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Pyruvate Fermentation by *Oenococcus oeni* and *Leuconostoc mesenteroides* and Role of Pyruvate Dehydrogenase in Anaerobic Fermentation

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The heterofermentative lactic acid bacteria *Oenococcus oeni* and *Leuconostoc mesenteroides* are able to grow by fermentation of pyruvate as the carbon source (2 pyruvate → 1 lactate + 1 acetate + 1 CO_2). The growth yields amount to 4.0 and 5.3 g (dry weight)/mol of pyruvate, respectively, suggesting formation of 0.5 mol ATP/mol pyruvate. Pyruvate is oxidatively decarboxylated by pyruvate dehydrogenase to acetyl coenzyme A, which is then converted to acetate, yielding 1 mol of ATP. For NADH reoxidation, one further pyruvate molecule is reduced to lactate. The enzymes of the pathway were present after growth on pyruvate, and genome analysis showed the presence of the corresponding structural genes. The bacteria contain, in addition, pyruvate oxidase activity which is induced under microoxic conditions. Other homo- or heterofermentative lactic acid bacteria showed only low pyruvate fermentation activity.

Oenococcus oeni and *Leuconostoc mesenteroides* are heterofermentative lactic acid bacteria (LAB) that are closely related to each other and have previously been grouped within the same family due to many physiological and genetic similarities (6, 8, 9). *O. oeni* is used in wine fermentation for degradation of malic acid, whereas *L. mesenteroides* is used for fermentation of vegetables. Both bacteria convert hexoses by heterolactic fermentation to lactate, ethanol, and CO_2. Ethanol formation from acetyl phosphate (or acetyl coenzyme A [acetyl-CoA]) is required for reoxidation of NAD(P)H, which is produced in the pentose phosphate pathway during hexose oxidation. In many heterolactic acid bacteria and in particular in *O. oeni*, NAD(P)H reoxidation by the ethanol pathway is slow because of low acetaldehyde dehydrogenase activity (16, 26, 27, 34). When, in addition, coenzyme A (HSCoA), and consequently acetyl-CoA, is limiting due to a shortage of the precursor pantothenate, the low capacity of NAD(P)H reoxidation by the ethanol pathway limits the metabolism and growth rate of the bacteria (26). For this reason, *O. oeni*, and to some extent also *L. mesenteroides*, uses alternative pathways for NAD(P)H reoxidation, such as the reduction of erythrose-4-phosphate to erythritol, to overcome the limitation (26, 34).

In addition to the alternative endogenous pathways of erythritol and glycerol formation for NAD(P)H reoxidation, the bacteria are able to use external electron acceptors for reoxidation of NAD(P)H. Fructose, O_2, and pyruvate can be used as electron sinks by *O. oeni* and *L. mesenteroides*, yielding mannitol, H_2O_2, and lactate as the reduced end products (16, 23, 27). *O. oeni*, which has very low capacities for NAD(P)H reoxidation in the ethanol pathway, gains significantly in metabolic and growth rates by the use of external electron acceptors.

When pyruvate was supplied in excess as an electron acceptor, there were indications that pyruvate is not only used as an electron acceptor but also oxidized to acetate (27, 28). As shown here, pyruvate can be disproportionated to lactate and acetate at substantial rates and can be used as the sole substrate for fermentation and growth by the bacteria. The fermentation process and the enzymatic reactions of this new fermentation pathway of *O. oeni* and *L. mesenteroides* are characterized here.

MATERIALS AND METHODS

Bacteria and growth. *Oenococcus oeni* B1 (26), *Leuconostoc mesenteroides* (DSMZ 20240), *Lactobacillus lactis* (DSMZ 20072), *Lactobacillus plantarum* (ATCC 8014), and *Lactococcus lactis* (DSMZ 20481) were used. Subcultures of the bacteria were grown in modified tomato juice medium (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], Braunschweig, Germany). Growth was performed at 30°C in anoxic Malo-Lactic Differential (MLD) medium (4) under N_2 in sealed bottles (50 ml or 400 ml as given for the specific experiments) at pH 5.9 with the modification of Richter et al. (26, 27). Anoxic conditions were checked by the inclusion of resazurin (1 mg/liter) in control experiments. The media were inoculated with 2% (vol/vol) of the subcultures.

