Sugar-Glycerol Cofermentations in Lactobacilli: the Fate of Lactate

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The simultaneous fermentation of glycerol and sugar by Lactobacillus brevis B22 and Lactobacillus buchneri B190 increases both the growth rate and total growth. The reduction of glycerol to 1,3-propanediol by the lactobacilli was found to influence the metabolism of the sugar cofermented by channelling some of the intermediate metabolites (e.g., pyruvate) towards NADH-producing (rather than NADH-consuming) reactions. Ultimately, the absolute requirement for NADH to prevent the accumulation of 3-hydroxypropionaldehyde leads to a novel lactate-glycerol cofermentation. As a result, additional ATP can be made not only by (i) converting pyruvate to acetate via acetyl phosphate rather than to the ethanol usually found and (ii) oxidizing part of the intermediate pyruvate to acetate instead of the usual reduction to lactate but also by (iii) reoxidation of accumulated lactate to acetate via pyruvate. The conversion of lactate to pyruvate is probably catalyzed by NAD-independent lactate dehydrogenases that are found only in the cultures oxidizing lactate and producing 1,3-propanediol, suggesting a correlation between the expression of these enzymes and a raised intracellular NAD/NADH ratio. The enzymes metabolizing glycerol (glycerol dehydratase and 1,3-propanediol dehydrogenase) were expressed in concert without necessary induction by added glycerol, although their expression may also be influenced by the intracellular NAD/NADH ratio set by the different carbohydrates fermented.

A few strains of lactobacilli have the ability to produce 1,3-propanediol (1,3-PDL) from glycerol while metabolizing glucose or fructose (18, 23, 25). These bacteria all have a coenzyme B$_{12}$-dependent dehydrogenase responsible for the dehydroxylation of glycerol to 3-hydroxypropionaldehyde (3-HPA), which is subsequently reduced by NADH to 1,3-PDL (19, 22, 27). Since glycerol is not metabolized as a sole energy source, its cofermentation with sugar was reported to affect the metabolism of glucose or fructose (but not of ribose) exclusively by reoxidizing NADH equivalent to that formed during the catabolism of the sugar via the 6-phosphogluconate pathway. Work with growing cultures by Schutz and Radler (18) supported this idea; by suppressing ethanol formation, glycerol allowed greater acetate production, the extra carbon flow through acetyl phosphate leading to consistently better growth of lactobacilli.

However, the metabolite balances obtained by these investigators show an unexplained disparity between the glucose used and the lactate and acetate plus ethanol formed and suggested to us a more complex effect of glycerol in the metabolism of glucose. In the present study a further investigation of this problem shows that, for Lactobacillus brevis B22 and Lactobacillus buchneri B190 growing with glycerol and growth-limiting concentrations of sugar, 1,3-PDL is not produced exclusively during growth with substrates metabolized via the 6-phosphogluconate pathway. Thus, glycerol reduction does not depend only on the necessity to recycle the NADH formed during the catabolism of hexoses, as was believed to be the case until now. The influence of glycerol on metabolism is apparently more extensive than was proposed by Schutz and Radler (18), because the cofermentation prompts a reoxidation of lactate to acetate.

MATERIALS AND METHODS

Bacterial strains. L. buchneri B190, originally isolated from sour dough, and L. brevis B22, isolated from spoiled wine, were both received from F. Radler from the culture collection in the Institut für Mikrobiologie und Weinfor-schung der Johannes Gutenberg-Universität Mainz, Mainz, Germany.

Media and growth. The cultures were maintained as stabs in solidified, complex MRS medium (5) and transferred monthly. For growth of liquid cultures, the MRS medium was modified by omitting Tween 80, sodium acetate, and glucose and was designated MLM. Concentrated sugar (or lactate) was autoclaved separately and added (final concentration, 20 mM) to the previously autoclaved MLM. When used, glycerol (final concentration, 100 mM) was added to MLM. All cultures were grown at 30°C after inoculation (1%) with a 48-h-old preculture. Anaerobic growth for the determination of the fermentation courses of the various sugars was conducted in static metal-capped tubes filled two-thirds full (15 ml). For each growth condition, a series of tubes was prepared in duplicate and inoculated and incubated as described above. Growth was monitored by measuring the A$_{600}$ of the cultures in paired tubes after their required incubation period. This practice was preferred over the alternative sampling of a single large culture because deposition of aggregated cells precluded repetitive sampling without strong disturbance of the culture’s growth conditions. For enzyme assays, the cultures were grown in stationary 500-ml bottles filled completely. Aerobically, cultures (200 ml) were grown with vigorous shaking in Erlemeyer flasks (1,000 ml) on a rotary shaker.

