Gastrointestinal and Microbial Responses to Sulfate-Supplemented Drinking Water in Mice

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There is increasing evidence that hydrogen sulfide (H_2S), produced by intestinal sulfate-reducing bacteria (SRB), may be involved in the etiopathogenesis of chronic diseases such as ulcerative colitis and colorectal cancer. The activity of SRB, and thus H_2S production, is likely determined by the availability of sulfur-containing compounds in the intestine. However, little is known about the impact of dietary or inorganic sulfate on intestinal sulfate and SRB-derived H_2S concentrations. In this study, the effects of short-term (7 day) and long-term (1 year) inorganic sulfate supplementation of the drinking water on gastrointestinal (GI) sulfate and H_2S concentrations (and thus activity of resident SRBs), and the density of large intestinal sulfomucin-containing goblet cells, were examined in C3H/HeJBir mice. Additionally, a PCR-denaturing gradient gel electrophoresis (DGGE)-based molecular ecology technique was used to examine the impact of sulfate-amended drinking water on microbial community structure throughout the GI tract. Average H_2S concentrations ranged from 0.1 m*M* (stomach) to 1 m*M* (cecum). A sulfate reduction assay demonstrated *in situ* production of H_2S throughout the GI tract, confirming the presence of SRB. However, H_2S generation and concentrations were greatest in the cecum and colon. Sulfate supplementation of drinking water did not significantly increase intestinal sulfate or H_2S concentrations, suggesting that inorganic sulfate is not an important modulator of intestinal H_2S concentrations, although it altered the bacterial profiles of the stomach and distal colon of 1-year-old mice. This change in colonic bacterial profiles may reflect a corresponding increase in the density of sulfomucin-containing goblet cells in sulfate-supplemented compared with control mice. Exp Biol Med 228:424–433, 2003

Key words: sulfate-reducing bacteria; hydrogen sulfide; gastrointestinal tract; sulfomucins

Sulfate-reducing bacteria (SRB) use sulfate as a terminal electron acceptor in the electron transport chain for the dissimilation of reduced organic substrates such as short-chain fatty acids or molecular hydrogen, produced during colonic fermentation (1). The end product of bacterial sulfate respiration is hydrogen sulfide (H_2S). There is increasing evidence that H_2S, produced by intestinal SRB, may be involved in the etiopathogenesis of chronic diseases such as inflammatory bowel disease (IBD), especially ulcerative colitis (UC) and colorectal cancer (2, 3).

Sulfate-reducing bacteria compete with methane (CH_4)-producing Archaea (methanogens) for hydrogen-reducing equivalents, and to a large extent, SRB and methanogens appear to be competitive in the human gut (4). Perhaps reflecting this ecological interaction, there is evidence that humans tend to predominantly harbor either SRB or methanogens. In a study of 87 healthy human volunteers, three SRB population groupings were recognized: Group I consisted of persons who were strong CH_4 producers and fecal SRB were completely absent (5). In Group 2, methanogenesis occurred and low numbers of SRB (approximtely 10^5/g wet weight feces) were detected, although SRB metabolic activities were negligible. The final group consisted of persons exhibiting high counts of fecal SRB (up to 10^{11}/g wet weight) and complete absence of methanogenesis (5). It has been proposed that sulfate reduction versus methanogenesis may be linked to the availability of sulfate in the large intestine. Dietary supplementation of a large dose of sodium sulfate inhibited methanogenesis and increased the intestinal SRB population in 50% of treated subjects (6). However, methanogenesis recovered after sulfate supplementation was halted, consistent with the idea that methanogen or SRB carriage and activity may be determined by a combination of both genetic and dietary factors.

Intestinal sulfate can be derived either from exogenous sources, namely sulfate in drinking water and dietary foodstuffs (8), or from endogenous sources such as taurocholic acid, chondroitin sulfate, and sulfated mucins (sulfomucin) (8, 9). Much of the sulfate present in the diet is derived from preservatives. Additionally, diets rich in dried fruits, Bras-

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sica vegetables, sausages, or beer contain substantial sulfate concentrations, consumption of which can result in the ingestion of more than 16 m*M* sulfate/day (7). Sulfate is also commonly found in drinking water (10), which may contain up to 20 m*M* of sulfate, when it is derived from private wells (11, 12). Willis and colleagues (13) proposed that 1.5–2.6 m*M* sulfate/day could also be derived from sulfomucins in the human large intestine, which represent concentrations adequate to support SRB growth (13). However, the extent of mucin sulfation varies among animal species and even among individual humans or animals (9, 14). Therefore, it is possible that the extent of host mucin sulfation dictates the carriage of SRB versus methanogens, and that dietary sulfate then influences overall bacterial activity. Consistent with this hypothesis is the observation that SRB concentrations were greatest in intestinal segments that harbored the greatest density of sulfomucins (15). However, little is known about the impact of dietary or drinking water sulfate on intestinal sulfate and SRB-derived H_2S concentrations.

In this study, the effects of short-term (7 day) and long-term (1 year) supplementation of the drinking water with 16.7 m*M* (1600 ppm) inorganic sulfate on intestinal sulfate and H_2S concentrations and thus activity of the SRB population, were examined in C3H/HeJBir mice (16). This sulfate concentration was chosen as it corresponds to the upper concentration range found in drinking water sources (11, 12).

Materials and Methods

Animals and Experimental Design. Colonies of inbred C3H/HeJBir mice were established in the Edward R. Madigan Laboratory Animal Care facility at the University of Illinois from breeding pairs imported from The Jackson Laboratory (Bar Harbor, ME) (17). Mice were weaned at 3 weeks of age to a standard laboratory chow diet (Teklad LM-485 7012; Harlan Teklad, Madison, WI). Mice were individually housed under a 12:12-hr light:dark cycle and were allowed unrestricted access to food and water. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Illinois and complied with the Guide for the Care and Use of Laboratory Animals.

