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# Chemical analyses of reservoir fluids from cattle waste, and identification of sulfate-reducing bacteria in the reservoir

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## Summary

Reservoir fluids were obtained from a sewage reservoir once every month from August to October, 1992. The chemical analyses of the reservoir fluids, and colony counts of anaerobic acid-producing bacteria and sulfate-reducing bacteria of the fluid were examined. We identified six strains of sulfate-reducing bacteria in the fluids. The fluids were slightly alkaline pH (8.7-9.4). The respective concentrations of sulfide, ammonium nitrogen, and total volatile fatty acids were 140-240 mg, 2,500-3,300 mg, and 22.0-71.2 mM, per liter of the fluids. The respective numbers of acids-producing and sulfate-reducing bacteria were abut $10^6$ and about $10^3$, per mℓ of the fluids. Six strains were identified as *Desulfovibrio vulgaris*.

## Introduction

In recent years, unpleasant odors generated by domestic animal wastes have become a main environmental pollutant, due to urbanization of rural communities and rural development. It is known that one of the malodorous compounds in animal wastewater treatment is hydrogen sulfide. Sulfate-reducing bacteria grow by sulfate respiration with hydrogen sulfide as a major end-product in anaerobic digestion of the waste[5),7),8)].

It seems important to clarify the distribution and role of sulfate-reducing bacteria in order to control production of hydrogen sulfide in the process of animal wastewater treatment.

This report contains the results of chemical analyses of the reservoir fluids from cattle waste and colony counts of acid-producing and sulfate-reducing bacteria in the fluids. Presumptive identification of isolates from the fluids are also presented.

## Materials and Methods

### Sampling of reservoir fluids

Reservoir fluid was obtained from a sewage reservoir in which 55 dairy cattle wastes were accumulated and stored. The samples were taken once a month at

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the Research Farm of Rakuno Gakuen University from August to October, 1992. The fluid was poured to fill a container to the top, and the container was tightly closed immediately. The chemical analyses of the fluids, and colony count of anaerobic acid-producing and sulfate-reducing bacteria of the fluid were examined after the container was transported to the laboratory.

**Chemical analyses**

The pH was determined by a glass electrode pH meter (Model M-11, Horiba Co.). Ammonium nitrogen was measured by semimicro-Kjeldahl procedure[1]. Sulfides were analyzed according to the method of Shiga[3].

The fatty acids were analyzed by a gas chromatograph {GC (Hitachi 163 gas chromatograph, Hitachi, Ltd.)} equipped with a flame inonization detector with $N_2$ as the carrier gas, by injecting deproteinized samples with 24% (w/v) meta-phosphoric acid in 5N-$H_2SO_4$ as described by Suto *et al.*[4]. The GC conditions were as follows. The volatile fatty acids were analyzed on a packed glass column (0.3 cm × 200 cm) with Chromosorb W/AW (60/80 mesh) coated with 20% ethylene glycol adipate and 2% (w/w) $H_3PO_4$. Column and inlet temperatures were 160°C and 190°C, respectively. Non-volatile fatty acids were analyzed on a packed glass column (0.3 cm × 200 cm) with Chromosorb W/AW (60/80 mesh) coated with 10% (w/w) FFAP. The column and inlet temperatures were 140°C and 190°C, respectively.

**Bacterial counts**

Anaerobic culture techniques and inoculum dilution methods were identical to those described previously[6].

Two media[7] were used for the enumeration of acid-producing bacteria. PY medium contained (per liter): salt solution I, 75 mℓ; salt solution II, 75 mℓ; trypticase peptone (BBL), 10 g; yeast extract (Difco) 5 g; resazurin-Na (0.1% solution), 1 mℓ; agar, 15 g; glucose, 0.25 g; cellobiose, 0.25 g; maltose, 0.25 g; soluble starch, 0.25 g; cystein-HCl-$H_2O$, 0.3 g; $Na_2CO_3$ (8% solution), 2.5 mℓ; pH 7.0-7.2. The composition of PYS medium was identical to that of PY medium except for the addition of supernatant of autoclaved sewage reservoir fluid[6),7] (300 mℓ/ℓ). For the enumeration of sulfate-reducing bacteria, a medium (Table 1) with lactate as the electron donor was used. Oxygen-free mixed gas (90% $N_2$ and 10% $CO_2$) was bubbled into the vessels containing each of these three media.

