APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1988, p. 2750–2755
0099-2240/88/112750-06$02.00/0

Vol. 54, No. 11

# Use of a Three-Stage Continuous Culture System To Study the Effect of Mucin on Dissimilatory Sulfate Reduction and Methanogenesis by Mixed Populations of Human Gut Bacteria

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Received 10 May 1988/Accepted 5 August 1988

**A mixed culture of human fecal bacteria was grown for 120 days in a three-stage continuous culture system. To reproduce some of the nutritional and pH characteristics of the large gut, each vessel had a different operating volume (0.3, 0.5, and 0.8 liter) and pH (6.0, 6.5, and 7.0). A mixture of polysaccharides and proteins was used as carbon and nitrogen sources. Measurements of $H_2$, $CH_4$, $S^{2-}$, sulfate reduction rates, sulfate-reducing bacteria (SRB), and volatile fatty acids were made throughout the experiment. After 48 days of running, porcine gastric mucin (5.8 g/day) was independently fed to vessel 1 of the multichamber system. The mucin was extensively degraded as evidenced by the stimulation of volatile fatty acid production. In the absence of mucin, sulfate-reducing activity was comparatively insignificant and methanogenesis was the major route for the disposal of electrons. The reverse occurred upon the addition of mucin; sulfate reduction predominated and methanogenesis was completely inhibited. This was attributed to release of sulfate from the mucin which enabled SRB to outcompete methanogenic bacteria for $H_2$. SRB stimulated by mucin were acetate-utilizing *Desulfobacter* spp., lactate- and $H_2$-utilizing *Desulfovibrio* spp., and propionate-utilizing *Desulfobulbus* spp. When the mucin pump was switched off, the multichamber system reverted to a state close to its original equilibrium. These data provide further evidence that sulfated polysaccharides such as mucin may be a source of sulfate for SRB in the human large gut.**

The major sources of carbon and energy for bacteria growing in the human large intestine are probably the polysaccharides that occur in plant cell walls, starch, meat (mucopolysaccharides), and endogenously produced substrates such as small intestinal secretions and shed epithelial cells (32).

In the large gut, these substances are fermented by populations of anaerobic bacteria, with the formation of a wide range of fermentation products of which acetate, propionate, and butyrate predominate (9). Hydrogen and $CO_2$ are the major gaseous products, but $CH_4$ is also produced in some individuals (37; G. R. Gibson, G. T. Macfarlane, and J. H. Cummings, J. Appl. Bacteriol., in press).

In many natural environments, methanogenesis and dissimilatory sulfate reduction are the principal terminal oxidative processes in the anaerobic degradation of organic matter (5, 6, 17, 18,. 26). It is well documented that under conditions of sulfate availability, sulfate-reducing bacteria (SRB) are able to outcompete methanogenic bacteria (MB) for $H_2$ (8, 20, 22, 30). Recent work in our laboratory has shown that only a minority of individuals (ca. 30%) in the United Kingdom carry significant numbers of MB in their colons and that SRB occur in the remaining 70% of the population (Gibson et al., in press). These data together with physiological studies (G. R. Gibson, J. H. Cummings, and G. T. Macfarlane, J. Appl. Bacteriol., in press) showed that SRB and MB were to a large extent mutually exclusive in the large gut and that SRB played an important role in $H_2$ metabolism in non-methane-producing individuals. These studies also demonstrated that SRB were able to outcompete MB for $H_2$ when sulfate-reducing and methanogenic fecal slurries were mixed, indicating that sufficient sulfate was present in feces to support the growth of SRB.

Sulfate availability is a significant factor controlling the growth of SRB, but few data are available concerning the levels of this metabolite in the human colon. Potential sources of sulfate may be either dietary or endogenous in origin. Our investigations (Gibson et al., in press) have indicated that the sulfated mucopolysaccharides, chondroitin sulfate and mucin, which are components of intestinal epithelial tissue (14, 31) can potentially support sulfate-reducing activity, in the colon. In this paper, we report the results of further studies on the effect of mucin on the activities of intestinal SRB and MB grown in a three-chamber continuous system.