Upon initiation of the sulfate supplementation study, mice were on average 3 weeks of age and did not manifest visible signs of intestinal inflammation (e.g., diarrhea or perianal inflammation). Mice were divided into two experimental groups (nine mice/treatment): one control group and one group receiving sulfate-supplemented drinking water. Inorganic sulfate, as anhydrous sodium sulfate (Sigma Chemical, St. Louis, MO), was dissolved in distilled, deionized drinking water. Mice were weighed weekly and were monitored for signs of inflammation and diarrhea. Neither weight loss nor external signs of inflammation were observed during the course of the study. Drinking behavior also did not differ between the two experimental groups.

Sampling of Gastrointestinal Contents. After 7 days of sulfate supplementation, three mice from both treatment groups were sacrificed by CO_2 asphyxiation. Immediately thereafter, the stomach, proximal, middle, and distal small intestine, cecum, and proximal and distal colon from each mouse were perfused with 2 ml of 0.1 *M* zinc acetate, which traps H_2S as zinc sulfide (ZnS), after which intestinal contents were extruded from each segment, and stored at –80°C. After 1 year of sulfate supplementation, the remaining six mice from both treatment groups were sacrificed by CO_2 asphyxiation. Hydrogen sulfide in the intestine of three mice per treatment group was trapped and stored as described above. For histological analysis, 0.5- to 0.75-cm tissue sections from the cecum and large intestine were taken from the three remaining mice per treatment. Tissue sections were fixed in Carnoy's solution for 2 hr on ice (18). Tissues were then dehydrated in fresh 100% ethanol and were prepared for histochemical analysis. Mucosal and lumenal contents of the intestine of these three mice per treatment were then collected by gently scraping the mucosal surface from each segment with a sterile glass slide for molecular analysis of bacterial composition. Thus, each mucosal sample contained intestinal contents plus mucus with entrapped bacteria. Samples were snap-frozen in liquid nitrogen and were stored at –80°C.

Anion Exchange Chromatographic Analysis of Sulfate Concentrations. Fecal samples with ZnS precipitate were well mixed and then pelleted and the supernatant was taken for sulfate concentration determinations. The supernatant was diluted between 11- or 15-fold and submitted to anion exchange chromatography as described previously (19). Sulfate concentrations were then calculated based on the average wet weight of intestinal contents from each intestinal region.

Microdistillation Analysis of H_2S Concentrations. Hydrogen sulfide concentrations were measured via a cold microdistillation procedure. A similar method was recently developed independently by Richardson *et al.* (20). The trap consists of two glass tubes, one for the test sample, which has an injection port into which 6 *M* HCl is injected to release H_2S. The other tube is used to trap H_2S as anionic sulfide in 1 *M* sodium hydroxide (NaOH) solution. The two tubes are joined by a head unit, which enables H_2S gas evolved to be carried into the trapping solution. The design also allows the introduction of an inert gas directly into the sample tube, which mixes the sample throughout the procedure. One milliliter of well-mixed sample was pipetted into the sample side of the unit. The head unit was then secured in position by sealing the joints with high-vacuum silicone grease and keck clips. Gas lines were attached to each unit and nitrogen was bubbled through the traps. After 5 min, 1 ml of concentrated HCl was added to the sample via the injection port. The units were then left for 15 min. The H_2S concentration was then determined via the methylene blue method of Cline (21). Spiking of the samples with different concentrations of ZnS yielded an average

recovery of 50% (data not shown). Longer incubation times yielded greater average recoveries, similar to those reported by Richardson *et al.* (20). However, we opted for a 15-min incubation period because of practical purposes related to foaming, and H_2S concentrations of the samples were therefore adjusted for a 50% recovery yield.

Determination of Sulfate Reduction Rates. A separate study was conducted with 30-day-old CD-1 mice ($n = 3$) to determine sulfate reduction rates in distinct intestinal compartments. Intestinal contents of the stomach, proximal, middle, and distal small intestine, cecum, and proximal and distal colon were collected and quickly placed in anaerobically sealed incubation tubes containing a deoxygenated solution of 5 m*M* sulfate in phosphate-buffered saline and were weighed. Five microliters of $^{35}S\text{-}SO_4^{2}$ (50 kBq; 1.35 μCi) was then injected in each incubation tube after which samples were incubated for 24 hr at 37°C. At the end of the incubation, 0.1 *M* zinc acetate was added to the sample and was mixed vigorously to trap H_2S and to suppress biological activity. To determine the sulfate reduction rate in each sample, it is essential to separate radiolabeled reduced sulfur compounds from the radiolabeled sulfate injected to start the rate determination. This was done by a combined acid chromium distillation (22) by which sulfur is liberated from the intestinal sample as H_2S, which is then removed by a constant stream of N_2 gas. Volatile H_2S was trapped again in a zinc acetate solution as ZnS, and the radioactivity in that pool was determined using a scintillation counter. The sulfate reduction rate (SRR) was then calculated according to the following equation:

$$SRR = (a/(A + a) \times t) \times (SO_4^{2-}) \times 1.06\ \mu M\ SO_4^{2-} \text{ per day}$$

with a being the radioactivity of the volatile sulfide; A, the radioactivity of SO_4^{2-} after the incubation; t the incubation time (days); (SO_4^{2-}) the 5 m*M* sulfate; and 1.06 is a constant that accounts for the percentage of fractionation between ^{35}S and ^{32}S by SRB (22).

PCR Amplification of 16S rDNA and Denaturing Gradient Gel Electrophoresis (DGGE). Genomic DNA was extracted from mucosal samples using a modified Tsai and Olson protocol (23) described in detail by Simpson *et al.* (24). These genomic DNA preparations were used as templates for PCR amplification of 16S rDNA and subjected to DGGE analysis as described (24). A DGGE ladder was created by mixing a collection of equal concentrations of species-specific 220-bp fragments obtained by PCR amplification of the 16S rDNA V3 region using the primers 341F and 534R (25). PCR template DNA was extracted from anaerobically grown pure cultures of *Bacteroides thetaiotaomicron* (VPI 5482), *B. fragilis* (VPI 2553), *Ruminococcus albus* strains 7 and 8 (laboratory collection), *Streptococcus bovis* (laboratory collection), *Desulfovibrio vulgaris* (ATCC 29579), *D. desulfuricans* (NCIB 8307), *Bilophila wadsworthia* (ATCC 49260), *Escherichia coli* K-12 NM522 (ATCC 47000), *Clostridium parvum* (laboratory collection), and *Clostridium perfringens* (laboratory collection).

