified using the general eubacterial primers fD1-FAM (Weisburg *et al.*, 1991) labelled at the 5′ end with FAM fluorescein, and 926r (Muyzer *et al.*, 1993). For amplification of *Bacteroides* spp., the reverse primer g-Bfra-R (Matsuki *et al.*, 2002) was used together with fD1-FAM (for primer sequences, see Table 1). The PCR products were digested with *Hae*III and the fluorescent terminal restriction fragments (TRFs) were separated according to size and quantified on an ABI sequencer as described previously (Jernberg *et al.*, 2005). The threshold value for TRFs was established at a relative abundance ⩾0.5% and TRFs differing by ±0.5 bp were grouped together. The General Rweb interface (http://pbil.univ-lyon1.fr/Rweb/) was used for Correspondence Analysis of the T-RFLP data, taking into account both TRF size and relative abundance values and scatter plots were created. The statistical significance of clustering patterns was determined by one-way ANOVA using Prism 4 software (GraphPad software, San Diego, CA, USA).

Real-time PCR

The presence of resistance genes *erm*(B), *erm*(F) and *erm*(G) was analysed in DNA extracted from faecal samples using the DNA extraction approach described above. The relative gene abundance in the DNA extracts was assessed using real-time PCR. The 16S rRNA gene was used as a reference gene for normalization of the levels of resistance genes in each sample (for primer sequences, see Table 1). Real-time PCR was performed using a LightCycler (Roche Diagnostics, Mannheim, Germany) and amplification was carried out in triplicate using 10 ng of extracted DNA as template for the reactions. Light Cycler-FastStart DNA Master SYBR green I kit (Roche Diagnostics) was used in a standard Light Cycler PCR according to the manufacturer's instructions. Primer sequences for the *erm* genes and

Table 1 Primers used for the different PCR reactions

Name	*Sequence*	*Reference*
REP1R-I	5′-IIIICGICGICATCIGGC-3′	Versalovic *et al.* (1991)
REP2-I	5′-ICGICTTATCIGGCCTAC-3′	Versalovic *et al.* (1991)
ERIC1R	5′-ATGTAAGCTCCTGGGGATTCAC-3′	Versalovic *et al.* (1991)
ERIC2	5′-AAGTAAGTGACTGGGGTGAGCG-3′	Versalovic *et al.* (1991)
BOXA1R	5′-CTACGGCAAGGCGACGCTGACG-3′	Versalovic *et al.* (1995)
fD1-FAM	5′-AGAGTTTGATCCTGGCTCAG-3′	Weisburg *et al.* (1991)
926r	5′-CCGTCAATTCCTTTRAGTTT-3′	Muyzer *et al.* (1993)
g-Bfra-R	5′-CCAGTATCAACTGCAATTTTA-3′	Matsuki *et al.* (2002)
Uni331F	5′-TCCTACGGGAGGCAGCAGT-3′	Nadkarni *et al.* (2002)
Uni797R	5′-GGACTACCAGGGTATCTATCCTGTT-3′	Nadkarni *et al.* (2002)

cycling conditions were as described previously (Löfmark *et al.*, 2006). The maximum C_t (threshold cycle) was set at 35 cycles and C_t levels above the threshold were considered as background. The Q-gene software (Muller *et al.*, 2002; from Biotechniques software library, www.Biotechniques.com) was used to calculate the levels of the three resistance genes. Using this software, the level of target genes (specific *erm* genes) were calculated relative to the 16S rRNA gene reference in the samples. The level of specific gene increase was recalculated as the fold increase compared to levels at day 0, before clindamycin administration.

Results

Clonal typing of Bacteroides *isolates*

Up to 20 *Bacteroides* isolates per sample from each individual and sampling date, that were collected previously from faecal samples and identified to the species level by biochemical tests (Löfmark *et al.*, 2006), were further typed to the clonal level by rep-PCR. This enabled us to follow how specific *Bacteroides* clones responded to a 7-day clindamycin administration and to monitor their persistence over a 2-year post-treatment period. Almost all clone types were unique for each specific subject except for *B. fragilis* that could not be genetically separated between the subjects, that is to say all the *B. fragilis* isolates had the same banding pattern, using the three different primer sets. This was also true for one specific transient clone type found in two subjects in the control group and these two subjects lived in the same household (data not shown). A large decrease in the collective number of *Bacteroides* clone types from all individuals in the exposed group was seen on the last day of clindamycin exposure (day 7) (Figure 1). This reduction in diversity persisted for up to 2 years post administration. By contrast, in the control group, only minor variations in the number of clone types were seen and individual clones and their abundances changed in an apparently random manner over time (Figure 1).

