Multiple lactate dehydrogenase activities of the rumen bacterium *Selenomonas ruminantium*

Martin Gilmour,¹ Harry J. Flint² and Wilfrid J. Mitchell¹

Author for correspondence: Wilfrid J. Mitchell. Tel: +44 31 451 3459. Fax: +44 31 451 3009.
e-mail: BBSWJM@CLUST.HW.AC.UK

¹ Department of Biological Sciences, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS, UK

² Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, UK

The lactate utilizing strain of *Selenomonas ruminantium* 5934e was found to contain three lactate dehydrogenase (LDH) activities in sonicated cell extracts. One activity, an NAD dependent L-LDH (L-nLDH) was measured at 15-fold greater levels in extracts of cells grown to mid-exponential phase on glucose compared to cells grown to the equivalent growth stage on DL-lactate. A second nLDH activity specific for D-lactate (D-nLDH) was detected at similar levels in both lactate-grown cell extracts and glucose-grown cell extracts. The third activity, an NAD independent D-LDH (D-iLDH) was very low in cells grown on glucose but was induced more than 10-fold when DL-lactate was used as the carbon source. The three LDH activities could be separated by gel filtration. Recovery of the activities was low due to the apparent instability of the enzymes at 4 °C, which was most pronounced in the case of the D-iLDH. A K_m for lactate of 0.5 mM was estimated for the D-iLDH and this was considerably lower than the values of 45 mM and 70 mM measured for L-nLDH and D-nLDH respectively. It is proposed that the D-iLDH may be largely responsible for the formation of pyruvate in lactate-grown cells of *S. ruminantium* strain 5934e. Three other lactate utilizing strains of *S. ruminantium*, HD4, 5521C1 and JW13 exhibited a similar profile of LDH activities to strain 5934e when grown on glucose and DL-lactate.

Keywords: *Selenomonas ruminantium*, lactate dehydrogenases, rumen, anaerobe

INTRODUCTION

Selenomonas ruminantium is an abundant member of the rumen community that utilizes a wide range of soluble substrates for growth (Hungate, 1966) including glucose which is metabolized mainly to lactate, propionate, acetate and CO₂ (Bryant, 1956). Furthermore, hydrogen can be consumed by *S. ruminantium* (Henderson, 1980) and can be produced in co-cultures with methanogenic bacteria (Scheifinger *et al.*, 1975b). Previous work concerning the metabolism of *S. ruminantium* has concentrated on glucose catabolism and the regulation of pathways leading to lactate, propionate and acetate, and from such studies most of the enzymes responsible for production of these acids have been demonstrated (Melville *et al.*, 1988a). Approximately 50% of isolated strains can also ferment

lactate (Flint & Bisset, 1990) producing propionate, acetate and CO₂. Little is known about the enzymes involved although the production of propionate from lactate has been shown to proceed via the 'succinate' pathway in studies using DL-[2-¹⁴C]lactate (Paynter & Elsdon, 1970). Of the other major lactate utilizing bacteria in the rumen, *Veillonella alcalescens* also produces propionate via the 'succinate' pathway (Johns, 1951), while *Megasphaera elsdenii* utilizes the 'acrylate' pathway (Baldwin *et al.*, 1965).

During rapid growth of *S. ruminantium* on glucose, lactate is the major product of fermentation, while at lower growth rates acetate and propionate become the main products (Hishinuma *et al.*, 1968; Scheifinger *et al.*, 1975a; Melville *et al.*, 1987). This difference appears to be due, at least in part, to homotropic activation of an NAD-dependent lactate dehydrogenase (nLDH) by its substrate, pyruvate (Wallace, 1978) and a higher V_{max} of the nLDH at higher growth rates may also be important (Melville *et al.*, 1988b).

Abbreviations: APAD, 3-acetylpyridine adenine dinucleotide; DCPIP, 2,6-dichlorophenol indophenol; LDH, lactate dehydrogenase; nLDH, NAD-dependent LDH; iLDH, NAD-independent LDH; PMS, phenazine methosulphate.

A recent study on the electrophoretic mobility of enzymes in 36 *S. ruminantium* strains detected four mobility variants of nLDH (Flint & Bisset, 1990), hinting at considerable genetic diversity among strains and the possibility of the existence of different LDH activities with different functions in the cell. An NAD-dependent LDH specific for L-lactate (L-nLDH) has been detected in some strains (Joyner & Baldwin, 1966; Scheifinger *et al.*, 1975a; Melville *et al.*, 1987, 1988a), whereas in WPL 151/1 the major LDH was NAD-dependent but specific for D-lactate (D-nLDH). In other bacteria, nLDH enzymes appear to be involved in lactate formation from pyruvate, rather than lactate oxidation *in vivo* (Garvie, 1980). The conversion of lactate to pyruvate is associated with a different, NAD-independent lactate dehydrogenase (iLDH). iLDH may be membrane-bound and linked to electron transport (Kemp, 1972; Kohn & Kaback, 1973), although in some bacteria which can grow on lactate soluble iLDHs have been reported (Molinari & Lara, 1960; Wittenberger & Haaf, 1966; Garvie, 1980). Among rumen bacteria, *M. elsdenii* possesses a constitutive D-iLDH involved in lactate oxidation (Brockman & Wood, 1975; Hino & Kuroda, 1993), and the lactate fermenter *Propionibacterium acnes* exhibits iLDH activity (Counotte *et al.*, 1980); on the other hand, the lactate producing strains *Peptostreptococcus productus* and *Actinomyces viscosus*, which do not ferment lactate, possess nLDH activity only (Counotte *et al.*, 1980).