## MATERIALS AND METHODS

**MCS.** A three-stage continuous culture system was constructed as shown in Fig. 1. Temperature (37°C) and pH were controlled as described by Macfarlane and Englyst (23). The pHs of cultures in vessels 1, 2, and 3 were 6.0, 6.5, and 7.0, respectively. Medium was added to vessel 1 by pump 1 and either distilled water or mucin (porcine gastric mucin, type II; Sigma Chemical Co., St. Louis, Mo.) was added (5.8 g/day) by pump 2. The culture medium contained (grams per liter): pectin, 0.6; xylan, 0.6; arabinogalactan, 0.6; amylopectin, 0.6; Lintner starch, 5.0; casein, 3.0; peptone water, 3.0; $K_2HPO_4$, 2.0; $NaHCO_3$, 0.2; NaCl, 4.5; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.45; cysteine, 0.4; $FeSO_4 \cdot 7H_2O$, 0.005; hemin, 0.01; bile salts, 0.05. Tween 80 (2 ml) and a vitamin solution (1 ml) were also added. The vitamin solution was filter sterilized separately and contained the following (milligrams per liter): menadione, 1; biotin, 2; vitamin $B_{12}$, 0.5; pantothenate, 10; nicotinamide, 5; *para*-aminobenzoic acid, 5; thiamine, 4. Individual vessels of

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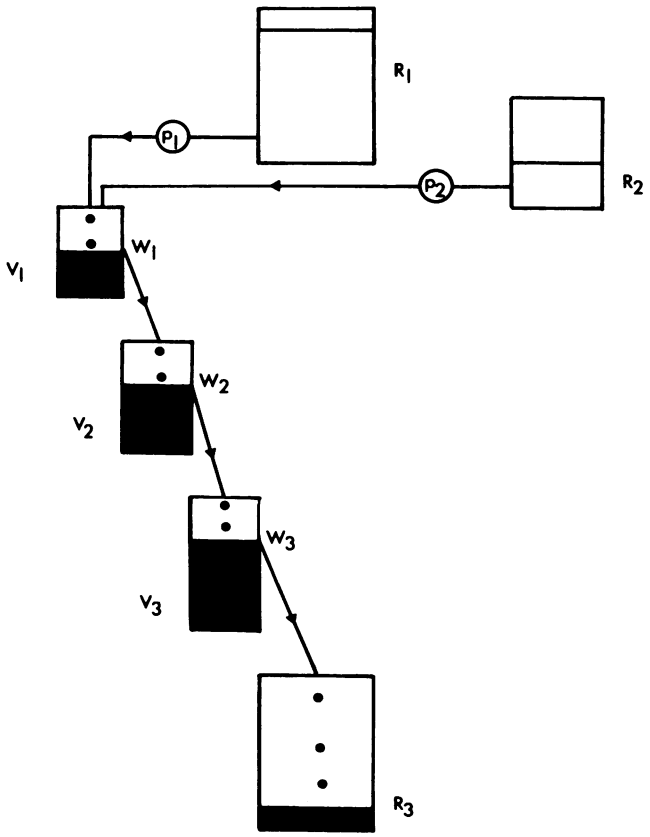


FIG. 1. Multichamber continuous culture system. Medium from reservoir R1 was fed to vessel 1 (V1) by pump 1 (P1). Distilled water or mucin from reservoir 2 (R2) was supplied by pump 2 (P2). Vessel 1 fed vessels 2 and 3 (V2 and V3) and the waste collection vessel (R3) sequentially by a series of angled glass tubes (W1, W2, W3). Vessels 1, 2, and 3 had respective operating volumes of 0.3, 0.5, and 0.8 liter with dilution rates of 0.08, 0.048, and 0.034/h. Total retention time in the system was 62.7 h.

the multichamber system (MCS) were not sparged with anaerobic gas, but each vessel was maintained under an atmosphere of oxygen-free nitrogen. The MCS was inoculated by adding 100 ml of a 20% (wt/vol) fecal slurry to each vessel. The slurry was prepared with anaerobic sodium phosphate buffer (0.1 mol/liter, pH 7.2). Feces used to inoculate the system were obtained from a putatively methanogenic individual. Samples were removed from each vessel of the MCS at regular intervals for analysis of $H_2$, $CH_4$, $S^{2-}$ production, $^{35}SO_4^{2-}$ reduction rates, organic acids, and enumerations of viable SRB populations. After 48 days of incubation, mucin was added to the system and this continued for a further 22 days when the mucin was again replaced by distilled water. One hundred and twenty days from the addition of the fecal inoculum, the experiment was terminated.

