APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1994, p. 1827–1831
0099-2240/94/$04.00+0

Vol. 60, No. 6

Substrate Preference in a Strain of *Megasphaera elsdenii*, a Ruminal Bacterium, and Its Implications in Propionate Production and Growth Competition

TSUNEO HINO,* KIMIOMI SHIMADA, AND TAKESHI MARUYAMA

Department of Agriculture, Meiji University, Higashimita, Tama-ku, Kawasaki 214, Japan

Received 17 September 1993/Accepted 5 March 1994

The NIAH 1102 strain of *Megasphaera elsdenii* utilized lactate in preference to glucose when the two substrates were present. Even when lactate was supplied to cells fermenting glucose, the cells switched substrate utilization from glucose to lactate and did not utilize glucose until lactate decreased to a low concentration (1 to 2 mM). Since substrate utilization was shifted gradually without intermittence, typical diauxic growth was not seen. The cyclic AMP content did not rise markedly with the shift in substrate utilization, suggesting that this nucleotide is not involved in the regulation of the shift. It was unlikely that propionate was produced from glucose, which was explicable by the fact that lactate racemase activity dropped rapidly with the exhaustion of lactate and cells actively fermenting glucose did not possess this enzyme. A coculture experiment indicated that *M. elsdenii* NIAH 1102 is overcome by *Streptococcus bovis* JB1 in the competition for glucose, mainly because *M. elsdenii* NIAH 1102 is obliged to utilize lactate produced by *S. bovis* JB1; i.e., glucose utilization by *M. elsdenii* NIAH 1102 is suppressed by the coexistence of *S. bovis* JB1.

Megasphaera elsdenii, an important propionate producer among rumen bacteria, produces propionate from lactate but not from glucose (7). We reported previously that this is primarily because cells fermenting glucose do not possess lactate racemase (LR), which was suggested to be induced by lactate (3).

Can *M. elsdenii* form propionate from glucose if LR is induced by lactate? To answer this question in a simple way, we supplied cultures with lactate and glucose simultaneously. As described below, however, the strain used in our study utilized lactate first and glucose was not fermented until lactate decreased to a low concentration. Even when both the substrates were fermented simultaneously at low lactate concentrations, propionate production corresponded exactly to lactate utilization. The utilization and fermentation of substrates appeared to be strictly regulated by complex mechanisms.

Therefore, we examined the shift in substrate utilization in detail and measured the activity of LR and NAD-independent D-lactate dehydrogenase (iD-LDH) in relation to the shift. We also determined the cyclic AMP (cAMP) content, because we considered the possibility that catabolite repression involving cAMP plays a role in the control of substrate utilization. In addition, to examine the ecological aspect of substrate preference, *M. elsdenii* was cocultured with *Streptococcus bovis* that produces lactate.

MATERIALS AND METHODS

Organisms and culture conditions. The NIAH 1102 strain of *M. elsdenii* (4) was grown by the procedure reported previously (4). The growth medium was essentially the same as that described previously (3) except that 10 mM acetate was added in order to stimulate initial growth (4). As energy substrates, glucose and/or DL-lactate were used, because D- and L-lactate supported similar growth (4). Hereafter, lactate refers to the DL form when it was added to media.

The JB1 strain of *S. bovis* (5) was cultured similarly in the glucose medium used for *M. elsdenii*. Culture incubation was performed in triplicate in all experiments.

Determination of enzyme activity. Cells were grown in a large scale (1 liter), and depending on cell density (A_{600}), 20 to 60 ml of the culture was harvested every 30 min during the period from h 4 to 12. Cells collected by centrifugation (10,000 × *g*, 5 min, 4°C) were disrupted by ultrasonication as described previously (3). The supernatant from centrifugation (15,000 × *g*, 10 min, 4°C) was used for enzyme assay. The activity of iD-LDH was measured by determining the reduction of ferricyanide by A_{420} (3). Since *M. elsdenii* NIAH 1102 always has iD-LDH (3), LR activity was measured by linkage to the iD-LDH reaction; i.e., L-lactate was used as a substrate, and the reduction of dichlorophenol indophenol was monitored by A_{600} (3). The assay for enzyme activity was conducted in duplicate for each sample; i.e., mean values were obtained by six determinations.

