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ROLE OF EXTRACELLULAR ACETATE IN THE FERMENTATION OF GLUCOSE BY A RUMINAL BACTERIUM, *MEGASPHAERA ELSDENII*

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In batch culture, addition of acetate stimulated the initial growth of *Megasphaera elsdenii* in medium containing glucose and Trypticase. As the initial concentration of acetate was increased, butyrate production increased. When acetate was used, the rate of butyrate production increased. Hydrogen production was decreased by adding acetate. Inhibition of hydrogen production by the hydrogenase inhibitor carbon monoxide inhibited the growth of *M. elsdenii*, but this inhibition was cancelled by adding acetate. In continuous culture, where the growth rate was kept relatively low by limiting the glucose supply, adding acetate similarly increased butyrate production. These results suggest that acetate serves as an electron sink in *M. elsdenii*.

Megasphaera elsdenii is a gram-negative obligately anaerobic bacterium normally found in the rumen, and is predominant when ruminants are fed high grain diets (*13*). Some metabolic characteristics include lactate utilization (*2*, *13*) as well as amino acid catabolism (*1*, *29*). Counotte et al. (*5*) suggested that *M. elsdenii* is the primary lactate-fermenting bacterium in cattle fed high levels of cereal grains. *M. elsdenii* may be an important organism for the prevention of rumen acidosis, since the bacterium actively utilizes lactate produced by *Streptococcus bovis* (*25*), an important contributor to rumen acidosis (*26*). *M. elsdenii* also ferments glucose, and produces mainly butyrate (*8*, *12*, *20*) rather than caproate as reported previously (*7*). Since not all of the electrons liberated by the oxidation of glucose can be used for the formation of butyrate and caproate, *M. elsdenii* enevitably evolves H_2 (*14*, *16*, *19*; Fig. 1). Production of H_2, which is converted to methane in the rumen, means a loss of energy to ruminants.

Previous research demonstrated that growth of *M. elsdenii* is stimulated by adding acetate (*3*). Forsberg (*8*) reported that acetate was needed for growth in a medium containing glucose, sulfate, and ammonia as the sole source of nitrogen,

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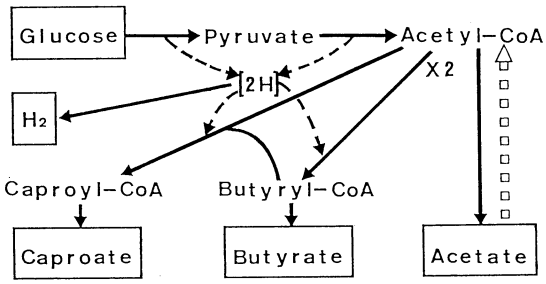

Fig. 1. Possible pathways from glucose to the main VFA and electron flow in *M. elsdenii*: $[2H] = 2e^- + 2H^+$ (reducing equivalent).

but when lactate served as the energy and carbon source, acetate was not required. It has also been reported that acetate stimulates growth on glucose in *Eubacterium limosum* (*9*), but no information is available at present on the role of acetate in the cellular function and physiology.

Stimulation of growth by acetate in the presence of glucose and Trypticase may indicate that acetate is not essential for the biosynthesis of cell components. It is reasonable to assume that acetate is metabolized by *M. elsdenii* as shown in Fig. 1. Obviously, acetate is not oxidized, i.e., acetate does not serve as the energy source. On the contrary, conversion of acetate to butyrate or caproate is an energy-consuming reaction. In fact, in preliminary experiments acetate never permitted growth without glucose or lactate. It seems likely that acetate serves as an electron sink. When acetate is metabolized to synthesize butyrate or caproate, electrons are consumed (Fig. 1). Acetate is converted to acetyl-CoA without donating or accepting electrons, while electrons are liberated in the formation of acetyl-CoA from glucose. Thus, if acetate is used to form butyrate or caproate, a net consumption of electrons would result. It is generally accepted that in anaerobic bacteria the readiness with which electrons are disposed of may influence the energy metabolism and consequently growth (*28*).

The purpose of our study was to determine whether acetate serves as a sink of electrons in *M. elsdenii*. Experiments were performed to examine the effect of acetate on: (1) the pattern of growth in batch culture when glucose, amino acids and other nutrients are sufficiently supplied, (2) the growth when hydrogen production is inhibited, and (3) the production of volatile fatty acids (VFA) in batch culture and in continuous culture where the glucose supply limits growth.