After the container was transported to the laboratory, another portion of the reservoir fluid was diluted by the one-tenth serial dilution technique with the anaeobic dilution solution[7] under a stream of $O_2$-free mixed gas (90% $N_2$ and $CO_2$). For the colony counts, the diluted fluids were inoculated into the media under a stream of $O_2$-free mixed gas (90% $N_2$ and $CO_2$). Then the roll tubes were formed. The number of colonies in the roll tubes was counted after 10 days incubation at 37°C. Black colonies appearing in the tubes were counted as those of sulfate-reducing bacteria.

**Identification of sulfate-reducing bacteria**

The bacteria were picked up from well-isolated colonies and transferred to the slants of the medium for subculture, as shown in Table 1. The ingredient of

**Table 1.** Compostion of media (g or mℓ/ℓ)

| | Enumeration | Subculture | Utility of substrate |
|---|---|---|---|
| $KH_2PO_4$ | 0.5 | 0.5 | 0.5 |
| $NH_4Cl$ | 1.0 | 1.0 | 1.0 |
| $CaCl_2 \cdot 2H_2O$ | 1.0 | 1.0 | 0.1 |
| Sodium lactate | 0.1 | 0.1 | 1.92[3)] |
| Yeast extract | 1.0 | 1.0 | 1.0 |
| Vitamin solution[1)] | 1.0 | 1.0 | 1.0 |
| Trace element solution[2)] | 10.0 | 10.0 | 10.0 |
| 0.1% Resazurin-Na | 1.0 | 1.0 | 1.0 |
| Agar | 20.0 | 20.0 | — |
| Sodium thioglycolate | 0.1 | — | 0.1 |
| Ascorbic acid | 0.1 | — | 0.1 |
| Cystein-$HCl \cdot H_2O$ | — | 0.5 | — |
| $Na_2S_2O_4$ | 0.025 | — | 0.025[4)] |
| $FeSO_4(NH_4)SO_4 \cdot 6H_2O$ | 0.28 | 0.006 | 0.28[4)] |
| 8% $Na_2CO_3$ | 2.5 | 2.5 | 2.5 |
| $Na_2SO_4$ | — | — | 0.1 |
| $MgSO_4 \cdot 7H_2O$ | — | — | 2.0[4)] |
| pH | 7.2~7.4 | 7.2~7.4 | 7.2~7.4 |
| Gas phase ($N_2:CO_2$) | 9:1 | 9:1 | 9:1 |

1) Vitamin solution (g/ℓ): biotin, 10; P-aminobenzoic acid, 40; nicotinic acid, 100.
2) Trace element solution (g or mℓ/ℓ): 25% HCl, 10; $FeCl_2 \cdot 6H_2O$, 1.5; $CoCl_2 \cdot 6H_2O$, 0.19; $MnCl_2 \cdot 4H_2O$, 0.1; $ZnCl_2$, 0.07; $H_3BO_3$, 0.062; $Na_2MnO_4 \cdot 2H_2O$, 0.36; $NiCl_2 \cdot 6H_2O$, 0.024.
3) Lactate, pyruvate, malate, choline, acetate and butyrate each was used as a sole electron donor in utility tests of substrate.
4) In tests of no sulfate,these reagents were omitted from this medium.

subculture and maintenance medium of sulfate-reducer was identical to the enumeration medium, except that sodium thioglycolate, ascorbic acid, and $Na_2S_2O_4$ were removed, while 0.5 g and 0.006 g of cystein-HCl and $FeSO_4(NH_4)SO_4 \cdot 6H_2O$ were added, respectively. Six isolates were cultured at 37°C for 2 or 3 days and then purity, Gram-stain reaction, and morphology were examined microscopically. Flagellar arrangement was examined by the method of Nishizawa and Sugahara[2]). Tests for substrate utilization were carried out using the liquid medium shown in Table 1. After incubation for 3 days at 37°C, substrate utilization was determined by observing the presence of blackness in the liquid medium. Acetic acid produced from lactate was analyzed using a gas chromatograph as described above. Primary identification of the isolates was carried out according to Bergey's Manual of Systematic Bacteriology[9)].