Quantitation of intracellular cAMP. A relatively large amount of cells was inoculated, and samples (1 to 2 ml, depending on cell density) were taken with syringes every 30 min. The samples, infused promptly into Eppendorf tubes, were cooled in an ice bath and then centrifuged at 10,000 × *g* for 5 min (4°C). After removal of the supernatant, 100 μl of cold 0.2 N HCl was added, and the mixture was vortexed vigorously and kept at 0°C for 30 min (12). Subsequently, 20 μl of 1 N KOH and 50 μl of potassium phosphate buffer (1 M, pH 7.0) were added, and the mixture was centrifuged at 5,000 × *g* for 5 min (0°C). The supernatant was assayed for cAMP with an enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, Mich.) according to the supplier's instructions. The assay was conducted in duplicate for each sample.

Quantitation of lactate, VFA, glucose, and protein. For the quantitation of lactate, volatile fatty acids (VFA), and glucose, samples were deproteinized by addition of sulfosalicylic acid (final concentration, 10%) followed by centrifugation at 10,000 × *g* for 10 min.

Lactate and VFA were measured by high-performance liquid chromatography (HPLC) using an apparatus equipped with an ion-exchange column (Ionpak KC-811) and UV detec-

* Corresponding author.

tor (Japan Spectroscopic Co., ltd.; model 800). The column was kept at 60°C, and 3 mM $HClO_4$ was allowed to flow at 1 ml/min as a mobile phase. The eluate from the column was combined with a solution containing 125 mg of bromthymol blue per liter in 15 mM Na_2HPO_4 infused at 1.5 ml/min. Organic acids were then measured by determining the A_{430}. Glucose was also assayed by HPLC in which the column and detector were replaced by Ionpak KS-801 and a refractive index detector (model 830-RI), respectively. As an eluent, distilled water was used at a flow rate of 1 ml/min.

The protein content in cell extracts was measured by using Coomassie brilliant blue (1), and enzyme activity was expressed per unit of protein. Assuming that the amount of protein in a cell extract reflects the amount of cells broken, this expression would give an approximate estimate for the amount of enzyme per cell mass. To estimate the cAMP content per cell mass, cellular protein was determined as follows. Samples (1 ml) withdrawn from cultures were centrifuged at 10,000 × *g* for 5 min, and the pellet was boiled for 15 min in 0.1 N NaOH. After recentrifugation, the supernatant was assayed for protein by the method of Lowry et al. (6).

Counting of bacterial cells. In a coculture experiment, the ratios of *M. elsdenii* to *S. bovis* in cell number were estimated as follows. After Gram staining, the two species were separately counted by microscopy. Counting was performed five times for each sample by moving the slide glass. On the basis of the ratios of the two species, the A_{600} values actually measured were proportionately divided and assigned to each species. Cells of *M. elsdenii* are larger than those of *S. bovis*, but suspensions with an equal cell count did not give great differences in A_{600}.

RESULTS

Growth and fermentation products. The energy-limited cultures of *M. elsdenii* indicated that 8 mM glucose was roughly equivalent to 40 mM lactate to support growth: the growth rate was slightly higher with 40 mM lactate, and final growth (A_{600}) was slightly higher with 8 mM glucose, but the differences were statistically insignificant (data not shown). The amounts of VFA produced per liter culture in 12 h were 6.8 mmol of acetate and 4.3 mmol of butyrate from 8 mmol of glucose and 13.5 mmol of acetate, 8.4 mmol of propionate, and 8.5 mmol of butyrate from 40 mmol of lactate. Negligible amounts of caproate and other VFA were produced in each case.