MATERIALS AND METHODS

Organism and growth conditions. *M. elsdenii* NIAH-1102 was a gift from Dr. H. Minato, National Institute of Animal Health, Tsukuba, Japan. Unless otherwise stated, the bacterium was grown in 120-ml serum bottles at 39°C under O_2-free CO_2 (initial pH 6.8). The basal medium for batch culture contained (g/liter):

KH_2PO_4, 0.9; K_2HPO_4, 0.9; Na_2CO_3, 2.5; $(NH_4)_2SO_4$, 1.8; NaCl, 1.8; $CaCl_2 \cdot 2H_2O$, 0.24; $MgSO_4 \cdot 7H_2O$, 0.38; cysteine·HCl, 0.6; glucose, 2; yeast extract (Difco), 1; and Trypticase (BBL, Microbiology Systems, Cockeysville, Md, USA), 1. Cell growth was estimated by measuring the optical density (OD) at 600 nm.

Glucose-limited continuous culture was conducted with 250 ml vessels (overflow type) under a stream of $CO_2 + N_2$ (1 : 9). The medium contained (g/liter): KH_2PO_4, 2.4; $Na_2HPO_4 \cdot 12H_2O$, 6.0; Na_2CO_3, 1.0; $(NH_4)_2SO_4$, 1.8; NaCl, 0.3; $CaCl_2 \cdot 2H_2O$, 0.06; $MgSO_4 \cdot 7H_2O$, 0.06; cysteine·HCl, 0.6; glucose, 1; yeast extract, 1; and Trypticase, 1. Glucose in the effluent from the continuous culture was checked by TLC separation (*17*): A cellulose TLC plate (Avicel) was developed with pyridine–ethyl acetate–acetic acid–water (36 : 36 : 7 : 21). Glucose was detected by spraying 0.5 M alcoholic NaOH on the plate after dipping it in $AgNO_3$–acetone (0.5% saturated aqueous solution of $AgNO_3$ in acetone). Glucose was never detected in the effluent (less than 10 mg/*l*), indicating that substantially all of the glucose was consumed.

Except for the continuous culture experiments, all incubations were performed in duplicate. Continuous culture was duplicated with essentially the same results each time.

Determination of VFA and H_2. VFA and H_2 were determined by GLC as described previously (*11*).

RESULTS

Effect of acetate on the growth of M. elsdenii

M. elsdenii grew in the absence of VFA in a medium containing glucose and Trypticase, but adding 6 mM acetate shortened the lag phase (Fig. 2) and the initial growth increased as the acetate concentration increased up to 25 mM (Fig. 3). When cells were incubated in the presence of 50% carbon monoxide (CO; $CO-CO_2 = 1 : 1$), a hydrogenase inhibitor, growth was inhibited more than 60%, but adding 25 mM

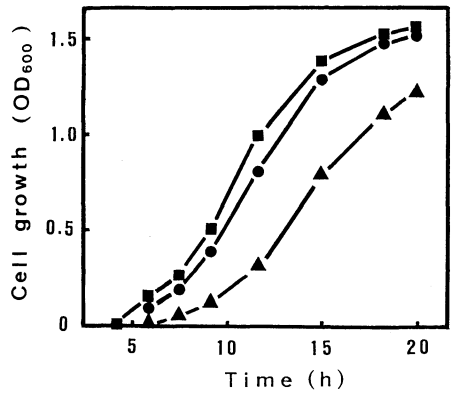

Fig. 2. Effect of acetate on the growth of *M. elsdenii* on glucose: Addition of acetate: 0 mM (▲), 6 mM (●), and 25 mM (■).

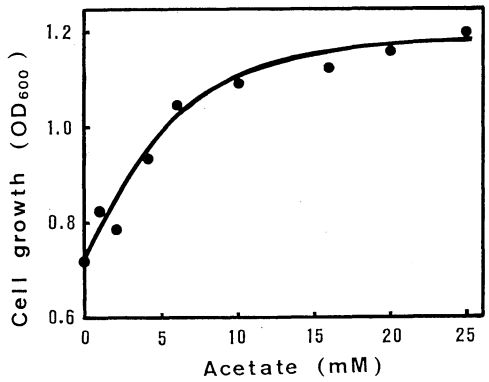

Fig. 3. Effect of acetate concentration on the growth of *M. elsdenii* on glucose: Graded concentrations of acetate were added, and incubated for 10 h.

