FEMS Immunology & Medical Microbiolog, Volume 65, Issue 1, pages 55–68, June 2012

Diversity and distribution of sulphate-reducing bacteria in human faeces from healthy subjects and patients with inflammatory bowel disease

Wenjing Jia[1], Rebekah N. Whitehead[1], Lesley Griffiths[1], Claire Dawson[2], Hao Bai[3], Rosemary H. Waring[1], David B. Ramsden[1], John O. Hunter[2], Michael Cauchi[4], Conrad Bessant[4], Dawn P. Fowler[4], Christopher Walton[4], Claire Turner[5] & Jeffrey A. Cole[1]

[1]School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK; [2]Gastroenterology Research Unit, Box 262, Addenbrookes Hospital, Cambridge CB2 0QQ, UK; [3]School of Electronic, Electrical and Computer Engineering, University of Birmingham, Birmingham B15 2TT, UK; [4]Cranfield Health, Vincent Building, Cranfield University, Bedfordshire MK43 0AL: [5]The Department of Life, Health and Chemical Sciences, Open University, Milton Keynes, MK7 6BJ, UK;

Correspondence: Jeffrey Cole, School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK. Tel.: +44 121 414 5440; fax: +44 121 4145925; e-mail: j.a.cole@bham.ac.uk

Running title: Sulphate-reducing bacteria in human faeces

Key words: faecal sulphate-reducing bacteria, diversity, 454 sequencing, Irritable Bowel Syndrome, Crohn's Disease, Ulcerative Colitis

Abstract

The relative abundance of different groups of sulphate reducing bacteria (SRB) in faecal DNA collected before and after therapy from patients suffering with Crohn's disease (CD), irritable bowel syndrome (IBS) or ulcerative colitis (UC) has been compared with that from healthy controls. Growth tests revealed that SRB were not more abundant in samples from CD patients before treatment than in the healthy control group. For most of the 128 samples available, these preliminary results were confirmed using degenerate PCR primers that amplify the *dsrAB* gene. However, some samples from CD patients before treatment contained a growth inhibitor that was absent from IBS or UC samples.

In-depth sequencing of PCR-generated *dsrB* fragments revealed that the diversity detected was surprisingly low, with only 8 strains of SRB and the sulphite reducing bacterium, *Bilophila wadsworthia*, detected above the 0.1% threshold. The proportion of the two major species detected, *B. wadsworthia* and *Desulfovibrio piger*, was as high as 93.5% of the total SRB population in the healthy control group, and lower in all patient groups. Four previously undescribed species were found: it is impossible to predict whether they are sulphate or sulphite reducing bacteria.

Introduction

A common feature of inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC) is the excessive immune response to bacteria that inhabit the intestinal lumen. Despite recent progress in defining factors that exacerbate or ameliorate these diseases, their precise causes remain poorly defined. Both can be treated with different degrees of success by immunosuppressive drugs such as corticosteroids, azathioprine or infliximab. Although enteral feeding to control dietary intake is effective in the treatment of Crohn's disease, this is not so in ulcerative colitis (King *et al*., 1997). Successful treatment of Crohn's disease is accompanied by substantial changes in the composition of gut microbiota and related immunoglobulins (van den Waaij *et al*., 2004) but no single group of bacteria has been implicated as the unequivocal source of these diseases. Irritable bowel syndrome (IBS) is a condition arising from a variety of causes whose symptoms are often similar to those of IBD. No intestinal inflammation is present, but as many as 50% of cases have food intolerances so that symptoms may be controlled successfully by diet (Wiesner *et al.*, 2009). These intolerances have been shown to be related to abnormal fermentation by the colonic microflora (King *et al.*, 1998, Dear *et al.*, 2005).

Two of the major groups of bacteria that dominate the human gut are those that ferment complex carbohydrates, lipids or protein to lactate; and those that convert lactate to propionate or butyrate. The production of butyrate is significant because it is the preferred energy source for colonocytes (Macpherson *et al.*, 1996; Aminov *et al.*, 2006; Louis & Flint, 2009; Mai & Draganov, 2009) and is both anti-inflammatory and anti-carcinogenic (Hamer *et al.*, 2008; Tazoe *et al.*, 2008). It is converted by gastroentestinal bacteria to other compounds that are also anti-inflammatory (Sokol *et al.*, 2008). Butyrate is generated from lactate by fermentative bacteria dominated by the *Firmicute* phylum, and a marked deficiency of one species, *Faecalibacterium prausnitzii,* has been suggested as

being correlated with Crohn's disease. However, this report is unconfirmed because successful treatment with enteral diet resulted in a decrease, not an increase, in *F. prausnitzii* in this patient group (Jia *et al.*, 2010). There was no significant deficiency in *F. prausnitzii* either before or after treatment of ulcerative colitis or irritable bowel syndrome patients.

It has been proposed that the production of an unidentified toxin by intestinal bacteria might play a significant role in provoking intestinal inflammation. Anecdotally, in an acute phase, Crohn's disease patients suffer from bad breath typical of hydrogen sulphide production, and the presence of high levels of sulphide in their faecal samples has been documented (Pitcher & Cummings, 1996). Some sulphate reducing bacteria (SRB) generate sulphide from sulphate using lactate as their preferred electron donor, and hence they compete with *Firmicutes* for their primary source of carbon and energy (Roediger *et al.*, 1997; Chapman *et al.*, 1994; Attene-Ramos *et al.*, 2007). It has therefore been proposed that sulphate reducing bacteria exacerbate gastrointestinal disease not only by generating a toxic product, hydrogen sulphide, but also by depleting the production of beneficial butyrate (Marquet *et al.*, 2009: but see also Shatalin *et al.*, 2011). If so, sulphate reducing bacteria might be less abundant in patients after successful treatment. Mills *et al.* (2008) reported that after continuous flow culture models of the human colonic microbiota were inoculated with faeces from UC and non-UC volunteers, changes in bacterial populations were observed, with elevated numbers of sulphate reducing bacteria in the microbiota from UC patients. Furthermore, it is possible that one or more species of sulphate reducing bacteria might be associated with gastrointestinal disease. A previous study has revealed that the prevalence of *Desulfovibrio piger* was significantly higher in inflammatory bowel disease patients as compared to healthy individuals (Loubinoux *et al.* 2002). However, results from the literature do not show a clear association between sulphate reducing bacteria and IBD (Zinkevich and Beech, 2000;

Pitcher *et al.*, 2000; Duffy *et al.*, 2002; Ohge *et al.*, 2005; Manichanh *et al.*, 2006; Collado *et al.*, 2007). This might reflect highly diverse microbiota within individual groups and differences in methodology.

To investigate the possible association between gut disease and SRB, we compared the relative abundance of sulphate reducing bacteria in faecal DNA collected before and after therapy from patients suffering with Crohn's disease, irritable bowel syndrome or ulcerative colitis; healthy subjects were also recruited as controls. In addition to conventional methods used in previous studies, such as recovering growth of SRB, PCR and denaturing gradient gel electrophoresis (DGGE), the diversity of SRB in human faeces was also studied by next-generation sequencing (454 sequencing, Roche), which allows the identification of SRB species at a DNA level.