We also correlated the minimal inhibitory concentration (MIC) values of clindamycin for each *Bacteroides* isolate, obtained previously (Löfmark *et al.*, 2006), to specific clone types. We found an immediate enrichment of highly clindamycin-resistant *Bacteroides* clones (MIC > 64 mg/l) on day 7, the last day of clindamycin administration and the majority of the different clone types remained highly resistant to clindamycin long after the administration (Figure 1). After 9 months the highest number of resistant clones was detected, with 88% of the different clone types shown to be highly resistant, and after 2 years 58% were still highly resistant to clindamycin. By contrast no highly resistant clones (MIC > 64 mg/l) were detected in the control group throughout the sampling period (Figure 1).

Figure 2 presents representative examples of the ecological stability of clone types in an exposed subject and a control subject (data for all of the individuals are included in Supplementary Figure S1). In two of the subjects in the exposed group, specific clones that were susceptible to clindamycin before exposure became resistant at day 7 and dominated the cultured *Bacteroides* community at subsequent sampling periods (see Figure 2a for an example and Supplementary Figure S1). For example, in subject 1, one specific clone type, *B. thetaiotaomicron* 1tIV, became dominant for 18 months and it was the only clone type identified for the first nine months post exposure to clindamycin (Figure 2a). Interestingly, in this subject there were two other clone types that were resistant to clindamycin on day 0, but they did not become dominant. However, at later sampling dates one of the prior resistant clones (*B. vulgatus* 1vI) was again detected and it was still resistant. In subject 2 a different clone that was susceptible to clindamycin before exposure, *B. thetaiotaomicron* clone (2tI), also became resistant after exposure and became dominant for 9 months (Supplementary Figure S1). In this particular subject, the *Bacteroides* community was so suppressed by clindamycin that it was

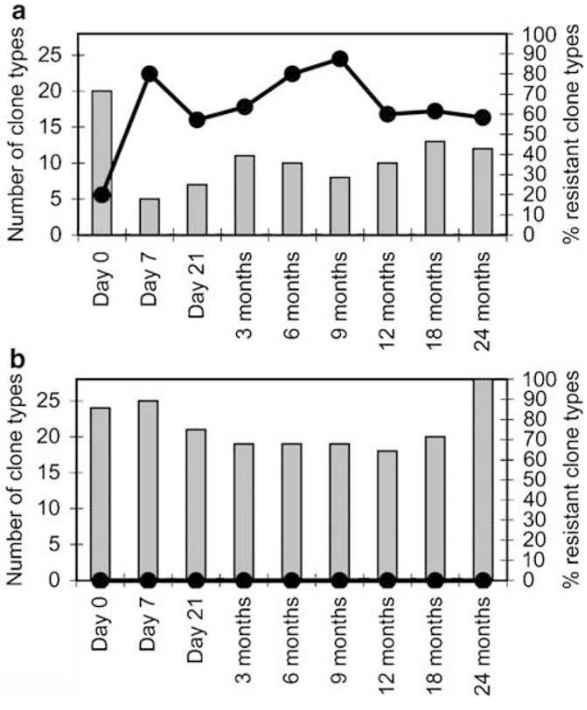

Figure 1 Number of *Bacteroides* sp. clone types as assessed by rep-PCR and percent of clones that are highly resistant to clindamycin over a 2-year sampling period: (**a**) clindamycin exposed group; (**b**) control group. Day 0: day before clindamycin administration. Bars represent total number of clone types and filled circles represent percent of highly clindamycin-resistant clones (>64 mg/l).