To analyze the resulting DGGE gel, the Diversity Database version 2.1 of "The Discovery Series" (Bio-Rad, Hercules, CA) was first used to determine the migration distances and intensity of individual bands within each gel. This yields a linear plot of selected gel lanes and allows the detection of bands that are common among different samples. The resulting data were then used to analyze banding patterns by applying Ward's algorithm as described previously (26) to develop dendograms of the different intestinal segments of control and sulfate-supplemented mice.

Cloning and Sequencing of 16S V3 rDNA PCR Amplicons. PCR products were excised from DGGE gels, reamplified, cloned in One Shot Competent *E. coli* using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). White colonies of ampicillin-resistant transformants were transferred to 5 ml of ampicillin-containing Luria-Bertani broth and grown overnight. Plasmid DNA was extracted by alkaline lysis as described previously (27). Plasmid DNA was digested by the restriction enzyme *EcoR*I and analyzed by electrophoresis in a 1.5% agarose gel to verify the insert size. Clones were then sequenced directly using an automated sequencing system (Applied Biosystems, Foster City, CA) at the W.M. Keck Center for Comparative and Functional Genomics (University of Illinois Biotechnology Center, Urbana, IL). Sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) family of programs to search for and align with similar nucleotide sequences in GenBank (www.ncbi.nlm.nih.gov/BLAST/).

Sulfomucin-Containing Goblet Cell Analysis. Tissues were stained for goblet cells, and specifically, sulfomucin-containing goblet cells as described previously (15). Briefly, tissues were trimmed, placed in fresh 100% ethanol for 10 min, cleared in xylene for 10 min, and placed in fresh xylene for 10 min. After clearing, tissues were embedded in paraffin and cut in 2-μm-thick sections for mucin histochemistry. For sulfomucin-containing goblet cell analysis, frosted microscope slides (Fisher Scientific, Pittsburgh, PA) supporting intestinal tissue sections from the cecum and large intestine were deparaffinized, incubated in double-distilled water for 5 min and stained for 16 hr in a high iron diamine (HID) solution (28). After HID staining, tissues were washed in running tap water for 5 min and stained with alcian blue (pH 2.5) for 5 min (28). After washing the tissues in tap water for 2–3 min, tissues were dehydrated in 95% ethanol for 5 min, dehydrated in 100% ethanol for 5 min, cleared in xylene for 5 min, and mounted with Permount (Fisher Scientific) on 1.5-mm-thick coverslips. For total goblet cell counts, tissues were treated essentially as described above, but were stained with periodic acid Schiff's reagent-alcian blue instead, as described (28). Tissues were analyzed using a Optiphot-2 microscope (Nikon, Melville, NY), and images were digitally captured using the Image-Pro Plus program, version 3.0 (Media Cybernetics, Silver Spring, MD). Results are expressed as the number of sulfomucin-containing goblet cells per millimeter squared epithelium. A minimum of three images per

intestinal segment per mouse were analyzed for both control and sulfate-supplemented mice.

Statistics. Statistical analysis of intestinal sulfate and H_2S concentrations, sulfate reduction rates, 16S V3 rDNA amplicon, and goblet cell numbers were performed using the General Linear Model Procedure of SAS (version 6.09; The SAS Institute, Cary, NC). The least significant difference test was used to make comparisons within treatments of the effects of sulfate supplementation on sulfate and H_2S concentrations with an assigned $P \leq 0.05$.

Results

Effect of Sulfate-Supplemented Drinking Water on Intestinal Sulfate Concentrations. Because intestinal sulfate concentrations for one of the sulfate-supplemented mice were consistently 1.5- to 7-fold greater (except the stomach) than the average of this treatment group, this animal was omitted for statistical analysis. Overall, sulfate concentrations ranged from 0.9 to 15.6 μmol sulfate/g wet weight with greatest sulfate concentrations in the middle and distal small intestine (Fig. 1). However, the relative increase in sulfate concentration in the small intestine may reflect the 2- to 2.5-fold lower average wet weight of the recovered intestinal contents compared with other intestinal segments. Sulfate concentrations throughout the GI tract of control and sulfate-supplemented mice were similar for both treatment periods, although measurable, but nonsignificant increases in sulfate concentrations of 1-year-old sulfate-supplemented mice were observed in the middle and distal small intestine, cecum, and distal colon compared with controls (Fig. 1).

Effect of Sulfate-Supplemented Drinking Water on Intestinal H_2S Concentrations. Supplementation of the drinking water with 16.7 m*M* sulfate did not significantly alter total H_2S concentrations in any intestinal segment of sulfate-supplemented mice (Fig. 2). Hydrogen sulfide concentrations in the stomach and large intestine were, however, greater in 1-year-old mice than in 30-day-old mice (data not shown), independent of treatment ($P < 0.05$). Except for the proximal small intestine of control 1-year-old mice, H_2S was detected in every GI segment. Hydrogen sulfide concentrations ranged from 0 to 0.1 μmol H_2S/g wet weight in the stomach and small intestine (Fig. 2). The greatest H_2S concentrations were found in the cecum ($P < 0.05$), averaging 1 μmol H_2S/g wet weight. Hydrogen sulfide concentrations were on average 0.18 and 0.29 μmol H_2S/g wet weight in the proximal and distal colon of control mice and, respectively, 0.25 and 0.37 μmol H_2S/g wet weight in sulfate-supplemented mice (Fig. 2). Overall, H_2S concentrations were 2-fold (cecum) to 100-fold (middle small intestine) lower than intestinal sulfate concentrations (Figs. 1 and 2).