Materials and methods

Patient recruitment and treatment

Faecal samples were collected from 21 IBS patients, 20 CD patients and 14 UC patients, as described previously (Jia *et al.*, 2010). As controls, 18 healthy subjects were also recruited. Briefly, all volunteers were recruited at Addenbrookes Hospital, Cambridge, which functions as a tertiary referral centre for IBD. Ethical permission to collect these samples was obtained from Leeds West Ethics Research Committee (Ref. 07/Q1205/39) and informed, written consent was obtained from each subject. The diagnosis of CD and UC was based on standard endoscopic, radiological and histological findings; all subjects with IBS fulfilled the Rome II criteria. The severity of CD symptoms was reflected by the Harvey and Bradshaw (1980) Index and an objective measure of inflammation was provided by the serum concentration of C-Reactive Protein (CRP), which was determined by the Clinical Biochemistry Department, Addenbrookes Hospital. The patients gave a faecal sample at the start of treatment and in

most cases again after treatment. Within 48 hours of collection, samples (at 4^{o}C) were sent by courier to the University of Birmingham for DNA extraction and preparation of faecal suspension.

CD patients were treated as described previously (Jia *et al.*, 2010). Apart from water *ad libitum*, their nutritional intake was limited to the elemental diet E 028 Extra (Nutricia, Liverpool UK) in quantities calculated individually by a registered dietician according to the Schofield (1985) equation until symptoms resolved, which took two to three weeks. Further faecal samples were collected when they reached remission. Eleven of the UC patients had received either immuno-modulation or 5-aminosalicylic acid therapy prior to this study, and this was continued or introduced for all of the UC group, albeit with changes in the drugs prescribed, and increases in the doses used. IBS patients received conventional treatment with either low-fibre diet or non-fermentable bulking agents such as sterculia. (Weisner *et al.*, 2009). Again, further faecal samples were obtained when the patients reached remission.

DNA extraction and templates preparation for 454 sequencing

DNA was extracted from each faecal sample and stored as reported previously (Jia *et al.* 2010). To amplify a DNA fragment that is definitive for SRB in environmental DNA, a semi-nested PCR strategy (the first round to amplify *dsrAB* followed by a second round to amplify *dsrB*) has proven to be much more effective for detecting the widest range of species than using only one round of PCR and was therefore employed by this study (Miletto *et al.* 2007). First, 5 forward and 6 reverse degenerate primers were used to amplify an approximately 1.9 kb *dsrAB* fragment from as many SRB as possible (Supplementary Table 1). In a total volume of 20 µl, 10 ng of DNA was used as a template with HotStarTaq Plus Master Mix Kit (Qiagen) plus 4 µl of Q-solution (Qiagen) according to the manufacturer's instructions. The reaction conditions were: initial denaturation (5 min at 95^{o}C); then 12

cycles of denaturation (40 s at 94°C), annealing at temperatures ranging from 60 to 48°C (decreasing 1°C per cycle, 40 s) and elongation (2 min at 72°C); followed by 23 cycles of denaturation (40 s at 94°C), annealing (40 s at 48°C) and elongation (2 min at 72°C); and a final extension (10 min at 72°C). The PCR product was then used as a template for a second round of PCR to amplify an approximately 430 bp *dsrB* fragment including a barcode (also called multiple identifiers, MID) that could represent a patient group and be recognised in the following analysis. The design of primers used for the second round of PCR was based on previous publications (Geets *et al.*, 2006; Miletto *et al.* 2007) and was made more degenerate to rescue as many SRB species as possible (Supplementary Table 1). The reaction conditions were: initial denaturation (5 min at 95°C); then 30 cycles of denaturation (40 s at 94°C), annealing (40 s at 55°C) and elongation (1 min at 72°C); and a final extension (10 min at 72°C).

Qualitative estimation of SRB abundance in faeces

By using a homogeniser, a 0.4 g faecal specimen from each subject was suspended in 3.6 ml of peptone water. The suspension was centrifuged at 100 x *g* for 40 s to remove any remaining solid matter and the supernatant was collected and centrifuged at 12,000 x *g* for 5 min. The pellet obtained was then washed twice in 1 ml of TE buffer (10 mM Tris-Cl pH 7.5 containing 1 mM EDTA). The final pellet was resuspended in 4 ml of TE buffer supplemented with 100 µl of 40% glycerol, and the suspension was aliquoted and stored at – 80°C. To recover the growth of SRB that are able to use lactate as their electron donor and sole source of carbon, 0.5 ml of faecal suspension was inoculated into a 9.5-ml sulphate-rich growth medium, Postgate B (Postgate, 1984). The cultures were grown anaerobically in sealed 10 ml serum bottles. After incubation at 30°C for 21 days, they were photographed and the digital pictures were analysed to measure the degree of blackening of the culture. The abundance of SRB was gauged according to the amount of black precipitate, ferrous sulphide, formed by SRB. The

142 pure black colour was defined as 99, whereas pure white was defined as 0. The black intensity of each
143 culture was determined accordingly, ranging from 4 to 73.

144 Because the capacity of Postgate B medium to recover all SRB is not known, the abundance of
145 SRB in faeces was also estimated by PCR. The production of a *dsrB* fragment following two rounds of
146 PCR was gauged. In the first round, 10 ng of faecal DNA was used as a template; and in the second
147 round, 0.5 µl of the first round PCR product was included in all photographs and used for
148 normalization. The final PCR product (10 µl) was loaded on a 0.8 % agarose gel to check for a specific
149 band with a length of approximately 430 bp. The samples were divided into five groups based on the
150 production of *dsrB*: score 1, no specific band could be detected; score 2, a weak band was detected
151 when the undiluted first round PCR product was used as a template; score 3, a strong band was
152 detected when the undiluted first round PCR product was used as a template; score 4, a strong band
153 was detected when a 10-fold dilution of first round PCR product was used as a template; and score 5, a
154 strong band was detected when a 1000-fold dilution of first round PCR product was used as a template.

155 **Denaturing Gradient Gel Electrophoresis**

156 The same nested PCR strategy was used to prepare template DNA for DGGE. The product from the
157 first round of PCR, *dsrAB*, was used as a template to amplify *dsrB* but with a 40-bp GC clamp (Miletto
158 *et al.* 2007). The design of primers used for second round of PCR was based on previous publications
159 (Geets *et al.*, 2006; Miletto *et al.* 2007) and was made more degenerate to rescue as many SRB species
160 as possible (Supplementary Table 1). An alignment of *dsrB* sequences has revealed that the improved
161 primers should be effective for over 95 % of all known SRB (allowing 2 mismatches). The reaction
162 conditions were: initial denaturation (5 min at 95^{o}C); then 30 cycles of denaturation (40 s at 94^{o}C),

annealing (40 s at 55^{o}C) and elongation (1 min at 72^{o}C); and a final extension (10 min at 72^{o}C). The *dsrB*-GC PCR product was purified by using QIAquick Gel Extraction Kit (Qiagen, Germany).