## Results and Discussion

### Chemical analyses of the reservoir fluids

pH values and ammonium nitrogen, sulfide, and organic acids concentrations

**Table 2.** Change of chemical parameters and colony counts of bacteria in the reservoir fluids of cattle waste

| | August | September | October |
|---|---|---|---|
| Acid-producing bacteria (Log No./mℓ reservoir fluid) | | | |
| PYS | 5.95 | 6.35 | 6.27 |
| PY | 6.07 | 6.26 | 6.23 |
| Sulfate-reducing bacteria (Log No./mℓ reservoir fluid) | 3.26 | 3.00 | 3.18 |
| Sulfide (mg/ℓ) | 240 | 180 | 140 |
| $NH_4$-N (mg/ℓ) | 3,300 | 3,000 | 2,500 |
| pH | 8.7 | 9.4 | 9.0 |
| Total volatile fatty acids (mM) | 71.2 | 47.8 | 22.0 |
| Acetic acid (mM) | 59.8 | 42.3 | 19.6 |
| Propionic acid (mM) | 8.7 | 4.5 | 0.1 |

in the fluids are shown in Table 2. We found the pH values to be slightly alkaline (8.7–9.4), while respective concentrations of $NH_4$-N, sulfide, and total acids ranged from 2,500 to 3,300 mg, 140 to 240 mg, and 22.0 to 71.2 mM, per liter of the reservoir fluids.

Among the detected organic acids, acetic acid had the highest concentration (19.6–59.8 mM/ℓ), and propionic acid ranged from 0.1 to 8.7 mM/ℓ. Non-volatile fatty acids (lactic,succinic and oxalic acid) were not detected in any fluid. However, two non-identified peaks were observed in non-VFA analyses (Fig. 1). The identification of these peaks is a question to be solved in the future.

The pH value and the amount of total acids obtained in August were higher than at other times due to higher temperatures and excessive fermentation. Indeed, pH then recorded its lowest value and fatty acids peaked.

Ueki *et al.*[7] reported the pH and chemical compositions, including $NH^4$-N, and organic acid in the reservoir fluid of cattle waste from April to December. They mentioned that pH values ranged from 6.9 to 7.5, and that concentrations of $NH_4$-N and total acids ranged from 833 to 1,270 mg and from 7.1 to 44.4 mM, per liter of cattle waste reservoir fluid, respectively.

Ueki *et al.*[7] also reported that the concentration of sulfide ranged from 6 to 60 mg per liter of the reservoir fluid for three years as mentioned above. In our study, higher values of pH seem to be due to higher amounts of $NH_4$-N than those reported by Ueki *et al.* However, the considerably higher amount of sulfide than Ueki *et al.* is assumed to be dependent on protein decomposition by acid-producing bacteria in addition to the change from sulfate by sulfate-reducing bacteria.

**Change of bacterial counts in the reservoir fluid**

Insignificant changes in counts of acid-producing bacteria (Sep.>Oct.>Aug.) and of sulfate-reducing bacteria (Aug.>Oct.>Sep.) were observed. Colony counts of anaerobic acid-producing bacteria were on the order of $\times 10^6$ per mℓ of the fluid.