Analysis of growth substrates and fermentation end products. Glucose, fructose, glycerol, and formate were estimated by using the appropriate test combination kits of Boehringer Mannheim Inc. Ribose was determined colorimetrically (30). D(-)-Lactate (9), L(+)-lactate (11), ethanol (3), and acetate (12) were determined enzymatically.

1,3-PDL was determined by using 1,3-PDL dehydroge-
nase from *Citrobacter freundii*. The enzyme was purified to apparent homogeneity by R. Scase. The assay mixture was as follows: 1 ml of buffer (75 mM pyrophosphate buffer [pH 8.7], 75 mM semicarbazide, 21 mM glycine), 15 µl of sample (0 to 50 mM 1,3-PDL), 70 µl of NAD (45 mM), and 2 µl of 1,3-PDL dehydrogenase (approximately 1.5 units per assay), activated before the assay by treatment with 0.5 mM MnCl₂ and 10 mM dithiothreitol for 10 min at 37°C. The reaction was started by adding the enzyme to the mixture, which had been warmed to 37°C. The change in A₃₄₀ was measured after 60 min of incubation of the standard solutions and the unknown samples. The concentration of 1,3-PDL was estimated by comparison of the increase in absorbance for the unknown samples with those given by known concentrations of 1,3-PDL. The determination was not affected by glyceral (100 mM), ethanol (30 mM), or mannitol (20 mM), compounds which themselves gave no response.

Mannitol was estimated by gas chromatography using a Varian 3600 gas chromatograph equipped with a hydrogen flame ionization detector; the column was a fused silica capillary SP-1000, 30 m by 2.5 mm. The mannitol was first converted to its trimethylsilyl derivative (24), inositol being used as an internal standard.

**Enzyme assays.** Cells were collected from 500-ml cultures and washed twice in the centrifuge (14,000 × g for 15 min) with 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, pH 7.2, containing 1 mM MnCl₂. Thick suspensions of the same buffer were treated for three 2-min periods with ultrasound (25 kHz; 600 W), and cell debris was removed by centrifugation before enzymes in the extracts were assayed.

The dehydrogenase was assayed by a colorimetric method (7) modified by using 50 mM potassium phosphate (pH 7.2) buffer and by omitting NH₄Cl. The modification was made because the activity of the dehydrogenase from labocubic acid is maximum in phosphate buffer in the pH range 7.0 to 7.5 and is increased equally by either NH₄⁺ or K⁺ (19).

1,3-PDL dehydrogenase was measured at 37°C in 100 mM Tris-HCl buffer (pH 9.0) containing 1 mM NAD and 155 mM 1,3-PDL by recording the change in A₃₄₀ caused by NADH production.

The NAD-dependent lactate dehydrogenase activity was measured at 37°C in 50 mM potassium phosphate, pH 7.5 (2). The NAD-independent lactate dehydrogenases (iLDHs) were measured at pH 6.0 by using d(-)- or l(+)-lactate (lithium salts) by the 2,6-dichlorophenol indophenol method (6) by using 13.8 cm⁻¹ mol⁻¹ × 10⁻³ as the molar extinction coefficient of the oxidized dye (1).

One unit of enzyme activity is the amount of enzyme that catalyzes the formation of 1 µmol of reaction product per minute under the specific conditions of the particular assay used. The protein in crude extracts was determined by using the biuret method (10) to allow calculation of specific activities.

