13C Nuclear Magnetic Resonance Analysis of Glucose and Citrate End Products in an ldhL-ldhD Double-Knockout Strain of Lactobacillus plantarum

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We have examined the metabolic consequences of knocking out the two ldh genes in Lactobacillus plantarum using 13C nuclear magnetic resonance. Unlike its wild-type isogenic progenitor, which produced lactate as the major metabolite under all conditions tested, ldh null strain TF103 mainly produced acetoin. A variety of secondary end products were also found, including organic acids (acetate, succinate, pyruvate, and lactate), ethanol, 2,3-butanediol, and mannitol.

Lactobacillus plantarum is a lactic acid bacterium that ferments glucose via the homofermentative pathway. Glucose is first converted to pyruvate through the Embden-Meyerhof-Parnas pathway, and most of the pyruvate is further reduced to D- and L-lactate by stereospecific NAD-dependent lactate dehydrogenases (LDHs). In addition to lactate, L. plantarum (and other lactic acid bacteria) can convert pyruvate to a variety of secondary end products (Fig. 1) whose distribution depends on strains and culture parameters. For instance, production of acetate from pyruvate via acetylphosphate has been largely reported for L. plantarum grown on glucose under aerobic conditions (14, 16, 33, 41, 42). The enzyme responsible for this conversion is pyruvate oxidase (15, 34-36, 41). Acetate production from glucose has also been observed in L. plantarum under anaerobiosis at neutral or alkaline pH (27, 33, 42), as well as in the presence of an external electron acceptor, such as citrate (25). Likewise, production of ethanol from glucose has also been reported for both aerobic and anaerobic cultures of L. plantarum (7, 28, 33). Ethanol can be formed from pyruvate via acetyl coenzyme A and acetaldehyde, with acetyl-CoA resulting from the anaerobic pyruvate formate-lyase reaction of citrate (25). Oxalacetate is usually decarboxylated to pyruvate, which is then converted to pyruvate via acetyl-CoA and acetaldehyde, with acetyl-CoA resulting from the anaerobic pyruvate formate-lyase pathway. The presence of this pathway in a few strains of L. plantarum has been suggested (12, 25), but pyruvate formate-lyase activity has not been demonstrated. Ethanol is also produced from external electron acceptors, such as citrate and acetate, during anaerobic catabolism of mannitol in L. plantarum (28). Moreover, during glucose fermentation, L. plantarum frequently produces C4 compounds from pyruvate via α-acetolactate. The formation of these metabolites (diacetyl, acetoin, and 2,3-butanediol) is enhanced by the addition of exogenous pyruvate (9, 16, 27, 30, 31) and is also stimulated by low pH values (30, 41).

Citrate is responsible for a large variety of fermentation profiles in lactic acid bacteria (17). Its catabolism usually generates additional pyruvate without reduction of the NAD cofactor, and pyruvate is further channeled towards the formation of the above-mentioned end products. Another pathway of citrate dissipation in L. plantarum and some other lactoba-
olism of glucose. We hypothesize that it does not result from pyruvate formate-lyase activity, since this pathway also leads to formate accumulation and has been reported for other species only under strict anaerobic conditions (13). Ethanol production from external electron acceptors (acetate, citrate, and oxalacetate) during anaerobic catabolism of mannitol in L. plantarum has been previously described (28, 37). According to these studies, ethanol derives from acetate via acetyl phosphate, acetyl coenzyme A, and acetaldehyde. All the enzymes involved in this pathway were shown to be constitutive in wild-type L. plantarum (7). Aerobic ethanol production in the LDH-defective strain therefore probably stemmed from acetyl phosphate arising from pyruvate oxidase activity. Likewise, part of this intermediate was also converted to acetate, since this metabolite was also produced under these conditions.

Low concentrations of mannitol were also detected. As mentioned above, L. plantarum is able to use this hexose as a carbon source (37). This pathway, which involves NAD$^+$ reduction, might be reversed in our strain. Under this condition, fructose 6-phosphate formed from glucose could be converted to mannitol (Fig. 1), lowering the NAD$^+$ debt. Small amounts of mannitol have been described as end products of Streptococcus mutans in the presence of large concentrations of sucrose or glucose (26). The presence of mannitol-1-phosphate dehydrogenase has been demonstrated for both TF103 and NCIMB8826 extracts (Table 2).