**Analysis of gases.** Samples (20 ml) of culture were removed from the MCS and placed into sterile 25-ml serum bottles. After 6 h of incubation at 37°C, headspace gas samples (1 ml) were removed from the bottles and $H_2$ and $CH_4$ were measured by gas chromatography (2a).

**Measurement of sulfide levels.** Sulfides were determined in spent media from the MCS by the colorimetric method of Cline (7) after precipitation of $S^{2-}$ in 10% (wt/vol) zinc acetate solution.

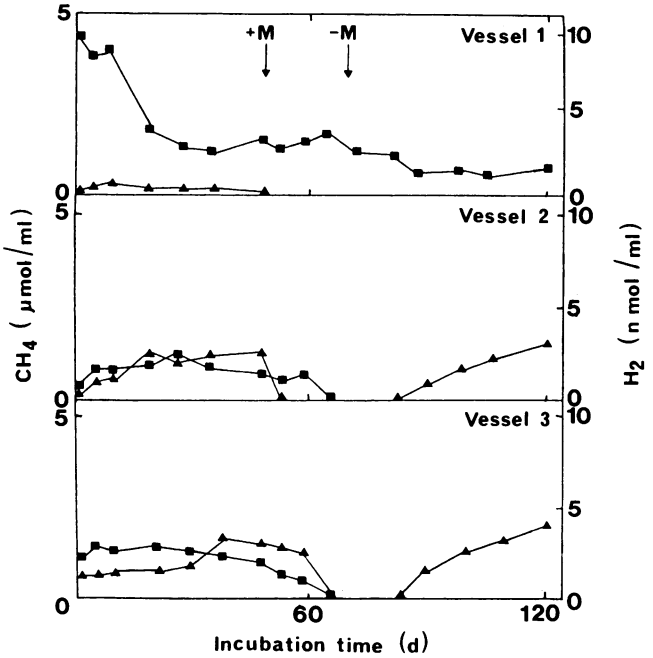


FIG. 2. Effect of mucin (m) on the production of $CH_4$ and $H_2$ by bacteria growing in the MCS. Symbols: ▲, $CH_4$; ■, $H_2$.

**Determination of sulfate reduction rates.** The $^{35}SO_4^{2-}$ radiotracer method of Jørgensen (16) was used to measure sulfate reduction rates in 5-ml subsamples of culture media from the MCS. The method measured the incorporation of $^{35}S^{2-}$ into acid-volatile sulfide but not pyrite sulfide.

**Enumeration of viable SRB populations.** Viable SRB were enumerated by the agar shake dilution method (27). Media, conditions of cultivation, and identification of SRB were those described by Gibson et al. (in press). Acetate, lactate, propionate, butyrate, and $H_2$-$CO_2$ were used as electron donors since these are the major substrates for the growth of human intestinal SRB (Gibson et al., in press).

**Measurement of organic acids.** Samples of culture (5 ml) were removed from the MCS, rapidly frozen with methanol-dry $CO_2$, and stored for later analysis. Acetate, propionate, butyrate, succinate, and lactate were extracted and measured by gas chromatographic procedures (13).

**Chemicals.** All chemicals and reagents were obtained from Sigma except for casein (BDH, Poole, England), Lintner starch (BDH), bile salts (Oxoid Ltd., London, England), peptone water (Oxoid), and $^{35}SO_4^{2-}$ (Amersham Corp., Arlington Heights, Ill.).