**Metabolism of whole cells.** The cells were grown anaerobically in bottles as described above, washed in 150 mM MOPS, pH 7.2, containing 75.0 µg of chloramphenicol ml⁻¹, and resuspended in the same buffer to about 50 mg ml⁻¹. An A₆0₀ of 1.0 was shown to correspond to 0.22 mg (dry weight) ml⁻¹ (*L. brevis*) or 0.18 mg (dry weight) ml⁻¹ (*L. buchneri*). The cell suspension (5 ml) was added to 15 ml of buffer with the various incubation substrates (50 mM glucose, fructose, or ribose with or without 50 mM glycerol; 20 mM Dl-lactate with or without 50 mM glycerol). The suspensions were incubated undisturbed for the required time at 30°C before centrifugation so that the fermentation products could be measured by the methods described for growing cultures.

**RESULTS**

**Effect of glycerol on growth.** Of the four strains of lactobacilli reported by Schutz and Radler (18) to produce significant amounts of 1,3-PDL from glycerol, the two used in the present work (*L. brevis* B22 and *L. buchneri* B190) were the ones that showed the higher growth responses (35 to 85% increase) upon addition of glycerol to the glucose fermentation. For *L. brevis* B18 and B20, the addition of glycerol increased growth by only 12 to 17%.

It was first established that 20 mM glucose, fructose, or ribose limited the final (70 h) extent of growth to about 30% of that which was reached with 200 mM sugar; the growth rate of the cultures was also limited. The detailed examination of the increase in biomass over a period of 70 h for the two strains grown with 20 mM sugar, with and without glycerol (100 mM), showed that the effect of glycerol during the growth of lactobacilli depended on several factors: (i) the organism, (ii) the sugar cofermented, and (iii) the stage of growth considered (Fig. 1). Considering the first periods of exponential growth, in *L. brevis* glycerol had very little effect but in *L. buchneri* glycerol increased the growth rate for each of the three sugars (by 30 to 40%). However, all of the growth rates with glycerol were biphasic, with a second 15-h period of exponential growth at a rate that was faster than the first (i.e., more than doubled for *L. brevis* with glucose), little changed (e.g., 30% reduced for *L. brevis* with fructose), or slower (e.g., reduced 2.5-fold for *L. buchneri* with ribose) and was delayed in the case of *L. brevis* with ribose. As a result of the second period of growth, an effect of glycerol was always to increase the final yield growth by 35 to 120%.

**Effect of glycerol on the metabolism of the sugars.** Substantial differences were observed in catabolic end products from cultures grown with and without glycerol. Both *L. brevis* and *L. buchneri* are heterofermentative (14), and we found them to produce from 20 mM glucose approximately equimolar lactate, some ethanol (12 to 14 mM), but unexpectedly large amounts of acetate (23 to 25 mM). During growth on fructose (20 mM), 35 to 40% of the sugar was reduced to mannitol (i.e., 7 to 8 mM), resulting in slightly more acetate (28 to 26 mM) and less ethanol (4 to 6 mM) per mol of fructose; as predicted (13), the lactate formed was equimolar with the fructose that had not been converted to mannitol, i.e., 12 to 13 mM. The fermentation of 20 mM ribose produced lactate (20 mM) and acetate (36 to 37 mM) but no ethanol. For these three fermentations, the acetate presumably does not arise solely from the sugar added.

When glycerol was added to cultures growing on glucose, the production of 1,3-PDL was accompanied not only by less ethanol but also by less lactate (e.g., Fig. 2A for *L. buchneri*). When all of the glucose had been consumed, there was a decline in the lactate concentration (from 9 mM for *L. brevis* and from 13 mM for *L. buchneri*). Simultaneously, the acetate concentration continued to rise (to 42 and 48 mM) with continuing 1,3-PDL production. This fermentation pattern was similar for both strains, although *L. buchneri* reduced more glycerol and consumed lactate to a greater extent (Fig. 2A).