Sulfate Reduction Rates in the Mouse Gastrointestinal Tract. In a pilot study with CD-1 mice, sulfate reduction rates were determined using a radiotracer (^{35}S-SO_4^{2}) technique as described in "Materials and Methods." Sulfate reduction rates in the cecum were significantly greater compared with other intestinal segments (Fig. 3). On average, 160 nmol H_2S was generated per gram of wet weight per day in the cecum. Sulfate reduction rates in the colon were on average 45 nmol H_2S per gram wet weight per day, but were not significantly greater than sulfate reduction rates in the stomach and small intestine. The luminal and mucosal content of the stomach generated on average 25 nmol H_2S per gram wet weight per day, similar to rates found in the proximal and distal small intestine. The

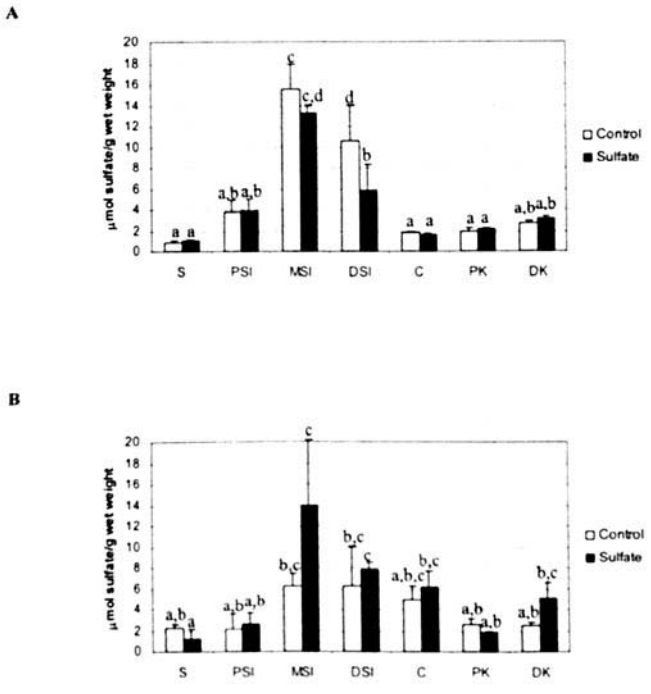


Figure 1. Sulfate concentrations in the stomach (S), proximal (PSI), middle (MSI), and distal (DSI) small intestine, cecum (C), and proximal (PK) and distal colon (DK) of postweaning control mice ($n = 3$) and mice offered 16.7 m*M* sulfate-supplemented drinking water for respectively 7 (A; $n = 3$) and 365 (B; $n = 3$ for control and $n = 2$ for sulfate-supplemented mice) days. Values are mean ± SEM. Columns not sharing a common letter are significantly different ($P < 0.05$).

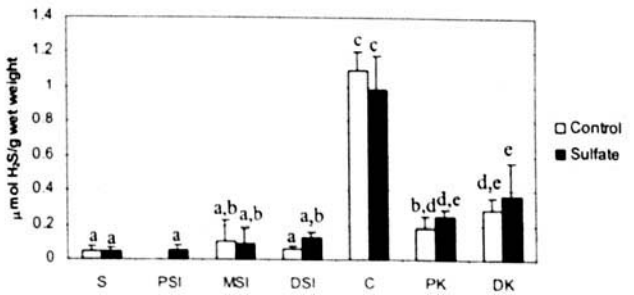


Figure 2. Hydrogen sulfide concentrations in the stomach (S), proximal (PSI), middle (MSI), and distal (DSI) small intestine, cecum (C), and proximal (PK) distal colon (DK) of postweaning control mice ($n = 3$) and mice ($n = 3$) offered 16.7 m*M* sulfate-supplemented drinking water for 1 year. Values are mean ± SEM. Columns not sharing a common letter are significantly different ($P < 0.05$).

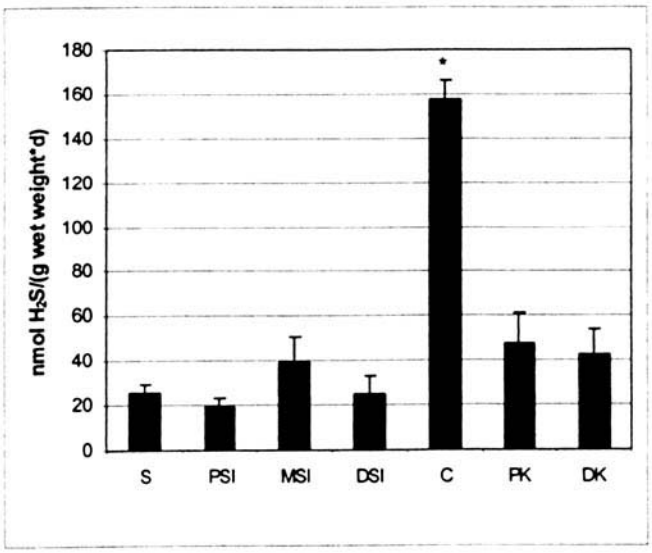


Figure 3. Hydrogen sulfide production rates by luminal and mucosal contents of the stomach (S), proximal (PSI), middle (MSI), and distal (DSI) small intestine, cecum (C), and the proximal (PK) and distal colon (DK) of 30-day-old CD-1 mice. All values are means ± SEM. *$P < 0.05$.

sulfate reduction rate in the middle small intestine was numerically greater than other small intestinal segments, generating on average 40 nmol H_2S per gram wet weight per day, although this difference was not significant. Because H_2S concentrations did not differ in control and sulfate-supplemented mice, sulfate reduction rates were not analyzed for the experimental C3H/HeJBir mice.

16S rDNA PCR-DGGE and Sequence Analysis of the Effect of Sulfate Supplementation on the Gastrointestinal Microbiota. To investigate the effect of sulfate supplementation on intestinal bacterial profiles, total genomic DNA was isolated from the stomach, proximal, middle, and distal small intestine, cecum, and proximal and distal colon of 1-year-old control and sulfate-supplemented mice, and was used as template for PCR amplification of the V3 region of 16S rDNA followed by DGGE analysis. Representative DGGE banding patterns are presented in Figure 4. The number of DGGE bands in any individual lane ranged from 15 to 31. A greater number of bands was detected in large intestinal (cecum, proximal, and distal colon; 28 ± 1) than in the stomach and small intestinal samples (16 ± 2) for both control and sulfate-supplemented mice ($P < 0.05$; Fig. 5). Band numbers were similar between intestinal samples from control compared with sulfate-supplemented mice.

