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Resistance of *Streptococcus bovis* to Acetic Acid at Low pH: Relationship between Intracellular pH and Anion Accumulation

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***Streptococcus bovis* JB1, an acid-tolerant ruminal bacterium, was able to grow at pHs from 6.7 to 4.5, and 100 mM acetate had little effect on growth rate or proton motive force across the cell membrane. When *S. bovis* was grown in glucose-limited chemostats at pH 5.2, the addition of sodium acetate (as much as 100 mM) had little effect on the production of bacterial protein. At higher concentrations of sodium acetate (100 to 360 mM), production of bacterial protein declined, but this decrease could largely be explained by a shift in fermentation products (acetate, formate, and ethanol production to lactate production) and a decline in ATP production (3 ATP per glucose versus 2 ATP per glucose). Y_{ATP} (grams of cells per mole of ATP) was not decreased significantly even by high concentrations of acetate. Cultures supplemented with 100 mM sodium acetate took up [^{14}C]acetate and [^{14}C]benzoate in accordance with the Henderson-Hasselbalch equation and gave similar estimates of intracellular pH. As the extracellular pH declined, *S. bovis* allowed its intracellular pH to decrease and maintained a relatively constant pH gradient across the cell membrane (0.9 unit). The decrease in intracellular pH prevented *S. bovis* from accumulating large amounts of acetate anion. On the basis of these results it did not appear that acetate was acting as an uncoupler. The sensitivity of other bacteria to volatile fatty acids at low pH is explained most easily by a high transmembrane pH gradient and anion accumulation.**

Short-chain volatile fatty acids are often end products of anaerobic fermentations, and they can be toxic if the medium is not well buffered. Bacteria differ greatly in their sensitivities to volatile fatty acids. Certain ruminal bacteria (24) and lactobacilli (10) are able to grow rapidly at low pH in the presence of volatile fatty acids, but *Escherichia coli* (6, 14, 27) and *Streptococcus cremoris* (18) are not resistant. *Clostridium acetobutylicum* produces volatile fatty acids at neutral pH but switches to solvent production as the pH declines (7).

The mechanism of volatile fatty acid toxicity at low pH has never been fully explained. Because undissociated volatile fatty acids can permeate cell membranes, it has been assumed that they dissipate the ΔpH in a manner analogous to that of uncouplers (1, 5, 8, 14, 16). This theory, however, does not explain why some organisms are resistant and others are sensitive. Furthermore, the analogy between uncouplers and volatile fatty acids has its flaws. Compounds such as carbonyl cyanide *m*-chlorophenylhydrazone, tetrachlorosalicylanilide, and SF6847 are lipid soluble in both the dissociated and undissociated forms, and their action as uncouplers arises from their ability to conduct a futile cycle of protons through the cell membrane (17). Since acetate anion should not be able to pass through cell membranes, such a cycle should not occur.

For many years, it was assumed that bacteria maintained a near-neutral intracellular pH, but it is now evident that some bacteria can allow their intracellular pH to decrease as a function of extracellular pH and can maintain a relatively constant and low pH gradient across the cell membrane (10, 25). Although there are only a few examples, it appears that bacteria which maintain a near-neutral intracellular pH (*Lactococcus lactis* and *L. cremoris* [19], *E. coli* [20], *C. acetobutylicum* [7] are more sensitive to volatile fatty acids than are those which allow intracellular pH to decrease (lactobacilli [10], *Streptococcus bovis* [25]). Results presented here indicated that acetate does not act as an uncoupler in *S. bovis* and that a decrease in intracellular pH protects this bacterium from the inhibitory effects of acetate anion accumulation.

