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Desulfovibrio of the Sheep Rumen

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A sulfate-reducing bacterium has been isolated in pure culture from sheep rumen contents. Its properties agree in all respects tested with those ascribed to *Desulfovibrio desulfuricans*. The populations observed (about 10^8/ml) are sufficient to account for published rates of ruminal sulfide production.

It has been known for many years that rumen contents, or washed suspensions of mixed rumen bacteria, have a considerable capacity for converting sulfate, and other oxyanions of sulfur, into sulfide (8). The extent to which large amounts of added sulfate can be reduced strongly suggests that a moderately large dissimilatory sulfate-reducing population is present. Coleman (3) isolated a sporeforming sulfate reducer from sheep rumen contents, now known (2) as *Desulfotomaculum ruminis*. Coleman's isolation procedure included a pasteurization step, and as *D. ruminis* occurs in very low numbers in rumen contents, about 100/ml, it is unlikely to be the main sulfate-reducing organism in the rumen. In the experiments described here, we succeeded in isolating what we believe to be the bacterium primarily responsible for sulfate reduction in the sheep rumen.

MATERIALS AND METHODS

Animals. Two sheep housed on the Lincoln College Research Farm were surgically prepared with rumen cannulae and were fed alfalfa pellets ad lib. Rumen liquor was collected in warmed vacuum flasks and was strained through cheesecloth before use.

Growth media. Isolating medium (medium 1). This was modified from the medium of Hungate (6) by replacing a portion of the sodium chloride by sodium sulfate and by including ferrous sulfate. The minerals were prepared as two stock solutions, A and B, which contained in g/liter: (A) Na_2SO_4, 5.0; NaCl, 2.0; $(NH_4)_2SO_4$, 3.0; KH_2PO_4, 3.0; $CaCl_2 \cdot 2H_2O$, 0.6; $MgSO_4 \cdot 7H_2O$, 0.6 and (B) contained K_2HPO_4, 3.0. To prepare a batch of basal medium, 60 ml each of solutions A and B were mixed with 230 ml of water, 0.4 ml of 0.1% resazurin, 0.35 g of yeast extract, and 1.75 g of $NaHCO_3$. The solution was warmed to about 90°C while being thoroughly gassed with O_2-free CO_2. Addition of 1.5 ml of 10% $FeSO_4 \cdot 7H_2O$ and 0.05 ml of thioglycolic acid caused reduction of the resazurin. The reduced medium was cooled to 40°C, dispensed under CO_2 in stoppered tubes (7), and sterilized under pressure at 121°C for 15 min. For roll tubes, 1.1% agar (Davis Gelatine Ltd, Christchurch, New Zealand) was also included.

Sulfate-free medium (medium 2). Medium 1 was modified by replacing the ammonium and sodium sulfates in solution A by 6.0 g of ammonium chloride and by replacing the ferrous and magnesium sulfates by the respective chlorides. A 5-ml portion of this medium gave a barely perceptible cloudiness with acidified $BaCl_2$.

Low-iron medium (medium 3). A modification of medium N of Saunders et al. (15) was used when growth with a minimum precipitation of FeS was desired. The stock solution contained, in g/liter: KH_2PO_4, 5.0; $(NH_4)_2SO_4$, 70.0; NaCl, 10.0; trisodium citrate $2H_2O$, 3.0; yeast extract, 10.0; $MgSO_4 \cdot 7H_2O$, 0.60; $CaCl_2 \cdot 2H_2O$, 0.60; $FeSO_4 \cdot 7H_2O$, 0.05. To prepare the medium, 25 ml of stock solution, 225 ml of water, 0.3 ml of 0.1% resazurin, 1.25 g of $NaHCO_3$, and appropriate oxidizable substrate were warmed together under thorough gassing with O_2-free CO_2. Addition of 1 drop of thioglycolic acid discharged the color of the resazurin, and the reduced medium was then dispensed and sterilized.