Because it is difficult to interpret bacterial community profiles with respect to both the number and relative diversity of distinct assemblages by visual inspection of the gel, DGGE patterns were subjected to cluster analysis using Ward's algorithm (26). Bacterial profiles clustered according to GI segment (Fig. 6). Distinct treatment clusters were only observed for the stomach and distal colon (Fig. 6). This result demonstrates that sulfate supplementation superimposed an additional level of bacterial species variation on animal-to-animal differences only in these two GI segments.

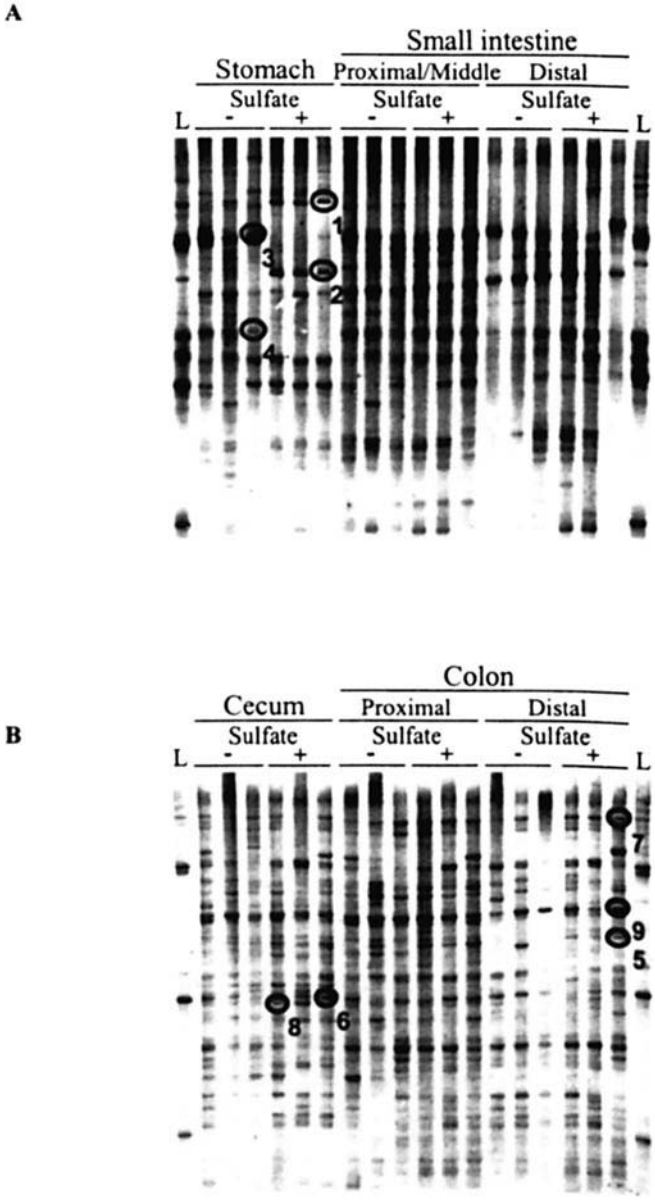


Figure 4. (A) DGGE profiles generated from PCR amplified V3-16S rDNA from the stomach, proximal and middle, and distal small intestine (A), cecum, and proximal and distal colon (B) of postweaning control mice ($n = 3$) and mice offered 16.7 m*M* sulfate-supplemented drinking water for 1 year ($n = 3$) as described in "Materials and Methods." The ladder (L) contains from top to bottom: *Bacteroides thetaiotaomicron, B. fragilis, Ruminococcus albus* strains 7 and 8, *Streptococcus bovis, Desulfovibrio vulgaris, D. desulfuricans, Bilophila wadsworthia, Escherichia coli* K-12 NM522, *Clostridium parvum,* and *Clostridium perfringens.* 1 through 9, sequenced amplicons (see text).

To identify bacterial species that were distinguished by treatment, 16S rDNA amplicons (V3 region) were cloned, sequenced, and analyzed using the BLAST family of programs to search for similar nucleotide sequences in GenBank. Amplicons 1 and 2 were cloned because of their unique presence in the stomach of sulfate-supplemented compared with control mice (Fig. 4A). Sequence analysis

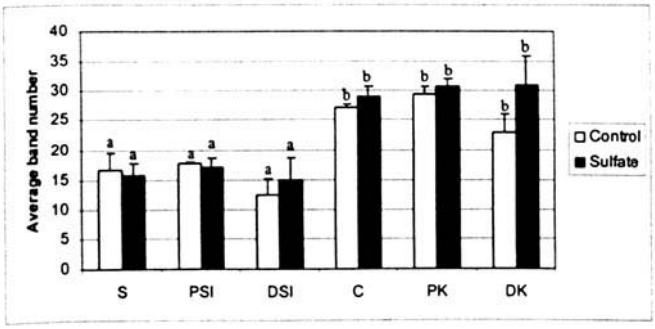


Figure 5. Average number of DGGE amplicons from the stomach (S), proximal and middle (PSI), and distal (DSI) small intestine, cecum (C), and proximal (PK) and distal (DK) colon of control mice (n = 3) and mice offered 16.7 m*M* sulfate-supplemented drinking water for 1 year (n = 3). Values are mean ± SEM. Columns not sharing a common letter are significantly different ($P < 0.05$).