MATERIALS AND METHODS

Growth conditions. *S. bovis* JB1 (23) was grown anaerobically in basal medium containing (per liter): 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 1,200 mg of NH_4SO_4, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4,000 mg of Na_2CO_3, 0.6 g of cysteine hydrochloride, 0.5 g of yeast extract, and 1.0 g of Trypticase (BBL Laboratories, Cockeysville, Md.). Glucose, NaCl, and sodium acetate were prepared separately and added to the medium after autoclaving (final concentrations: 72, 100, and 100 mM, respectively). The pH of batch cultures (500 ml, continuously purged with 0_2-free carbon dioxide) was decreased by the addition of concentrated HCl. Growth rate was determined from the increase in optical density (600 nm, 1.0-cm cuvettes, Gilford 260 spectrophotometer). Continuous cultures were grown anaerobically with 4 mM glucose in a New Brunswick Scientific Co. F1000 fermentor that was modified to operate as a pH-controlled chemostat by the addition of concentrated HCl. Anaerobic NaCl or sodium acetate was added to the medium reservoir to achieve the desired concentration.

Proton motive force. Internal pH was determined by an acid distribution method (21). Growing cultures (0.5 to 0.8 mg of protein per ml) were incubated with [7-^{14}C]benzoate (1.0 μCi; 21.8 μCi/μmol), [2-^{14}C]acetate (10 μCi; 55 μCi/μmol), [U-^{14}C]taurine (1.0 μCi; 115 μCi/μmol), or ^3H_2O (4.0 μCi; 3.6 μCi/μmol) for 5 min and then centrifuged through silicon oil (mixture [equal parts] of Dexter Hysol 550 and Dexter Hysol 556; Hysol Co., Olean, N.Y.) in a microcentrifuge (13,000 × *g*, 5 min). Supernatant samples (20 μl) were removed, and bottoms of tubes containing cell pellets were removed with dog nail clippers after tubes were frozen.

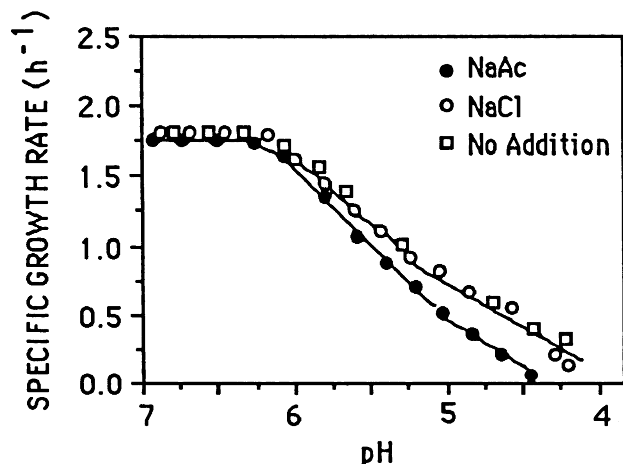

FIG. 1. Effect of pH on the growth rate of *S. bovis* in medium containing an additional 100 mM sodium acetate (NaAc) or 100 mM NaCl. A control without any added salt is also shown.

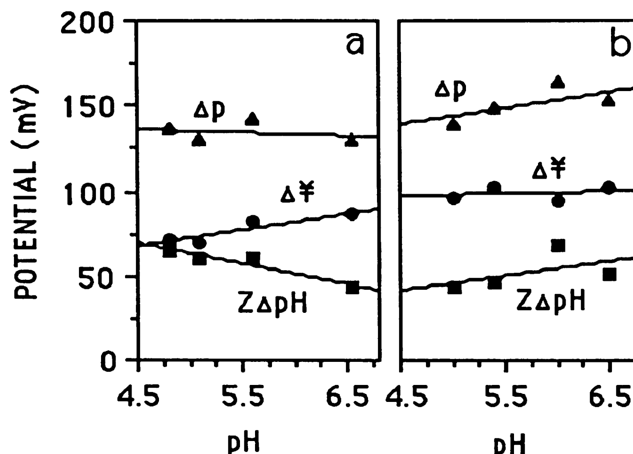

FIG. 2. Proton motive force of batch cultures of *S. bovis* in medium containing an additional 100 mM NaCl (a) or 100 mM sodium acetate (b).