Medium 4 and medium 5. The medium of Postgate (13) and medium C of Pankhurst (10), respectively, with resazurin as the Eh indicator, were sterilized in stoppered tubes under N_2.

Materials. Chemicals used were of the best available reagent grade, and media were always made up with distilled water. Aseptic and anaerobic addition of substrates to the basal medium was carried out as follows. Substances were prepared normally as 10% solutions in water containing 0.0001% resazurin and adjusted where necessary to pH 7. These solutions, contained in stoppered tubes, were gassed with O_2-free CO_2, reduced by adding the minimum amount of ferrous sulfate or thioglycolic acid, and sterilized by autoclaving or filtration. The amount of sterile solution needed to bring the final concentration of substrate to 0.3% was injected into the tubes of basal medium before use.

Some carbon sources, and some sulfur compounds, which might possibly be inhibitory or poisonous, were tested as follows. Tubes of 5 ml of agar medium, complete except for the test substance, were inoculated with a well-grown suspension of the organism and allowed to set as "deeps." The test substance was then added as a dilute solution to lie

over the agar, and the tubes were incubated. Diffusion of the test substance into the agar allowed the opportunity for growth in concentrations of it too low to be inhibitory.

Incubation of cultures was at 38°C unless stated otherwise.

RESULTS

Growth of dissimilatory sulfate reducers in medium 1 was evidenced by precipitation of black ferrous sulfide. In liquid medium this precipitate settled out as growth subsided, leaving a gray turbid suspension of the bacteria. In roll tubes the precipitation usually occurred in and around the colonies.

Tenfold dilutions of sheep rumen fluid in liquid medium 1 containing a variety of carbon compounds showed, after incubation for periods between 1 and 7 days, intense blackening in dilutions up to 10^8. Substances that served as substrates for the reduction of sulfate included lactate, formate, malate, fumarate, citrate, glucose, glycerol, mannitol, ethanol, and methanol. Medium containing no added substrate showed no blackening in tubes beyond the first dilution, and media containing acetate, propionate, butyrate, succinate, oxalate, or glutamate also failed to support sulfate reduction.

Repeated samplings showed no evidence that the populations of sulfate reducers varied in size or in properties, either between sheep or at different times. The results that follow were all obtained using a single sample of rumen fluid from one sheep.

Transfer to roll tubes from the highest positive dilutions of rumen fluid in liquid medium 1 yielded tubes from which well-separated colonies could be picked. Repetition of this process eventually gave pure cultures of several isolates of sulfate-reducing organisms. Strains were isolated with lactate, formate, ethanol, or methanol as the carbon source. The strain isolated on lactate was used for further work. When grown on liquid medium 3 containing lactate as the energy source, it appeared under the microscope as a gram-negative curved rod. Under the electron microscope negatively stained or shadowed preparations showed uniform curved rods (2.0 × 0.8 to 2.5 by 1.1 μm), each with a single polar flagellum (Fig. 1). Colonies on solid medium 3 containing lactate as energy source are small, round, clear, and colorless to pale gold. Colonies on solid medium 1 are usually dense black, but occasionally (especially when ethanol or methanol is being oxidized) colonies are pale colored, surrounded by a clear halo, with blackening in more distant parts of the agar.

In medium 1 the bacterium will grow and reduce sulfate at the expense of hydrogen gas, lactate, pyruvate, formate, fumarate, malate, choline, ethanol, and methanol. It cannot grow on medium 1 containing no added substrate, nor on glucose, mannitol, glycerol, citrate, oxamate, glycine, D-alanine, L-alanine, DL-serine, D-aspartate, L-phenylalanine, L-tyrosine, formaldehyde, propan-1-ol, propan-2-ol, isobutyl alcohol, *n*-butan-1-ol, *n*-butan-2-ol, amyl alcohol, nor on any of the substances found not to support sulfate-reduction by the initial dilutions of rumen liquor.