Little work has been done to determine whether a specific LDH activity is responsible for lactate oxidation in *S. ruminantium*. The fact that L-nLDH has a far greater affinity for pyruvate than for lactate (Scheifinger *et al.*, 1975a; Melville *et al.*, 1988a) suggested that another enzyme would be responsible for lactate utilization. The purpose of this study was to investigate in detail the LDH activities present in *S. ruminantium* when utilizing lactate as compared to glucose as an energy source in an effort to establish the mechanism of lactate assimilation. The lactate utilizing strain *S. ruminantium* 5934e (Flint & Bisset, 1990) was used because in our laboratory this strain grows much more efficiently on lactate in batch culture than the commonly studied strain HD4. The results show the existence of at least three distinct LDH activities in *S. ruminantium* 5934e which differ in their cofactor requirements, substrate affinities, stabilities and relative activities in lactate and glucose grown cells.

METHODS

Organisms. *Selenomonas ruminantium* 5934e, 5521C1, JW13 and FB322 were isolated from rumen fluid at the Rowett Research Institute (Wallace & Brammall, 1985; Flint *et al.*, 1988; Flint & Bisset, 1990). Strain HD4 was originally isolated by Bryant (1956), strain D by John *et al.* (1974), and strain GA192 by Bryant (1984).

Anaerobic media and culture conditions. Anaerobic methods followed those of Bryant (1972). Cultures were maintained in basal M2 medium + 0.2% (w/v) glucose, starch and cellobiose. This medium was similar to medium 2 of Hobson (1969) but with 30 ml clarified rumen fluid and 0.1 g cysteine.HCl added per 100 ml medium. Stock cultures were stored at -20 °C on

this medium containing 0.75% agar. Basal M2 refers to the above medium with no added energy source. Basal M2 was supplemented with 0.5% (w/v) glucose or 2% D,L-lactate for the growth of cells in certain experiments (see Results). S-medium was identical to basal M2 except with no rumen fluid and 0.03 ml n-valerate added per 100 ml.

D-medium contained (per 100 ml final volume): Bacto-Casitone, 1 g; mineral solution (a) (Hobson, 1969), 15 ml; mineral solution (b) (Hobson, 1969) lacking (NH₄)₂SO₄, 15 ml; trace metals solution (Clark & Holms, 1976), 0.5 ml; ammonium chloride, 0.1 g; 0.1% (w/v) resazurin, 0.1 ml; NaHCO₃, 0.4 g; haemin, 0.001 g; L-methionine, 0.0045 g; volatile fatty acid solution (Caldwell & Bryant, 1966), 0.31 ml; cysteine.HCl, 0.01 g; vitamin solution, 1 ml. The composition of the vitamin solution was such that the concentration of the components in the medium was the same as the medium of Scott & Dehority (1965). Either 0.5% glucose or 2% D,L-lactate were added as the carbon/energy source. All media were adjusted to pH 6.8 before being dispensed into 7.5 ml aliquots in Hungate tubes, or 400 ml aliquots in 1000 ml Duran bottles (Gallenkamp) with crimp-top attachments, and autoclaved.

Growth of bacteria. Batch cultures were grown under O₂-free CO₂ in 7.5 ml of medium in Hungate tubes from an inoculum of 0.2 ml from an overnight culture. Cultures were incubated at 39 °C and the cell density was estimated by gently inverting the tubes before measuring optical density at 650 nm in a Bausch & Lomb spectronic 20 spectrophotometer. Larger volume batch cultures were in 1000 ml Duran bottles (Gallenkamp) with crimp-top attachments. Samples were removed aseptically by syringe and transferred to sterile, CO₂-filled Hungate tubes for optical density measurements.

Fermentation analyses. Fermentation products were measured in the supernatant fluid from centrifuged culture samples by the capillary gas chromatography method of Richardson *et al.* (1989) using a 5890 series II gas chromatograph (Hewlett Packard).

To measure D-lactate and L-lactate separately in supernatant fluid, samples were first deproteinized by adding trichloroacetic acid (TCA) to a final concentration of 5% and leaving at 4 °C for 5 min. The precipitate was removed by filtration before the samples were neutralized with potassium hydroxide. D-Lactate was then measured using the method described by Bergmeyer & Gahwehn (1974) using D-LDH from *Leuconostoc mesenteroides* (EC 1.1.1.28) and L-lactate was measured by the automated enzymatic procedure of Hochella & Weinhouse (1965). Glucose was measured by the Lever assay (Lever, 1977).