Pellets and supernatants were dissolved in aqueous-compatible scintillation fluid. Internal volume (4.3 μl/mg of protein) was estimated from the difference between ^3H_2O and [^{14}C] taurine. The membrane potential (Δψ) was determined from the distribution of the lipophilic cation [phenyl-^{14}C]tetraphenylphosphonium bromide (TPP^+) (1.0 μCi; 30 μCi/μmol) according to the Nernst equation. Cells (0.19 mg of protein per ml) were incubated for 5 min with 0.5 μCi of TPP^+ and processed as described above. Nonspecific TPP^+ binding was estimated from cells which had been treated with 10 μM valinomycin plus 10 μM nigericin.

Other analyses. Glucose was analyzed by an enzymatic method using hexokinase and glucose-6-phosphate dehydrogenase (2). Protein from NaOH-hydrolyzed cells (0.2 N NaOH, 100°C, 15 min) was assayed by the method of Lowry et al. (13). The ratio of protein to optical density was 160 μg of protein per ml per optical density unit (1-cm cuvettes, 600 nm). Lactate, ethanol, and fermentation acids in cell-free supernatant samples were analyzed by high-pressure liquid chromatography with a Beckman 334 liquid chromatograph which was equipped with a model 156 refractive index detector and a Bio-Rad HPX-87H organic acid column (20-μl sample, 0.013 N H_2SO_4; flow rate, 0.5 ml/min, 50°C).

RESULTS

When *S. bovis* was grown anaerobically in a medium containing an additional 100 mM sodium acetate or 100 mM NaCl and the pH was decreased with concentrated HCl, there was no decline in growth rate until the extracellular pH was less than 6.0 (Fig. 1). As the pH decreased further, there was a nearly linear decline in growth rate. Cultures containing the sodium acetate were somewhat more sensitive to the decline in pH than those containing added NaCl, but this difference was not dramatic. Both cultures were able to grow at pHs less than 4.7.

At pH 6.7, the culture with an additional 100 mM NaCl had a transmembrane chemical gradient of protons (ZΔpH) of 45 mV, a Δψ of 85 mV, and a total proton motive force (Δp) of approximately 130 mV (Fig. 2a). As extracellular pH was decreased from 6.7 to 4.55, the cultures with added NaCl increased ZΔpH and decreased Δψ by approximately 25 mV, but the Δp remained relatively constant. Cultures receiving 100 mM sodium acetate had a slightly higher ZΔpH and Δp at pH 6.7, and there was little decrease in ZΔpH, Δψ, or Δp when the pH was decreased from 6.7 to 4.55 (Fig. 2b).

When sodium acetate or NaCl was added to glucose-limited continuous cultures (dilution rate, 0.1 h^{-1}; pH 5.2), sodium acetate caused a greater decrease in bacterial protein than did NaCl (data not shown). However, nearly all of this difference could be explained by a shift in fermentation end products and a decrease in ATP availability (Fig. 3a). Acetate, formate, and ethanol were initially the primary fermentation end products, but lactate eventually accumulated when more than 100 mM sodium acetate was added to the medium reservoir (Fig. 3b). Lactate was not detected in the continuous cultures which received added NaCl (data not shown).

Benzoate anion accumulates intracellularly in response to a ZΔpH, and this accumulation provided the basis for the intracellular pH determinations. When concentrated HCl was added to *S. bovis* cultures containing 100 mM sodium acetate, [^{14}C]acetate and [^{14}C]benzoate gave a similar estimate of intracellular pH (Fig. 4). These results indicated that both benzoate and acetate were equilibrating across the cell membrane in accordance with the Henderson-Hasselbalch equation. The cells had an approximately 10-fold concentration gradient of acetate anion across the cell membrane over the pH range 6.7 to 4.55 (Fig. 4b). Cells which were treated with nigericin and valinomycin did not accumulate [^{14}C] acetate (data not shown).