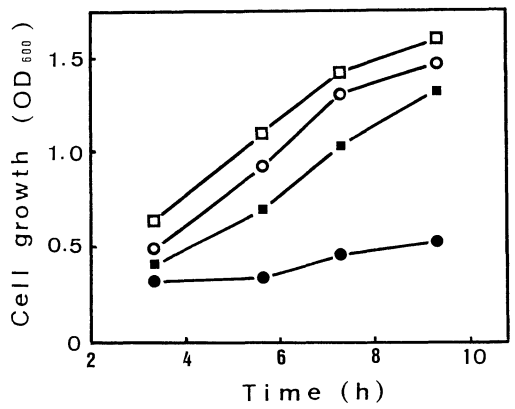

Fig. 4. Effect of acetate on the growth of *M. elsdenii* in the presence or absence of CO: Addition of 0 (○) or 25 mM (□) acetate. Open and closed symbols indicate addition of 0 and 50% CO, respectively.

acetate improved the amount of growth under CO (Fig. 4). These results suggest that some of the electrons liberated from glucose oxidation is used in the metabolism of acetate.

Effect of acetate on the VFA and H_2 production by M. elsdenii

When increasing concentrations of acetate were added to the growth medium, more butyrate was produced (Table 1). Below 4 mM acetate (initial concentration), net acetate production decreased, and net acetate consumption increased above 4 mM acetate. But caproate production decreased as acetate utilization increased. These trends were also followed in the production or consumption per unit amount of cell growth, i.e., final OD_{600}. The effects of extracellular acetate on the production of acetate, butyrate, and caproate were evaluated more precisely. When 15 mM

Table 1. Effect of acetate on VFA production by *M. elsdenii* grown on glucose.[a]

Initial acetate (mM)	VFA production[b] (mM)						Cell growth (OD_{600})
	Acetate	Isobutyrate	Butyrate	Isovalerate +2-methylbutyrate	Valerate	Caproate	
0	2.33	0.14	4.46	0.47	0.04	0.43	0.72
	(3.24)	(0.19)	(6.19)	(0.65)	(0.06)	(0.60)	
1	1.99	0.14	5.64	0.63	0.07	0.41	0.75
	(2.65)	(0.19)	(7.52)	(0.84)	(0.09)	(0.55)	
2	1.47	0.13	5.96	0.48	0.13	0.40	0.80
	(1.84)	(0.16)	(7.45)	(0.60)	(0.16)	(0.50)	
4	0.54	0.14	6.87	0.46	0.05	0.46	0.90
	(0.60)	(0.16)	(7.63)	(0.51)	(0.06)	(0.51)	
6	−0.13	0.14	8.05	0.50	0.06	0.43	1.05
	(−0.12)	(0.13)	(7.76)	(0.48)	(0.06)	(0.41)	
10	−2.08	0.13	9.54	0.50	0.03	0.35	1.15
	(−1.81)	(0.11)	(8.30)	(0.43)	(0.03)	(0.30)	
15	−5.07	0.14	9.92	0.65	0.13	0.29	1.17
	(−4.33)	(0.12)	(8.48)	(0.56	(0.11)	(0.25)	
20	−5.90	0.15	11.12	0.52	0.02	0.26	1.18
	(−5.00)	(0.13)	(9.42)	(0.44)	(0.02)	(0.22)	
25	−6.12	0.14	11.84	0.55	0.02	0.19	1.20
	(−5.10)	(0.12)	(9.87)	(0.46)	(0.02)	(0.16)	

Figures in parentheses indicate the values per final OD_{600}.

[a] *M. elsdenii* was grown for 10 h.

[b] Final concentration minus initial concentration.

acetate was added to the growth medium, the acetate concentration began to decrease after 9 h (Fig. 5-A). Before this point, utilization of acetate was not apparent, but the number of cells was small during this period, so the acetate used per cell may have been similar. When the growth rate slowed down, possibly due to a decrease of glucose concentration (after 15 h), the acetate concentration began to increase gradually. Acetate utilization was closely related to butyrate production (Fig. 5-B). Caproate production was reduced by the presence of acetate (Fig. 5-C). Hydrogen production from glucose fermentation was almost parallel to cell growth, but adding 15 mM acetate decreased H_2 production, though the rate of growth was increased (Fig. 5-A, Fig. 6).