Preparation of cell free extract. Cells were collected by centrifugation (4 °C, 3000 g, 20 min), washed once in 50 mM sodium phosphate buffer (pH 6.8) and resuspended in the same buffer. All transfers of cell suspensions were carried out anaerobically. Cells were disrupted using an MSE Soniprep 150 at full power for 5 min with alternate 30 s on/off periods and keeping the samples on ice throughout. Cell debris was removed by centrifugation (13400 g, 4 °C, 1 h) and the supernatant was either used immediately or stored at -70 °C until required.

Zymograms. Approximately 20 µl of sample was loaded onto a non-denaturing polyacrylamide gel containing a 2.7% stacking gel and a 7.5% running gel. Electrophoresis was at 10 mA with 0.25 M Tris/glycine pH 8.3, with constant cooling using circulating cold water. Gels were stained until bands appeared by incubation at 37 °C in the reaction mixture: 25 ml 0.1 M Tris/HCl pH 8.0, 1.5 ml 2 M D- or L-lactate, 72 mg NAD, 3.6 mg nitroblue tetrazolium and 2 mg phenazine methosulphate (PMS).

Enzyme assays. The nLDH activity in samples was assayed using pyruvate as the substrate as described by Wallace (1978) but at pH 6.5: therefore the reaction mixture contained NADH, 0.3 mM; sodium pyruvate, 10 mM; potassium phosphate buffer, 50 mM (pH 6.5); and sample. The reaction was performed at 25 °C and the absorbance change at 340 nm measured. nLDH assays with lactate as the substrate were performed with 3-acetylpyridine adenine dinucleotide (APAD) as the cofactor and cofactor reduction measured at 365 nm as described by Wittenberger (1966): i.e. Tris/HCl, 100 mM (pH 8.0); lithium D- or L-lactate, 87.3 mM; APAD, 2 mM; and sample at 25 °C. Assays for nLDH were also carried out by the method of Scheifinger *et al.* (1975a) with the following reaction mixture: potassium phosphate buffer, 50 mM (pH 8.5); lithium D- or L-lactate, 87.3 mM; NAD, 0.3 mM; and sample at 25 °C.

Assays for iLDH essentially followed the method described by Macy *et al.* (1978) but were performed at pH 6.5: i.e. potassium phosphate buffer, 50 mM (pH 6.5); lithium D- or L-lactate, 5 mM; 2,6-dichlorophenol indophenol (DCPIP), 0.15 mM; and sample at 30 °C and measuring absorbance at 600 nm. All absorbance measurements for assays were performed using a Cecil CE 595 double beam UV spectrophotometer with a path length of 1 cm.

Lactate racemase activity was estimated using the procedure described by Dennis & Kaplan (1963). Reaction mixtures contained 0.1 M sodium acetate (pH 5.0), 0.2 M lithium D- or L-lactate and cell extract in a final volume of 1 ml. The reaction was allowed to proceed at 37 °C and at specific times aliquots were removed and boiled for 5 min. Following cooling on ice, samples were centrifuged at 13400 g for 20 min at 4 °C and the supernatant withdrawn for analysis of D- and L-lactate.

For the purpose of specific activity calculations protein was measured in all samples by the Lowry method.

Separation of enzyme activities. Gel filtration was carried out at 4 °C on a 2.5 × 100 cm Ultrogel AcA 34 column (IBF Biotechnics) equilibrated with 50 mM sodium phosphate, pH 6.8. The column was calibrated with blue dextran, sweet potato β -amylase (M_r 200000), yeast alcohol dehydrogenase (150000), bovine serum albumin (66000) and bovine erythrocyte carbonic anhydrase (29000). Samples were run at a flow rate of 20 ml h⁻¹ and 3 ml fractions were collected for assaying purposes. Samples were also run on a Sephadex G-100 column of the same size with 50 mM sodium phosphate buffer, pH 6.8 at a flow rate of 14 ml h⁻¹, collecting 5 ml fractions. This column was calibrated with blue dextran, bovine serum albumin, bovine erythrocyte carbonic anhydrase, horse heart cytochrome *c* (M_r 12400) and bovine lung aprotinin (6500). Protein in the eluent was detected by measuring absorbance at 280 nm on an LKB 8300 Uvicord.

RESULTS

Growth rates and fermentation

The abilities of four lactate utilizing strains of *S. ruminantium* (5934e, HD4, 5521C1 and JW13) to metabolize lactate as the sole carbon and energy source were compared. Strain 5934e reached the highest maximum OD₆₅₀ of 1.2, and exhibited the highest growth rate (generation time 2.4 h), and was therefore selected as the most appropriate strain for further study. This strain grown in batch culture on D-medium + 0.5% glucose as the sole carbon/energy source initially produced mainly lactate with a low level of propionate and little or no

acetate as fermentation end-products (Fig. 1a); succinate was not detected at significant levels. When all the glucose was utilized and the culture had entered stationary phase, accumulated lactate was converted to propionate and acetate, as shown previously with *S. ruminantium* strain HD4 (Scheifinger *et al.*, 1975a; Melville *et al.*, 1987) and strain B (Hishinuma *et al.*, 1968). Strain 5934e showed no apparent preference for D- or L-lactate when grown on media with an equal concentration of the isomers as the sole carbon/energy source; after 15 h more than 90% of the lactate initially present in the medium had been taken up by the cells (Fig. 2). Also, both isomers of lactate were found to be produced from glucose (data not shown).