For the *L. brevis* culture with glucose, the rate of growth may be limited by the rate of energy generation. If so, the effect of glycerol, to increase the growth rate in the second phase (after 35 h of growth), could arise from an ability of glycerol either to generate additional energy itself or to promote an increased rate of glucose use. Even in the absence of glycerol, only 3.5 mM glucose was found to have
LACTOBACILLUS SUGAR-GLYCEROL COFERMENTATION

FIG. 1. Effect of glycerol on growth of L. brevis (A to C) and L. buchneri (D to F). The cultures were grown statically in MLM with 20 mM sugar and 100 mM glycerol. Growth was monitored by measuring the culture’s A600, as described in Materials and Methods.

been consumed by 35 h yet 20 mM was consumed by 60 h. Therefore, the effect of glycerol on the metabolism of the sugars was examined initially by analysis of the end products of catabolism.

When the sugar was changed to fructose, the ability to reduce glycerol did not affect mannitol formation by L. brevis (7 mM maximum; Fig. 2B) but did depress the mannitol concentration (to 4 mM) for L. buchneri, which also began making 1,3-PDL about 10 h earlier than the other organism. With either lactobacillus, glycerol did not depress ethanol formation (in contrast to the glucose-grown cultures), but less ethanol was made again as 1,3-PDL appeared. Later, either organism consumed both the lactate and mannitol that had been formed during the initial growth period and continued to produce acetate (Fig. 2B). The metabolism of mannitol accumulated during the early stages

FIG. 2. Effect of glycerol on the metabolism of various sugars by growing cultures of L. brevis (B and D) and L. buchneri (A and C). The end products, lactate (□), acetate (■), ethanol (▲), 1,3-PDL (○), and mannitol (●), were measured as described in Materials and Methods. The arrow indicates when all of the sugar added to the medium was used. One culture (A) was grown in MLM with glucose and glycerol, another (B) was grown with fructose and glycerol, and two (C and D) were grown with ribose and glycerol.
of heterolactic fermentations has also been reported for \textit{Lactobacillus plantarum} when in the presence of a suitable electron acceptor (other than glycerol) (15).

During growth on ribose, glycerol caused \textit{L. buchneri} to start consuming accumulated lactate (16 mM) as soon as most of the sugar in the medium was used (Fig. 2C). \textit{L. brevis}, on the other hand, removed only lactate and reduced glycerol about 24 h after ribose was completely fermented (Fig. 2D). The lactate removal was accompanied by almost twice as much acetate being produced. Ethanol was still absent when glycerol was cofermented with the ribose.

A strong implication from these data is that the availability of glycerol to generate an electron acceptor allows an eventual conversion of any lactate that has been made into either acetate (an oxidation) or the chemically dissimilar 1,3-PDL (a reduction); the latter case seems extremely unlikely.

The cultures in which the accumulated lactate was less than equimolar with the sugar used were tested for the production of formate as an alternative fate for pyruvate, but not even traces of that acid could be detected. Therefore, a pyruvate-formate lyase is not likely to be competing with the lactate dehydrogenase.

After 70 h of growth with the various supplements, all of the sugar carbon could be accounted for in the end products measured, except that the acetate was always about 17 mM too high. However, when washed cells metabolized similar combinations of sugars and glycerol under nongrowth conditions (100 mM MOPS, pH 7.0), all of the acetate produced could be calculated as arising from the sugar. The combination of these two observations strongly suggests that the excess acetate (17 mM) produced during growth resulted from the metabolism of an MLM component(s), such as amino acids (4).

**Metabolism of lactate.** During glycerol metabolism, the cells will need NADH to reduce the potentially toxic 3-HPA to 1,3-PDL. During the earlier growth period, this requirement is met by decreasing the NADH consumed in the production from glucose of lactate and ethanol (of mannitol from fructose). The diversion of each mole of lactate to acetate conserves directly 1 mol of NADH; also, each mole of pyruvate diverted to acetate not only conserves 2 mol of NADH but also can generate 1 mol of ATP (via acetyl phosphate and phosphotransacetylase) and so account for the increased growth yield.