Table 2 shows the effect of acetate on VFA and H_2 production in the presence of CO. When CO was present, there was no growth, even after 48 h in the absence of acetate. When 25 mM acetate was added, the growth was similar to that in the absence of CO. In the presence of acetate, CO inhibited H_2 production and enhanced acetate utilization, which led to the increase in butyrate production. In this case caproate was also increased. These results suggest that extracellular acetate is used as an electron-accepting substance in the formation of butyrate, and this process increases when the electron flow to H_2 is inhibited by CO (see Fig. 1).

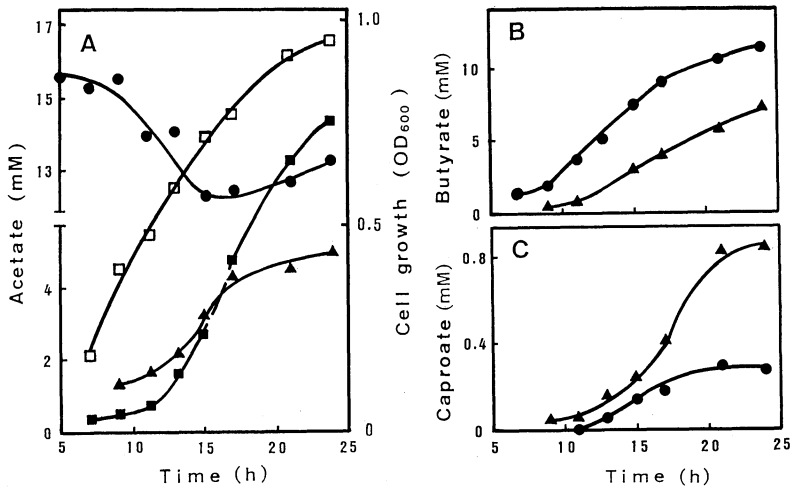

Fig. 5. Effect of acetate on VFA production by *M. elsdenii* grown on glucose: A. Acetate, B. Butyrate, and C. Caproate. Addition of 0 (▲) or 15 mM (●) acetate. Cell growth in the presence (□) or absence (■) of 15 mM acetate.

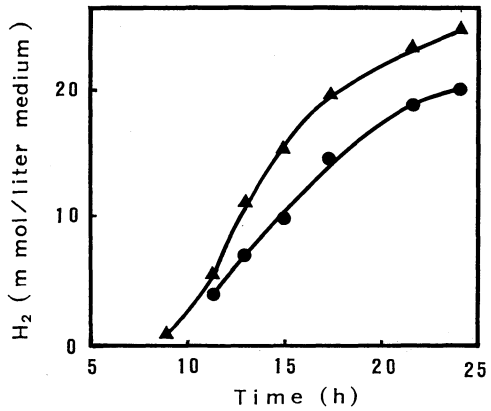

Fig. 6. Effect of acetate on H_2 production by *M. elsdenii* grown on glucose: Addition of 0 (▲) or 15 mM (●) acetate.

The effect of adding acetate on VFA production by *M. elsdenii* grown in glucose-limited continuous culture is shown in Table 3. Even when growth was kept at a low rate by limiting the supply of glucose, i.e., the rate of electron flow was kept low, adding 15 mM acetate decreased the net production of acetate with a concomitant increase in butyrate production. In the steady state, adding acetate gave no significant difference in the OD_{600}, since 5 mM acetate was constantly present without adding acetate.

Table 2. Effect of CO on H_2 and VFA production by *M. elsdenii* grown on glucose in the presence or absence of acetate.[a]

CO (%)	Acetate (mM)	H_2 production (mmol/liter medium)	VFA production (mM)		
			Acetate	Butyrate	Caprate
0	0	20.1	4.1	6.6	0.6
50	0		no growth		
0	25	16.7	−5.5	17.3	0.3
50	25	0.6	−14.3	20.8	1.3

[a] Incubation was continued for 48 h (Final OD_{600} was similar among the three treatment groups).

Table 3. Effect of acetate on VFA production by *M. elsdenii* grown in glucose-limited continuous culture.[a]

Acetate (mM)	VFA production (mM)		
	Acetate	Butyrate	Caproate
0	5.0	5.7	0.9
15	2.0	6.9	0.8

[a] Dilution rate was 0.1 (/h).

DISCUSSION

Adding acetate to the growth medium shortened the lag phase and stimulated the initial growth of *M. elsdenii*. Microorganisms never grow at constant rates in the rumen: Usually there are lag periods for most organisms. Therefore, stimulation of initial growth may have a significant effect on overall rumen fermentation. Adding acetate increased butyrate production, and there was a close relationship between acetate utilization and butyrate production. These results strongly support the assumption that acetate serves as an electron sink.