## RESULTS

**Gas production in MCS.** Measurements of $CH_4$ and $H_2$ production during the first 48 days of the experiment showed that little methane was produced by bacteria in vessel 1 of the MCS (Fig. 2). Methane production rates were approximately fivefold higher in vessels 2 and 3, indicating that MB preferred the less acidic conditions and lower dilution rates in these vessels. Methanogenesis was strongly inhibited when mucin, which contained approximately 3% (wt/vol) sulfate (12), was added to the system (days 48 to 70). During this time, $H_2$ still accumulated in samples removed from vessel 1 but did not occur in samples from vessels 2 and 3. When the mucin addition was stopped, methanogenesis recovered in vessels 2 and 3 but not in vessel 1.

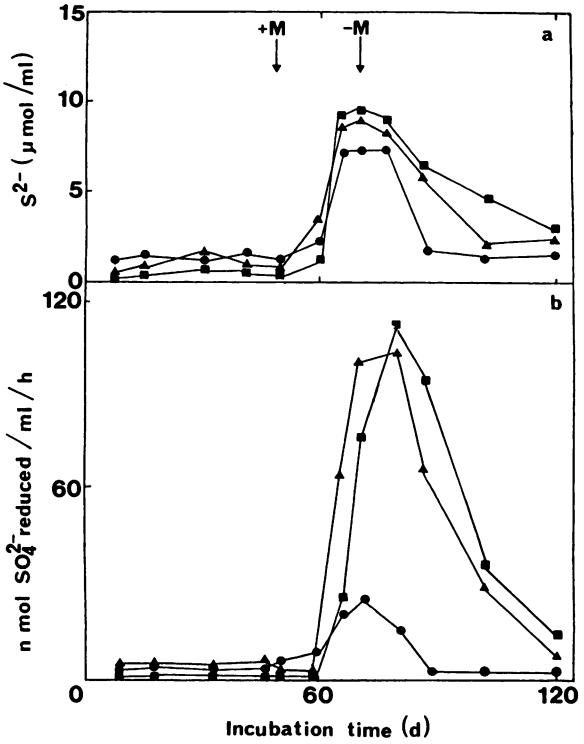


FIG. 3. Sulfide concentrations (a) and sulfate reduction rates (b) in the MCS in the presence and absence of mucin (m). Symbols: ●, vessel 1; ▲, vessel 2; ■, vessel 3.

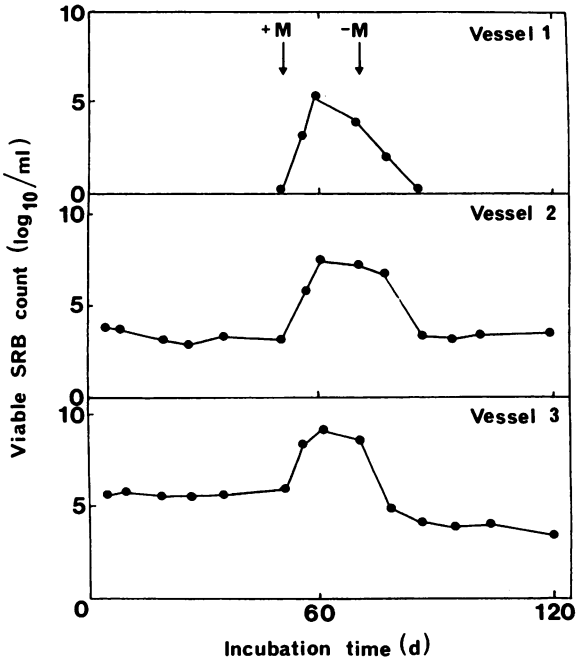


FIG. 4. Enumeration of SRB production in the MCS. Results are presented as sum of viable counts obtained with acetate, lactate, propionate, and $H_2$ as electron donors. M, Mucin; d, days.

