Effect of pH on Dissimilatory Sulfate Reduction by Porcine Cecal Microflora

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Abstract The pH optima of dissimilatory sulfate reduction by porcine cecal microflora were determined. Digesta were sampled from a cecally fistulated pig and washed bacterial cell suspensions were prepared. They were incubated in anaerobic culture tubes under nitrogen atmosphere with 20 mM sulfate and an electron donor for 12 h at 37°C. Either hydrogen (100% in head space gas) or lactate (50 mM) was used as the electron donor. The initial culture pH was adjusted to 14 levels from 4.5 to 7.4. Hydrogen sulfide concentration in the head space gas was determined at the end of incubation by FPD (flame photometric detector)-gas chromatography after addition of 6N HCl to the culture. Organic acids were determined by ion exclusion HPLC. The pH optimum for hydrogen sulfide production with hydrogen was as low as 6.0 and those with lactate placed between 6.1 and 6.4. The pH optimum for lactate consumption was slightly lower than that for hydrogen sulfide production, while that for acetate production was close to that for hydrogen sulfide production.

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Dissimilatory sulfate reduction is one of the major electron disposals in the large intestine of human and pig^{1,15)}. Although sulfate reduction is an important metabolic process for anaerobic microflora, providing an electron sink for fermentative hydrogen producers, the resultant hydrogen sulfide is toxic¹²⁾ and a relationship with colonic ulcerative colitis has been suggested⁶⁾. Accordingly the importance of controlling hydrogen sulfide production in the large intestine has been stressed⁷⁾.

Colonic sulfate reduction was shown to increase when turnover of digesta was low under neutral to weakly alkaline conditions in a multiple vessel-continuous culture experiment⁵⁾. The results appeared to be consistent with those reviewed by Pfennig *et al.*¹¹⁾ who indicated that sulfate reducing bacteria (SRB)

grew well under slightly alkaline conditions (pH 7.0-7.8). However, research on sudden death victims suggested that colonic sulfate reduction was most active in the ascending and transverse colons⁹⁾ where the usual digesta pH ranges from 5.4 to 6.2 due to short chain fatty acid production²⁾. The effect of pH on dissimilatory sulfate reduction is still controversial⁴⁾. Tuttle *et al.*¹⁴⁾ reported that sulfate reduction occurred even in a highly acidic environment (pH 3.0), although the strains of SRB isolated, belonging to the genera *Desulfovibrio* and *Desulfotomaculum*, could not reduce sulfate when pH was below 5.5. The effect of milieu pH on sulfate reduction is further complicated by the electron donors. Thus hydrogen gas oxidation with sulfate anion requires a proton, whereas oxidative

decarboxylation of lactate coupled with sulfate reduction releases a proton (see below).

The present experiment was therefore conducted to determine the pH optima of sulfate reduction with two major electron donors, hydrogen and lactate, in the hind gut of pig.

Materials and Methods

A cecally-fistulated, Large White $\times$ Landrace cross-bred female pig (BW = 110 kg) was used as a donor of cecal digesta. The pig was fed 1,000 g commercial concentrate free from antibiotics (Ultrabreed 72, Kumiai Shiryō Co., Kobe, Japan) and 500 g alfalfa (*Medicago sativa*) meal twice a day (09 : 00 and 17 : 00). The diet contained a relatively large amount of lucerne meal to stimulate hind gut fermentation. Water and a mineral block were always available. Digesta (ca. 200 ml) were sampled 2 h after the morning feed and squeezed through a nylon mesh of 250 μ m pore. Subsamples of the filtrate were centrifuged in nitrogen-filled tubes at 25,000 $\times$ g for 10 min at 4°C. Each resultant pellet was washed twice with one of following buffers: 0.05 M MES (2-(*N*-morpholino) ethanesulfonic acid) at pH 5.2 and 6.0 and 0.05 M HEPES (*N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid) at pH 7.2. One centrifuge tube was allotted to each of the three buffers. Bacterial pellets were finally suspended in the same washing buffers of which the volumes were twice as much as original filtrate volume so that bacterial concentration was adjusted to be half that of the original digesta. A portion (1 ml) of washed cell suspension was introduced into an anaerobic culture tube (Aluminum seal type; Bellco Biotechnology, Vineland, NJ, USA) in which was placed 5 ml of buffer of different pH containing 20 mM (final concentration) of sodium sulfate and 50 mM (final concentration) of lithium lactate. The following three buffers with 14 different pHs were used: 0.05 M citrate buffer (pH 4.5, 5.0, 5.2, 5.5), 0.05 M MES (pH 5.2, 5.5, 6.0, 6.2, 6.4, 6.6), and 0.05 M HEPES (pH 6.8, 7.0, 7.2,

7.4). Washed cell suspensions prepared with MES (pH 5.2), MES (pH 6.0) and HEPES (pH 7.2) were introduced into the tubes with respectively citrate, MES, and HEPES buffer. The buffers were boiled and gassed with O₂-free nitrogen before use. Three culture tubes were allotted for each buffer (3 $\times$ 14 = 52 tubes in total) and one of the three tubes was used for the measurement of the pH at zero time using a card type pH meter (C-1, Horiba). Culture tubes were filled with O₂-free nitrogen and closed with butyl rubber septa and aluminum caps. When hydrogen was used as the electron donor, lithium lactate was omitted and nitrogen was replaced by hydrogen. Tubes were incubated at 37°C for 12 h and activity was terminated by the introduction of 1 ml of 6 N HCl. Incubation continued for another 30 min at 37°C, then a portion (0.5 ml) of head space gas was sampled by a gas-tight syringe. The gas was analyzed for acid volatile sulfur compounds (AVS) using a gas chromatograph (GC-14 B, Shimadzu) fitted with a flame photometric detector. AVS were separated by DB-23 capillary column (0.25 mm $\times$ 25 m, J & W Scientific, Folsom, CA, USA) at 80°C with nitrogen as a carrier gas. Split injection (1/100) was done at 200°C. Quantification of hydrogen sulfide was performed using a C-R6 A integrator (Shimadzu). This followed the official analytical method for environmental samples established by the Environment Agency of the Japanese government¹⁰⁾ except for the GC conditions.