Lactate dehydrogenase activities in strain 5934e

Previous work on strain HD4 revealed one major LDH activity which was an L-nLDH (Scheifinger *et al.*, 1975a; Melville *et al.*, 1987). A single band of LDH activity was detected previously in *S. ruminantium* strain 5934e by non-denaturing PAGE (Flint & Bisset, 1990). In the present work zymograms were stained with L-lactate and D-lactate separately and activity was shown to be due to an L-nLDH. The L-nLDH band was found to be more intense with glucose-grown cell extracts than it was with lactate-grown cell extracts (Fig. 3).

Fig. 1. (a) Growth and fermentation products of *S. ruminantium* strain 5934e grown on glucose in batch culture: ■, OD₆₅₀; ○, glucose; ●, D-lactate; □, propionate; ▲, acetate. (b) Lactate dehydrogenase activities in cell extracts of the same batch culture: ●, D-iLDH; ○, L-nLDH; ■, D-nLDH. Activity is expressed as nmol DCPIP reduced min⁻¹ (mg protein)⁻¹ for iLDH and nmol APAD reduced min⁻¹ (mg protein)⁻¹ for nLDH.

Fig. 2. Utilization of DL-lactate by *S. ruminantium* strain 5934e in batch culture. ■, OD₆₅₀; ○, D-lactate; ●, L-lactate.

LDH assays were carried out using cells growing exponentially on basal M2+glucose, which are utilizing glucose and producing lactate (Fig. 1a). NADH-dependent reduction of pyruvate was readily observed in cell free extracts; however, assays of reduction of L- or D-lactate with NAD as cofactor gave low or undetectable activities (Table 1). These activities could be stimulated at least threefold by replacing NAD with the analogue APAD. This preference of some nLDH enzymes for APAD over NAD has been discussed by previous authors (Holzer & Soling, 1962; Wittenberger, 1966) and appears to be a property of dehydrogenases related to the higher redox potential of APAD.

The assays were repeated with *S. ruminantium* strain 5934e cells grown to the mid-exponential phase on basal M2

Fig. 3. Non-denaturing PAGE of *S. ruminantium* strain 5934e cell extract, stained for L-nLDH activity. L, Lactate-grown cells; G, glucose-grown cells.

medium+DL-lactate. In assays with pyruvate as the substrate the level of LDH activity was approximately 10-fold lower in extracts prepared from lactate-grown cells compared to glucose-grown cells, and lower activity was also found for the L-nLDH using L-lactate as substrate and APAD as the cofactor. The lower L-nLDH activity present in the lactate-grown cells could not be detected when NAD was used as the cofactor (Table 1). These

Table 1. Lactate dehydrogenase activities in *S. ruminantium* strain 5934e

Values given are means of triplicate determinations, with calculated standard deviation. Extracts were prepared from cells grown to mid-exponential phase on basal M2+glucose or +DL-lactate.

Extract	LDH activity*					
	Pyr → Lac	L-Lac → Pyr		D-Lac → Pyr		
		NADH ^a	APAD ^b	NAD ^c	APAD ^b	NAD ^c
Glucose	2424 ± 897	13.2 ± 1.9	< 5	14.4 ± 5.0	ND	< 2
DL-Lactate	284 ± 156	< 5	ND	16.4 ± 3.5	ND	22.5 ± 11.1

* Activity is expressed as (a) nmol NADH oxidized min⁻¹ (mg protein)⁻¹; (b), nmol APAD reduced min⁻¹ (mg protein)⁻¹; (c), nmol NAD reduced min⁻¹ (mg protein)⁻¹; (d), nmol DCPIP reduced min⁻¹ (mg protein)⁻¹.

ND, Not detectable [< 0.4 nmol substrate reduced or oxidized min⁻¹ (mg protein)⁻¹].

measurements are consistent with the results of the LDH zymograms (Fig. 3) showing greater L-nLDH activity in glucose extracts. D-Lactate specific nLDH activity was similar in extracts of lactate-grown cells and glucose-grown cells, but was only detectable when APAD was used as cofactor (Table 1). The relative differences in the two nLDH activities between glucose-grown cells and lactate-grown cells suggested that *S. ruminantium* strain 5934e may possess two distinct nLDH enzymes. Although lactate racemase activity was detected in crude extracts, it was similar in cells grown on either glucose or lactate (data not shown), and therefore could not explain the observed differences in lactate oxidation.

In lactate-grown cells a significant NAD-independent D-LDH (D-iLDH) activity was detected, which was approximately 10-fold higher than in glucose-grown cells (Table 1). This activity was apparently induced when *S. ruminantium* cells were utilizing lactate and suggested the existence of a third LDH distinct from the nLDH activities already identified. Unlike the L-iLDH activity previously detected in strain HD4 (Scheifinger *et al.*, 1975a), addition of NAD or PMS to D-iLDH assay mixtures did not increase the activity. Only trace levels of L-iLDH activity [< 0.5 nmol DCPIP reduced min^{-1} (mg protein) $^{-1}$] could be detected in any cell extracts of strain 5943e.