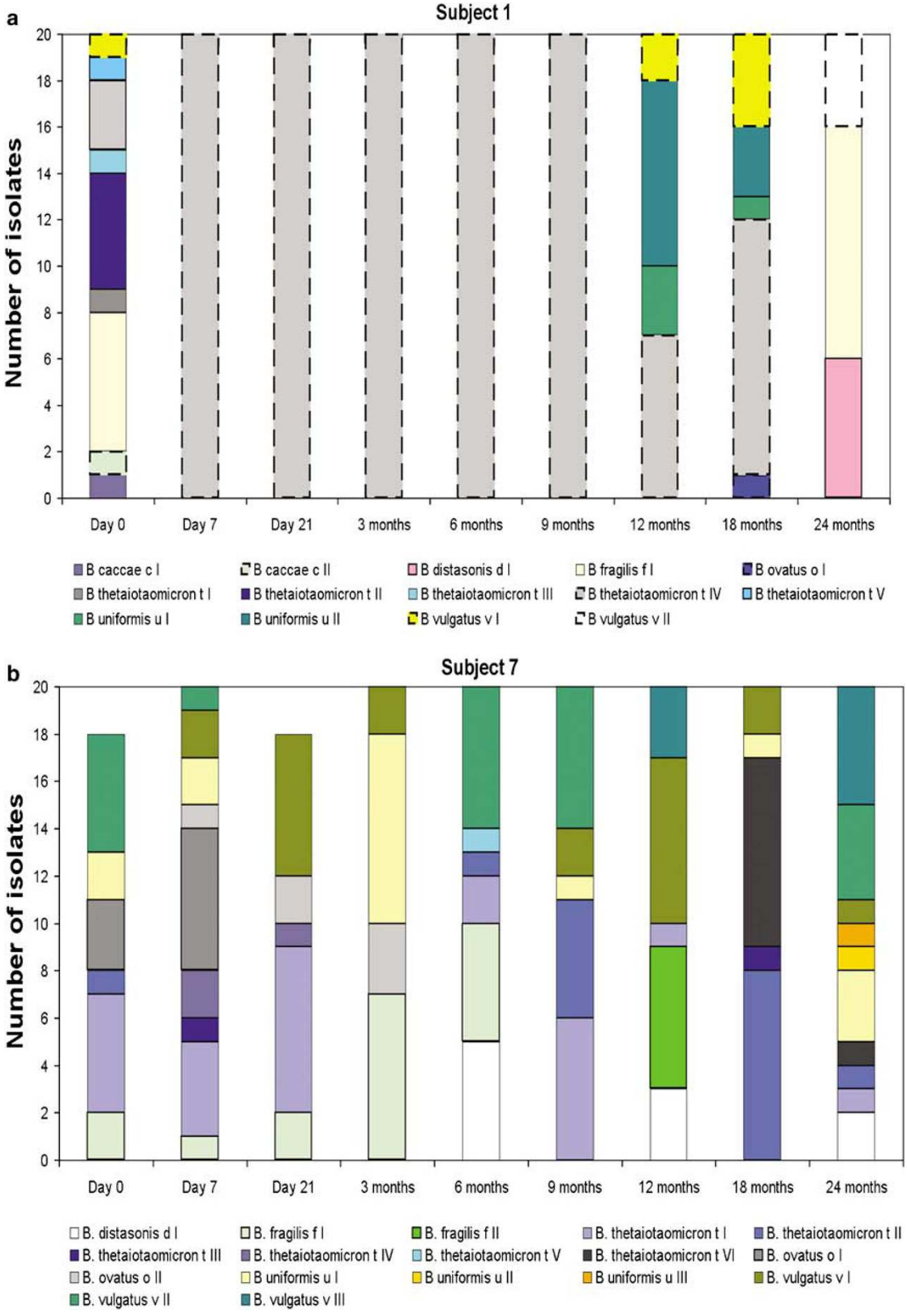

Figure 2 Designation of *Bacteroides* sp. clone types as determined by rep-PCR over a 24-month period. (**a**) representative subject 1 (clindamycin exposed) and (**b**) representative subject 7 (control). Each clone type is designated by a specific colour and species designations obtained by prior biochemical tests (Löfmark *et al.*, 2006) are shown. Bars representing clindamycin resistant clones (MIC > 8 mg/l) are designated with hatched lines and those representing clindamycin susceptible clones by solid lines. Data for all remaining subjects are included in Supplementary Figure S1.

not possible to pick 20 colonies from the faecal samples on sampling days 7 and 21 (Löfmark *et al.*, 2006).