In medium 2, which lacks sulfate, only pyruvate and choline supported growth. When incubated in deep agar medium 2 containing lactate as carbon source, growth and FeS formation occurred when sulfate, sulfite, thiosulfate, or dithionite was added. With sulfate and thiosulfate, the zone of blackening spread downwards from the interface as incubation proceeded; with sulfite and dithionite, blackening started in the lower half of the agar, and spread upwards.

Liquid medium 1, containing lactate but without yeast extract, did not support growth of the organism, but growth occurred if this medium was supplemented with either yeast extract or Trypticase, dissolved and sterilized separately. The lowest concentrations tested, 0.02 and 0.05%, respectively, both supported visible growth.

Washed cells of the organism showed a red fluorescence under ultraviolet light when freshly suspended in 3 N NaOH (12). Cell-free extracts, obtained from cells broken in a Braun homogenizer, had the spectral properties shown in Table 1.

Cultures of the organism in medium 1 containing lactate failed to grow after heating to 80°C for 5 min, when inoculated into fresh medium. No growth occurred in medium 1 containing lactate, incubated at 45°C or higher temperatures.

The effects of selenate, molybdate, and hibitane on growth of the organism are shown in Table 2.

The strains isolated on media in which formate, ethanol, or methanol was the energy source were indistinguishable from the strain isolated on lactate when grown on lactate-containing medium, by the following criteria: appearance of colonies, appearance of Gram-stained smears under the light microscope, the energy sources supporting growth and sulfate reduction in medium 1, and the energy sources supporting growth in medium 2.

In preliminary experiments at Davis, Calif., the rumen fluid of a cow fed alfalfa hay ap-