Separation of LDH activities from strain 5934e

In order to demonstrate that the three LDH activities in *S. ruminantium* strain 5934e were due to different enzymes, a cell extract prepared from mid-exponential cells grown on D-medium + 2% DL-lactate was fractionated by gel filtration on Ultrogel AcA 34. The elution profile from the gel filtration column showed three distinct activity peaks (Fig. 4). The D-iLDH peak corresponded to a protein with an approximate M_r of 280 000 and the fractions making up this peak contained no D-nLDH activity. An approximate M_r of 140 000 was found for the L-nLDH and this was in agreement with the M_r of 150 000 estimated from non-denaturing PAGE (results not shown). The third enzyme, the D-nLDH, was separated from the other two LDH activities but this enzyme was eluted from the column at the same elution volume as NaCl suggesting an M_r less than the lower exclusion limit of the gel matrix, i.e. < 20 000. A small peak of D-nLDH activity was also detected in the fractions containing L-nLDH, perhaps due to non-specificity of the L-nLDH. Sephadex G-100 gel filtration chromatography of a sample prepared in the same way indicated that the D-nLDH had an M_r of approximately 10 000.

The recoveries of the three LDH activities from gel filtration were low (11%, 28% and 1% for L-nLDH, D-nLDH and D-iLDH respectively). These recoveries correlated with stability of the activities after storage at 4 °C for 48 h (37%, 76% and 10% L-nLDH, D-nLDH and D-iLDH activity retained respectively). Addition of the protease inhibitors EDTA (1 mM) or PMSF (100 $\mu\text{g ml}^{-1}$) to samples of crude extract did not increase the stability of LDH activities on incubation at various

Fig. 4. Elution profile of the three lactate dehydrogenase activities of *S. ruminantium* strain 5934e, DL-lactate-grown, cell extract, after gel filtration on Ultrogel AcA 34. ●, D-iLDH; ○, L-nLDH; ■, D-nLDH. Activity is expressed as nmol DCPIP reduced min^{-1} by each fraction for iLDH and nmol APAD reduced min^{-1} by each fraction for nLDH.

temperatures from 4 °C to 37 °C, and the presence of 0.5 mM dithiothreitol during gel filtration did not significantly increase the recovery of the enzyme activities (data not shown). Due to the low recoveries of activity, no further purification was attempted.

The preference of the semi-purified nLDHs for APAD over NAD as the cofactor was the same as for the corresponding activities in crude extracts (results not shown). The rate of pyruvate reduction by the L-nLDH was again approximately 200-fold greater than that of L-lactate oxidation. No activity could be measured with the D-nLDH fraction using pyruvate as the substrate with either NADH or APADH as cofactor indicating that all the pyruvate to lactate activity in crude extracts is due to the L-nLDH. This was confirmed by the direct demonstration that only L-lactate was produced during pyruvate oxidation (data not shown).

Approximate K_m values with respect to the relevant isomer of lactate for the semi-purified L-nLDH, D-nLDH and D-iLDH enzymes were estimated at 45 mM, 70 mM and 0.5 mM respectively; therefore the D-iLDH has the highest affinity for lactate. The K_m value of the L-nLDH for pyruvate was 0.5 mM, which is considerably lower than the K_m for L-lactate.

LDH activities in strain 5934e at different growth phases

Cell extracts of *S. ruminantium* 5934e obtained from different stages of growth on glucose were assayed for all three LDH activities (Fig. 1b). Both nLDH activities peaked during exponential growth, when glucose was being converted to lactate, although activity could be

Table 2. Lactate dehydrogenase activities in extracts of different strains of *S. ruminantium* grown on glucose or DL-lactate

Values given are means from two determinations. Extracts were prepared from cells grown to mid-exponential phase on basal M2+0.5% glucose (Glc) or on basal M2+2% DL-lactate (Lac).

Strain	LDH activity*							
	L-nLDH ^a		D-nLDH ^a		D-iLDH ^b		L-iLDH ^b	
	Glc	Lac	Glc	Lac	Glc	Lac	Glc	Lac
5934e	28.0	9.2	14.7	13.5	0.7	15.5	ND	ND
HD4	16.3	10.9	6.1	25.7	0.3	32.1	ND	3.8
5521C1	25.9	2.1	13.9	22.9	0.4	6.2	ND	ND
JW13	23.0	2.0	15.4	9.0	0.5	14.1	ND	ND
FB322	17.2	—	10.3	—	ND	—	ND	—
GA 192	16.1	—	4.9	—	ND	—	ND	—
D	42.9	—	15.3	—	ND	—	ND	—

* Activity is expressed as (a) nmol APAD reduced min⁻¹ (mg protein)⁻¹; (b) nmol DCPIP reduced min⁻¹ (mg protein)⁻¹.