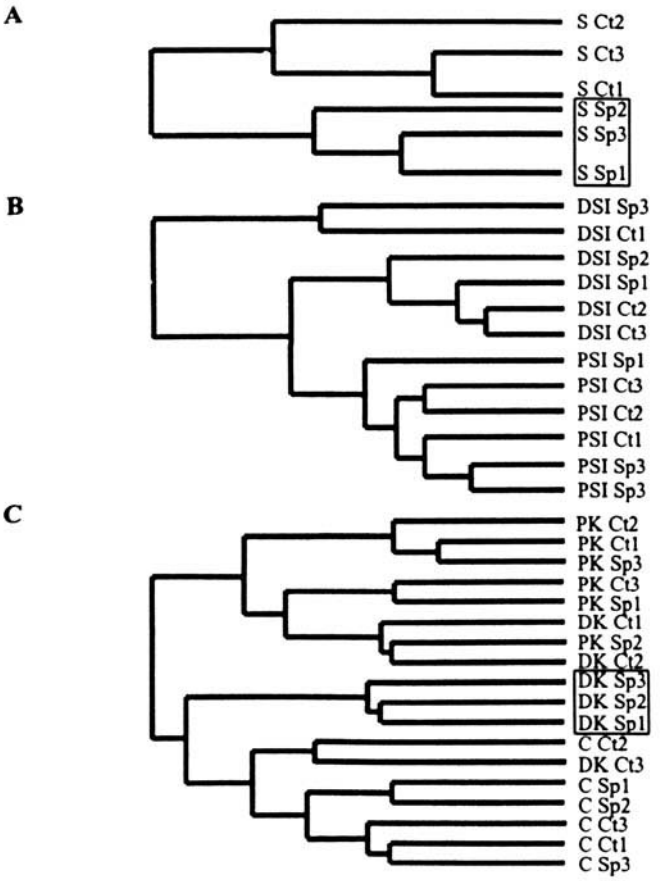
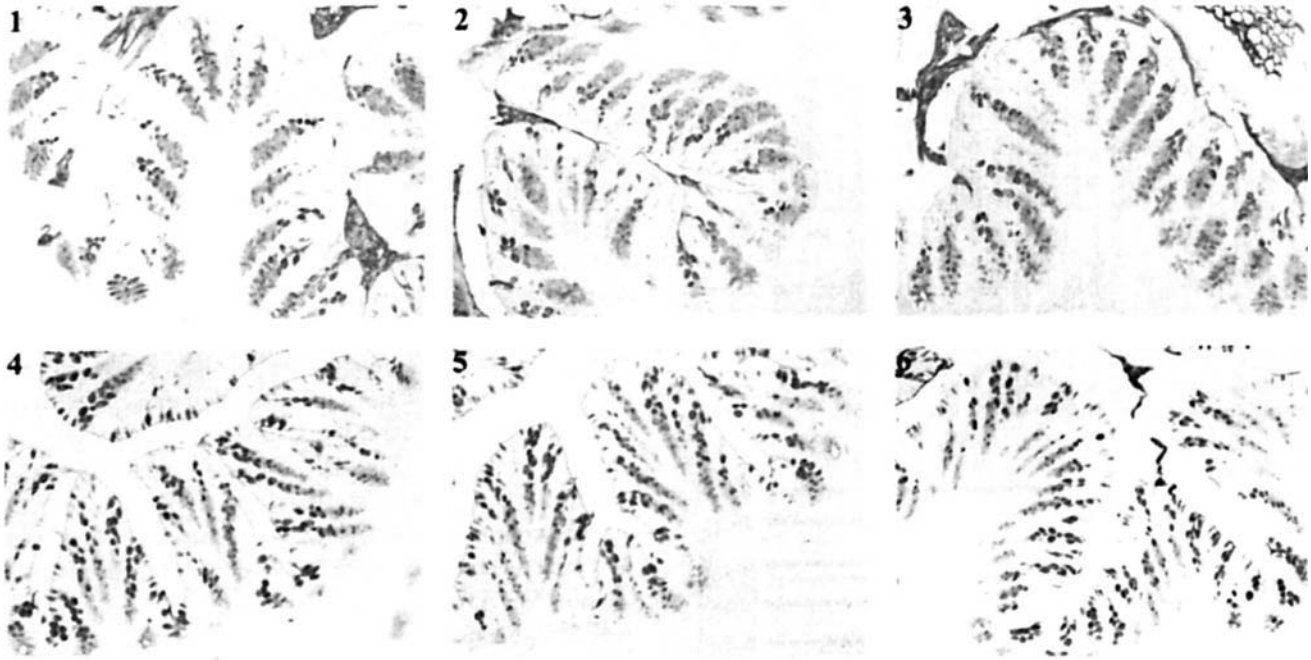


Figure 6. Dendrograms of 16S V3 rDNA PCR-DGGE banding patterns from (A) the stomach (S), (B) proximal (PSI) and distal (DSI) small intestine, (C) cecum (C), and proximal (PK) and distal (DK) colon from control (Ct) and sulfate-supplemented (Sp) mice. The dendrograms were constructed using Ward's algorithm and the Diversity Database software. Distances are measured in arbitrary units. Rectangles indicate treatment clusters.

revealed that Clone 1 was most closely related (95%) to a recently discovered large taxon of mostly uncultured, low G-C, gram-positive mouse intestinal bacteria (GenBank accession No. AJ400266), whereas Clone 2 showed closest homology (98%) to *Lactobacillus murinus*. In contrast, Amplicons 3 and 4 were sequenced because of their dominant presence in the stomach of control, but not sulfate-supplemented mice (Fig. 4A), and were tentatively identified as *Bacteroides putredinis* (97%) and *Lactobacillus gasseri* (98%), respectively. Amplicon 5 was cloned because of its unique presence in the large intestine of sulfate-supplemented compared with control mice (Fig. 4B). Sequence analysis revealed that this 16S rDNA-V3 clone was identical (100%) to an uncultured bacterium from the pig intestine, grouped within the *Clostridium coccoides* cluster (29). Several large intestinal amplicons (6–8) were also observed whose presence was unique because of sulfate supplementation or whose intensities were greater in bacterial profiles from sulfate-supplemented compared with control mice, although the differences were not as pronounced as for Amplicon 5 (Fig. 4B). Sequence analysis revealed that Clone 6 was most closely related (99%) to members of a new taxon of mouse intestinal bacteria harboring mostly uncultured, low G-C, gram-positive bacteria (GenBank accession No. AJ400239), similar to Amplicon 1 of the stomach. Amplicon 7 was also most closely related (99%) to *Lactobacillus murinus*. Amplicon 8 was similar (98%) to the same cluster of species as Clone 5. Amplicon 9 was sequenced because of its presence throughout the mouse GI tract, and was most closely related (95%) to *C. leptum*. The molecular ecological analysis did not reveal SRB that were distinguished by treatment. However, studies with SRB-specific 16S rDNA probes would be required to conclude that intestinal SRB populations are not modulated by inorganic sulfate in drinking water.