Butyrate production was proportional to the concentration of added acetate (Table 1): As the extracellular acetate concentration increased, net acetate production decreased, and above a certain level (initial concentration of 4 mM in this case) consumption increased. It appears that a certain equilibrium exists between acetate and acetyl-CoA under certain conditions, and the concetration of acetyl-CoA may affect butyrate formation. The acetyl-CoA/CoA quotient may also influence butyrate formation by affecting the electron flow. In clostridia this quotient allosterically regulates NADH: ferredoxin oxidoreductase (*28*).

In *Butyrivibrio fibrisolvens*, net production or consumption of acetate occurs: Whether acetate is produced or consumed depends on the strains, although that was not examined in relation to the extracellular acetate concentration (*4*, *23*, *27*). *Clostridium acetobutyricum* ferments sugars in two distinct phases: Initially at high

glucose levels growth is rapid with copious acetate and butyrate production and H_2 evolution. When growth becomes slower due to low glucose levels and low pH, acetate and butyrate accumulated in the medium are taken up and acetone, ethanol, and butanol are formed with decreased H_2 evolution (*21*, *24*, *30*).

Since the acetate utilization by *M. elsdenii* was increased by the inhibition of H_2 formation, accumulation of reducing equivalents appears to stimulate the reactions from acetyl-CoA to butyrate. In *Clostridium acetobutyricum* the inhibition of hydrogenase by CO leads to an increase in the pool of NADH, which in turn leads to an increased formation of products that require NADH at the expense of the accumulated acids (*6*, *15*, *22*).

Unexpectedly adding acetate decreased caproate production, but increased it only when hydrogenase was inhibited. It is likely that the regulation of caproate formation differs from that of butyrate formation. *Closridium kluyveri* has two distinct β-hydroxybutyryl-CoA dehydrogenase, i.e., a soluble NADPH-specific enzyme and a particulate NADH-specific enzyme (*10*, *18*), and it was suggested that the former enzyme functions in butyrate formation whereas the latter one is involved in caproate formation (*18*). On the other hand, since caproate production by *M. elsdenii* was initiated after butyrate was produced and adding butyrate markedly increased caproate production (data not shown), caproate seems to be formed, at least partly, by using butyrate.

Why acetate stimulates the initial growth of *M. elsdenii* is still not known. However, the role of acetate as an electron sink may indicate that electrons are not readily disposed of during the initial stage of growth, possibily due to the delay in the synthesis of hydrogenase or other electron-flow systems.

We thank Dr. H. Minato for donating the bacterial strain.

REFERENCES

1) Allison, M. J., Production of branched-chain amino acids by certain rumen anaerobic bacteria. *Appl. Environ. Microbiol.*, **35**, 872–877 (1978).
2) Baldwin, R. L., Pathways of carbohydrate metabolism in the rumen. *In* Physiology of Digestion in The Ruminant, ed. by Dougherty, R. W., Butterworths, London (1965), p. 379–398.
3) Bryant, M. P., and Robinson, I. M., Some nutritional characteristics of predominant culturable ruminal bacteria. *J. Bacteriol.*, **84**, 605–614 (1962).
4) Bryant, M. P., and Small, N., The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. *J. Bacteriol.*, **72**, 16–21 (1956).
5) Counotte, G. H., Prins, R. A., Janssen, R. H. A. M., and DeBie, M. J. A., Role of *Megasphaera elsdenii* in the fermentation of DL-[2-^{13}C]lacetate in the rumen of dairy cattle. *Appl. Environ. Microbiol.*, **42**, 649–655 (1981).
6) Datta, R., and Zeikus, J. G., Modulation of acetone-butanol-ethanol fermentation by carbon monoxide and organic acids. *Appl. Environ. microbiol.*, **49**, 522–529 (1985).
7) Elsden, S. R., Volcani, B. E., Gilchrist, F. M. C., and Lewis, D., Properties of a fatty acid forming organism isolated from the rumen of sheep. *J. Bacteriol.*, **72**, 681–689 (1956).
8) Forsberg, C. W., Nutritional characteristics of *Megasphaera elsdenii*. *Can. J. Microbiol.*, **24**, 981–985 (1978).

9) Genthner, B. R. S., Davis, C. L., and Bryant, M. P., Features of rumen and sewage sludge strains of *Eubacterium limosum*, a methanol- and H_2-CO_2-utilizing species. *Appl. Environ. Microbiol.*, **42**, 12–19 (1981).