ND, Not detectable [< 0.4 nmol substrate reduced or oxidized min⁻¹ (mg protein)⁻¹].

detected throughout the stationary phase of growth. The D-iLDH activity, on the other hand, was present at very low levels during the exponential phase, but was markedly stimulated in stationary phase cells which were actively utilizing lactate (Fig. 1).

LDH activities in other strains of *S. ruminantium*

Three other lactate utilizing strains of *S. ruminantium* were tested for nLDH and iLDH activities by assaying extracts of both glucose-grown and lactate-grown cells. LDH activities in strains 5521C1, JW13 and HD4 followed a similar pattern to strain 5934e (Table 2) except that strain HD4 exhibited low levels of L-iLDH activity when grown with DL-lactate as the sole energy source. This may be the same L-iLDH activity found previously at low levels in strain HD4 (Scheifinger *et al.*, 1975a; Melville *et al.*, 1987) or could be due to a highly active lactate racemase in strain HD4 converting L-lactate in the assay to D-lactate.

Three lactate non-utilizing strains of *S. ruminantium*, FB322, GA192 and D, were grown on glucose and cell extracts examined for nLDH and iLDH activities. The activities found were similar to those in lactate utilizing strains grown on glucose, and the iLDH activities were absent or insignificant (Table 2).

DISCUSSION

This work shows for the first time that multiple LDHs are present in strains of *Selenomonas ruminantium*. The D-iLDH of strain 5934e was only detected at significant levels in cells grown with lactate as the sole carbon and energy source (Table 1) and the enzyme had a higher affinity for lactate than the cofactor dependent LDH activities, suggesting that it is the enzyme most likely to

be involved in lactate utilization *in vivo*. In *S. ruminantium* strain HD4, Joyner & Baldwin (1966) found low levels of both D-iLDH and L-iLDH, while Wallace (1978) found low levels of iLDH in strain WPL 151/1, but in both these studies cells were grown only on glucose. Therefore this is the first report of a LDH activity induced by lactate in *S. ruminantium*.

Strain 5934e was found to utilize L-lactate and D-lactate at approximately the same rate when grown on DL-lactate (Fig. 2), presumably due to the presence of lactate racemase, which was detected in extracts of cells grown on either glucose or lactate. The ability to use both D- and L-lactate may enhance the survival of *S. ruminantium* within the rumen where D- and L-lactate are both present. However, the fact that only D-iLDH activity and no L-iLDH activity was detected in extracts of 5934e and two other strains indicates that the racemase does not contribute to the *in vitro* LDH assays under the conditions used.

D-iLDH activity, which was found in all lactate utilizers, was particularly unstable. Overnight dialysis of cell extract of lactate-grown strain 5934e resulted in the loss of over 90% of the D-iLDH activity (results not shown), which may explain why it was not detected in previous studies examining lactate-grown cells and cells grown at different dilution rates on glucose (Scheifinger *et al.*, 1975a; Melville *et al.*, 1987). Further purification and characterization of the enzyme will depend on a means of overcoming the problem of the rapid loss of activity after cell disruption.

The normal electron acceptor for the D-iLDH *in vivo* is not known in *S. ruminantium* but it is tempting to speculate that this may involve the anaerobic respiratory chain. *S. ruminantium* strains WPL 151/1, 6, 21 and GFA are known

to contain a membrane associated cytochrome *b* which in its reduced form is oxidizable by fumarate (De Vries *et al.*, 1974; Henderson, 1980), and it is possible that lactate oxidation could be linked to fumarate reduction *in vivo*.

The L-nLDH detected in strain 5934e appears to be similar to the enzyme previously described in strain HD4 (Scheifinger *et al.*, 1975a; Melville *et al.*, 1987, 1988a), having a similar M_r . The higher levels of L-nLDH activity found in glucose-grown cells compared with lactate-grown cells (Table 1, Fig. 3) and the much lower (90-fold) K_m value for pyruvate than for lactate suggest that this enzyme is more important in glucose metabolism than in lactate metabolism. Assays with L-lactate as the substrate were more sensitive when APAD was used instead of NAD as the cofactor (Table 1) due to the greater electron accepting power of APAD (Kaplan & Ciotti, 1956).

The third activity detected in all four lactate utilizing strains, D-nLDH, was only measurable in a quantitative manner when APAD and D-lactate were used as cofactor and substrate in the assays (Table 1). However, in colorimetric assays containing D-lactate, NAD, nitroblue tetrazolium and PMS activity was detected in crude extracts (results not shown) suggesting that under some conditions NAD/NADH can be used as a cofactor by this enzyme. The role of this enzyme *in vivo* is as yet unclear. It had a relatively high K_m value for D-lactate but a method of assaying the enzyme in the pyruvate to lactate direction is needed to estimate the K_m value for pyruvate. The small size of this D-nLDH is unusual compared to other bacterial D-nLDH enzymes which have M_r values of 70 000–80 000 or larger (Garvie, 1980), although it is possible that the D-nLDH activity measured after gel filtration results from denaturation of the enzyme into individual subunits. The only other report of a D-nLDH activity in *S. ruminantium* was that of Wallace (1978) in strain WPL 151/1. However, this activity was assayed using pyruvate as the substrate and NADH as the cofactor and therefore may be of a different type from the enzyme in strain 5934e.