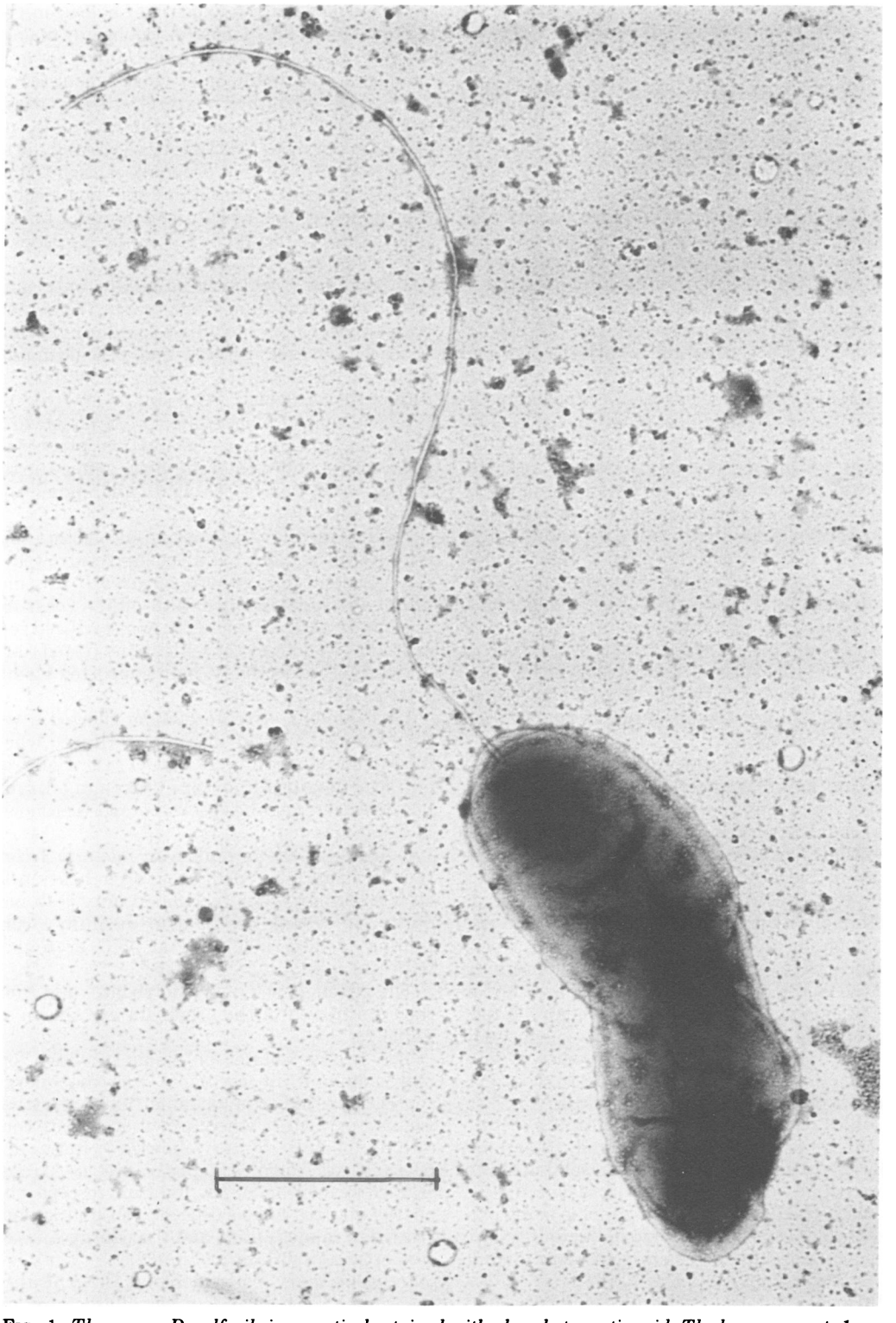

FIG. 1. *The rumen Desulfovibrio, negatively stained with phosphotungstic acid. The bar represents 1 μm.*

TABLE 1. *Spectral properties of cell-free extracts of the rumen Desulfovibrio*

Treatment of extract	Absorption maxima (nm)			
As prepared in equilibrium with atmosphere	410			630
After immersion in boiling water for 5 min	410			
After reduction with dithionite	418	525s	551	630

TABLE 2. *Growth of the rumen Desulfovibrio in the presence of inhibitors*[a]

Inhibitor	Highest concn not inhibitory	Partially inhibitory concn	Lowest concn completely inhibitory
Sodium selenate	20 μM	200 μM	2 mM
Ammonium molybdate[b]	10 μM	100 μM	1 mM
Hibitane	16 mg/liter	20 mg/liter	25 mg/liter

[a] The organism was incubated in liquid medium 1 containing lactate and various concentrations of the inhibitors. Growth was observed after 1 week.

[b] Concentration calculated as MoO_3.

peared to contain a population of about 10^8/ml of a sulfate-reducing organism having very similar properties to the New Zealand isolate. No pure culture was obtained in this work, and the results are therefore not presented in detail.

DISCUSSION

The organism from sheep rumen contents has all the properties expected of a member of the genus *Desulfovibrio* as defined by Postgate and Campbell (14). It is a nonsporeforming, mesophilic, gram-negative vibrio, motile by means of a single polar flagellum. It is obligately anaerobic and reduces sulfate to sulfide at the expense of a limited range of oxidizable substrates. The positive fluorescence test (12) and the absorption spectra of cell-free extracts in the oxidized and reduced states, and after heating, show the cells to contain the thermostable cytochrome c_3 and the thermolabile desulfoviridin, both characteristic of *Desulfovibrio*.

On morphological grounds alone, our organism might be assigned to any of the three species *D. desulfuricans*, *D. vulgaris*, or *D. salexigens*. The last named is eliminated on physiological grounds, since our organism lacks the salt requirement (2.5% NaCl) and the hibitane resistance (1.0 g/liter) characteristic of *D. salexigens*. Because our organism can grow on pyruvate or choline without sulfate, on malate with sulfate, and in presence of up to 16 mg of hibitane per liter, it resembles *D. desulfuricans* rather than *D. vulgaris*.