DISCUSSION

Mitchell (16) used the action of compounds such as dinitrophenol as evidence for the chemiosmotic model of energy coupling, and since this time the action of uncouplers has been studied in some detail (12). Uncouplers are highly lipid-soluble weak acids or bases, and these compounds act in a cyclic fashion to translocate either H^+ or OH^- across the cell membrane (Fig. 5). The protonated species travels across the cell membrane and releases a proton in response to pH gradient. The anion is driven to the external surface of the membrane by the electrical potential (negative inside). The anion is then protonated, and the cycle continues (17). Since the action of membrane-bound ATPases counteracts this futile cycle of protons through the membrane, the cell is

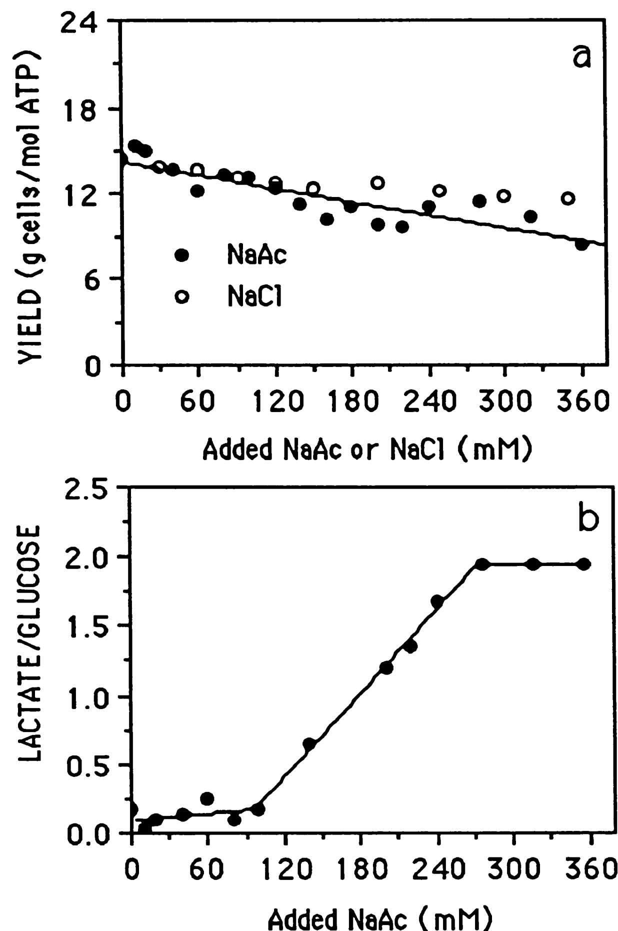

FIG. 3. (a) Effect of sodium acetate (NaAc) and NaCl addition on the growth *S. bovis* in continuous culture ($D = 0.1h^{-1}$) at a pH of 5.2. (b) Effect of sodium acetate addition on lactate production. Because *S. bovis* uses a phosphotransferase system or a facilitated diffusion mechanism to take up glucose (22), the conversion of glucose to lactate yields 2 ATP and acetate, formate, and ethanol production yields 3 ATP. Y_{ATP} was calculated on the basis of either 2 or 3 ATP per glucose.