In order to reveal the diversity of SRB, the *dsrB*-GC fragments (with the GC clamp) derived from different species in each patient were separated by DGGE using the DCode Universal Mutation Detection System (Bio-Rad, USA). A gradient of 40 to 70% denaturant was constructed in a 1 mm thick 8% (w/v) polyacrylamide gel by mixing a high density solution (70%) with a low density solution (40%) using the Gradient Delivery System. The 100% (w/v) denaturant solution contains 7 M urea and 40% (v/v) formamide. To visually check the formation of the gradient, 300 µl of DCode dye solution was added into 15 ml of high density solution. The purified PCR product was mixed with 6 x bromophenol blue loading buffer and loaded into the parallel gradient gel. Prior to loading, the gel had been pre-heated to 60^{o}C in 7 litres of 0.5 x TAE buffer. The gel was initially run at 150 V for 5 min without circulating the TAE buffer in the tank to facilitate the access of PCR products into the gel, and then at 75 V for 21 h at 60^{o}C with the buffer circulating continuously. Following electrophoresis, the gel was stained for 30 min in 250 ml of 0.5 x TAE buffer containing 1 µg.ml^{-1} ethidium bromide. The gel was photographed in a UV transilluminator and each *dsrB*-GC band representing a different SRB resource was removed and soaked in 130 µl of nuclease-free water. After eluting DNA into water overnight, a solution containing 50 ng of DNA was evaporated down to 7 µl and sequenced by using primer DSR4R. The sequencing data were analysed using NCBI blast.

Identification of SRB in human faeces by 454 sequencing

Four groups of subjects were recruited in this study to produce seven groups of samples: patients with IBS before and after treatment; patients with CD before and after treatment; patients with UC before and after treatment; and one healthy control group. Unlike the control group, in which a *dsrB* fragment

was amplified successfully from every sample, some patients did not carry any SRB that could be amplified and therefore 454 sequencing could not be undertaken. As a result, only 9 subjects in each group were selected and equimolar amounts of template from each patient were pooled. When a before-treatment group was compared with the corresponding after-treatment group, the sequencing data were not for completely paired patients. For the before- and after-treatment UC groups, 7 out of 9 patients were paired; for the 2 CD groups, 5 out of 9 patients were paired; and for the 2 IBS groups, 3 out 9 patients were paired. The 7 patient pools were sequenced by the Functional Genomics and Proteomics Laboratory at the University of Birmingham, and 200,133 sequences were extracted.

The sequencing data were analysed by using software Geneious® (Biomatters Ltd, New Zealand). The sequences with low quality (shorter than 150 bp or longer than 440 bp, 15.3% of total) were removed before further analysis. For each pool, 24,220 high-quality sequences were produced on average (169,539 sequences in total). In order to handle the data efficiently, the data produced for one pool were further divided into batches, each comprising approximately 2,500 sequences. They were then aligned by using software MUSCLE, and a neighbour joining phylogenetic tree was constructed based on the alignment result. The distance between clusters in a tree reflects the diversity of SRB found within a patient pool. Sequences within one cluster (diverging distance less than 0.03) were extracted and a consensus sequence was produced. Consistent with the report of Kjeldsen *et al.* (2007), clusters whose consensus amino acid sequences were over 97% identical were considered as one phylotype. Duplicate samples of high quality sequences were analysed from 7 of the pools to check that sufficient data had been analysed to generate a reproducible result. In total, 55,708 out of 169,539 sequences were analysed as 21 datasets, which generated 21 phylogenetic trees (Supplementary figures 1 – 21). A further comparison between the trees revealed that there were 16 phylotypes, i.e. 16 sulphate- or sulphite-reducing bacterial species. In addition, 10 to 20% of the sequences within each

bacterial group were analysed using NCBI blast, and the identification reports validated the grouping method based on alignment. The DNA consensus sequences were also translated into amino acid sequences, which again confirmed the grouping method. Furthermore, each patient group could be considered as two sub-groups, because four samples from one group were pooled and labelled with a unique barcode whereas the other five were labelled with a different barcode. Comparison of the two subgroups revealed whether the diversity of SRB was consistent. By combining data for the two subgroups, it was possible to determine whether one patient group as a whole was different from other groups.

In addition to the study based on pooled samples, 6 before-treated CD patients were analysed individually by 454 sequencing. To investigate whether the composition of SRB populations had changed in response to the ED therapy, the treated samples from 4 out of the 6 patients were also sequenced. Samples from the three control subjects were also sequenced to test whether the composition of SRB was similar between healthy controls.

Statistical analysis of the data

Results were assessed by non-parametric methods: Mann-Whitney U (MWU) test to compare the healthy control group with patient groups; and Spearman r for correlation analysis. These analyses were carried out using the Instat statistical package (GraphPad).

Results

The abundance of SRB in faecal suspensions from different patient groups

In this study, 128 faecal samples were donated by 7 groups of patients: CD, UC and IBS patients both before and after treatment, and one healthy control group. For the 128 samples, the abundance of SRB

was estimated by assessing the amounts of ferrous sulphide produced by SRB after aliquots of faecal suspensions had been grown anaerobically in Postgate medium B. Depending on the abundance of SRB in each inoculum, a black precipitate of iron sulphide had accumulated after 1 to 14 days. After 21 days at 30°C, the intensity of the black precipitate in each culture was measured by densitometry (Table 1). The resulting data for each patient group were analysed using the Mann-Whitney test to detect differences between groups. This analysis revealed that there was significantly less growth of SRB in samples from CD patients before treatment than in the healthy control group (Mann Whitney test, $p=0.017$). There had been little change in SRB abundance following enteral diet treatment, but almost certainly due to the small number of samples available for analysis, the P value for the difference compared with the healthy control group had increased to 0.065. Samples from UC patients before treatment also appeared to give less SRB growth compared with the healthy control group, but due to the small number of samples available, this apparent difference was not statistically significant. In contrast, there were no significant differences between the healthy controls and the IBS patients either before or after treatment.

The abundance of SRB DNA in faecal DNA determined by PCR analysis

Dissimilatory sulphite reductase encoded by *dsrAB* is an essential and highly conserved enzyme in sulphate reducing bacteria. The occurrence of similar sequences is limited to organisms that reduce other sulphur compounds such as organosulphates or sulphite and to sulphur oxidizing that are unlikely to be abundant in human faeces. Degenerate primer sets have been developed that amplify a 1.9 kb fragment of the *dsr* operon from virtually all known SRB (Zverlov *et al.*, 2005). These primers were used to amplify PCR products using faecal DNA as template. The abundance of SRB in each sample was estimated on the basis of the quantity of DNA template required to amplify a PCR product that was visible by gel electrophoresis (Table 1). This independent qualitative method for estimating the

abundance of SRB also revealed significantly less SRB DNA in faecal samples from CD patients and in the healthy control group. Statistical analysis again revealed that this difference compared with the healthy control group was statistically significant before treatment (Mann Whitney test p=0.0136): the p value for the comparison of the post-treatment samples with the control group was 0.066. As for the growth-dependent assays, there were no significant differences in the abundance of *dsrB* DNA in samples from UC and IBS patients and the healthy control group either before or after treatment.

Comparison of data for individual samples obtained by growth experiments and PCR analysis

There was a significant correlation between the estimated relative abundance of SRB obtained for each individual sample by using the two independent methods (Supplementary Fig. S22: Spearman r= 0.3994, $p< 0.01$, number of XY pairs = 125; culture data were not available for 3 patients). It was therefore concluded that, to a first approximation, both methods provide valid qualitative estimates of the relative abundance of SRB in faecal samples, and therefore that the PCR approach could be extended to compare the diversity of SRB within and between samples.