We believe that the isolated organism is chiefly responsible for the sulfate reduction in the rumen. Bray and Till (1) calculated that a population of dissimilatory sulfate reducers of 5.8×10^8/ml would be required to maintain the normal concentration of sulfide sulfur of 5 μg per ml of rumen liquor. This, in view of the approximations involved, can be taken as consistent with our demonstration of sulfate reduction by dilutions of rumen liquor of up to 1 in 10^8. It should be noted that some workers in the field of ruminal sulfate metabolism have supplemented the diet of their experimental animals with sulfate, which increases the sulfate-reducing activity of the rumen contents (e.g., 8), presumably by permitting an increase in population of the sulfate-reducing organisms. Our own experimental animals received no supplement.

Several observations by Lewis (8) are consistent with the proposition that *D. desulfuricans* is the predominant ruminal sulfate reducer: (i) sulfate reduction was associated with the bacterial, rather than the protozoal fraction of the rumen microbial population; (ii) sulfite and thiosulfate were also reduced to sulfide by suspensions of rumen microbes; (iii) mixed rumen microbes reduced sulfate in the presence of hydrogen, lactate, pyruvate, formate, ethanol, and malate, but not with fatty acids, mannitol, or glycerol.

Discrepancies between the properties of mixed rumen microbes (8) and of our organism are the following: (i) the inability of the mixed organisms to reduce dithionite; (ii) the ability of mixed rumen microbes to reduce sulfate in the presence of glucose, fructose, and citrate; (iii) the ability of the mixed rumen microbes to reduce sulfate in the presence of succinate. (i) Discrepancy may be due either to the poisoning of the organisms in Lewis's experiments by exposure to 2 mM dithionite or to the spontaneous decomposition of the dithionite into sulfite during our own longer term incubations (11). (ii) Discrepancy may be convincingly explained by the known abilities of other members of the mixed rumen population to convert the substances mentioned into substrates for the *Desulfovibrio*. (iii) Discrepancy is difficult to reconcile with other observations, even before our isolation of the *Desulfovibrio* that cannot use succinate. Succinate added to rumen contents or produced as a fermentation product by certain species of rumen bacteria is considered to be wholly and rapidly decarboxylated to propionate (7), which itself is inert in sulfate reduc-

tion in both Lewis's experiments and our own. In our experiments, rumen fluid diluted into medium 1 containing succinate did not initiate sulfate reduction. We are unable to explain Lewis's observation in the light of present knowledge.

The properties of our organism can explain some minor biochemical reactions, otherwise puzzling, which occur in rumen contents. Ethanol, which is produced by some rumen bacteria in pure culture and may be formed in small quantities in the slightly acid rumen, is slowly metabolized when added to the rumen. Part of that which disappears is converted to acetate (9). Methanol, which is liberated from pectin in the rumen by the action of microbial pectinmethylesterase, is also metabolized (4), presumably by oxidation. The oxidation of organic compounds in the strongly reducing environment of the rumen appears paradoxical; the presence of a population of *Desulfovibrio* able to oxidize these substances at the expense of sulfate could explain the process.

After this work was commenced, a description of a sulfate-reducing bacterium from sheep rumen was published by Huisingh et al. (5). The organism, assigned to the genus *Desulfovibrio*, was a nonmotile, straight rod, which would grow, and reduce sulfate, on a wide range of sugars as sole carbon source and would grow on glucose in the absence of sulfate. Most of the other properties were typical of a conventional *Desulfovibrio*. A culture of this organism, ATCC 27882, was obtained from the American Type Culture Collection. Inoculation into liquid medium 1 containing lactate or glucose gave rapid sulfate reduction, but dilution into agar roll tubes, and picking of well-separated colonies, yielded two subcultures. One of these would grow and reduce sulfate on lactate but not glucose; the other could not reduce sulfate in any of the media used in the present work, but grew rapidly on glucose and slowly or not at all on lactate. Under the microscope the former appeared as gram-negative vibrios, and the latter as gram-variable rods. The properties of the culture of Huisingh et al. (5) are consistent with the hypothesis that it is a mixture of a *Desulfovibrio* and an obligately anaerobic rumen bacterium, possibly a strain of *Bacteroides*. The latter grows on sugars, converting them to lactate, H_2, or other products oxidized by the *Desulfovibrio* with sulfates.

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