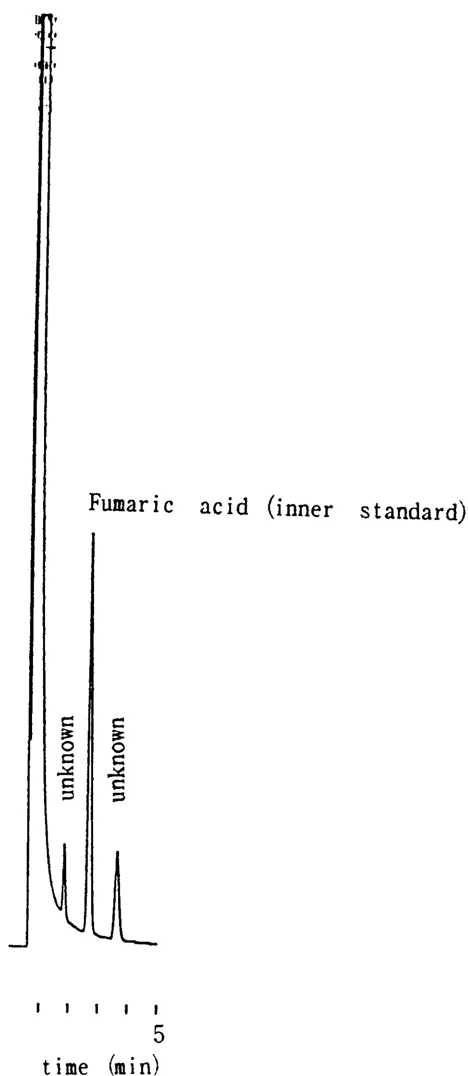


**Fig. 1.** Gas chromatogram of n-volatile fatty acid in the reservoir fluids of cattle waste (August).

Nitrogen was used as the carrier gas at a flow rate of 30 mℓ/min. Other condition of gas chromatography are described in the text.

Colony counts of acid-producing bacteria by medium PY and PYS were almost the same. The addition of supernatant of the reservoir fluid did not show any effect on colony counts of acid-producing bacteria in the fluids. Colony counts of sulfate-reducing bacteria were on the order of $\times 10^3$ per mℓ of the fluids. No relation between sulfate-reducer count and amount of sulfide was found (Table 2).

On the other hand, Ueki *et al.*[7] mentioned that the colony counts of acid-producer and sulfate-reducer were on the order of $\times 10^7$-$10^8$, and $\times 10^6$, per mℓ of the fluid, respectively. Lower values of two kinds of bacteria in our study were presumed to be due to higher values of pH and higher amounts of $NH_4$-N than

those of Ueki *et al.*.

Sulfate-reducing bacterial counts were less than 1% of the acid-producing bacterial counts in all the cases. From these results, it is assumed that sulfate-reducing bacteria are not considered as major bacteria in the reservoir fluid. This is as described by Ueki *et al.*[7].

### Identification of sulfate-reducing bacteria

Six strains isolated from the reservoir fluid were vibroid, their cell sizes were $0.5 \times 2.0$–$5.0\ \mu m$ and their flagellar arrangements were single polar. Their growths were supported by the presence of sulfate, with lactate or pyruvate, while no growth was observed by the presence of malate, choline, acetate and butyrate. From these results, these strains were identified as *Desulfovibrio vulgaris*.

Hydrogen and acetate were formed by anaerobic decomposition of organic matter in digester of animal waste[5),8)]. It is known that these two substances are the substrate for methanogenesis and are also consumed as the main electron donor for sulfate-reduction. In order to clarify the role of sulfate-reducing bacteria in anaerobic treatment processes of animal wastes, the role of methanogens coexisting in the system must also be made clear.

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## 要　　約

1992年8～10月にかけて各月1回，酪農学園大学付属農場第一牛舎の貯留槽から醱酵液を採取し，pH，硫化物濃度等の分析及び酸生成嫌気性細菌数及び硫酸塩還元細菌数の変化につ

いて調べた。分離した6株の硫酸還元菌は，同定を行った。

pHは，8.7～9.4で弱アルカリ性を示した。醗酵液1リットル当たりの硫化物は140～240 mg，アンモニア態窒素は2,500～3,200 mgであった。揮発性脂肪酸は，19.6～59.8 mMと変化し，主として酢酸が検出された。

醗酵液1 mℓ当たりの酸生成嫌気細菌の菌数は，約$10^6$個，硫酸還元細菌のそれは，約$10^3$個であった。硫酸還元細菌6株は，*Desulfovibrio vulgaris* と同定された。