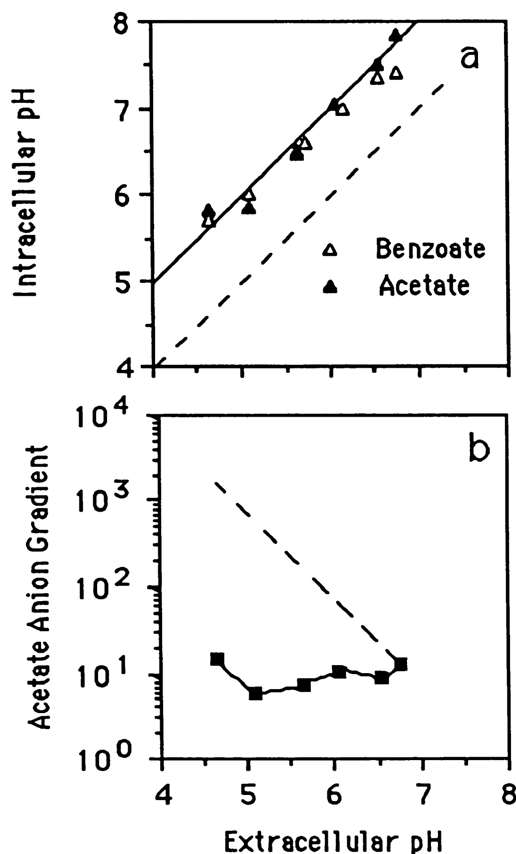

FIG. 4. (a) Intracellular pHs for *S. bovis* determined with radioactively labeled benzoate or acetate. (b) Concentration gradient of acetate anion across the cell membrane. The dashed line in panel b represents the concentration of acetate anion at a constant intracellular pH. In all cases, the extracellular acetate concentration was 100 mM.

eventually deenergized. Many of the synthetic uncouplers are effective at micromolar concentrations.

Phenolic acids such as benzoate and salicylate can permeate the cell membrane, and these acids accumulate when the intracellular environment is more alkaline than the exterior. The accumulation of benzoate or salicylate anion has provided a basis for intracellular pH determinations (9, 22). If these compounds acted as uncouplers, then both $Z\Delta pH$ and $\Delta\psi$ would be destroyed. Short-chain volatile fatty acids (e.g., acetate) are also lipid soluble when they are protonated, but the anions are lipophobic. The flux of undissociated acid into the cell and the release of protons in the more alkaline interior would dissipate $Z\Delta pH$, but this dissipation would be minor if the anion did not recross the cell to pick up another proton (no cycle).

S. bovis has an internal volume of 4.3 μl/mg of protein, and a 100 mM increase in intracellular acetate would release only 0.43 μmol of H^+ per mg of protein. Nongrowing, nitrogen-limited *S. bovis* cultures which were treated with the protonophore, tetrachlorosalicylanilide, fermented glucose at a rate of approximately 20 μmol/mg of protein per h, and under these conditions virtually all of the ATP utilization could be explained by the activity of the membrane-bound ATPase (26). Assuming that the ATPase has an H^+/ATP stoichiometry of 3 (4), the *S. bovis* cultures should have been able to pump as many as 120 H^+ per mg of protein per h. The observation that even high concentrations of acetate at pH 5.2 did not cause a significant decrease in Y_{ATP} supports the assumption that acetate-mediated H^+ influx and "uncoupling" were trivial (Fig. 3a).

When *S. bovis* was incubated with [^{14}C]acetate or [^{14}C] benzoate, the label equilibrated across the cell membrane according to its pK_a value and the Henderson-Hasselbalch equation (Fig. 4a). This result indicated that acetate anion accumulated intracellularly and that the cell did not have a means of expelling acetate anion. Böenigk et al. (3) recently presented evidence that *Acetobacterium woodii* has a carrier-mediated acetate transport system and that rapidly growing cells have an internal acetate concentration higher than the amount predicted from intracellular pH determinations. At this time it is not known whether *S. bovis* has a carrier protein for acetate, but the studies with [^{14}C]acetate provide convincing evidence that acetate anion is accumulated in response to a $Z\Delta pH$.

S. bovis accumulated a similar amount of [^{14}C]acetate over the pH range of 6.75 to 4.55 (Fig. 4b). This accumulation was consistent with the ability of the organism to