Cell homogenates and enzyme activities. MLD medium (400 ml) containing the respective C source (80 mM pyruvate, 40 mM glucose, or 40 mM glucose plus 80 mM pyruvate) was inoculated with 8 ml of a subculture, made anoxic by degassing and gassing with N_2 for three cycles and grown to the late-logarithmic growth phase. The bacteria were harvested (17,700 × *g* for 25 min at 4°C), washed, and resuspended in 10 ml potassium-phosphate buffer (50 mM at pH 7). The cells were broken in a cell mill (Vibrogen VI; Bühler) or by three passages through a French pressure cell (1,200 lb/in^2). For treatment in the cell mill, the bacterial suspension (10 ml) was mixed with 20 g of zirconia/silica beads (0.1 mm; Roth) and incubated for three cycles of 3 min, with 30-s intervals between the cycles, at 4°C and 75 Hz. The cell homogenates were cleared by centrifugation at 11,400 × *g* for 15 min, and the supernatant was either frozen in aliquots at −20°C or used immediately. Protein was determined by the Bradford assay (3) using Rothiquant (Roth) in a microassay.

(i) PDH. The assay of Reed and Willms (25), which depends on the conversion of pyruvate to acetyl-CoA, NADH, and CO_2 in the presence of NAD (acetyl-

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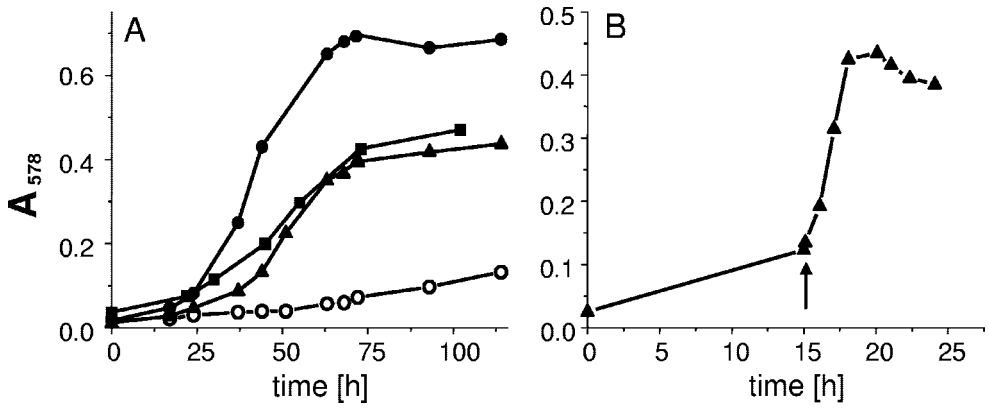

FIG. 1. Growth on pyruvate in MLD medium (A) and in "predigested" MLD medium (B). The substrates for growth were pyruvate (40 mM in panel A and 20 mM in panel B) (▲), 40 mM glucose (■), 10 mM glucose plus 20 mM pyruvate (●), or no additional carbon source (○). Experiment A is shown for *Oenococcus oeni*, experiment B for *Leuconostoc mesenteroides*. In panel B, pyruvate was added (arrow) after growth of the bacteria in MLD medium for 15 h without an additional carbon source.

CoA formation via pyruvate dehydrogenase [PDH]), was used. Acetyl-CoA is converted in a coupled assay by phosphotransacetylase to acetyl phosphate, and NAD is regenerated by inclusion of lactate dehydrogenase. Formation of acetyl phosphate is determined colorimetrically by reaction with hydroxylamine and Fe(III), which results in formation of a reddish brown dye that was measured discontinuously at 540 nm (14). The formation of acetyl phosphate (or of the dye complex) corresponds to the activity of pyruvate oxidation and is expressed in μmol of acetyl-CoA or acetyl phosphate formed/min/g of protein. In the same setup, degradation of pyruvate was tested by high-performance liquid chromatography (HPLC) (see below), and it was verified that pyruvate degradation depended on the presence of NAD.

(ii) Pox. Pyruvate oxidase (acetyl phosphate-forming, or Pox) activity (pyruvate + O_2 + P_i → acetyl phosphate + H_2O_2 + CO_2) was tested by the formation of acetyl phosphate in the presence of O_2 and pyruvate, but without added phosphotransacetylase or lactate dehydrogenase. The requirement for O_2 and the independence from HSCoA allow differentiation from pyruvate dehydrogenase. Acetyl phosphate formation was tested with hydroxylamine and Fe(III) as described above for pyruvate dehydrogenase.

(iii) Other enzyme assays. Lactate dehydrogenase was assayed photometrically as the pyruvate-dependent oxidation of NADH (34). Phosphotransacetylase activity was followed photometrically at 233 nm by the formation of acetyl-CoA from acetyl phosphate plus HSCoA ($E_{\text{acetyl-CoA}} = 4.4\ \text{mM}^{-1}\ \text{cm}^{-1}$) using 260 nm as a reference wavelength (Specord S10; Zeiss, Jena, Germany) according to Klotzsch (13). Acetate kinase was tested by the decrease of NADH in a coupled assay with pyruvate kinase and lactate dehydrogenase (2).