**Sulfide levels and sulfate reduction rates in MCS.** Low levels of $S^{2-}$ (ca. 2 μmol/ml) were detected in each vessel of the MCS prior to the addition of mucin (Fig. 3a). When mucin was fed to the system, however, $S^{2-}$ production was stimulated in all vessels but most markedly in vessel 3. After distilled water replaced mucin, $S^{2-}$ concentrations in the MCS slowly decreased. Sulfate reduction rates correlated well with those of $S^{2-}$ formation (Fig. 3b). Relatively low activities (2 to 8 nmol of $SO_4^{2-}$ reduced per ml per h) were present before the addition of mucin; however, rates in vessels 2 and 3 increased 100-fold when mucin was added to the system, but a considerably smaller and more transient increase in sulfate reduction rates was detected in vessel 1.

**SRB populations in MCS.** Bacteriological studies showed that SRB populations increased substantially when mucin was fed to the MCS (Fig. 4). SRB were never detected in vessel 1 unless mucin was available. However, relatively low numbers (ca. $10^3$ to $10^6$/ml) were recorded in vessels 2 and 3 in the absence of mucin. The highest cell population densities of SRB were always found in vessel 3, irrespective of whether mucin was present. To identify the SRB growing in the MCS, individual colonies from the highest-dilution agar shakes were purified and identified by criteria described by Gibson et al. (in press). Lactate-utilizing *Desulfovibrio* spp. were always numerically predominant in the MCS (Table 1). However, during the period when mucin was fed to the system, the relative proportions of acetate-oxidizing *Desulfobacter* spp. and propionate-oxidizing *Desulfobulbus* spp. increased. SRB able to grow solely on $H_2$-$CO_2$ constituted only a small proportion of the total viable SRB count (5 to 13%), while butyrate oxidizers were never detected in the system.

**Organic acid production.** Baseline measurements of fermentation acids in the MCS showed that volatile fatty acid

TABLE 1. Effect of mucin on the percent distribution of SRB in the MCS[a]

| Substrate used for enumeration of SRB | SRB isolated | % Total SRB metabolizing each substrate | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Vessel 1 | | | Vessel 2 | | | Vessel 3 | | |
| | | No Mucin | Mucin | No Mucin | No Mucin | Mucin | No Mucin | No Mucin | Mucin | No Mucin |
| Acetate | *Desulfobacter* spp. | 0 | 26 ± 2 | 0 | 17 ± 6 | 24 ± 1 | 42 ± 3 | 25 ± 1 | 30 ± 3 | 47 ± 1 |
| Propionate | *Desulfobulbus* spp. | 0 | 32 ± 1 | 0 | 19 ± 6 | 22 ± 0 | 0 | 25 ± 2 | 28 ± 2 | 0 |
| Lactate | *Desulfovibrio* spp. | 0 | 42 ± 0 | 0 | 65 ± 10 | 41 ± 0 | 58 ± 3 | 39 ± 5 | 37 ± 0 | 53 ± 2 |
| Butyrate | NA[b] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $H_2$-$CO_2$ | *Desulfovibrio* spp. | 0 | 0 | 0 | 0 | 13 ± 0 | 0 | 11 ± 4 | 5 ± 4 | 0 |

[a] The MCS was run for 48 days without mucin. Over this period, SRB were continuously enumerated. Results presented from this initial period are the mean ± standard error of the mean (SEM) from counts made on days 9, 22, and 48. Mucin was added to the system from days 48 to 70. SRB counts given for this period are the mean ± SEM from days 55, 59, and 65. The addition of mucin was stopped on day 50, and the system was operated for a further 50 days without this substrate. SRB counts here are those from days 82, 92, and 120 (mean ± SEM).

[b] NA, Not applicable.