The rapid enrichment of resistant clones in the remaining subjects was partially due to selection of a few clones already resistant to clindamycin at day 0 which then persisted for the remainder of the study period. For example, in subject 3, one clone, *B. thetaiotaomicron* 3tIII, was identified throughout the study period and was the dominant clone from 9 months and up to 24 months post exposure to clindamycin. Finally, in subject 4, a specific resistant clone, *B. thetaiotaomicron* 4tI dominated the *Bacteroides* community at almost all sampling occasions for up to 24 months and this particular clone was identified throughout the study period (Supplementary Figure S1).

By contrast, the number of clone types and their abundances varied over time in the control group (Figure 2b). In most subjects in the control group there were specific clone types identified at all nine sampling occasions, for example clone *B. thetaiotaomicron* 6tI in subject 6 (Supplementary Figure S1). This demonstrated that some *Bacteroides* clones persist for long periods of time in the human gut in the absence of antibiotic disturbance. Also more transient clone types were identified in the control group, for example clone *B. thetaiotaomicron* 8tII in subject 8 that could only be detected in one sample at 3 months (Supplementary Figure S1). Other clones were detected repeatedly, but not consistently throughout the sampling period (see subject 7, Figure 2b). These might represent resident members of the gut microbiota that were detected, or not, based on the detection limitations of the sampling procedure used (i.e. random collection of up to 20 colonies per sample).

Real-time PCR analysis of erm *genes*

We also quantified the relative levels of functional genes encoding antibiotic resistance in DNA extracted directly from the faecal samples. The levels of the *erm* genes, *erm*(B), *erm*(F) and *erm*(G) were quantified in community DNA by real-time PCR. The data were compared to levels found at day 0, before clindamycin administration, to enable relative differences in antibiotic resistance gene levels to be quantified in all samples, including from individuals from the exposed group that had some antibiotic resistant *Bacteroides* isolates before treatment. For each clindamycin exposed subject one specific *erm* gene increased in abundance and became dominant (Figure 3). For subjects 2 and 3, *erm*(F) showed the largest increases. After 24 months the increase in *erm*(F) was still over 1000-fold pre-clindamycin administration levels in these two subjects (Figure 3). By contrast, the largest increases in *erm*(G) levels were seen in subjects 1 and 4. For subject 1 the increase was not as dramatic as in the three other subjects in the exposed group. Subject 4 was the only subject where the relative amount of *erm* genes returned to baseline levels at 18 months. By contrast, *erm*(B) gene levels did not increase to as great an extent in any of the four clindamycin-exposed subjects (Figure 3). For the subjects in the control group the resistance genes were either under the detection limit or varied around the baseline except for one gene in one subject (*erm*(F) in subject 8), with the highest increase (624-fold for *erm*(F)) in the sample collected at 18 months. However, this increase is minor compared to the high increases we found in members of the exposed group, for example increase by almost six orders of magnitude for subject 2, *erm*(F).

T-RFLP analysis

Changes in the relative abundance of individual members of the total *Bacteroides* community were specifically assessed by T-RFLP using a *Bacteroides* specific reverse primer during PCR amplification of the 16S rRNA genes. We found that three of four subjects in the exposed group had marked differences in the compositions of their *Bacteroides* communities before and after clindamycin administration (see Figure 4a showing representative subject 2; data for all individuals are included in Supplementary Figure S2 and Supplementary Table S1). There was a large shift in the *Bacteroides* community composition at day 0 compared to all of the samples that were taken post-clindamycin administration and no tendencies towards normalization to a preadministration community structure were seen. In subject 4, the *Bacteroides* community was so suppressed after the administration (days 7, 21 and 3 and 9 months) that no PCR products could be obtained on these sampling dates (Supplementary Figure S2) and this was the same subject that we had difficulty in obtaining *Bacteroides* isolates from. By contrast, in the control group the differences between the samples were not as large as in the exposed group and the clustering of samples was instead more time dependent (see Figure 4a, representative subject 8).