All activities were assayed at least in duplicate (variation, <30%), and average values are given. The activities correspond to the conversion of 1 μmol of pyruvate (pyruvate dehydrogenase, pyruvate oxidase, and lactate dehydrogenase), the formation of 1 μmol of acetyl-CoA (phosphotransacetylase), or the conversion of 1 μmol of acetate or acetyl phosphate (acetate kinase) per g of protein/min at 30°C.

Fermentation substrates and products. For determination of fermentation balances in growing bacteria, MLD medium (50 ml) was inoculated with 2% (vol/vol) of a subculture of the bacteria. Growth was performed under anoxic conditions, which were controlled by inclusion of resazurin. At time zero (inoculation) and at various times after inoculation, samples were withdrawn and centrifuged at 11,000 × *g* for 5 min. The cell-free supernatants were analyzed for the substrates and products by HPLC separation on an Aminex HPX87H column (26). The assays were performed in duplicate (or repeated if the values differed by more than 15%), and the mean values are given. In addition, D- and L-lactate were determined photometrically by the D- and L- lactate dehydrogenase-dependent reduction of NAD at the expense of lactate (34). For fermentation balances, cultures that had converted >7 mM pyruvate were used. All fermentation reactions were performed twice or more, and typical results are shown. In some experiments, a medium without a C source was inoculated and incubated with the bacteria for 12 to 24 h under anoxic conditions to ensure degradation of fermentable substrates in the medium. Growth was then started by addition of the substrate after cessation of bacterial growth in the medium (generally at an optical density at 578 nm [OD_{578}] of 0.2 to 0.3).

For measurement of fermentation balances in resting cells, the bacteria were first grown in MLD medium to the late-exponential growth phase with the respective substrate. The bacteria were then washed in morpholinepropanesulfonic acid (MOPS) salts medium (pH 7.4) and resuspended in the same medium under anoxic conditions (26, 27). The bacteria (OD_{578}, ~6) were incubated in the medium under anoxic conditions with the respective carbon source. At time zero and after various times, samples were withdrawn, and the substrates and products were determined from the supernatant by HPLC analysis.

Database search and identification of genes encoding the relevant enzymes. Proteome data of the partly and fully sequenced genomes were obtained from the U.S. Department of Energy Joint Genome Institute (JGI) and the European Bioinformatics Institute (EBI). Identification of the genes includes functional domain searches and BLAST analysis. Functional domains characteristic for each enzyme were determined using the reference sequences from the following organisms: *L. mesenteroides* (PDH, Pox, and pyruvate formate lyase [PFL]), *Escherichia coli* (phosphotransacetylase, acetate kinase, and D-lactate dehydrogenase), and *Enterococcus faecium* (pyruvate ferredoxin oxidoreductase [PFOR]). The proteomes were screened for the functional domains identified. The analysis was carried out by means of InterProScan (18). For verification, the hit sequences were analyzed using BLAST. The presence of an open reading frame (ORF) indicates that the corresponding domain is present and that the BLAST hit showed the functional annotation.

RESULTS AND DISCUSSION

Growth of *Oenococcus oeni* and *Leuconostoc mesenteroides* with pyruvate. *Oenococcus oeni* and *Leuconostoc mesenteroides* are able to use pyruvate as an electron acceptor during growth on hexoses, and the pyruvate is then reduced to lactate (23, 27, 28). When pyruvate was supplied in a two- to fourfold excess over hexoses, as much as one-quarter of the pyruvate was converted to acetate (27, 28). Therefore, we tested whether pyruvate can serve as a substrate for growth. *O. oeni* showed significant growth in MLD medium with pyruvate compared to growth without an added carbon source (Fig. 1A). Due to the nutritional requirements of *O. oeni* and *L. mesenteroides*, complex media are required for growth. The MLD medium that was used for growth is supposed to supply only essential nutrients but no fermentable substrate. Growth in the MLD medium depended on pyruvate (Fig. 1B). The medium was first depleted by incubation with bacteria, but without addition of pyruvate, which resulted in very poor growth (OD_{578}, ≤0.15). After addition of pyruvate, the growth rate was stimulated dramatically, and an additional OD_{578} of 0.3 to 0.4 was obtained for *L. mesenteroides* (Fig. 1B) and *O. oeni* (not shown). The stimulation of the growth rate with pyruvate was compa-