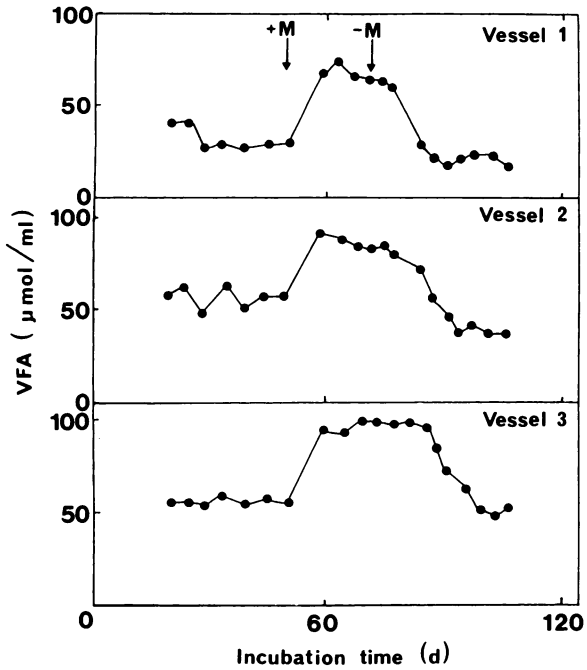


FIG. 5. Effect of mucin (m) on VFA production by bacteria in the MCS.

(VFA) production was maximal in the first two vessels of the system and that fermentation was stimulated by mucin (Fig. 5). The proportion of acetate produced always increased from vessels 1 to 3 regardless of whether mucin was present (Table 2). However, when mucin was added to the MCS, acetate formation was strongly stimulated with proportionate decreases in propionate and, to a lesser extent, butyrate production. When mucin was removed from the MCS, VFA molar ratios returned to values which approximated original premucin steady-state levels. Throughout the experiment, concentrations of the fermentation intermediates lactate and succinate never exceeded 1 µmol/ml in any vessel (data not shown).

## DISCUSSION

The large intestine of humans is essentially an unbranched tube into which material containing fermentable substrates enters from the small bowel. The digesta are mixed and retained for a period in the cecum, after which portions are ejected and pass from the right to the left colon for storage in the sigmoid colon and eventual excretion (35).

The rationale for using a three-vessel system to study the activities of the gut microflora in vitro arises from observations which have shown that the environments in the right and left colon are different. The cecum and right colon are comparatively nutrient rich and acidic, owing to strong fermentation, whereas the left colon is usually more nutrient depleted and its pH is neutral or slightly alkaline (9). Conditions in the large gut result, in a large part, from the activities of the microflora, but these activities are in turn influenced by the architecture and physiology of the gut.

In an attempt to introduce elements of heterogeneity characteristic of the gut fermentation to studies in vitro with respect to spatial, temporal, nutritional, and pH factors, we developed a three-stage continuous culture system. The advantage of the system was that in vessel 1, bacterial activities could be examined under high nutrient, fast growth, and low pH conditions similar to those in the cecum, while in vessels 2 and 3 the processes could be investigated in a more nutrient-depleted, slow growth, and neutral pH environment similar to that in the transverse and left colon.

In the absence of mucin, the polysaccharides starch, xylan, pectin, arabinogalactan, and amylopectin provided the sole sources of carbohydrate. Starch was included as the major polysaccharide, since recent investigations have indicated that considerably more of this carbohydrate reaches the large gut compared with other, nonstarch polysaccharides (10, 11).

The feces used to inoculate the MCS were obtained from a methane-producing individual. Attempts to detect SRB in these feces were unsuccessful (minimum level of detection, $10^2$/g). However, this person was unusual in that SRB could be enriched by adding sulfate (20 mmol/liter) to the feces in vitro (data not shown).

When the MCS was operated in the absence of mucin, low levels of sulfate-reducing activity were recorded and methanogenesis was the major route of electron disposal. However, the mucin had profound effects on the activities of SRB and MB. Methanogenesis was completely inhibited, whereas dissimilatory sulfate reduction was stimulated. We attribute this effect to release of sulfate from the polysaccharide since the mucin preparation did not contain free sulfate or substances inhibitory to MB (G. R. Gibson, unpublished data). Mucins are highly sulfated acidic mucopolysaccharides (12, 14, 32), and it has been shown that they are synergistically degraded by certain components of the gut microflora (15, 29). In this study, porcine gastric mucin was extensively degraded in the MCS as evidenced by the elevated levels of VFA production (Fig. 5). The increase in $S^{2-}$ production and sulfate reduction rates indicates that sulfate released by the breakdown of mucin stimulated SRB activity. As a consequence, MB were metabolically displaced as the major $H_2$-utilizing species, but did not completely wash out of the MCS.