Inhibition of SRB growth by faecal suspensions from some CD patients

Despite the significant correlation between results of growth tests and PCR analysis of faecal DNA, clear discrepancies were apparent in the results from the two methods for some of the CD samples. For each of the 18 healthy controls, both growth tests and PCR amplification data indicated a high abundance of SRB, and the ratios of the two scores ranged between 11 and 18 (on average 15). In contrast, in 8 out of the 22 samples from CD patients before treatment, PCR analysis revealed a high level of SRB DNA, but relatively low SRB growth occurred when suspensions were used to inoculate Postgate medium B. The ratios of the two scores for these 8 samples were all below 11, including a ratio of 2 for sample 82 and less than 1 for sample 148. This lack of growth despite the presence of

SRB DNA might indicate either that the SRB in these samples were no longer viable, or that the major SRB species present grew poorly in Postgate medium B. A further possibility was that the faeces contained an inhibitor of SRB growth. To investigate whether samples 82 and 148 from CD patients before treatment contain an inhibitor of SRB growth, 0.5 ml of faecal suspension from these two samples was incubated in Postgate medium B with samples 116 or 126 in which SRB growth was rapid and abundant. Both samples strongly inhibited the growth of the positive controls (Fig. 1a and 1b). Dilution of these samples resulted in progressively less growth inhibition of the positive controls, confirming that inhibition was concentration-dependent. Growth inhibition was also lost on subsequent subculturing, presumably also due to dilution of the growth inhibitor in the original faecal sample (Fig. 1c). Sample #82 was from patient CG before treatment: the post-treatment sample #88 from this patient was also incubated with SRB-positive controls: it did not inhibit SRB growth (Fig. 1b). Further investigation established that 6 out of the 8 samples that gave poor SRB growth contained inhibitory factors for the growth of SRB, and in all six cases, growth inhibition was lost or had decreased substantially in samples from the same patient post-treatment. Samples from IBS and UC patients in which SRB DNA was abundant but growth in Postgate medium B was poor were also tested for the presence of growth-inhibitory factors. None of these samples inhibited growth of the positive controls.

DGGE investigation and sample selection for 454 sequencing

In initial experiments, the 1.9 kb *dsrAB* fragments were used as templates to generate 390 bp PCR products with clamps suitable for analysis by DGGE. To provide standards to calibrate DGGE gels, PCR products were also generated from DNA isolated from pure cultures of well-characterised sulphate reducing bacteria, and also from *B. wadsworthia*. Multiple bands following electrophoresis revealed the presence of PCR products of different GC content, suggesting the presence of a range of different SRB in these samples (Supplementary figure S23). However, multiple bands were also

obtained from PCR products generated using the same set of degenerate primers and chromosomal DNA purified from pure cultures of known SRB. Bands extracted from some of these gels were sequenced, and their origins were confirmed. By using NCBI blast, three species, *B. wadsworthia*, *D. vulgaris* Hildenborough and *Desulfovibrio* sp. NY682 were found in the samples tested. However, extensive experiments with this technique gave data too variable for reliable use in detecting minority populations or in determining the relative abundance of different SRB groups. The method was also too insensitive to detect minority populations that might be relevant to disease. The DGGE analysis was therefore abandoned in favour of in-depth DNA sequencing of PCR products.

Diversity of sulphate reducing bacteria in human faecal DNA

The 1.9 kb *dsrAB* fragment was readily generated using faecal DNA from all of the 18 samples from the control group, and all of these faecal samples gave abundant growth of SRB in Postgate medium B. Nine of these samples were used as templates to generate two pools (one pool from five samples and the other from four samples) of 430 bp bar-coded PCR products suitable for in-depth DNA sequencing. A further 12 pools of 430 bp bar-coded PCR products were also generated from faecal DNA from CD, UC and IBS patients before and after treatment (Table 2). As a result, the 454 sequencing data would reflect the most abundant SRB species present in the samples. However, the profile for CD and UC patients would not be quite complete because it was impossible to include samples from which *dsrB* fragments could not be amplified.

Equal quantities of DNA from each of the 14 resulting pools were mixed and sequenced. The 14 pools yielded 169,539 DNA sequences of good quality, of which over 99% could unequivocally be assigned to a *dsrB* fragment from an SRB in an identified pool of PCR products. The numbers of

sequences obtained from individual pools ranged from 1,591 in one of the pools of samples from IBS patients after treatment to 24,100 sequences from a pool of UC patients after treatment.

As a first step in data analysis, the diversity and relative numbers of SRB amongst over 2,500 randomly selected sequences were then analysed and compared with a further 2,500 sequences from the same sample. The results of these duplicate analyses were essentially identical (Supplementary Fig. S24), suggesting that it was sufficient to analyse 2,500 sequences from each pool to detect species present at more than 0.1% of the total SRB population.

The diversity of SRB in each pool was then determined, as described in detail in the Methods section. As shown in Table 2, each of the 7 patient groups could be considered as two sub-groups. The two sub-groups were compared to determine whether the diversity of SRB was consistent within one patient group; and, when the results of the two sub-groups were combined, whether one patient group as a whole was different from others (Fig. 2 - comparison between 7 patient groups; Table 3- comparison between 2 subgroups within one patient group).

The diversity and relative distribution represented in the 14 pooled samples were then determined by analysis of 55,708 sequences out of the 169,539 good-quality sequences available following 454 sequencing. There were two key observations from this initial series of experiments.

First, the diversity detected was surprisingly low, with only 8 groups of *dsrB* sequences other than the sulphite reducing bacterium, *B. wadsworthia*, detected above the 0.1% threshold (Fig. 3). Four of the SRB are either identical or closely related to known species: *D. piger*; *D. vulgaris* Hildenborough; *D. sp. NY682;* and *D. desulfuricans* F28-1. Note that the remaining four species might not be SRB, but bacteria able to reduce sulphite or organic oxidized sulphur compounds.

Secondly, four previously undescribed species designated strains A, B, C and D were found: strains A and B were more widely distributed than strains C and D. Because these results were from pools of DNA samples from 4 or 5 individuals, these strains might have been carried by only one or two people. Database searches revealed that on the basis of the *dsrB* sequences, the closest relative to species A is *D. desulfuricans* F28-1 (93% sequence identity); *dsrB* from species B is 84% identical to that from *D. oxamicus*; *dsrB* from C is 80% identical to *Desulfotomaculum sp. Lac2*; and D is 88% identical to *D. simplex*. It is impossible to predict whether the 4 new bacterial groups have sulphate or sulphite reducing capacity as they are so different from any known bacteria (Fig. 3; Table S2).

Relative abundance of SRB in faeces

The data described above enabled the relative abundance of the various strains to be calculated. *D. piger* was the most abundant SRB present in 9 of the 14 pools. However, its abundance varied widely from pool to pool and no consistent trends were apparent. For example, *D. piger* was not detected in one of the samples, pool 11, from IBS patients before treatment but constituted 44.7% of the *dsrB* sequences in the other pool of patients (pool 4; Table 3). There was similar variation in the abundance of *D. piger* in pools from IBS patients post-treatment, with none detected in pool 12, but 36% in pool 5. *B. wadsworthia* was more abundant than all of the SRB in 9 of the 14 pooled samples, but again its relative abundance varied widely between pools (Table 3).