TABLE 1. Parameters for growth of *Oenococcus oeni* and *Leuconostoc mesenteroides* on pyruvate and comparison to growth on glucose[a]

Carbon source	μ (h^{-1})		Y (g [dry wt]/mol)	
	O. oeni	*L. mesenteroides*	*O. oeni*	*L. mesenteroides*
Pyruvate	0.053	0.42	4.0	5.3
Glucose	0.024	0.28	8.3	10.5
Glucose + pyruvate	0.083	0.47	17.9	22.0

[a] Growth was performed under anoxic conditions in MLD medium with the carbon source indicated. Growth rate (μ) and growth yield (Y) were determined from the experiments for which results are shown in Fig. 1. Each value is the mean for two independent experiments.

rable to that with glucose (Table 1), but the cell densities achieved with glucose were higher. Supply of pyruvate plus glucose, where pyruvate is used as an electron acceptor for glucose oxidation, resulted in high growth rates, as described previously (27, 28). The response of *L. mesenteroides* to pyruvate as a carbon source or as an electron acceptor was very similar to that of *O. oeni* (Table 1). The molar growth yields of these bacteria with pyruvate amounted to 4 and 5.3 g (dry weight)/mol pyruvate, respectively, about half the growth yields with glucose (Table 1). Therefore, the ATP yield with pyruvate can be estimated as 0.5 mol of ATP/mol of pyruvate.

Lactate and acetate as the end products of pyruvate fermentation. Fermentation balances were determined with bacteria growing in MLD medium on pyruvate after preceding consumption of fermentable substrates in the medium. This precaution should help to avoid the formation of additional products from components of the MLD medium. Under these conditions, *O. oeni* as well as *L. mesenteroides* fermented pyruvate to approximately stoichiometric amounts of lactate and acetate (and presumably CO_2) (Table 2), suggesting disproportionation of 1 mol of pyruvate to 0.5 mol of lactate and of acetate. Neither ethanol, erythritol, glycerol, nor other fermentation products were detected in significant amounts. The recovery of 83 to 97% of the carbon in lactate, acetate, and CO_2 confirms that no other products were formed in substantial amounts and that pyruvate was the only substrate for growth.

TABLE 2. Fermentation balances of *O. oeni* and *L. mesenteroides* for growth on pyruvate or glucose under anoxic conditions or in resting cells[a]

Condition	Fermentation balance (mol of product/mol of pyruvate consumed)				% C[c]
	Lactate	Acetate	Ethanol	CO_2[b]	
Growing bacteria					
O. oeni B1	0.44	0.47	<0.04	0.47	91
L. mesenteroides	0.50	0.33	<0.04	0.33	83
Resting bacteria					
O. oeni B1	0.38	0.47	<0.04	0.47	85
L. mesenteroides	0.45	0.52	<0.04	0.52	97

[a] Fermentation balances of growing bacteria were determined in MLD medium after consumption of fermentable substrates in the medium by the bacteria. Pyruvate (20 mM) was added as the substrate for growth after digestion of the medium without added substrate. The fermentation balance of the resting bacteria was determined by incubating washed bacteria (OD_{578}, 6) in MOPS buffer with 10 mM pyruvate under anoxic conditions.

[b] Calculated from the amounts of acetate formed.

[c] Carbon yield of the fermentation products (lactate, acetate, CO_2) compared to the amount of pyruvate consumed.

Similar fermentation experiments were performed with resting bacteria in cell suspensions. Bacteria pregrown on pyruvate degraded pyruvate to lactate and acetate (and presumably CO_2) as the major products. The products were found in ratios very similar to those of the growing bacteria (Table 2), confirming pyruvate disproportionation to lactate, acetate, and CO_2 by the bacteria. An enzymatic assay showed that the lactate excreted by *O. oeni* was exclusively (>98%) D-lactate.