Substrate preference. When both glucose and lactate were provided for growth, *M. elsdenii* utilized lactate in preference to glucose and glucose utilization began after lactate had decreased to 1 to 2 mM (Fig. 1B). While the substrate was switched from lactate to glucose, growth was obviously decelerated (Fig. 1A). It appears that the rate of energy supply to cells decreases when cells are preparing for the utilization of another substrate. However, growth did not stop, because glucose utilization was initiated before the complete exhaustion of lactate. Apparently, the growth observed in this experiment is not typical diauxic growth. Propionate production was roughly parallel to lactate utilization (Fig. 1A): even though propionate was produced from glucose at low lactate concentrations, the amount must have been negligible. The pattern of VFA production reflected the substrates utilized: the composition of VFA at each time, even during the shift in substrate utilization, was exactly as expected from the amounts of lactate and glucose utilized (Fig. 1).

Figure 2A shows the growth when glucose was added to cultures grown on lactate. Regardless of whether glucose was added with a relatively high or low concentration of lactate

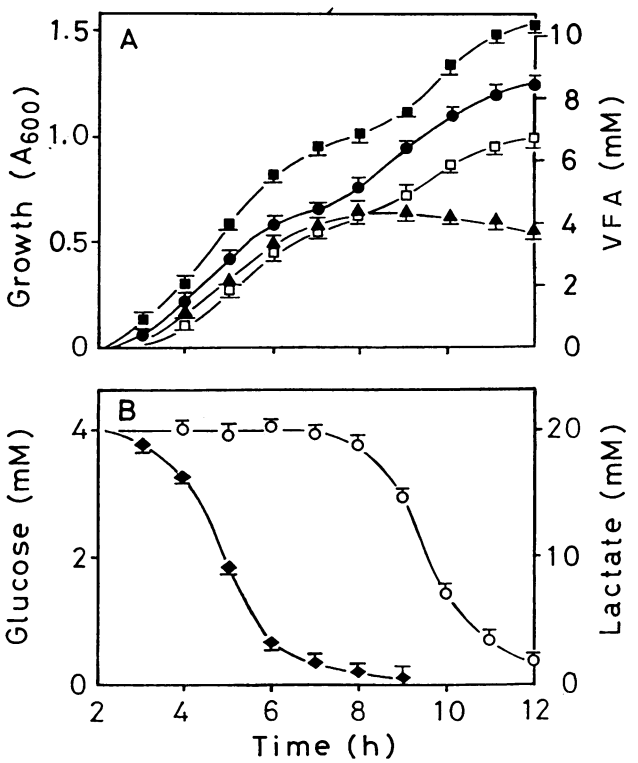

FIG. 1. Utilization by *M. elsdenii* of glucose (4 mM) and lactate (20 mM) given simultaneously. ●, growth; ■, acetate; ▲, propionate; □, butyrate; ○, glucose; ◆, lactate. Vertical bars indicate standard errors (n = 3).

remaining, growth was similar (Fig. 2A, a and b), confirming that glucose utilization began at a low concentration of lactate. When glucose was added after lactate was completely exhausted (growth ceased), it took a longer time to resume growth, i.e., to initiate glucose utilization (Fig. 2A, c). Even when glucose was added to the cultures in which a low concentration of lactate remained, the amount of propionate produced could be accounted for by the amount of lactate utilized (Fig. 2B, b). Again, it appeared that propionate was not formed from glucose, even when LR is considered to be present. This suggests that the equilibrium for the iD-LDH reaction strongly favors pyruvate formation (3).

Figure 3B shows the utilization of substrates when lactate was added to cultures fermenting glucose. Lactate utilization was initiated between 1 and 2 h after the addition of lactate, and glucose utilization was suppressed while lactate was actively utilized. The resumption of glucose utilization after the consumption of lactate was essentially the same as described above. The growth rate decreased during the shifts both from glucose to lactate and from lactate to glucose (Fig. 3A).

Change in iD-LDH and LR activity with the shift in substrate utilization. Figure 4 shows the change in activity of LR and iD-LDH in the cells grown by switching the substrates from glucose to lactate and then to glucose again. The addition of lactate induced the synthesis of LR in 1 to 2 h, and LR activity reached the maximum rapidly. Then the activity dropped abruptly as the lactate concentration declined and disappeared with the exhaustion of lactate.