Distribution of SRB in individuals

The most significant differences in SRB populations revealed by PCR analysis were between CD patients before treatment and the healthy controls. In order to identify any differences in SRB populations before and after treatment, 13 individual samples were investigated, including 3 healthy controls, 4 pairs of CD patients both before and after treatment, and 2 other CD patients before

361 treatment (Table 4). In the 3 healthy individuals, the proportion of the 2 major species contributed over
362 90% of all *dsrB* sequences, similar to the pooled data. However, the ratios of *B. wadsworthia* to *D.*
363 *piger* varied over a wide range. Among the 6 CD patients before treatment, 4 patients carried a much
364 lower proportion of *B. wadsworthia* (below 27.8%) compared to the average level in the healthy
365 control group (67.7%). Subsequent analysis of the 2 exceptional CD patients (Sample #47 and #128)
366 with high levels of *B. wadsworthia* revealed that the severity of disease in patient 128 was very low (2
367 mg/ml CRP), while in patient #47, no other sulphate or sulphite reducing bacterium was detected apart
368 from *B. wadsworthia*. This result is consistent with the results from the pooled data: a low proportion
369 of *B. wadsworthia* is likely to correlate with CD. However, based on the observation of these four
370 patients, effective treatment determined either by the Harvey and Bradshaw Index or by the
371 concentration of C-Reactive Protein (Table 5) does not always lead to an increase in the proportion of
372 *B. wadsworthia*. Clearly many more data from individuals before and after treatment will be required
373 for any statistically significant conclusions to be drawn. Based only on the 13 tested individuals, there
374 was no obvious association between the presence of species A and an abnormal gut environment. No or
375 very low levels of species C were found in CD patients, but it was present only in one of the 3 healthy
376 individuals investigated. Based only on the 13 tested individual samples, there was no obvious
377 association between the presence of species A or C and a healthy gut environment. The relatively high
378 proportion (approximately 6%) of species D observed in the CD patient pool was likely contributed by
379 one individual (patient sample #140), carrying species D at a level of 60% of all SRB. Again, no
380 statistically meaningful conclusion could be drawn from such a small sample.

381 **Discussion**

382 To our knowledge, this is the first attempt to define the relative abundance and diversity of SRB in the
383 human gut. Our primary objective was to determine by in depth sequencing of individual DNA

molecules whether the presence of one or more species, or their relative abundance, could be correlated with gut disease. Once they had been screened for variation due to sequencing errors, highly significant data were obtained. The results are therefore important in that they demonstrate that for bacteria for which the more traditional method of analysing 16S-ribosomal DNA sequences is unreliable, a combination of bar-coded primers and DNA sequencing of genes for a highly conserved metabolic enzyme can provide a useful alternative approach to analyse complex bacterial communities that might be associated with human disease.

Three major caveats should be considered in interpreting the data obtained from this study. First, two rounds of PCR were required to generate the template molecules that were sequenced. In any PCR reaction involving degenerate primers and community DNA, some templates will be amplified more than others. Secondly, primer design is critical because only those templates recognised by the primers can be amplified. For these reasons, it was essential to provide independent data to confirm that at least the major groups of SRB present in human faeces can be detected using the methods described in this study. The third caveat is that samples that failed to generate sufficient PCR product could not be included in the sequencing. Thus it is conceivable, but we believe unlikely, that new SRB species might occur only in these samples, and hence would have been overlooked in this study. With these caveats in mind, several important conclusions can be drawn from our data.

Due to the scarcity of SRB in human faeces, the 1.9 kb *dsrAB* fragments produced from the first round of PCR was hardly detected on an agarose gel whereas the second round PCR product, *dsrB*, was visible for most samples. Thus, the amount of SRB in each patient was scored by the amount of first round PCR product required to produce a clearly visible *dsrB* band on an agarose gel. The faecal samples were classified into 5 groups according to the PCR scores. To explore the possibility that our data were invalidated during the first round of PCR, the abundance of SRB in each individual faecal

sample was first assessed for its ability to generate a black precipitate of iron sulphide, which is indicative of SRB growth. The data correlated significantly with the PCR scores (Supplementary Fig. S22), which suggests that most of the major groups of SRB had been detected. However, there were two types of deviation from the general trend. First, 6 out of 8 of the samples from CD patients before treatment were shown to contain an inhibitor of SRB growth. The PCR assay was therefore more reliable than the growth assay for these samples. Conversely, two out of 28 UC samples (#14 and #32), two out of the 41 CD samples (#123 and 124) and 6 out of 42 IBS samples (#17, #64, #86, #90, #72 and #68) gave abundant formation of iron sulphide, but the PCR primers failed to detect the *dsrAB* genes in these samples. In this context it was surprising that no *D. fairfieldensis* was detected in any of our sample (see, for example, Loubinoux *et al*., 2002). This bacterium has recently been implicated in human disease (Gaillard *et al*., 2011). Thus despite the generally good correlation between growth tests and the PCR assay, further improvements to the primer set are required for the PCR assay to be completely reliable. An example of such an improvement was published after this work had been completed (Steger *et al.*, 2011).

The DGGE experiments provided an indication of the likely reliability of the second round of PCR using two independent sets of primers, but identical DNA templates. Comparable data for the relative abundance of *B. wordsworthii* and *D. piger* were obtained from both DNA sequencing and analysis of DGGE gels. This correlation extended to the three other previously characterized SRB found in individual samples. However, the DGGE method was insufficiently sensitive to be used to detect minority SRB populations

If all sulphate and sulphite reducing bacteria in each faecal DNA sample were defined as 100%, then *B. wadsworthia* and *D. piger* contributed 86% on average to the overall SRB (Table 3 and Fig 2) and were the major species of sulphate or sulphite reducing bacteria detected in this study. The

proportion of the two species was as high as 93.5% in the healthy control group, and lower in all patient groups. After treatment, the proportion of the two species had increased slightly in all three disease groups. In the CD patient group before treatment, the proportion of *B. wadsworthia* (41.9%) was much lower than that in the healthy group (67.7%) or any other patient group; after treatment, the proportion of *B. wadsworthia* had increased to a level similar to the healthy group. Also in this group before treatment, the proportion of *D. piger* (36.7%) was higher than that in the healthy group (25.8%) or any other patient group; the corresponding figure decreased to a level similar to the healthy group in response to treatment. In all of the 3 patient groups, after treatment, there was always an increase, to different extents, in the ratio of *B. wadsworthia* to *D. piger*. It is notable that the level of new species A is very low in healthy controls (0.48%) compared to patients with any of the 3 diseases (in a range of 6 to 12%). Following treatment, the proportion of species A decreased in every disease group, especially in CD patients (from 8.7% to 1.6%). New species C was found almost only in the healthy group; and new species D was found almost only in the CD group. If these preliminary results can be confirmed in a much more extensive study, they raise many interesting questions. For example, could a relatively low level of *B. wadsworthia* or high level of *D. piger* be used as an indicator to distinguish CD from the other 2 gut diseases? Does an effective treatment always lead to a decrease in the proportion of *D. piger* or an increase in the proportion of *B. wadsworthia*? Could the presence of new species A be used as an indicator for abnormal gut environment, or the presence of new species C be used as an indicator for healthy gut environment? Does the presence of new species D contribute to the aetiology of CD? Are species A B, C and D sulphate reducing bacteria, or prokaryotes that reduce organosulphur compounds, or sulphite? Does the observation in pooled data reflect the distribution of SRB in each individual? Finally, could the presence of a growth inhibitor in CD patients be developed as a useful tool in diagnosis?