It has been estimated that the major contribution (60–80%) to lactate fermentation in the rumen is made by *M. elsdenii* via the 'acrylate' pathway, and a lower contribution is made by organisms such as *S. ruminantium* that employ the alternative 'randomizing' pathway for propionate formation from lactate (Counotte *et al.*, 1981). One of the main reasons for the relatively low contribution of *S. ruminantium* is likely to be repression of lactate utilization in the presence of readily utilizable sugars, which is not observed in *M. elsdenii*. In addition, many *S. ruminantium* strains are not lactate utilizers, and we also found that there is considerable variation between lactate utilizing strains in their growth responses with lactate as substrate. There may be scope for using specially selected strains of *S. ruminantium* such as 5934e, that show high lactate utilizing ability, to enhance lactate utilization in the rumen, but only if such strains could be modified so as to abolish the repression of lactate utilization by sugars. Now that the likely route for lactate assimilation in *S. ruminantium* has been established, further investigations

can be expected to elucidate the regulation of lactate utilization in this organism.

ACKNOWLEDGEMENTS

This work was supported by an SERC-CASE award to M.G., with additional support from SOAFD (Scottish Office Agriculture and Fisheries Department). We are grateful to Dr R. J. Wallace for valuable discussions and advice.

REFERENCES

- Baldwin, R. L., Wood, W. A. & Emery, R. S. (1965). Lactate metabolism by *Peptostreptococcus elsdenii*: evidence for lactyl co-enzyme A dehydrase. *Biochim Biophys Acta* **97**, 202–213.
- Bergmeyer, H. U. & Gahwehn, K. (1974). D-Lactate. In *Methods of Enzymatic Analysis*, 2nd edn, vol. 3, pp. 1492–1495. Edited by H. U. Bergmeyer. New York: Academic Press.
- Brockman, H. L. & Wood, W. A. (1975). D-Lactate dehydrogenase of *Peptostreptococcus elsdenii*. *J Bacteriol* **124**, 1454–1461.
- Bryant, M. P. (1956). The characteristics of strains of *Selenomonas* isolated from bovine rumen contents. *J Bacteriol* **72**, 162–167.
- Bryant, M. P. (1972). Commentary on the Hungate technique for culture of anaerobic bacteria. *Am J Clin Nutr* **25**, 1324–1328.
- Bryant, M. P. (1984). *Selenomonas*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 650–653. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Caldwell, D. R. & Bryant, M. P. (1966). Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. *Appl Microbiol* **14**, 794–801.
- Clark, B. & Holms, W. H. (1976). Control of the sequential utilization of glucose and fructose by *Escherichia coli*. *J Gen Microbiol* **95**, 191–201.
- Counotte, G. H. M., De Groot, M. & Prins, R. A. (1980). Kinetic parameters of lactate dehydrogenases of some rumen bacterial species, the anaerobic ciliate *Isotricha prostoma* and mixed rumen microorganisms. *Antonie van Leeuwenhoek* **46**, 363–381.
- Counotte, G. H. M., Prins, R. A., Janssen, R. H. A. M. & Debie, M. J. A. (1981). Role of *Megasphaera elsdenii* in the fermentation of DL-[2-¹³C]lactate in the rumen of dairy cattle. *Appl Environ Microbiol* **42**, 649–655.
- Dennis, D. & Kaplan, N. O. (1963). Lactic acid racemization in *Clostridium butylicum*. *Biochem Z* **338**, 485–495.
- De Vries, W., Van Wijck-Kapteyn, W. M. C. & Oosterhuis, S. K. H. (1974). The presence and function of cytochromes in *Selenomonas ruminantium*, *Anaerovibrio lipolytica* and *Veillonella alcalescens*. *J Gen Microbiol* **81**, 69–78.
- Flint, H. J. & Bisset, J. (1990). Genetic diversity in *Selenomonas ruminantium* isolated from the rumen. *FEMS Microbiol Ecol* **73**, 351–360.
- Flint, H. J., Duncan, S. H., Bisset, J. & Stewart, C. S. (1988). The isolation of tetracycline resistant strains of strictly anaerobic bacteria from the rumen. *Lett Appl Microbiol* **6**, 113–115.
- Garvie, E. I. (1980). Bacterial lactate dehydrogenases. *Microbiol Rev* **44**, 106–139.
- Henderson, C. (1980). The influence of extracellular hydrogen on the metabolism of *Bacteroides ruminicola*, *Anaerovibrio lipolytica* and *Selenomonas ruminantium*. *J Gen Microbiol* **119**, 485–491.
- Hino, T. & Kuroda, S. (1993). Presence of lactate dehydrogenase and lactate racemase in *Megasphaera elsdenii* grown on glucose or lactate. *Appl Environ Microbiol* **59**, 255–259.