On the other hand, *M. elsdenii* had iD-LDH activity before lactate was added, and the activity was nearly doubled after the

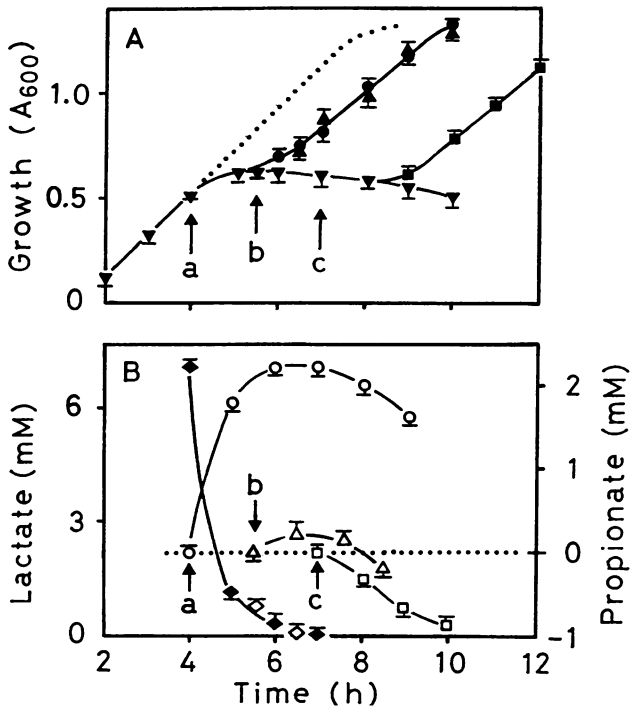

FIG. 2. Growth and propionate production in the cultures of *M. elsdenii* given lactate initially and glucose subsequently. (A) Growth when 4 mM glucose was added to cultures growing on 20 mM lactate at arrows a (●), b (▲), and c (■) and not added (▼). The dotted line indicates the growth when 20 mM lactate was added at arrow a. (B) Propionate production after glucose was added at arrows a (○), b (△), and c (□) (the propionate concentration at each arrow is expressed as 0 mM); lactate utilization after glucose addition at arrows a (◆) and b (◇).

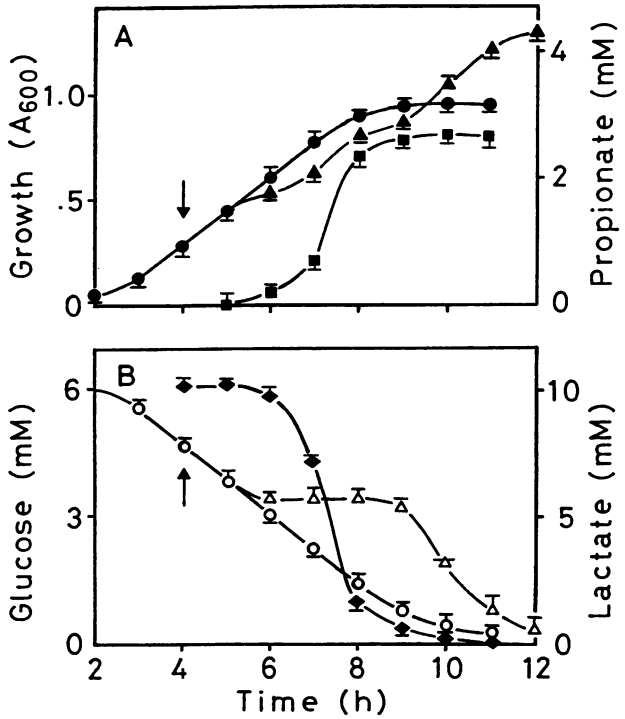

FIG. 3. Lactate utilization by *M. elsdenii* growing on glucose. *M. elsdenii* was grown on 6 mM glucose, and 10 mM lactate was added after 4 h. (A) Growth with (▲) or without (●) the addition of lactate and propionate production (■) when lactate was added; (B) glucose utilization when lactate was added (△) or not added (○) and lactate utilization (◆) when lactate was added.

addition of lactate. The enzyme decreased slowly to a basal level as lactate was consumed. The activity of iD-LDH appears to be retained at the basal level in the absence of lactate. These results are consistent with our previous observations that cells grown on glucose had approximately half as much iD-LDH activity as those grown on lactate (3). The present data support our previous speculation that LR and iD-LDH are inducible and constitutive, respectively (3).