T-RFLP with general bacterial primers was also used to assess the impact of clindamycin exposure on the dominant members of the total bacterial flora. A large shift in the composition of the bacterial community was observed immediately after clindamycin exposure at days 7 and 21 (Figure 4b; Supplementary Figure S2) that was highly statistically significant ($P<0.001$) in all exposed subjects. However, after 3 months the clustering of samples was more time dependent or random. In the control group, the samples clustered more tightly together in the COA plots thereby showing a higher similarity than the samples in the exposed group, although there were some time dependent shifts, such as that seen for the last sampling date in subject 8 (Figure 4b; Supplementary Figure S2).

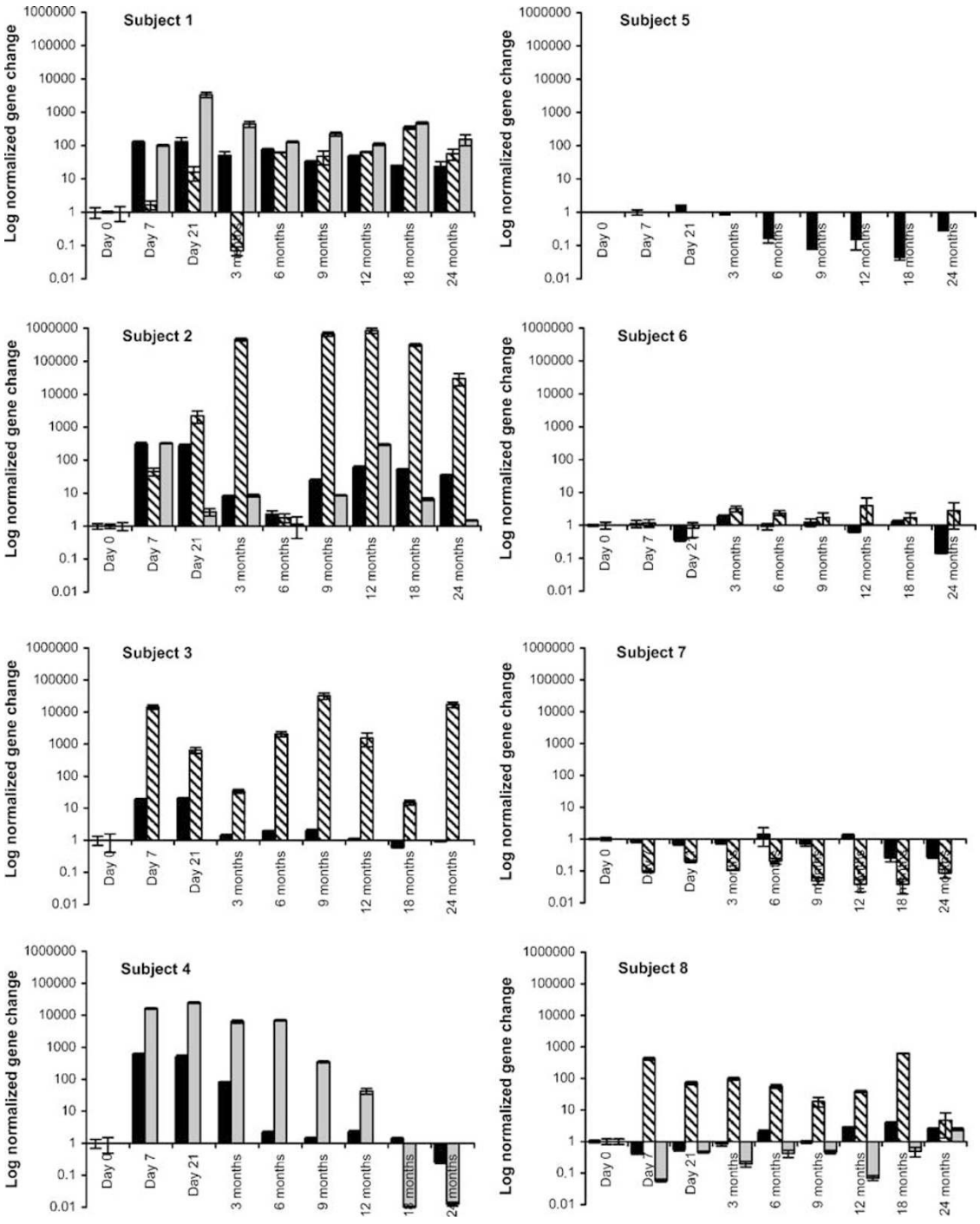

Figure 3 Normalized relative gene increase of *erm*(B), (F) and (G) genes compared to day 0 in community DNA extracted from faecal samples for the subjects in the exposed group (subjects 1–4) and control group (subjects 5–8). Black bars *erm*(B), striped bars *erm*(F) and grey bars *erm*(G). The error bars represent the s.e.m. of three replicate faecal samples.