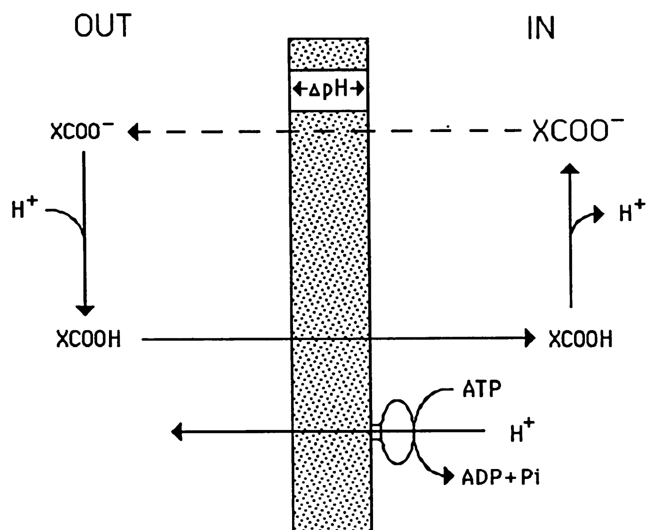

FIG. 5. Schematic representation of acetate movement in bacteria in response to a ZΔpH. The protonated species (XCOOH) should freely permeate the cell membrane, and the concentration inside and outside should be the same. If the anion ($XCOO^-$) cannot penetrate the cell membrane, it will accumulate inside the cell in response to the ZΔpH. In fermentative species such as *S. bovis*, intracellular protons are expelled by membrane-bound ATPases.

decrease its intracellular pH as a function of extracellular pH and to maintain a relatively constant pH gradient across the cell membrane (Fig. 4a). If the organism had been able to maintain a constant intracellular pH, the concentration gradient of acetate anion across the cell membrane would have been as much as 1,500-fold. At an external acetate concentration of 100 mM, the internal acetate concentration would have been more than 5 M!

When *S. bovis* was grown in glucose-limited chemostats at pH 5.2 the addition of greater than 100 mM acetate caused a shift from acetate, formate, and ethanol production to lactate production (Fig. 3b). The lactate dehydrogenase of *S. bovis* is regulated by the intracellular concentration of fructose 1,6 bisphosphate (28), and this effect can account for increased lactate production at rapid growth rates (25). At low pH, intracellular pH declines, the lactate dehydrogenase loses its requirement for fructose 1,6 bisphosphate, and lactate is produced even at slow growth rates (25). Since the growth rate was always slow and it did not appear that acetate was causing a decrease in intracellular pH, neither of these parameters could explain the increase in lactate (Fig. 3b). Since the cells concentrated acetate anion 10-fold (Fig. 4b), it is possible that end product inhibition may have limited the conversion of pyruvate to acetate. In *Acetogenium kivui* the pyruvate-ferridoxin oxidoreductase and acetate kinase were inhibited by 800 mM acetate (11). The effect of acetate on pyruvate-formate-lyase has not been examined.

The rumen always has high concentrations of volatile fatty acids (80 to 120 mM), but not all ruminal bacteria are able to grow at low pH. *Fibrobacter* (*Bacteroides*) *succinogenes* attempts to maintain a near-neutral intracellular pH as extracellular pH declines and is unable to take up cellobiose or grow at pHs less than 5.8 (21). *Bacteroides ruminicola* B_14, an amylolytic bacterium that produces the same fermentation products as *F. succinogenes*, grew at pH 5.1 (24). *B. ruminicola* lets its intracellular pH drop when extracellular pH declines (data not shown). These latter comparisons support the assumption that volatile fatty acid toxicity may be more closely correlated with intracellular pH regulation and anion accumulation than "uncoupling" per se.