Low concentrations of lactate were also measured. Both D and L isomers were observed with a large excess of L-lactate (80%). However, no LDH activity was present in the mutant strain (Table 2). Other NAD-dependent dehydrogenases able to reduce α-keto acids to the corresponding α-hydroxy acids in vitro in lactic acid bacteria have been previously described (19). Some of these stereospecific enzymes (known as hydroxyisocaproate dehydrogenases) are able to weakly reduce pyruvate to lactate in vitro (1, 23, 24). It was reported that during anaerobic mannitol fermentation, L. plantarum was able to use several α-keto acids as electron acceptors for NAD$^+$ regeneration (α-ketobutyric acid, α-ketovaleric acid, and α-ketocapry-
FIG. 2. $^{13}$C nuclear magnetic resonance spectra of end products from cell suspensions of wild-type *L. plantarum* NCIMB8826 (A) and LDH-defective *L. plantarum* TF103 (B) under the following conditions: aerobiosis and 50 mM glucose (a); aerobiosis and 50 mM glucose plus 10 mM citrate (b); anaerobiosis and 50 mM glucose (c); and anaerobiosis and 50 mM glucose plus 10 mM citrate (d). ACN, acetoin; ACT, acetate; BUT, 2,3-butanediol; CIT, citrate; black triangles, resonance of residual glucose; ETH, ethanol; LAC, lactate; MAN, mannitol; PYR, pyruvate; SUC, succinate. Spectra are presented from 15 to 75 ppm. Carbon atoms of the carbonyl groups were also detected, but under our experimental conditions with a 6-s repetition time, those signals were not quantitative.
The production of CO$_2$ and volatile compounds was not measured during this study.

Aerobic and anaerobic glucose-citrate cometabolism in LDH-deficient strain TF103. A typical anaerobic pathway frequently observed in lactic acid bacteria is pyruvate formate-lyase (if any) is likely to have been inactivated, since the enzyme is reported to be highly sensitive to oxygen (8). Most of the end products detected during aerobic and anaerobic glucose-citrate cometabolism were the same as those derived from glucose alone (Table 1). Acetoin remained the major metabolite. We also identified trace amounts of 2,3-butanediol resulting from acetoin reduction. Lactate production was largely increased, and all the additional lactate was in the form of the L isomer. This fits the above-described hypothesis of l-lactate production through malolactic fermentation; under these conditions, oxalacetate would arise from citrate (Fig. 1). On the other hand, levels of ethanol and mannitol production were lower in the presence of citrate, which could reflect NAD$^+$ regeneration via other pathways owing to citrate utilization as the external electron acceptor. Small amounts of pyruvate were also detected. Wild-type Lactococcus lactis and Leuconostoc spp. have been reported to excrete pyruvate under conditions in which C$_3$ compounds are formed, i.e., during citrate fermentation (18, 38), and the presence of these compounds has been correlated with an internal accumulation of high concentrations of pyruvate, which very likely occurs in the LDH-defective strain TF103 of L. plantarum.

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REFERENCES


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TABLE 1. End product concentrations from glucose and glucose-citrate cometabolism by cell suspensions of wild-type (NCIMB8826) and LDH-defective (TF103) strains of L. plantarum

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Strain</th>
<th>Carbon source</th>
<th>Glucose</th>
<th>Citrate</th>
<th>Lactate</th>
<th>Acetate</th>
<th>Ethanol</th>
<th>Acetoin</th>
<th>Mannitol</th>
<th>2,3-Butanediol</th>
<th>Succinate</th>
<th>Pyruvate</th>
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<tbody>
<tr>
<td>Aerobic</td>
<td>NCIMB8826</td>
<td>Glucose</td>
<td>8</td>
<td>72</td>
<td>6</td>
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<tr>
<td></td>
<td>NCIMB8826</td>
<td>Glucose plus</td>
<td>6</td>
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<td>77</td>
<td>12</td>
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<td></td>
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<tr>
<td></td>
<td>TF103</td>
<td>Glucose</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td>&lt;1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TF103</td>
<td>Glucose plus</td>
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<td></td>
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<tr>
<td>Anaerobic</td>
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<td>Glucose</td>
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<tr>
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<td>Glucose plus</td>
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<td>20</td>
<td>6</td>
<td>3</td>
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</table>

* The production of CO$_2$ and volatile compounds was not measured during this study.

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TABLE 2. Enzymatic activities detected in cell extracts from the TF103 and NCIMB8826 strains of L. plantarum

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sp act (U/mg of protein)*</th>
<th>Reference(s) for assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TF103</td>
<td>NCIMB8826</td>
</tr>
<tr>
<td>Pyruvate carboxylase</td>
<td>6 × 10$^{-3}$</td>
<td>5 × 10$^{-3}$</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>173 × 10$^{-3}$</td>
<td>NA$^*$</td>
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<tr>
<td>Malolactic enzyme</td>
<td>25 × 10$^{-3}$</td>
<td>NA</td>
</tr>
<tr>
<td>Mannitol-1 phosphate dehydrogenase</td>
<td>23 × 10$^{-3}$</td>
<td>29 × 10$^{-3}$</td>
</tr>
<tr>
<td>LDH</td>
<td>ND$^*$</td>
<td>14</td>
</tr>
</tbody>
</table>

* Cell extracts were prepared according to the method described in reference 10.

* One unit of activity corresponds to the conversion of 1 μmol of substrate per min.

* NA, not applicable because of the high level of LDH activity and large amount of lactate in the wild-type strain, which interfere with malate dehydrogenase and malolactic enzyme assays, respectively.

* ND, not detected.