Intracellular cAMP levels. The levels of cAMP were measured in cells when substrate was shifted from glucose to lactate and then to glucose. As shown in Fig. 5, cAMP content increased slightly during the shift in substrate utilization, but a much greater increase was seen when growth ceased. The increase in cAMP content appeared to be coincident with the deceleration of growth. When glucose or lactate was supplemented after the complete exhaustion of glucose, the cAMP content rose to approximately four times the basal level at the cessation of growth (data not shown). These results indicate that the rise in cAMP content is associated mainly with the decline of growth rate, not the shift in substrate utilization.

Coculture of *M. elsdenii* with *S. bovis*. In monocultures, *S. bovis* grew faster than *M. elsdenii* on glucose but the final cell concentration was lower for *S. bovis* than for *M. elsdenii* (Fig. 6A). This is because *S. bovis* produced much lactate, which means a lower ATP yield per glucose. When *S. bovis* and *M. elsdenii* were cocultured by simultaneous inoculation, *S. bovis* grew faster than *M. elsdenii* and the final cell concentration of the former was higher than that of the latter (Fig. 6B). Propionate production indicated that *M. elsdenii* utilized lactate produced by *S. bovis* during the period later than 3 to 4 h. The growth of *S. bovis* suggested that most of the glucose was utilized by *S. bovis*, and *M. elsdenii* utilized little glucose while lactate was available. This implies that the growth of *M. elsdenii* was greatly affected by lactate produced by *S. bovis*.

When *S. bovis* was inoculated 2 h after the inoculation of *M. elsdenii*, *M. elsdenii* obviously utilized glucose until lactate was accumulated to a certain concentration, presumably 1 to 2 mM (Fig. 6C). Thereafter, the growth of *M. elsdenii* appeared to be dependent largely on lactate produced by *S. bovis*. As a result, *M. elsdenii* was overtaken by *S. bovis*.

DISCUSSION

The NIAH 1102 strain of *M. elsdenii* preferred lactate to glucose as a substrate. In this respect, a wide variety of strains have been reported. Marounek et al. (7) reported that some strains of *M. elsdenii* utilized lactate earlier than glucose, whereas in other strains such a preference was not distinctly seen: lactate utilization lasted as long as growth continued, although a decrease in lactate concentration appears to have accelerated the utilization of glucose. Apparently, the strain used in our study showed a more distinct preference in substrate utilization than the strains used by Marounek et al. On the other hand, the strain used by Russell and Baldwin utilized lactate, glucose, and maltose simultaneously (8).

It is rather strange that some strains of *M. elsdenii* prefer lactate to glucose, because lactate provides much less ATP than glucose, not only on a molar basis but also on a weight basis. However, growth rates did not differ greatly, irrespective