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REFERENCES

1. **Baronofsky, J. J., W. J. A. Schreurs, and E. R. Kashket.** 1984. Uncoupling by acetic acid limits growth of and acetogenesis by *Clostridium thermoaceticum*. Appl. Environ. Microbiol. **48:** 1134–1139.
2. **Bergmeyer, H. U., and H. Klotsch.** 1965. Sucrose, p. 99–102. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis. Academic Press, Inc., New York.
3. **Böenigk, R., P. Durre, and G. Gottschalk.** 1989. Carrier-mediated acetate transport in *Acetobacterium woodii*. Arch. Microbiol. **152:**589–593.
4. **Harold, F. M.** 1986. The vital force: a study of bioenergetics, p. 105. W. H. Freeman and Co., New York.
5. **Herrero, A. A., R. F. Gomez, B. Snedecor, C. J. Tolman, and M. F. Roberts.** 1985. Growth inhibition of *Clostridium thermocellum* by carboxylic acids: a mechanism based on uncoupling by weak acids. App. Microbiol. Biotechnol. **22:**53–62.
6. **Hollowell, C. A., and M. J. Wolin.** 1965. Basis of the exclusion of *Escherichia coli* from the rumen ecosystem. Appl. Microbiol. **13:**918–924.
7. **Huang, L., L. N. Gibbins, and C. W. Forsberg.** 1985. Transmembrane pH gradient and membrane potential in *Clostridium acetobutylicum* during growth under acetogenic and solventogenic conditions. Appl. Environ. Microbiol. **50:**1043–1047.
8. **Hugenholtz, J., R. Splint, W. N. Konings, and H. Veldkamp.** 1987. Selection of protease-positive and protease-negative variants of *Streptococcus cremoris*. Appl. Environ. Microbiol. **53:**309–314.
9. **Kashket, E. R.** 1985. The proton motive force in bacteria: a critical assessment of methods. Annu. Rev. Microbiol. **39:**219–242.
10. **Kashket, E. R.** 1987. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiol. Rev. **46:**233–244.
11. **Klemps, R., S. Schoberth, and H. Sahm.** 1987. Production of acetic acid by *Acetogenium kivui*. Appl. Microbiol. Biotechnol. **27:**229–234.
12. **Krulwich, T. A., P. G. Quirk, and A. A. Guffanti.** 1990. Uncoupler-resistant mutants of bacteria. Microbiol. Rev. **54:**52–65.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
14. **Luli, G. W., and W. R. Strohl.** 1990. Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. Appl. Environ. Microbiol. **56:**1004–1011.
15. **Menzel, U., and G. Gottschalk.** 1985. The internal pH of *Acetobacterium wieringae* and *Acetobacter aceti* during growth and production of acetic acid. Arch. Microbiol. **143:**47–51.
16. **Mitchell, P.** 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature (London) **191:**144–148.
17. **Nicholls, D. G.** 1982. Bioenergetics, p. 32–33. Academic Press, Inc., New York.
18. **Otto, R.** 1981. Ph.D. Thesis, University of Groningen, Groningen, The Netherlands.
19. **Poolman, B., A. J. M. Driessen, and W. N. Konings.** 1987. Regulation of solute transport in streptococci by external and internal pH values. Microbiol. Rev. **51:**498–508.
20. **Ramos, S., and H. R. Kaback.** 1977. The electrochemical proton gradient in *Escherichia coli* membrane vesicles. Biochemistry **16:**848–854.
21. **Russell, J. B.** 1987. Effect of extracellular pH on growth and

proton motive force of *Bacteroides succinogenes*, a cellulolytic ruminal bacterium. Appl. Environ. Microbiol. **53:**2379–2383.

22. **Russell, J. B.** 1990. Low-affinity, high-capacity system of glucose transport in the ruminal bacterium *Streptococcus bovis*: evidence for a mechanism of facilitated diffusion. Appl. Environ. Microbiol. **56:**3304–3307.
23. **Russell, J. B., and R. L. Baldwin.** 1978. Substrate preferences in rumen bacteria: evidence of catabolite regulatory mechanisms. Appl. Environ. Microbiol. **36:**319–329.
24. **Russell, J. B., and D. B. Dombrowski.** 1980. Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture. Appl. Environ. Microbiol. **39:**604–610.
25. **Russell, J. B., and T. Hino.** 1985. Regulation of lactate production in *Streptococcus bovis*: a spiraling effect that leads to rumen acidosis. J. Dairy Sci. **68:**1712–1721.
26. **Russell, J. B., and H. J. Strobel.** 1990. ATPase dependent energy spilling by the ruminal bacterium, *Streptococcus bovis*. Arch. Microbiol. **153:**378–383.
27. **Wallace, R. J., M. L. Falconer, and P. K. Bhargava.** 1989. Toxicity of volatile fatty acids at rumen pH prevents enrichment of *Escherichia coli* by sorbitol in rumen contents. Curr. Microbiol. **19:**277–281.
28. **Wolin, M. J.** 1964. Fructose 1,6 diphosphate requirement of streptococcal lactic dehydrogenases. Science **146:**775–777.