In sedimentary ecosystems that contain sufficient sulfate, SRB are able to outcompete MB for mutual substrates such as $H_2$ and acetate (1, 2, 36). Hydrogen is essential for the

TABLE 2. Effect of mucin on VFA molar ratios in the MCS[a]

| Vessel | No Mucin | | | Mucin | | | No Mucin | | |
|---|---|---|---|---|---|---|---|---|---|
| | Acetate | Propionate | Butyrate | Acetate | Propionate | Butyrate | Acetate | Propionate | Butyrate |
| 1 | 64 ± 1 | 27 ± 2 | 9 ± 2 | 74 ± 3 | 14 ± 2 | 12 ± 1 | 64 ± 0 | 25 ± 2 | 11 ± 2 |
| 2 | 68 ± 0 | 23 ± 1 | 9 ± 1 | 78 ± 2 | 14 ± 1 | 8 ± 0 | 68 ± 1 | 22 ± 2 | 10 ± 1 |
| 3 | 72 ± 0 | 21 ± 0 | 7 ± 0 | 84 ± 5 | 9 ± 3 | 7 ± 2 | 72 ± 1 | 20 ± 1 | 8 ± 1 |

[a] See Table 2, footnote *a*, for details of system operation. Results of VFA analysis from the first nonmucin period are the mean of data ± SEM from samples taken on days 40, 44, and 48. VFA molar ratios from the mucin period are the mean of results ± SEM from days 61, 63, and 70. Results from the postmucin period are mean ± SEM from days 90, 95, and 100.

growth of colonic methanogens (24, 25), and while many types of SRB (3, 4, 33, 34) can utilize $H_2$ as an electron donor, SRB, unlike intestinal MB, do not have an obligate requirement for this gas.

Different types of SRB, especially *Desulfovibrio* spp., were stimulated during the fermentation of mucin (Fig. 4; Table 1), suggesting that these bacteria were the major $H_2$-scavenging species. Before mucin was added to the MCS, low counts of SRB were recorded in vessels 2 and 3. The presence of 2 mmol of sulfate per liter in the growth medium was therefore sufficient to maintain these bacteria in the system, but was not sufficient to facilitate competitive inhibition of MB.

*Desulfovibrio* spp. were always the numerically predominant SRB in the MCS (Table 1), demonstrating that, as in the large gut (Gibson et al, in press), these bacteria were the most successful SRB in competing for sulfate. The enhanced ability of *Desulfovibrio* spp. to compete for sulfate has been demonstrated by Laanbroek et al. (21), who showed that pure cultures of these bacteria outcompeted *Desulfobulbus* spp. and *Desulfobacter* spp. for limiting amounts of sulfate. When mucin was added to the MCS, the relative proportions of *Desulfobulbus* spp. and *Desulfobacter* spp. increased, presumably as a result of more efficient competition for sulfate, as the electron acceptor became more available.

Clearly, a number of factors other than sulfate availability would be expected to influence the growth of SRB in the MCS, for example, pH and dilution rate. The highest numbers and activities of SRB were always found in vessel 3. This is not too surprising since studies on SRB in other environments have shown that their growth is favored by high pH, low dilution rates, and high concentrations of organic acids (19, 27). The results obtained in this study suggest that growth of SRB in the human large gut would be optimal in the prevailing conditions of pH occurring in the left colon.

The effect of sulfate-reducing activity on the health of the colon is an area of potential interest. Hydrogen sulfide is a major product of SRB metabolism and is extremely toxic (28). In some individuals, the capacity for $H_2S$ formation must be considerable, since their feces can contain very high numbers of SRB; indeed, viable counts in excess of $10^{10}$/g (dry weight) are frequently recorded (Gibson et al., in press). This may be of significance with respect to the etiology of some intestinal disorders. A number of colonic diseases such as diverticular disease, ulcerative colitis, and bowel cancer favor the left colon, and it is tempting to speculate whether SRB may be involved.

This is clearly an area of some challenge, and studies to determine possible clinical effects of SRB in the large gut are now under way in our laboratory.

## ACKNOWLEDGMENT

This work was supported by a grant from Johnson Wax, Racine, Wis.

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