In summary, despite its acknowledged limitations, this study has indicated some clear objectives for future research, and methods applicable to answer the many questions raised.

Acknowledgement

This work was funded by The Wellcome Trust (Grant No. WT080238MA).

References

Aminov RI, Walker AW, Duncan SH, Harmsen HJM, Welling GW & Flint HJ (2006) Molecular diversity, cultivation, and improved detection by in situ hybridization of a dominant group of human gut bacteria related to *Roseburia* spp. or *Eubacterium rectale*. *Appl Environ Microbiol* **72:** 6371-6376.

Chapman MAS, Grahn MF, Boyle MA, Hutton M, Rogers J & Williams NS (1994) Butyrate oxidation is impaired in colonic mucosa of sufferers of quiescent ulcerative colitis. *Gut* ***35*****:** 73-76.

Collado MC, Calabuig M & Sanz Y (2007) Difference between the faecal microbiota of coeliac infants and healthy controls. *Curr Issues Intest Microbiol* **8:** 9-14.

Dear KLE, Elia M & Hunter JO (2005) Do interventions which reduce colonic fermentation improve symptoms of irritable bowel syndrome? *Dig Dis Sci* **50:** 758-766.

Duffy M, O'Mahony L, Coffey JC, Collins JK, Shanahan F, Redmond HP & Kirwan WO (2002) Sufate-reducing bacteria colonize pouches formed for ulcerative colitis but not for familial adenomatous polyposis. *Dis Colon Rectum* **45:** 384-388.

Gaillard T, Pons S, Darles C, Beausset O, Monchal T & Brisou P (2011) *Desulfovibrio fairfieldensis* bacteremia associated with acute sigmoiditis. *Med Mal Infect* **41**: 267-268.

473 Geets J, Borrenans B, Diels L, Springael D, Vangronsveld J, van der Lelie D & Vanbroekhoven K
474 (2006) DsrB gene-based DGGE for community and diversity surveys of sulphate-reducing bacteria.
475 *J Microbiol Methods* **66:** 194-205.

476 Hamer HM, Jonkers D Venema K, Vanhoutvin S, Troost FJ & Brummer RJ (2008) Review article: the
477 role of butyrate in colonic function. *Aliment Pharmacol Ther* **27:** 104-119.

478 Harvey RF & Bradshaw JM (1980) A simple index of Crohn's disease activity *Lancet* **1:** 54.

479 Jia W, Whitehead RN, Griffiths L, Dawson C, Waring RH, Ramsden DB, Hunter JO & Cole JA (2010)
480 Is the abundance of *Faecalibacterium prausnitzii* relevant to Crohn's disease? *FEMS Microbiol*
481 *Lett* **310**: 138-144.

482 King TS, Woolner JT & Hunter JO (1997) Dietary treatment of Crohn's disease. *Aliment Pharm*
483 *Toxicol* **11:** 17-31.

484 King TS, Elia M & Hunter JO (1998) Abnormal colonic fermentation in irritable bowel syndrome.
485 *Lancet* **352:** 1187-1189.

486 Kjeldsen KU, Loy A, Jakobsen TF, Thomsen TR, Wagner M & Ingvorsen K (2007) Diversity of
487 sulphate-reducing bacteria from an extreme hypersaline sediment. Great Salt Lake (Utah). *FEMS*
488 *Microbiol Ecol* **60**: 287-298.

489 Loubinoux J, Bronowscki J-P, Pereira IAC, Mougenel J-L & Faou AEL (2002) Sulphate-reducing
490 bacteria in human feces and their association with inflammatory bowel disease. *FEMS Microbiol*
491 *Ecol* **40:** 107-112.

492 Louis P & Flint HJ (2009) Diversity, metabolism and microbial ecology of butyrate-producing bacteria
493 from the human large intestine. *FEMS Microbiol Lett* **294:** 1-8.

494 Louis P, Young P, Holtrop G & Flint HJ (2009) Diversity of human colonic butyrate-producing
495 bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. *Environ Microbiol*
496 In press.

497 Macpherson A, Khoo UY, Forgacs I, Philpott-Howard J & Bjarnason I (1996) Mucosal antibodies in
498 inflammatory bowel disease are directed against intestinal bacteria. Gut **38:** 365-375.

499 Mai V & Draganov PV (2009) Recent advances and remaining gaps in our knowledge of associations
500 between gut microbiota and human health. *World J Gastroenterol* **15:** 81-85.

501 Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, Nalin R, Jarrin C,
502 Chardon P, Marteau P, Roca J & Dore J (2006) Reduced diversity of faecal microbiota in Crohn's
503 disease revealed by a metagenomic approach. *Gut* **55:** 205-211.

504 Marquet P, Duncan S, Chassard C, Bernalier-Donadille A & Flint HJ (2009) Lactate has the potential
505 to promote hydrogen sulphide formation in the human colon. *FEMS Microbiol Lett* **299:** 128-134.

506 Miletto M, Bodelier PL & Laanbroek HJ (2007) Improved PCR-DGGE for high resolution diversity
507 screening of complex sulfate-reducing prokaryotic communities in soils and sediments. *J Microbiol*
508 *Methods* **70:** 103-111.

509 Mills DJS, Tuohy KM, Booth J, Buck M., Crabbe MJC, Gibson GR & Ames JM (2008) Dietary
510 glycated protein modulates the colonic microbiota towards a more detrimental composition in
511 ulcerative colitis patients and non-ulcerative colitis subjects. *J Appl Microbiol* **105:** 706-714.

512 Ohge H, Furne JK, Springfield J, Rothenberger DA, Madoff RD & Levitt MD (2005) Association
513 between fecal hydrogen sulphide production and pouchitis. *Dis Colon Rectum* **48:** 469-475.

514 Pitcher MCL & Cummings JH (1996) Hydrogen sulphide: a bacterial toxin in ulcerative colitis? *Gut*
515 **39:** 1-4.

516 Pitcher MCL, Beatty ER & Cummings JH (2000) The contribution of sulphate reducing bacteria and 5-
517 aminosalicylic acid to faecal sulphide in patients with ulcerative colitis. *Gut* **46:** 64-72.

518 Postgate JR (1984) Genus *Desulfovibrio* Desulfovibrio Kluyver and van Niel 1936. In NL Krieg & JG
519 Holt (ed.) Bergey's manual of systematic bacteriology, vol.1, p. 666-672. The Williamms and
520 Wilkins Co., Baltimore, MD.

521 Roediger WEW, Moore J & Babidge W (1997) Colonic sulphide in pathogenesis and treatment of
522 ulcerative colitis. *Dig Dis Sci* **42:** 1571-1579.

523 Schofield WN (1985) Predicting basal metabolic rate, new standards and review of previous work.
524 *Hum Nutr Clin Nutr* **39C**: 5-41.