10) Hillmer, P., and Gottschalk, G., Particulate nature of enzymes involved in the fermentation of ethanol and acetate by *Clostridium kluyveri*. *FEBS Lett.*, **21**, 351–354 (1972).

11) Hino, T., Action of monensin on rumen protozoa. *Jpn. J. Zootech. Sci.*, **53**, 171–179 (1981).

12) Hobson, P. N., Mann, S. O., and Oxford, A. E., Some studies on the occurrence and properties of a large gram-negative coccus from the rumen. *J. Gen. Microbiol.*, **19**, 462–472 (1958).

13) Hungate, R. E., The rumen and Its Microbes. Academic Press Inc., New York (1966), p. 76–77.

14) Joyner, A. E., Winter, W. T., and Godbout, D. M., Studies on some characteristics of hydrogen production by cell-free extracts of rumen anaerobic bacteria. *Can. J. Microbiol.*, **23**, 346–353 (1977).

15) Kim, B. H., Bellows, P., Datta, R., and Zeikus, J. G., Control of carbon and electron flow in *Clostridium acetobutylicum* fermentations: Utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yield. *Appl. Environ. Microbiol.*, **48**, 764–770 (1984).

16) Ladd, J. N., and Walker, D. J., The fermentation of lactate and acrylate by the rumen micro-organism LC. *Biochem. J.*, **71**, 364–373 (1959).

17) Lewis, B. A., and Smith, F., *In* Thin-Layer Chromatography, A Laboratory Handbook, 2nd ed., ed. by E. Stahl, Springer-Verlag, Berlin, New York (1969), p. 813–815.

18) Maden, V. K., Hillmer, P., and Gottschalk, G., Purification and properties of NADP-dependent L(+)-3-hydroxybutyryl-CoA dehydrogenase from *Clostridium kluyveri*. *Eur. J. Biochem.*, **32**, 51–56 (1973).

19) Marounek, M., and Bartos, S., Interactions between rumen amylolytic and lactate-utilizing bacteria in growth on starch. *J. Appl. Bacteriol.*, **63**, 233–238 (1987).

20) Marounek, M., Fliegrova, K., and Bartos, S., Metabolism and some characteristics of ruminal strains of *Megasphaera elsdenii*. *Appl. Environ. Microbiol.*, **55**, 1570–1573 (1989).

21) McNeil, B., and Kristiansen, B., The acetone butanol fermentation. *Adv. Appl. Microbiol.*, **31**, 61–92 (1986).

22) Meyer, C. L., Roos, J. W., and Papoutsakis, E. T., Carbon monoxide gassing leads to alcohol production and butyrate uptake without acetone formation in continuous cultures of *Clostridium acetobutyricum*. *Appl. Microbiol. Biotechnol.*, **24**, 159–167 (1986).

23) Miller, T. L. and Jenesel, S. E., Enzymology of butyrate formation by *Butyrivibrio fibrisolvens*. *J. Bacteriol.*, **138**, 99–104 (1979).

24) Rao, G. and Mutharasan, R., Altered electron flow in continuous cultures of *Clostridium acetobutyricum* induced by viologen dyes. *Appl. Environ. Microbiol.*, **53**, 1232–1235 (1987).

25) Russell, J. B., Cotta, M. A., and Dombrowski, D. B., Rumen bacterial competition in continuous culture: *Streptococcus bovis* versus *Megasphaera elsdenii*. *Appl. Environ. Microbiol.*, **41**, 1394–1399 (1981).

26) Russell, J. B. and Hino, T., Regulation of lactate production in *Streptococcus bovis*: A spiraling effect that contributes to rumen acidosis. *J. Dairy Sci.*, **68**, 1712–1721 (1985).

27) Shane, B. S., Gouws, L., and Kistner, A., Cellulolytic bacteria occurring in the rumen of sheep conditioned to low-protein teff hay. *J. Gen. Microbiol.*, **55**, 445–457 (1969).

28) Thauer, R. K., Jungermann, K., and Decker, K., Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.*, **41**, 100–180 (1977).

29) Wallace, R. J., Catabolism of amino acids by *Megasphaera elsdenii* LC1. *Appl. Environ. Microbiol.*, **51**, 1141–1143 (1986).

30) Zeikus, J. G., Chemical and fuel production by anaerobic bacteria. *Annu. Rev. Microbiol.*, **34**, 423–464 (1980).