Whenver lactate was being consumed (after sugar exhaustion), 1,3-PDL and acetate were produced (Fig. 2). The suggestion that lactobacilli can grow by cofermenting lactate and glycerol as a secondary process was confirmed when cells grown on glucose plus glycerol were used to inoculate fresh medium with appropriate supplements and incubated for 2 days at 30°C. When the pH of the fresh medium had been initially adjusted to 5.8, either \textit{L. brevis} or \textit{L. buchneri} grew with lactate alone ($A_{500}$ of 1.0 and 1.2, respectively) and grew much better ($A_{500}$ of 1.9 and 3.0, respectively) when glycerol had also been supplied; glycerol alone supported relatively little growth ($A_{500}$ of 0.3). At the more usual initial medium pH of 7.2, the growth was poorer ($A_{500}$ of 0.3 and 0.6, respectively) and was not improved by glycerol. This correlates well with the finding that in the fermentations depicted in Fig. 2 lactate oxidation was first observed only after the pH of the media had dropped to approximately 5.2.

To test directly the ability of cells to couple lactate and glycerol metabolism, cells grown with glucose plus glycerol until the onset of the second period of exponential growth were washed, resuspended, and incubated (12 h at 30°C) in lactate-NaOH buffer (20 mM), pH 5.5, with and without glycerol (100 mM). A significant amount of lactate (3.2 mM for \textit{L. brevis} and 5.0 mM for \textit{L. buchneri}) was oxidized to an equimolar amount of acetate only when glycerol was present. Because only 3.9 and 6.3 mM 1,3-PDL were found to be produced, it is likely that only one of the reactions during the conversion of lactate to acetate generates NADH.

There are two published studies in which the lactate formed during the earlier period of growth was metabolized further, by the homofermentative \textit{L. plantarum} (16, 17). However, that metabolism was aerobic, as was the partial conversion of lactate to acetate by one heterofermentative strain of \textit{L. brevis} (29). The effect of aeration on the metabolism of lactate was tested here by growing \textit{L. brevis} and \textit{L. buchneri} in strongly aerated MLM with glucose with and without glycerol and by determining the metabolic products. Although \textit{L. brevis} did not grow aerobically at all, the \textit{L. buchneri} cell density reached 70% of the anaerobic cell density value after 64 h. Aerobically, the \textit{L. buchneri} culture did convert to acetate part of the lactate that had first accumulated to 18.5 mM from glucose. But aeration actually suppressed lactate oxidation (irrespective of the presence of glycerol); e.g., aerobically 60% of the lactate was still found 40 h after glucose catabolism ended, yet anaerobically all the lactate had gone within 15 h (Fig. 2A).

**Enzymes responsible for lactate metabolism.** In \textit{L. plantarum}, the enzyme catalyzing the conversion of lactate to pyruvate is an iLDH (EC 1.1.99) (17). We have found, by using both D-(-) and L- (+)-lactates, that this activity was also present in our cell extracts, but only in those from cultures in which lactate was being metabolized (Tables 1 and 2). These activities contrast with the very much greater NAD-dependent lactate dehydrogenase (EC 1.1.1.27 and EC 1.1.1.28) activities that are still present even when lactate production has stopped.

In \textit{L. plantarum}, the activities of the iLDH and pyruvate oxidase (EC 1.2.3.3) are together enhanced in the presence of oxygen and reduced in the presence of glucose (17, 20, 21). In \textit{L. brevis} and \textit{L. buchneri}, the activity of iLDH (Tables 1 and 2) was detected only in extracts from cultures cofermenting sugar and glycerol; the cultures without glycerol had no iLDH activity. When present, the iLDH activity became significant only after the sugar was exhausted and lactate oxidation began. Because iLDH could not be found even after all of the sugar was used in cultures without glycerol, another factor apart from the exhaustion of sugar and the availability of lactate as an alternative metabolizable carbon source is required for the expression of the enzyme.