525 Shatalin K, Shatalina A, Mironov A & Nudler E (2011) H_2S: A universal defense against antibiotics in
526 bacteria. *Science* **335**: 986-990.

527 Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ *et al.* (2008)
528 *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut
529 microbiota analysis of Crohn's disease patients. *Proc Natl Acad Sci USA* **105:** 16731-16736.

530 Tazoe H, Otomo Y, Kaji I, Tanaka R, Karaki SI & Kuwahara A (2008) Roles of short chain fatty acid
531 receptors, GPR41 and GPR43 on colonic functions. *J Physiol Pharmacol* **59 (Suppl. 2):** 251-262.

532 van der Waaij LA, Kroese FG, Visser A, Nelis GF, Westerveld BD, Jansen PLM & Hunter JO (2004)
533 Immunoglobulin coating of faecal bacteria in inflammatory bowel disease. *Eur J Gastroenterol*
534 *Hepatol* **16**: 669-674.

Wiesner M, Naylor SJ, Copping A, Furlong A, Lynch AG, Parkes M & Hunter JO (2009) Symptom classification in irritable bowel syndrome as a guide to treatment. *Scand J Gastro* **44:** 796-803.

Zinkevich V & Beech IB (2000) Screening of sulphate-reducing bacteria in colonoscopy samples from healthy and colitic human gut mucosa. *FEMS Microbiol Ecol* **34:** 147-155.

Zverlov V, Klein M, Lucker S, Friedrich MW, Kellermann J, Stahl DA, Loy A & Wagner M (2005) Lateral gene transfer of dissimilatory (bi)sulfite reductase revisited. *J Bacteriol* **187:** 2203-2208.

Figure legends.

Fig. 1. Inhibition of SRB growth by faecal suspensions prepared from samples donated by some CD patients. In a sealed serum bottle, 9.5 ml of Postgate medium B was inoculated with 0.5 ml of faecal suspension. Photographs were taken after 21 days of growth at 30^{o}C. (a) Cultures inoculated with sample #38, #116 and #139 were used as positive controls. The culture inoculated with sample #148 did not form a black precipitate of FeS possibly due to the presence of inhibitors of SRB growth. When the positive controls were mixed with sample #148, growth and FeS formation were severely inhibited. (b) A 0.5 ml inoculum from the cultures with sample #148 alone, or #148 plus #38, #116 or #139 were subcultured into 9.5 ml of fresh Postgate B medium. After a further 21 days, cultures were photographed and compared with the starting cultures. (c) Samples obtained from the same patient before treatment (#82) and after treatment (#88) were mixed with a positive control #126, and the effect on growth was tested. Growth SRB in the positive control samples was inhibited by 6 out of the 8 faecal samples from CD patients before treatment that gave a poor correlation between the level of growth and abundance of *dsrB* DNA estimated by PCR.

Fig. 2. Distribution of 4 types of SRB in 7 patient groups. The histogram shows relative abundance in the seven sample groups of *dsrB* DNA from four species (the sulphite reducing bacterium, *Bilophila wadsworthia*, *Desulfovibrio piger* and two unknown species) expressed a percentage of the total number of SRB sequences obtained.

Fig. 3. Consensus sequences of the 9 phylotypes found in this study and the 8 reference species. The phylotype labels 100% *B. wadsworthia*, 99% *D. piger*, 98% *D. desulfuricans* F28-1 and 98% *Desulfovibrio* NY682 overlap the labels of their reference species, as they are too similar to be distinguished on the dendrogram.

Table 1. Abundance of SRB in human faeces estimated by recovering growth and PCR

Patient	Sample No. Before	FeS formed	PCR score	Sample No. After	FeS formed	PCR score	Patient	Sample No. Before	FeS formed	PCR score	Sample No. After	FeS formed	PCR score
			Healthy							**UC**			
AA	11	73	5				**DA**	13	17	2	22	15	3
AB	15	66	5				**DB**	14	4	4	24	63	4
AC	16	71	5				**DC**	23	29	3	35	73	3
AD	37	71	5				**DD**	28	22	2	32	9	4
AE	40	56	5				**DE**	30	37	4	60	73	5
AF	42	73	4				**DF**	31	54	5	34	72	5
AG	43	66	5				**DG**	38	71	5	49	72	5
AH	44	72	5				**DH**	51	34	4	62	19	2
AI	50	71	4				**DI**	81	n/a	4	170	n/a	3
AJ	54	n/a	4				**DJ**	98	58	5	108	62	4
AK	71	68	5				**DK**	102	72	4	111	58	4
AL	74	71	4				**DL**	116	72	5	120	72	5
AM	95	64	4				**DM**	139	72	5	155	69	4
AN	114	65	4				**DN**	160	60	5	165	71	4
AO	145	70	5										
AP	146	58	5										
AQ	168	71	5										
AR	169	69	5										
Mean		**68**	**4.7**						**46**	**4.1**		**56**	**3.9**
STDV		**5.0**	**0.5**						**24**	**1.1**		**24**	**0.9**
			IBS							**CD**			
BA	8	73	1	9	72	4	**CA**	18	56	4	27	8	2
BB	12	60	4	29	38	4	**CB**	39	42	5	66	24	3
BC	17	18	5	25	62	5	**CC**	46	32	3	56	13	3
BD	20	72	4	45	73	5	**CD**	47	71	4	73	43	4
BE	36	71	4	90	21	4	**CE**	53	69	4	61	71	5
BF	48	71	5	69	72	5	**CF**	57	61	5	65	65	5
BG	52	70	4	72	16	4	**CG**	82	9	4	88	18	4
BH	55	36	4	159	70	4	**CH**	93	5	5	127	73	5
BI	58	18	4	76	59	4	**CI**	117	58	2	123	56	1
BJ	63	42	4	70	50	1	**CJ**	121	29	3	141	48	4
BK	64	16	5	68	21	5	**CK**	124	58	1	138	47	3
BL	84	69	5	89	66	5	**CL**	126	66	5	163	71	5
BM	86	29	5	106	55	5	**CM**	128	71	5	133	72	5
BN	100	64	5	134	70	5	**CN**	131	7	3	136	25	1
BO	104	72	4	129	72	5	**CO**	137	35	1	157	59	4
BP	105	65	5	150	59	1	**CP**	140	72	4	147	70	5
BQ	110	70	4	115	68	4	**CQ**	142	70	5	149	70	5
BR	112	71	4	176	69	4	**CR**	143	68	4	154	72	5
BS	113	66	4	118	68	4	**CS**	164	71	5	172	69	5
BT	122	35	4	125	70	4	**CT**	166	13	3	175	19	4
BU	162	72	5	167	71	4	**CU**[1]	148	4	5			
Mean		**55**	**4.2**		**58**	**4.1**			**48**	**3.8**		**50**	**3.9**
STDV		**21**	**0.9**		**18.5**	**1.1**			**25**	**1.3**		**23**	**1.3**

Note 1. Patient CU did not donate a follow-up sample, thus his/her sample was not used for the calculation of mean and standard deviation.

n/a: not assayed.