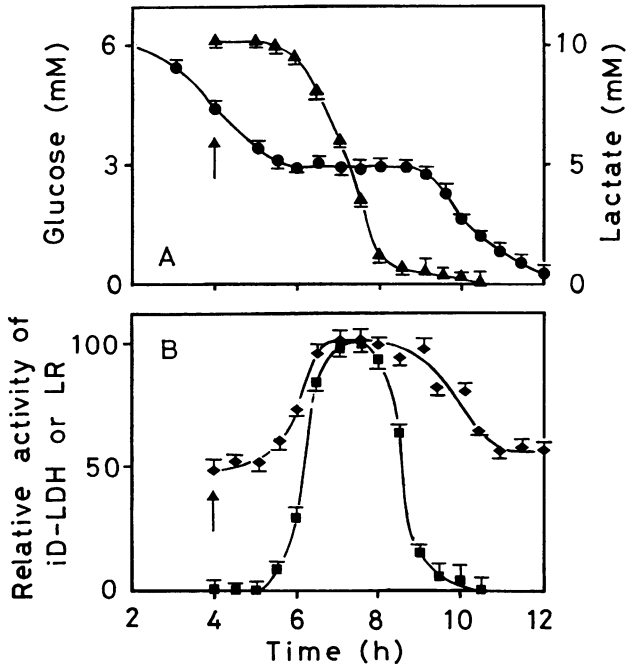

FIG. 4. Activity of iD-LDH and LR in *M. elsdenii* fermenting glucose or lactate. Cells were grown on 6 mM glucose, and 10 mM lactate was added after 4 h (arrows). The activity of iD-LDH (◆) and LR (■) is expressed as relative values based on the change in absorbance per minute per milligram of protein. The utilization of glucose (●) and lactate (▲) also is shown.

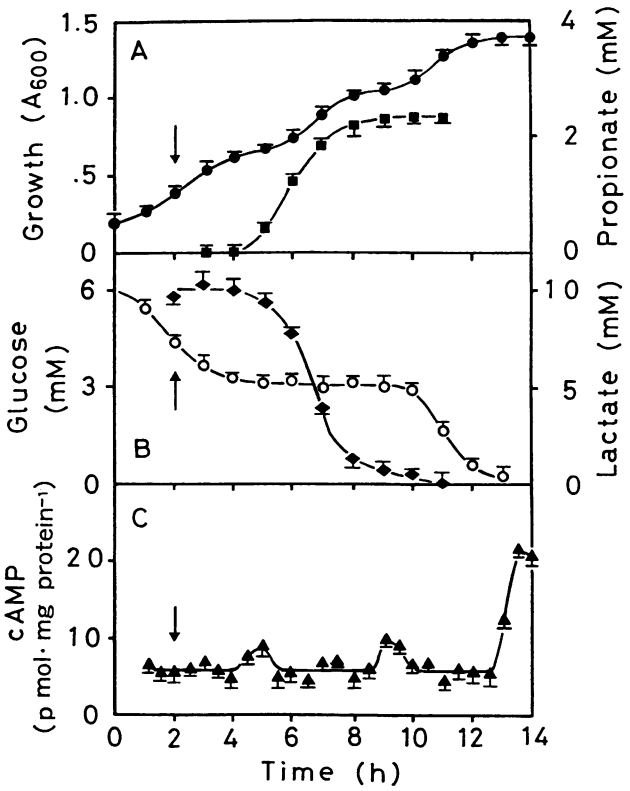

FIG. 5. cAMP levels in *M. elsdenii* shifting substrate utilization. Cells were inoculated on a glucose (6 mM) medium, and lactate (10 mM) was added after 2 h (arrows). ●, growth; ■, propionate; ○, glucose; ◆, lactate.

of whether lactate or glucose was utilized (Fig. 1). It can be inferred that lactate was fermented five to six times faster than glucose, because five to six times as much lactate as glucose on a molar basis was needed for similar growth (3). Theoretical values of ATP generated in *M. elsdenii* by the fermentation of energy substrates can be calculated from VFA produced. Because the amounts of branched-chain VFA were negligible and little propionate was produced in cultures with glucose, the fermentation of amino acids must have been negligible. Assuming that ATP is generated in the reactions from pyruvate to acetate and to butyrate (1 mol of ATP per mol of acetate or butyrate formed) (10, 11), the total amounts of ATP generated (± standard error) should have been 26.5 ± 1.2 mmol from 8 mmol of glucose and 22.0 ± 1.7 mmol from 40 mmol of lactate. This difference is statistically insignificant. Therefore, it is unlikely that lactate is energetically more efficient than glucose, although energy expenditure for the transport of glucose and lactate is not known sufficiently. Presumably, lactate is a more readily utilizable substrate for *M. elsdenii* NIAH 1102 because of the uptake system and metabolic pathway.