**Enzymes responsible for glycerol metabolism.** In contrast to a previous report on \textit{L. brevis} B18 (18), in both \textit{L. brevis} B22 and \textit{L. buchneri} B190 the two enzymes converting glycerol to 1,3-PDL (glycerol dehydratase and 1,3-PDL dehydrogenase) were expressed in concert without needing to be induced by added glycerol (Tables 1 and 2). These results were extended when washed cells that had been grown either with or without glycerol produced similar concentrations of 1,3-PDL when incubated in a buffered mixture of glycerol and a sugar (Table 3). These cells had been harvested at the end of the period of exponential growth, i.e., once all the sugar had been used. It is not surprising, therefore, that the cultures of \textit{L. brevis} with ribose made no 1,3-PDL. This agrees with the finding that the metabolism of glycerol by this organism began only 24 h after all of the ribose was used (Fig. 2D) and with the measurements of the specific activities in extracts of the two enzymes responsible.
TABLE 1. Expression of the enzymes involved in the metabolism of lactate and glycerol by *L. brevis* B22a

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Growth (A1000)</th>
<th>Sp act (mU mg⁻¹)b</th>
<th>nLDH</th>
<th>iD-(−)-LDH</th>
<th>iL-(+)-LDH</th>
<th>Glycerol dehydratase</th>
<th>1,3-PDL dehydrogenase</th>
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</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.6</td>
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<td>0</td>
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<td>0</td>
<td>30.0</td>
<td>190</td>
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<td></td>
<td>2.3</td>
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<td>0</td>
<td>7.5</td>
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<tr>
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<td>0</td>
<td>47.0</td>
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<tr>
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<td>0</td>
<td>0</td>
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<td>8</td>
<td>8</td>
<td>59.0</td>
<td>280</td>
</tr>
<tr>
<td>Ribose</td>
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<td>0</td>
<td>0</td>
<td>3.0</td>
<td>40</td>
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<td>8</td>
<td>8</td>
<td>16.0</td>
<td>130</td>
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<tr>
<td></td>
<td>3.6</td>
<td>5,000</td>
<td>9</td>
<td>12</td>
<td>12</td>
<td>24.0</td>
<td>170</td>
</tr>
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</table>

a The cells were grown as described in Materials and Methods. After 20 and 60 h (and 80 h in the case of ribose), growth was measured, the cultures were harvested, and the respective cell extracts were assayed for the indicated enzyme activities as described in Materials and Methods.
b nLDH, iD-(−)-LDH, and iL-(+)-LDH, NAD-dependent lactate dehydrogenase and NAD-independent d-(−)– and l-(+)-lactate dehydrogenase, respectively.

for the reduction of glycerol to 1,3-PDL, for they increased only in cells harvested after 40 h of growth.

When *L. brevis* was grown without added glycerol, the activity of the two enzymes was highest in cells grown on glucose, followed by the activity in cells grown on fructose, and very low when the growth substrate was ribose (Table 1). For *L. buchneri* (Table 2), the effect of the sugar on the expression of the glycerol-metabolizing enzymes is not so clear. However, the latter organism generally induced more of the glycerol dehydratase and 1,3-PDL dehydrogenase, e.g., during growth on ribose, in which not even ethanol is made as a way of recycling NADH.

DISCUSSION

For these heterofermentative lactobacilli, the ability to use glycerol was believed until now to provide the cell with 3-HPA as an alternative hydrogen acceptor for recycling the NADH that is generated during hexose catabolism via the 6-phosphogluconate pathway. However, the sugar-glycerol co fermentations by *L. brevis* and *L. buchneri* clearly show that at a lower sugar concentration (a condition likely to prevail in nature), this extra ability to reoxidize NADH also allows lactate to be scavenged for ATP generation during a second, slow growth phase. The glycerol-supported removal of mannitol subsequent to fructose fermentation further suggests that after its initial role in the oxidation of hexose intermediates the production of 1,3-PDL may allow a range of metabolites to be scavenged. This second role can also be considered as a cellular detoxication, because the simultaneous presence of both glycerol dehydratase and excess glycerol should cause the lactobacilli to accumulate toxic 3-HPA (26) unless there was a means either to continue the supply of NADH for 1,3-PDL production or to inactivate the dehydratase.

The anaerobic conversion of lactate to acetate is assumed to occur via pyruvate and acetyl phosphate, with acetate kinase generating ATP to support growth. The conversion in vivo of lactate to pyruvate is not likely to be catalyzed by the effectively irreversible NAD-dependent lactate dehydrogenase, however high its specific activity (8), but rather only by the iLDH(s).