- Hishinuma, F., Kanegasaki, S. & Takahashi, H. (1968). Ruminal fermentation and sugar concentration: a model experiment with *Selenomonas ruminantium*. *Agric Biol Chem* **32**, 1325–1330.
- Hobson, P. N. (1969). Rumen bacteria. *Methods Microbiol* **3B**, 133–139.
- Hochella, N. J. & Weinhouse, S. (1965). Automated lactic acid determination in serum and tissue extracts. *Anal Biochem* **10**, 304–317.
- Holzer, H. & Soling, H. D. (1962). Bestimmung von L-lactat, L-malat, L-glutamat und athylalkohol im enzymatisch-optischen test mit hilfe des DPN-analogen 3-acetylpyridin-DPN. *Biochem Z* **336**, 201–214.
- Hungate, R. E. (1966). *The Rumen and its Microbes*. New York: Academic Press.
- John, A., Isaacson, H. R. & Bryant, M. P. (1974). Isolation and characterisation of a ureolytic strain of *Selenomonas ruminantium*. *J Dairy Sci* **57**, 1003–1014.
- Johns, A. T. (1951). The mechanism of propionic acid formation by *Veillonella gazogenes*. *J Gen Microbiol* **5**, 326–336.
- Joyner, A. E. & Baldwin, R. L. (1966). Enzymatic studies of pure cultures of rumen microorganisms. *J Bacteriol* **92**, 1321–1330.
- Kaplan, N. O. & Ciotti, M. M. (1956). Chemistry and properties of the 3-acetylpyridine analogue of diphosphopyridine nucleotide. *J Biol Chem* **221**, 823–832.
- Kemp, M. B. (1972). D- and L-lactate dehydrogenases of *Pseudomonas aeruginosa*. *Biochem J* **130**, 307–309.
- Kohn, L. D. & Kaback, H. R. (1973). Mechanisms of active transport in isolated bacterial membrane vesicles. XV. Purification and properties of the membrane-bound D-lactate dehydrogenase from *Escherichia coli*. *J Biol Chem* **248**, 7012–7017.
- Lever, M. (1977). Carbohydrate determination with 4-hydroxybenzoic acid hydrazide (PAHBAH): effect of bismuth on the reaction. *Anal Biochem* **81**, 21–27.
- Macy, J. M., Ljungdahl, L. G. & Gottschalk, G. (1978). Pathway of succinate and propionate formation in *Bacteroides fragilis*. *J Bacteriol* **134**, 84–91.
- Melville, S. B., Michel, T. A. & Macy, J. M. (1987). Involvement of D-lactate and lactic acid racemase in the metabolism of glucose by *Selenomonas ruminantium*. *FEMS Microbiol Lett* **40**, 289–293.
- Melville, S. B., Michel, T. A. & Macy, J. M. (1988a). Pathway and sites for energy conservation in the metabolism of glucose by *Selenomonas ruminantium*. *J Bacteriol* **170**, 5298–5304.
- Melville, S. B., Michel, T. A. & Macy, J. M. (1988b). Regulation of carbon flow in *Selenomonas ruminantium* grown in glucose-limited continuous culture. *J Bacteriol* **170**, 5305–5311.
- Molinari, R. & Lara, F. J. S. (1960). The lactic dehydrogenase of *Propionibacterium pentosaceum*. *Biochem J* **75**, 57–65.
- Paynter, M. J. & Elsdon, S. R. (1970). Mechanism of propionate formation by *Selenomonas ruminantium*, a rumen micro-organism. *J Gen Microbiol* **61**, 1–7.
- Richardson, A. J., Calder, A. G., Stewart, C. S. & Smith, A. (1989). Simultaneous determination of volatile and non-volatile acidic fermentation products of anaerobes by capillary gas chromatography. *Lett Appl Microbiol* **9**, 5–8.
- Scheifinger, C. C., Latham, M. J. & Wolin, M. J. (1975a). Relationships of lactate dehydrogenase specificity and growth rate to lactate metabolism by *Selenomonas ruminantium*. *Appl Microbiol* **30**, 916–921.
- Scheifinger, C. C., Linehan, B. & Wolin, M. J. (1975b). H₂ production by *Selenomonas ruminantium* in the absence and presence of methanogenic bacteria. *Appl Microbiol* **29**, 480–483.
- Scott, H. W. & Dehority, B. A. (1965). Vitamin requirements of several cellulolytic rumen bacteria. *J Bacteriol* **89**, 1169–1175.
- Wallace, R. J. (1978). Control of lactate production by *Selenomonas ruminantium*: homotropic activation of lactate dehydrogenase by pyruvate. *J Gen Microbiol* **107**, 45–52.
- Wallace, R. J. & Brammall, M. J. (1985). The role of different species of bacteria in the hydrolysis of protein in the rumen. *J Gen Microbiol* **131**, 821–832.
- Wittenberger, C. L. (1966). Unusual kinetic properties of a DPN-linked lactate dehydrogenase from *Butyrivacterium rettgeri*. *Biochem Biophys Res Commun* **22**, 729–736.
- Wittenberger, C. L. & Haaf, A. S. (1966). Isolation of NAD-independent lactate dehydrogenase from extracts of *Butyrivacterium rettgeri*. *Biochim Biophys Acta* **122**, 393–405.

Received 21 March 1994; accepted 27 April 1994.