The average number of TRFs was significantly lower ($P<0.01$) at day 7 for the individuals in the exposed group (20) compared to the individuals in the control group (34). Furthermore, when comparing the total number of TRFs in the exposed group to the control group over the 2 year study period the average number of TRFs was highly significantly lower in the exposed group ($P<0.001$) compared to the control group. However, the exposed group already had a lower average number of TRFs on day 0 before antibiotic exposure (26, compared to 29 for the control group) and the difference in average number of TRFs from day 0 to day 7 was not significant for either the exposed or the control group. Therefore, although the T-RFLP data suggests a lower diversity (richness) in the exposed group,

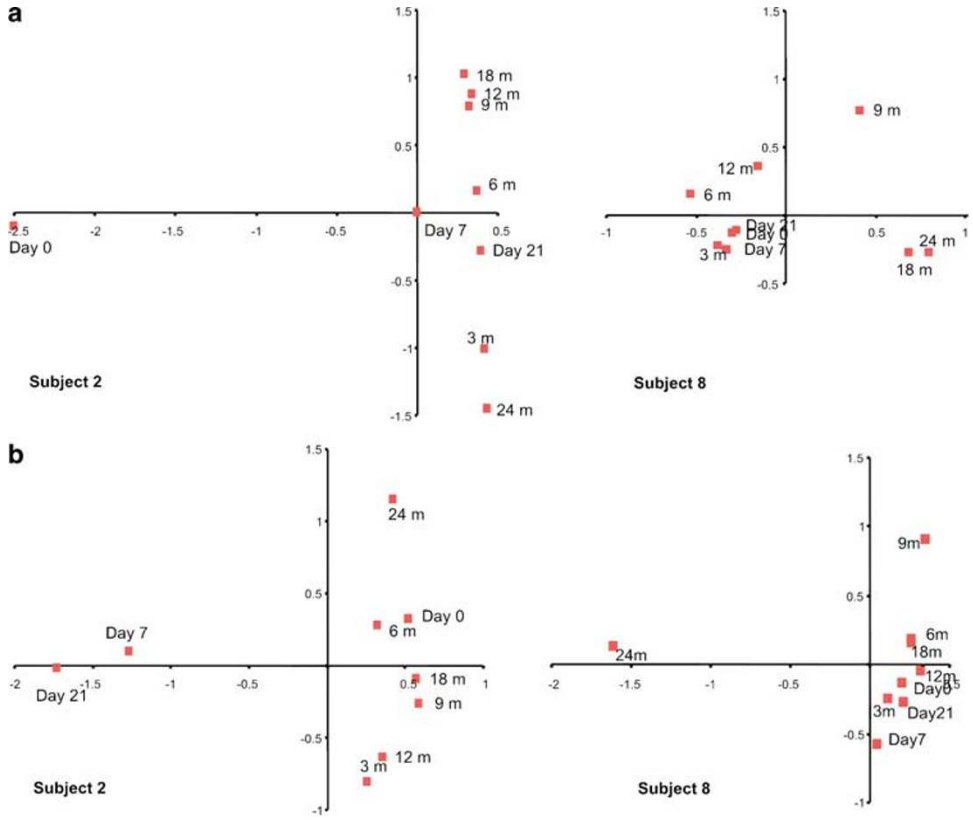

Figure 4 Correspondence analysis plots of T-RFLP fingerprints (TRF size and relative abundance values) over time for (**a**) the *Bacteroides* community and (**b**) the bacterial community. Each plot represents one representative subject per study group: subject 2 (clindamycin exposed); subject 8 (control) (see Supplementary Figure S2 for data from all subjects and Supplementary Table S1 for eigen values for the COA plots).

it is not possible to correlate this to antibiotic exposure.