Effect of Sulfate-Supplemented Drinking Water on the Density of Sulfomucin-Containing Goblet Cells in the Cecum and Colon. To compare the effect of sulfate supplementation on the density of sulfomucins, tissue sections of the cecum and proximal and distal colon from control and sulfate-supplemented mice were stained for both total and sulfomucin-containing goblet cells using conventional histochemical techniques as described in "Materials and Methods." There were no differences in the density of total goblet cells between the cecum, proximal and distal colon, or between treatments. However, the density of sulfomucin-containing cells was signficantly greater in the distal colon compared to the cecum and proximal colon (Fig. 7), consistent with previous observations (15). As also evidenced by visual inspection of histological samples (Fig. 7A), sulfate supplementation significantly increased the density of sulfomucin-containing goblet cells in the cecum and distal colon compared with control mice, whereas differences were not observed in the proximal colon (Fig. 7B). The density of sulfomucin-containing goblet cells in the cecum, proximal, and distal colon of control (versus sulfate-supplemented) mice was 793 (1153), 377 (312), and 1757 (2874) per mm2 epithelium, respectively.

A



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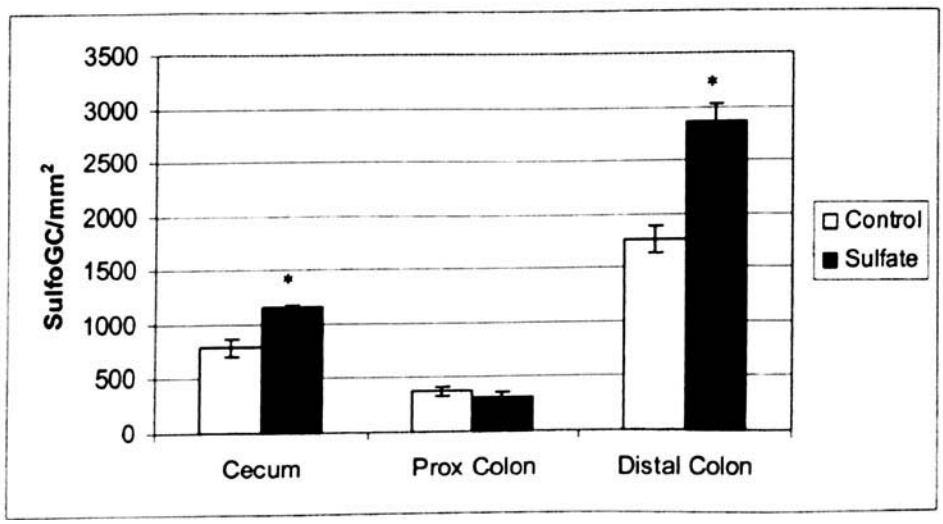

Figure 7. (A) Representative photomicrographs of distal colon sections of 1 year-old control (1–3) and sulfate-supplemented (4–6) C3H/HeJBir mice stained with high iron diamine/alcian blue (pH 2.5) to detect sulfated mucins (brown stain; 200x). (B) Bar graph of the number of sulfomucin-containing goblet cells (SulfoGC) per millimeter-squared epithelium in the cecum and colon of control and sulfate-supplemented mice. Values are mean ± SEM of a minimum of three images per intestinal segment per mouse ($n = 3$). The asterisk represents a significant difference relative to the respective control value ($P < 0.05$).

Discussion

The data presented demonstrate that H_2S is present throughout the gastrointestinal tract of adult mice. Hydrogen sulfide concentrations were greatest in the cecum and colon, which corresponds to the observation that H_2S-producing SRB are most abundant in these mouse intestinal segments (15). Hydrogen sulfide concentrations averaged 1 m*M* in the cecum, and, respectively, 0.2 and 0.4 m*M* in the proximal and distal colon. Although H_2S concentrations throughout the gastrointestinal tract have to our knowledge not been determined previously, the reported colonic con-

centrations fall within a previously described range of human fecal H_2S concentrations, varying between 0.29 and 3.4 m*M* H_2S (30–32). However, cecal concentrations were about 10-fold lower than the H_2S concentrations observed in the ceca of rats, orally treated with bismuth subnitrate, which avidly binds sulfide (33). This result may indicate that the zinc acetate perfusion method used in this study and designed to minimize H_2S loss may still not have prevented the loss of a significant proportion of volatile H_2S, suggesting that mouse intestinal H_2S concentrations are likely even greater. Results from the sulfate reduction rate analyses also confirmed the earlier observation of wide-spread SRB presence throughout the mouse gastrointestinal tract (15). The greatest sulfate reduction activity was observed in the cecum, consistent with the detection of greatest H_2S concentrations in this intestinal segment. However, the observed sulfate reduction rates are an order of magnitude lower than fecal H_2S release rates reported by Levitt and colleagues (33). This apparent discrepancy may be simply explained by the fact that inorganic sulfate reduction constitutes only one way by which H_2S can be generated in the intestine. In fact, these results suggest that H_2S generated from inorganic sulfate reduction may represent only a relatively minor fraction of total intestinal H_2S, with H_2S generated from sulfur amino acid dissimilation likely constituting the major fraction (32).