571 **Table 2.** Pooled and individual samples used in two 454 sequencing experiments

Barcode Pool No.	Patient No.	Sample No. Before	FeS formed[1]	PCR score	Barcode Pool No.	Patient No.	Sample No. After	FeS formed[1]	PCR score
Pooled samples used for the first 454 sequencing experiment									
Healthy									
1	AA	11	73	5					
	AC	16	71	5					
	AD	37	71	5					
	AH	44	72	5					
8	AE	40	56	5					
	AG	43	66	5					
	AM	95	64	4					
	AN	114	65	4					
	AP	146	58	5					
IBS Before treatment					**IBS After treatment**				
4	BF	48	71	5	5	BD	45	73	5
	BL	84	69	5		BF	69	72	5
	BP	105	65	5		BN	134	70	5
	BU	162	72	5		BO	129	72	5
11	BC	17	18	5	12	BB	29	38	4
	BI	58	18	4		BE	90	28	4
	BK	64	16	5		BG	72	16	4
	BM	86	29	5		BK	68	21	5
	BT	122	35	4		BM	106	55	5
CD Before treatment					**CD After treatment**				
6	CL	126	66	5	7	CL	163	71	5
	CM	128	71	5		CM	133	72	5
	CQ	142	70	5		CP	147	70	5
	CP	140	72	4		CQ	149	70	5
13	CA	18	56	4	14	CD	73	43	4
	CB	39	42	5		CG	88	18	4
	CF	57	61	5		CJ	141	48	4
	CG	82	9	4		CO	157	59	4
	CH	93	5	5		CT	175	19	4
UC Before treatment					**UC After treatment**				
2	DG	38	71	5	3	DE	60	73	5
	DL	116	72	5		DF	34	72	5
	DM	139	72	5		DG	49	72	5
	DN	160	60	5		DL	120	72	5
9	DB	14	4	4	10	DB	24	63	4
	DE	30	37	4		DD	32	9	4
	DF	31	54	5		DJ	108	62	4
	DH	51	34	4		DK	111	58	4
	DJ	98	58	5		DM	155	69	4

572

573

Samples sequenced individually in the second 454 experiment

CD Before treatment					**CD After treatment**				
3	CQ	142	70	5	4	CQ	149	70	5
5	CD	47	71	4	6	CD	73	43	4
7	CG	82	9	4	8	CG	88	18	4
9	CH	93	5	5	10	CH	127	73	5
11	CM	128	71	5					
12	CP	140	72	4					
Healthy									
13	AD	37	71	5					
15	AE	40	56	5					
16	AM	95	64	4					

574 [1]: Equal quantities of DNA from 4 or 5 samples from each subgroup were sequenced. The first of the two pools
575 was from samples that generated most FeS; the second from samples that generated slightly less FeS.

Table 3. Variation of SRB in 14 patient pools including 2 subgroups for each of the 7 patient groups

	Healthy		**UC Before**		**UC After**		**IBS Before**		**IBS After**		**CD Before**		**CD After**	
Intensity of FeS in culture	High	High	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low
Barcode Pool No.	1	8	2	9	3	10	4	11	5	12	6	13	7	14
No. of sequence analysed	5,729	3,141	2,204	5,326	5,672	6,003	4,001	5,639	1,593	6,078	2,061	2,290	2,990	2,981
B. wadsworthia (100% identical)	67.7	67.7	70.4	64.6	72.7	68.65	29.3	94.2	39.8	99.4	45.5	39.1	24.5	96.98
D. piger (> 99% identical)	27.25	24.5	24.2	7.1	22.8	4.5	44.7	0	36.1	0	32.2	40.3	52.2	0.1
New species A	0.95	0.1	1.3	17.35	0.7	11.8	24.7	1.9	16.7	0.65	1.4	14.5	0	2.92
New species B	0.6	3.9	4.1	10.55	3.8	1.4	0.2	0	1.3	0	6.5	2.6	0.4	0
New species C	2.75	3.2	0	0	0	0	0.2	0.1	0	0	0	0.1	0	0
D. vulgaris Hildenborough (> 97% identical)	0	0	0	0	0	14.1	0	3.4	0	0	0	0	0.7	0
Desulfovibrio NY682 (> 98% identical)	0.55	0	0	0	0	0	0.0	0	0	0	0	2.6	9.9	0
D. desulfuricans F28-1 (> 98% identical)	0	0	0	0	0	0	0.6	0	6.0	0	0.0	0.6	0	0
New species D	0	0	0	0	0	0	0.0	0	0	0	15	0	12.1	0
Other	0.2	0.6	0	0.4	0	0.04	0.3	0.4	0.1	0	0	0.2	0	0
All SRB %	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

Table 4. Diversity of SRB in individual patients

	Patients with Crohn's disease										**Healthy controls**		
Patient No.	CQ		CD		CG		CH		CM	CP	AD	AE	AM
Sample No.	Before 142	After 149	Before 47	After 73	Before 82	After 88	Before 93	After 127	Before 128	Before 140	37	40	95
Intensity of black FeS in culture	High	High	High	Low	Low	Low	Low	High	High	High	High	High	High
No. of sequences analysed	2,999	2,985	1,238	2,976	3,001	2,987	2,993	2,975	3,000	2,999	3,000	2,998	3,010
B. wadsworthia (100% identical)	27.81	6.47	100	99.66	3.97	100	5.15	10.18	88.3	0	90.77	100	37.77
D. piger (> 99% identical)	70.92	92.13	0	0.34	0	0	94.29	88.84	0	37.91	0	0	62.19
New species A	0	0	0	0	91.2	0	0.13	0	0.9	1.63	3.2	0	0
New species B	0	0	0	0	0	0	0	0	10.8	0	0	0	0
New species C	0	0	0	0	0	0	0.17	0	0	0	5.73	0	0
D. vulgaris Hildenborough (> 97% identical)	0	0	0	0	0	0	0	0	0	0	0	0	0
Desulfovibrio NY682 (> 98% identical)	0	0	0	0	0	0	0	0	0	0	0	0	0
D. desulfuricans F28-1 (> 98% identical)	0	0	0	0	4.83	0	0	0	0	0	0	0	0
New species D	1.27	1.41	0	0	0	0	0.27	0.34	0	60.42	0	0	0.33
Other	0	0	0	0	0	0	0	0.64	0	0.03	0.3	0	0
All SRB %	100	100	100	100	100	100	100	100	100	100	100	100	100

Table 5. Clinical response of patients with Crohn's disease to treatment as measured by the analysis of C-reactive protein (CRP) and the Harvey & Bradshaw Index.

Patient No.	Sample No. Before	Harvey & Bradshaw Index	CRP (mg/l)	Sample No. After	Harvey & Bradshaw Index	CRP (mg/l)
CA	18	9	96	27	7	7
CB	39	5	6	66	0	1
CC	46	7	5	56	3	2
CD	47	7	76	73	0	10
CE	53	6	n/a	61	3	n/a
CF	57	8	n/a	65	2	n/a
CG	82	15	33	88	2	1
CH	93	12	5	127	1	3
CI	117	6	5	123	2	2
CJ	121	4	10	141	0	12
CK	124	8	n/a	138	2	n/a
CL	126	5	n/a	163	5	40
CM	128	4	2	133	0	6
CN	131	8	97	136	2	14
CO	137	3	4	157	3	3
CP	140	11	2	147	3	n/a
CQ	142	8	27	149	2	5
CR	143	4	6	154	1	4
CS	164	9	56	172	2	n/a
CT	166	12	n/a	175	8	n/a