The concentrations of organic acids in the 12 h culture were determined by ion exclusion HPLC¹⁶⁾. Briefly, organic acids were separated by two tandem Waters Organic acid columns (30 cm $\times$ 2, Nihon Waters, Osaka) at 80°C with an isocratic elution of *p*-toluene sulfonic acid aqueous solution.

The results suggested that day to day variation was insignificant; accordingly the results from three incubations in three weeks were combined.

Results and Discussion

Figures 1 and 2 show the pH dependence of dissimilatory sulfate reduction in pig cecal flora for, respectively, the two electron donors, hydrogen gas and lactate. Hydrogen sulfide production was observed between pH 5.5 and 7.4 with both electron donors. This pH range

Fig. 1. Hydrogen sulfide production (ppm) by pig caecal flora incubated *in vitro* with hydrogen. ■ Citrate buffer (pH 4.5-5.5) ● MES buffer (pH 5.2-6.6) ▲ HEPES buffer (pH 6.8-7.4)

Fig. 2. Hydrogen sulfide production (ppm) by pig caecal flora incubated *in vitro* with lactate. ■ Citrate buffer (pH 4.5-5.5) ● MES buffer (pH 5.2-6.6) ▲ HEPES buffer (pH 6.8-7.4)

corresponds to the growth pH of colonic SRB, mainly *Desulfovibrio* spp.⁶⁾. The pH optimum for dissimilatory sulfate reduction with hydrogen gas (around pH 6.0) was slightly acidic than that with lactate (pH 6.1-6.4). Such a small difference was somewhat unexpected, because the equations of dissimilatory sulfate reduction suggest that proton concentration might inversely affect hydrogen sulfide production with hydrogen and lactate. According to Thauer *et al.*¹³⁾, following equations can be proposed for the dissimilatory sulfate reduction with hydrogen or lactate ;

Equations (1) and (2) suggest that dissimilatory sulfate reduction with hydrogen gas may proceed more efficiently under acidic conditions and vice versa for that with lactate. As the mixed cecal population was used in this experiment, the competition and cooperation among various bacteria may complicate the situation making the pH effect on sulfate reduction less clear than expected. However apparently the pH optima of dissimilatory sulfate reduction were acidic, between pH 6.0 and pH 6.4, at least for the two major electron donors in the pig cecal microflora. Apparent lactate consumption during 12-h incubation is shown in Fig. 3. Maximal lactate consumption was recorded at pH 5.7 (Fig. 3), slightly lower than the pH at which maximal hydrogen sulfide production with lactate was recorded (pH 6.2, Fig. 2). Since apparent acetate production (Fig. 3) showed a similar pH dependence profile to that of sulfide production (Fig. 2), acetate was therefore suggested to be produced mainly from lactate by sulfate reducers in this experiment.

The pH optima of sulfate reduction in pig cecal digesta may be placed around pH 6.2 that are similar to those observed in human large intestine⁹⁾. The pH of cecal digesta of the

Fig. 3. Acetate and propionate production and lactate consumption (mM) by pig caecal flora incubated *in vitro* with lactate.

Apparent production of acetate (●) and propionate (○), apparent consumption of lactate (▲) in the lactate-culture of pig caecal microflora are shown.

Buffers and pH, see text.

experimental pig was 6.6 that was higher than pH optima of sulfate reduction. Colonic digesta pH (6.8 to 7.2) was higher than that in cecum (6.7) when pigs were fed only presently used concentrate (Ushida and Ohashi, unpublished). Therefore sulfate reduction seems not to proceed at the maximum efficiency under such condition. However, the use of probiotics such as live *Lactobacilli* and prebiotics such as oligosaccharide becomes common in the pig industry as evidenced by commercialization of probiotics- or prebiotics-containing concentrates. Since the production of lactate provides a major electron donor to the colonic sulfate reducers, a dietary supplementation of indigestible oligosaccharides might be expected to cause hydrogen sulfide production in the colon. Such oligosaccharides are considered to be bifidogenic³⁾ because they promote accumulation of lactic acid in the large intestine⁸⁾. A concomitant decrease in pH has been observed after the ingestion of indigestible oligosaccharides⁸⁾. If digesta pH is decreased

lower than 5.5 due to lactate accumulation, sulfate reduction will be substantially inhibited. If digesta pH ranges around 6.2, sulfate reduction will be stimulated. This point requires further elucidation.

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ブタ盲腸内硫酸還元の pH 依存性

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異化型硫酸還元の結果, 大腸で発生する硫化水素は生体にとって有害であり, 発生を制御する必要があるが, 大腸における硫酸還元菌の生理や生態はあまり知られていない. この実験では, 大腸で起こる異化型硫酸還元の pH 依存性について知ることを目的とした. ブタ盲腸液より調整した細菌懸濁液を pH 4.5 から 7.4 の範囲で 14 段階に調整した緩衝液中で 12 時間培養し, 発生した硫化水素を定量した. このとき硫酸イオンと電子供与体 (乳酸あるいは水素) は十分量供給した. 至適 pH は乳酸基質の場合 6.1~6.4 で, 水素基質の場合の 6.0 よりやや高かった.

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