It is unlikely that *M. elsdenii* NIAH 1102 produces propionate from glucose. This is probably because glucose is not readily utilized when cells are utilizing lactate at high rates and LR activity drops rapidly in response to the decrease in lactate concentration. In other words, glucose is not fermented at high rates until LR activity is substantially lost. This may mean that intracellular pyruvate does not accumulate to high concentrations when cells have significant LR activity. Since the equilibrium position of the iD-LDH reaction is considered to favor pyruvate formation (3), this situation is unfavorable for the flow from glucose to propionate. Thus, *M. elsdenii* appears unable to produce propionate from glucose, at least as far as the strain used in our study is concerned.

The cAMP content in cells sharply rose as growth stopped. A similar trend was observed for *Escherichia coli* by Buettner et al. (2). A short supply of energy may trigger the rise in cAMP. In this context, Buettner et al. have shown that cultures utilizing a source of carbon that supported growth relatively poorly had consistently higher concentrations of cAMP than did cultures utilizing sugars that supported rapid growth (2). In *M. elsdenii* NIAH 1102, cAMP levels were similar irrespective of whether glucose or lactate was utilized, possibly because the two substrates supported similar growth rates.

The failure of the level of cAMP to rise markedly with the shift in substrate utilization may be because the change occurred gradually without intermittence and the energy supply did not stop even temporarily. The results of cAMP content measurements exclude the possibility that regulation of the shift in substrate utilization in *M. elsdenii* NIAH 1102 is mediated through a mechanism involving cAMP. Possibly, a more complicated mechanism is involved in the regulation of transcription of DNA. Since substrates are changed without an accompanying complete intermittence of growth, *M. elsdenii* NIAH 1102 may have more developed machinery to keep optimal growth.

In coculture, *M. elsdenii* was overcome by *S. bovis* in the competition for glucose. As a result, *S. bovis* grew faster than *M. elsdenii*, even when *S. bovis* was inoculated later. Russell et al. have reported that the ratio of the two species in continuous culture could be explained by their relative affinities for substrate, although maltose was used as a substrate in their study (9). In our case, however, a difference in the maximum

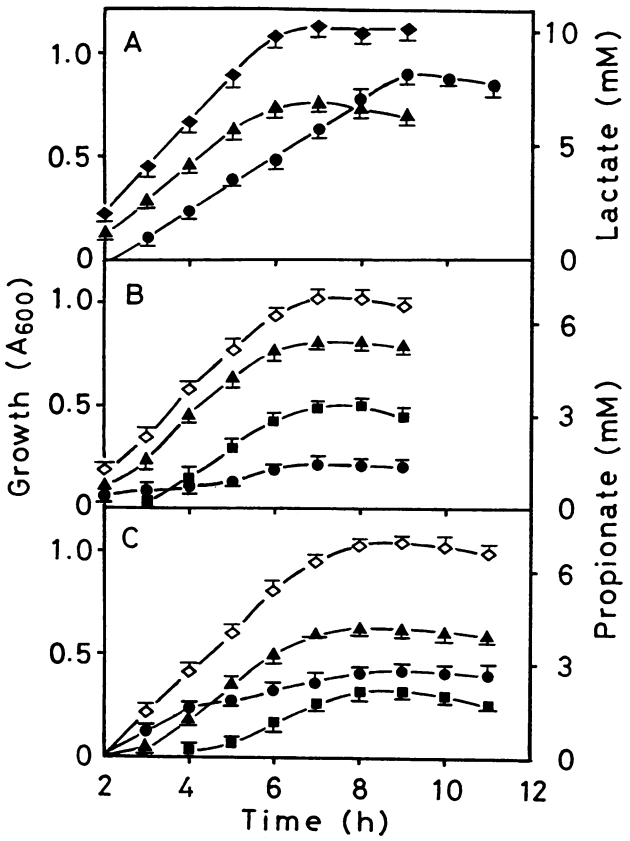

FIG. 6. Coculture of *M. elsdenii* and *S. bovis*: utilization by *M. elsdenii* of lactate produced by *S. bovis*. (A) Monoculture; (B) coculture in which the two species were inoculated simultaneously; (C) coculture in which *S. bovis* was inoculated 2 h after the inoculation of *M. elsdenii*. Shown are the growth of *M. elsdenii* (●) and *S. bovis* (▲) on 6 mM glucose, the sum of the growth of the two species (◇), lactate in *S. bovis* culture (◆), and propionate in coculture (■).