Because the extracted iLDH activity of cells grown on ribose plus glycerol appeared only after 40 h of incubation in

TABLE 2. Expression of the enzymes involved in the metabolism of lactate and glycerol by *L. buchneri* B190a

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Growth (A1000)</th>
<th>Sp act (mU mg⁻¹)b</th>
<th>nLDH</th>
<th>iD-(−)-LDH</th>
<th>iL-(+)-LDH</th>
<th>Glycerol dehydratase</th>
<th>1,3-PDL dehydrogenase</th>
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<td>Glucose</td>
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<td>0</td>
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<td>100</td>
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<tr>
<td>Fructose + glycerol</td>
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<td>0</td>
<td>75.0</td>
<td>120</td>
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<td>12,000</td>
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<td>245</td>
<td>245</td>
<td>68.0</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Ribose + glycerol</td>
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<td>0</td>
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<td>90.0</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>9,000</td>
<td>90</td>
<td>190</td>
<td>190</td>
<td>70.0</td>
<td>70</td>
</tr>
</tbody>
</table>

a Results were derived as described in Table 1.
b See Table 1, footnote b.
TABLE 3. Production of 1,3-PDL by whole cells of *L. brevis* and *L. buchneri* grown in the presence and in the absence of glycerol

<table>
<thead>
<tr>
<th>Organism and incubation substrate</th>
<th>1,3-PDL (mM) after 3 h of incubation at 30°C with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose Fructose Ribose</td>
</tr>
<tr>
<td><em>L. brevis</em> B22</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Glucose + glycerol</td>
<td>38 38 0</td>
</tr>
<tr>
<td>Fructose</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Fructose + glycerol</td>
<td>40 38 0</td>
</tr>
<tr>
<td><em>L. buchneri</em> B190</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Glucose + glycerol</td>
<td>46 46 50</td>
</tr>
<tr>
<td>Fructose</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Fructose + glycerol</td>
<td>48 51 51</td>
</tr>
</tbody>
</table>

* The incubation of the cells and the determination of the 1,3-PDL formed are described in Materials and Methods. The results were essentially the same when glycerol was included in the medium.

MLM, i.e., when the production of 1,3-PDL began, the expression of iLDH seems to be independent of the presence of glycerol per se and more directly linked to the NADH-dependent reduction of 3-HPA. Linkage of the expression of iLDH to a raised intracellular NAD/NADH ratio would account for the appearance of the enzyme not only during the anaerobic growth of *L. brevis* and *L. buchneri*, when glycerol is being reduced to 1,3-PDL, but also during the aerobic metabolism of *L. plantarum* when O2 is used to recycle NADH through NADH oxidase. Furthermore, the investigations on the metabolism of lactate showed that the latter is better oxidized at pH 5.8 that at pH 7.2 and that aerobic cultures of *L. plantarum* produced acetate when growing at pH 5.5 but not at pH 7.7 (28), suggesting that the activity of the enzyme is best at low pH. Since the conversion of lactate to acetate causes a rise in pH, the drop in the pH due to the production of acid from sugar may be a necessary, but not a sufficient, condition for the expression of iLDH. The similar values of pH 5 measured in the culture medium after the metabolism of the sugar with and without glycerol suggest that the induction of the iLDH is not likely to be related to a different degree of dissociation of the lactate (and therefore ability to penetrate the cell) when the cultures are grown in the presence or absence of glycerol.

The apparent constitutivity of the glycerol dehydratase and 1,3-PDL dehydrogenase is surprising but may reflect inductive control by a glycerol metabolite such as glycerol 3-phosphate derived from carbohydrate. This could also provide an indirect mechanism for the intracellular NAD/NADH ratio to influence enzyme expression; the three sugars used here would be expected to set different NAD/NADH ratios and did affect differently the formation of the enzymes; e.g., ribose should give the cell the least difficulty in maintaining a higher NAD/NADH ratio, and as shown for *L. brevis* it is best at suppressing the enzymes of glycerol reduction.

**ACKNOWLEDGMENTS**

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**REFERENCES**