Discussion

In this study a polyphasic approach was used to monitor the long-term ecological effects of 7 days clindamycin administration on the human intestinal microbiota. The different analysis methods were complementary and provided information on the general bacterial community structure, the *Bacteroides* community specifically and the levels and persistence of resistance genes. This polyphasic approach was necessary because of the extreme complexity of the intestinal microbiota. Also, although the composition of the intestinal microbiota is generally perceived to be relatively stable in healthy subjects, it can fluctuate owing to external influences, such as diet (Gibson and Roberfroid, 1995). A previous study has shown that the *Bacteroidetes* division, encompassing *Bacteroides* species, has the largest subject-to-subject variability of all divisions (Eckburg *et al.*, 2005). Therefore, the study of the control group in this study provided valuable insight into the natural fluctuations in the intestinal microbiota at large and the *Bacteroides* community specifically.

Previous studies have shown that the administration of clindamycin often leads to major short-term ecological disturbances (Nord *et al.*, 1997; Sullivan *et al.*, 2003). The antibiotic is excreted in bile at high concentrations and thereby it leads to strong suppression of the anaerobic community (Nord *et al.*, 1997; Sullivan *et al.*, 2001). In this study, using the anaerobic *Bacteroides* spp. as an indicator organism, the impact of clindamycin on the flora was monitored on a clonal level as well as on a genus specific 16S rRNA gene level. Furthermore, the changes in relative abundance of the dominant members of the faecal microbiota were monitored and finally, the faecal samples were analysed on a functional gene level measuring the relative increase of specific *erm* genes in directly extracted DNA. Clindamycin was shown to have a profound and persistent impact on the faecal microbiota according to all analysis methods used in this study.

Previously Löfmark *et al.*, 2006 obtained *Bacteroides* isolates from the faecal samples and typed them using biochemical tests to the species level. At the same time, the levels of resistance to clindamycin (MIC values) were determined with the finding that antibiotic resistant isolates could still be obtained 2 years post-clindamycin treatment. However, it was not determined in that study whether the same strains/clones were detected throughout

the sampling period or not. Therefore, we used rep-PCR in this study to type the strains to the clonal level and to monitor their long-term persistence. Our results support the hypothesis that each individual harbours a specific gut microbiota. This was at least the case for the *Bacteroides* community as each individual had their own unique set of *Bacteroides* clone types except for *B. fragilis*. This species could not be genetically separated using rep-PCR or, alternatively, this species does not easily become host-specific. In the control group, many of the same clones were found repeatedly throughout the sampling period in the same individual, thus supporting the hypothesis that the gut microbiota is relatively stable over time. There was, however, an ebb and flow of clone types detected over time and these changes could be due to environmental factors, such as diet, or to detection limitations associated with only picking up to 20 *Bacteroides* colonies per sample. In another study, to investigate the stability of the *Bacteroides* community, six samples from a healthy subject were analysed by TGGE over a 22-month period (Pang *et al*., 2005). A small shift in the population could be detected but the overall TGGE pattern during the sampling period maintained relatively stable. We also found some temporal shifts in the *Bacteroides* community in healthy, non-exposed individuals by T-RFLP, but these differences were relatively minor judging by the small variation seen in the COA plots (Figure 4a; Supplementary Figure S2).

In this study, we also monitored the impact of antibiotic treatment on the dynamics of the *Bacteroides* community over time, both by monitoring individual clones and by T-RFLP of DNA extracted from the faecal samples. Both of these approaches showed that the antibiotic treatment had a dramatic impact on the *Bacteroides* community resulting in large and persistent changes. The marked decrease in clonal diversity within the *Bacteroides* community was due to selection of specific clindamycin resistant clones that came to dominate the community, e.g. subject 1 (Figure 2a). In most individuals resistant *B. thetaiotaomicron* clones became dominant and the clone types were host specific. In all exposed subjects, clones that dominated the *Bacteroides* community after exposure were not new colonizers taking advantage of the change in the intestinal environment created by clindamycin. Instead they could all be found in the day 0 samples and thereby already belonged to the dominant members of this community within the different subjects. Three subjects in the exposed group had clone types that were already resistant to clindamycin at day 0, five clones in total. However, in subject 1, harbouring two of these clones, a prior susceptible clone became resistant and dominated the community. In another study the long-term persistence of erythromycin resistant enterococci (Sjölund *et al*., 2003) was investigated, using pulsed field gel electrophoresis to type individual clones. Similar to our findings, they found that three out of five subjects carried highly resistant enterococci clones 1 year post administration. Taken together with our findings, these results strengthen the importance of monitoring the impact of different antibiotic treatments on specific bacterial groups.