rate of growth or glucose utilization rather than the affinity for glucose should have been critically important at least during the initial period of incubation, because sufficient levels of glucose were supplied in batch culture. That the growth of *M. elsdenii* decelerated during the shift from glucose to lactate (Fig. 3) may be one of the reasons why the growth of *M. elsdenii* was delayed in coculture (Fig. 6). Thereafter, the rate of lactate supply by *S. bovis* may have retarded the growth of *M. elsdenii*. It appears that *M. elsdenii* NIAH 1102 is unable to utilize glucose at high rates when lactate is present even at low concentrations.

Thus, in the competition with *S. bovis* for glucose, *M. elsdenii* has a disadvantage: that it utilizes lactate in preference to glucose. However, these conclusions may be restricted to the strain used in the present study. As described above, there must be a variety of strains in the rumen that would behave in different manners. In the usual rumen, glucose concentration is considered to be quite low. In such conditions, affinity to substrate must have greater significance, as pointed out by Russell et al. (9).

In conclusion, *M. elsdenii* is ecologically important in the rumen in that it utilizes lactate to produce propionate, as emphasized previously (3).

ACKNOWLEDGMENT

This work was supported in a part by a grant-in-aid (Bio-Cosmos Program) from the Ministry of Agriculture, Forestry, and Fisheries.

REFERENCES

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Chem. **72:**248–254.
2. **Buettner, M. J., E. Spitz, and H. V. Rickenberg.** 1973. Cyclic adenosine 3′,5′-monophosphate in *Escherichia coli*. J. Bacteriol. **114:**1068–1073.
3. **Hino, T., and S. Kuroda.** 1993. Presence of lactate dehydrogenase and lactate racemase in *Megasphaera elsdenii* grown on glucose or lactate. Appl. Environ. Microbiol. **59:**255–259.
4. **Hino, T., K. Miyazaki, and S. Kuroda.** 1991. Role of extracellular acetate in the fermentation of glucose by a ruminal bacterium, *Megasphaera elsdenii*. J. Gen. Appl. Microbiol. **37:**121–129.
5. **Hino, T., H. Mukunoki, K. Imanishi, and K. Miyazaki.** 1992. Necessity of ready electron disposal and interspecies hydrogen transfer for the utilization of ethanol by rumen bacteria. Asian Australas. J. Anim. Sci. **5:**511–517.
6. **Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with phenol reagent. J. Biol. Chem. **193:**265–275.
7. **Marounek, M., K. Fliegrova, and S. Bartos.** 1989. Metabolism and some characteristics of ruminal strains of *Megasphaera elsdenii*. Appl. Environ. Microbiol. **55:**1570–1573.
8. **Russell, J. B., and R. L. Baldwin.** 1978. Substrate preferences in rumen bacteria: evidence of catabolite regulatory mechanisms. Appl. Environ. Microbiol. **36:**319–329.
9. **Russell, J. B., M. A. Cotta, and D. B. Dombrowski.** 1981. Rumen bacterial competition in continuous culture: *Streptococcus bovis* versus *Megasphaera elsdenii*. Appl. Environ. Microbiol. **41:**1394–1399.
10. **Russell, J. B., and R. J. Wallace.** 1988. Energy yielding and consuming reactions, p. 185–215. *In* P. N. Hobson (ed.), The rumen microbial ecosystem. Elsevier Science Publishers Ltd., Essex, England.
11. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41:**100–180.
12. **Unden, G., and A. Duchene.** 1987. On the role of cyclic AMP and Fnr protein in *Escherichia coli* growing anaerobically. Arch. Microbiol. **147:**195–200.