Mice were subjected to 16.7 m*M* sulfate-supplemented drinking water on both a short-term (7 day) and long-term basis (1 year), after which GI sulfate and H_2S concentrations of control and sulfate-supplemented mice were analyzed. Colonic sulfate concentrations were on average 2-fold greater than previously reported human fecal sulfate concentrations (30, 32, 34). This difference may reflect bacterial use of sulfate in the colon for sulfate reduction or assimilation purposes, which would likely reduce fecal sulfate output (30). Distal small intestinal concentrations were in a similar range as sulfate concentrations observed in effluent of ileostomy patients (30). However, sulfate supplementation did not significantly alter H_2S concentrations in any GI segment, as a likely consequence of the failure of sulfate supplementation to significantly increase intestinal sulfate concentrations. Together, these findings suggest that inorganic sulfate provided via the drinking water is not a determining factor in SRB-associated H_2S production in the mouse GI tract. In contrast, dietary intake of sulfur amino acids was correlated significantly with human fecal H_2S concentrations (32), which may indicate that overall protein consumption may be more important than inorganic sulfate intake in the modulation of intestinal H_2S concentrations.

The present data are consistent with sulfate provided via the drinking water being rapidly absorbed in the upper GI tract, agreeing with results from a study in which radiolabeled sulfate was administered to rats and monitored during a subsequent 24-hr period (35). ^{35}S-Radioactivity was measurable in plasma after 15 min and reached a peak after about 1.5–2 hr. The total radioactivity found in the plasma after 2 hr indicated a rapid and almost complete absorption of inorganic sulfate in amounts up to 5 m*M* (35), which exceeds the daily sulfate supplementation in this study by several orders. Additionally, earlier reports demonstrated similar absorptive capacities by the upper GI tract in rats and dogs (36–38), and similar findings were more recently reported in humans (30, 39). However, the sulfate absorptive capacity in the upper GI tract is not infinite because ingestion of single doses of 30 m*M* sodium sulfate or greater resulted in severe diarrhea (39, 40), implying that a threshold for dietary sulfate intake exists. A study of sulfate absorption in ileostomy patients defined this threshold as approximately 7 m*M*/day, above which a significant amount of sulfate spills into the colonic sulfate pool (30). Obviously, this plateau was not reached in this study, and the direct contribution of supplemented sulfate to the sulfate pool in distal parts of the intestine was therefore likely insignificant.

Phylogenetic analysis revealed that sulfate supplementation superimposed a level of bacterial species variation on animal-to-animal differences, as bacterial community profiles from the stomach and distal colon of sulfate-supplemented mice clustered tightly compared with those from control mice. This observation indicates that, although sulfate supplementation did not alter stomach sulfate concentrations, it did have a direct impact on bacterial profiles in the stomach. Sulfate can be used by bacteria to synthesize the sulfur amino acids methionine and cysteine when environmental cysteine and methionine concentrations are low (41), or can be used as an electron acceptor and may thus, based on the present data, provide a selective advantage to certain bacteria in the stomach. Specifically, the observed enrichment of uncultured, low G-C, gram-positive bacteria in both the stomach and distal colon of sulfate-supplemented mice may indicate that these bacteria have an affinity for sulfate, which may prove valuable for future cultivation attempts.

The clustering of bacterial profiles from the distal colon of sulfate-treated mice is as intriguing, and may indicate that the measurable increase in sulfate concentration in this segment of sulfate-supplemented mice is physiologically relevant. As indicated above, sodium sulfate ingestion rapidly increases the serum sulfate concentration (42). Whereas a significant portion of sulfate is subsequently excreted in the urine (40), both *in vivo* and *in vitro* observations also demonstrate a correlation between increased serum sulfate concentrations and extent of mucin sulfation (43–45). Sulfomucins are synthesized by specialized goblet cells and are most prevalent in the distal parts of the intestine, particularly the distal colon (15, 46). Lane *et al.* (47) observed that 1 hr after the administration of radiolabeled sulfur (^{35}S) in the form of sodium sulfate to rats, ^{35}S-sulfate was confined exclusively to goblet cells of colonic crypts, indicating that supplemented sulfate was used almost solely for the synthesis of sulfated glycoproteins. Results from the current study corroborate those findings, as a significant increase in

the density of sulfomucin-containing goblet cells was observed in both the cecum and distal colon of sulfate-supplemented compared with control mice, indicating increased sulfation of large intestinal mucins and thus increased sulfate availability. This phenomenon may explain the measurable increase in sulfate concentrations in the distal colon given the significant contribution of sulfomucin-containing goblet cells to the mucin pool in this segment, and may account for the tight clustering of bacterial community profiles in the distal colon of sulfate-supplemented versus control mice. Specifically, the unique presence of a bacterial species in the colon of sulfate-supplemented mice tentatively identified as *C. coccoides* may indicate that this bacterial group has an affinity for sulfomucins. This would be consistent with recent studies demonstrating the ability of *C. septicum* and *C. perfringens* to grow on sulfated carbohydrates (48, 49).

In summary, the present study complements previous findings regarding SRB ecology in the mouse GI tract (15). Hydrogen sulfide generation and concentrations were greatest in the mouse cecum and colon, consistent with the finding that SRB are most abundant in these intestinal segments. Average H_2S concentrations ranged between 0.1 m*M* (stomach) and 1 m*M* (cecum), the latter being well above the H_2S toxicity limit of 50 μM. The sulfate reduction assay also demonstrated *in situ* production of H_2S in the stomach and small intestine, confirming the presence of SRB in these intestinal regions. Supplementation of inorganic sulfate in the upper concentration range found in drinking water did not significantly increase intestinal sulfate or H_2S concentrations. Therefore, these findings suggest that sulfate is readily absorbed, and that exogenous inorganic sulfate is not an important modulator of intestinal H_2S concentrations. Accordingly, it becomes important to determine the contributions of dietary and endogenous sources of sulfate to intestinal SRB activity as large intestinal H_2S concentrations appear to be well in excess of toxic levels for eukaryotic cells, and may thus represent an epithelial insult. Consistent with this hypothesis is the observation of increased intestinal H_2S concentrations in patients suffering from active UC or colorectal cancer (2, 3).

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