The T-RFLP results also demonstrated a large shift in the *Bacteroides* community structure after clindamycin administration. The samples at day 0 were clearly separated from the rest of the samples collected during the 2-year study period on COA plots. Therefore, the *Bacteroides* communities had been impacted by antibiotic administration in these four subjects for a long period of time and this could even lead to a permanent change as no tendencies were observed to return to a pre-administration community structure.

By contrast, when general primers were used for T-RFLP the microbiota was observed to stabilize to preclindamycin administration levels after only three months post exposure. These results are in accordance with previous culture-based studies showing that the impacts of antibiotics such as clindamycin (Sullivan *et al*., 2001) last a few weeks after which the microbiota is normalized. The general primers, characterizing a much larger part of the faecal community, were unable to illuminate the otherwise long-term changes that the more specific analysis tools could visualize. Furthermore, the variations in the faecal microbiota seen in the control group are probably due to environmental factors, such as diet and when these variations are compared to the large shift observed in the exposed group the dramatic impact of the antibiotic is even further strengthened.

The effect of clindamycin exposure on specific resistance genes, *erm*(B), *erm*(F) and *erm*(G), in the total microbiota was assessed by real-time PCR. As a reference gene the 16S rRNA gene was amplified using universal primers. However, 16S rRNA gene copies cannot directly be converted into cell counts because some bacteria can have more than one *rrna* operon (Farrelly *et al*., 1995). Using the 16S rRNA gene as a reference gene was still considered an appropriate choice of reference, considering that our aim was not to use this gene for absolute quantification purposes. Furthermore, Nadkarni *et al*. (2002) showed that the estimation of bacterial load determined by colony counting or real-time PCR in an artificial mixture was similar despite the fact of differences in rRNA copies in the different species. In this study, we found that one specific *erm* gene, either *erm*(F) or *erm*(G), increased in relative abundance for each exposed subject in DNA extracted from the faeces. This could be owing to selection of a particular resistant member of the microbiota, carrying a particular *erm* gene, but here we could not couple the resistance genes to any particular organism. Previously, we showed that the *erm*(B) gene was not detected in any of the *Bacteroides* isolates (Löfmark *et al*., 2006), which

supports the findings in the present study where the *erm*(B) gene showed the lowest level of increase in all the exposed subjects.

All three *erm* genes encode erythromycin methylases catalysing the same reaction and therefore exhibit functional redundancy. The *erm* genes are located on transposable elements and not connected to specific operons. What influences the host range of these genes is not clear. It may be either connected to the host range of the specific mobile element or to basic differences in bacterial physiology. Resistance in *Bacteroides* spp. is known to be mainly associated with the *erm*(F) gene, and *erm*(G) to a lesser extent *erm*(B) has also been described in *Bacteroides* spp., but it is more commonly found in Gram-positive aerobic strains (Roberts, 2004). Therefore, the predominant increase in levels of *erm*(F) and *erm*(G) genes in the community DNA extracted from faeces of exposed individuals suggests that the genes are mainly harboured in the *Bacteroides* community.

Strikingly, long after the selection pressure of the antibiotic subsided we could still detect high levels of particular *erm* genes in the faecal samples, even after 2 years time. This finding has important clinical implications, for example by providing extended opportunities for transmission of resistance genes to other species (Salyers and Amabile-Cuevas, 1997). The persistence of resistance genes found in the DNA directly extracted from the faecal samples was reflected in the selection and persistence of resistant *Bacteroides* clones, demonstrating a good correlation between these data.

Currently, it is widely accepted that the indigenous intestinal microbiota stabilizes a few weeks after an antibiotic administration. However, when we analysed the bacterial flora in more detail, we found that this is not the case, and that a short-term antibiotic exposure can have persistent consequences for the individual treated. These results urge caution for a more restricted use of antimicrobial agents.

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