Use of pyruvate by other homo- and heterolactic acid bacteria. In a similar way, *Lactobacillus plantarum*, *Lactobacillus lactis*, and *Lactococcus lactis* were incubated under anaerobic conditions in MLD medium with pyruvate as the sole C source. The bacteria showed only poor growth stimulation by pyruvate (OD_{578}, ≤0.2). *L. plantarum* and *Lactobacillus lactis* metabolized pyruvate at low rates. The products were lactate and acetate; other products, such as formate, were not detected in significant amounts. When pyruvate was supplied in the presence of glucose, *Lactobacillus lactis* and *L. plantarum* cometabolized comparable amounts of glucose and pyruvate. Lactate was the major end product, suggesting that pyruvate was used predominantly as an electron acceptor. *Lactococcus lactis*, on the other hand, showed no growth stimulation by pyruvate as the sole substrate in MLD medium, and the pyruvate was not metabolized when pyruvate was supplied as the only substrate or in addition to glucose. In cell suspensions, neither *L. plantarum*, *Lactobacillus lactis*, nor *Lactococcus lactis* was able to convert pyruvate as the sole substrate at a rate comparable to that of *O. oeni* or *L. mesenteroides*. In summary, the *Lactobacillus* strains can use pyruvate as an electron sink and for pyruvate fermentation, but the rates, in particular that of pyruvate fermentation, are very low. *Lactococcus*, on the other hand, was not able to use pyruvate for fermentation or as an electron acceptor.

Genes for potential enzymes of pyruvate fermentation. The genome sequences of *O. oeni* (strain PSU-1) and *L. mesenteroides* (strain ATCC 8293) were screened for genes encoding potential enzymes of a pyruvate fermentation pathway (Table 3). In *O. oeni* and *L. mesenteroides*, genes homologous to genes encoding pyruvate dehydrogenase, phosphotransacetylase, acetate kinase, and D-lactate dehydrogenase were found. The sequence predicted a classical NAD-dependent enzyme with an E1α E1β E2 E3 subunit composition (*pdhABCD* genes) typical for gram-positive bacteria (11). The genes encoding phosphotransacetylase (*pta* homologous), acetate kinase (*ackA* homologous), and D-lactate dehydrogenase (*ldhA* homologous) shared similarity with the corresponding genes of *E. coli* and other bacteria. For some of the enzymes, more than one gene cluster was found, suggesting the presence of isoenzymes (see Table 3).

In both bacteria, in addition, genes encoding pyruvate oxidase of the *Leuconostoc* Pox type were found. Lactic acid bacteria are known to contain pyruvate oxidase which produces acetyl phosphate from pyruvate (pyruvate + O_2 + P_i → acetyl phosphate + H_2O_2 + CO_2) (29, 30). In *O. oeni* genes for four or five Pox type pyruvate oxidases were present, whereas in *L. mesenteroides* only one ORF could be identified. Genes encoding other enzymes for pyruvate decarboxylation, such as pyruvate ferredoxin oxidoreductase from anaerobic bacteria,

TABLE 3. Genes encoding potential enzymes of pyruvate fermentation by *O. oeni* and *L. mesenteroides*[a]

Enzyme	Gene(s) in:		Orthologous gene in *E. coli*
	O. oeni	*L. mesenteroides*	
PDH			
E1α	S3 G1158	S56 G1496	NP[b]
E1β	S3 G1159	S56 G1497	NP
E2	S3 G1160	S56 G1498	*aceF*
E3	S3 G1161	S56 G1499	*lpdA*
		S6 G1574	
Phosphotransacetylase	S2 G766	S25 G712	*pta*
Acetate kinase	S6 G1856	S36 G1026	*ackA*
		S42 G1163	
D-Lactate dehydrogenase	S5 G1762	S24 G696	*ldhA*
	S1 G56	S49 G1324	
Pyruvate oxidase (acetyl phosphate forming)	S37 G1370	S1 G63	*poxB*
	S17 G518		
	S25 G968		
	S25 G969		
	S25 G970		

[a] The genes of *O. oeni* and of *L. mesenteroides* were identified by functional domain searches and BLAST analysis using orthologous genes of *E. coli* or the other bacteria encoding the enzymes. The table gives the gene designations in *O. oeni* PSU-1 and *L. mesenteroides* ATCC 8293 according to the genomic database (http://www.jgi.doe.gov/) including the paralogs.

[b] NP, no homologous genes present.

pyruvate:quinone oxidoreductase (Pqo from *Corynebacterium glutamicum* or PoxB from *E. coli*), PFL, or pyruvate decarboxylase, were not detected in the genome of *O. oeni* or *L. mesenteroides*.

Enzyme activities of the pyruvate fermentation pathway. The genes found in *O. oeni* and *L. mesenteroides* (Table 3) suggest a pathway for pyruvate fermentation as shown in Fig. 2, including pyruvate decarboxylation by pyruvate dehydrogenase, phosphoryl transfer from acetyl phosphate to ADP by acetate kinase, and NADH reoxidation by lactate dehydrogenase. The presence of the corresponding enzyme activities was tested in cell extracts of the bacteria after anoxic growth (Table 4). *O. oeni* contained high activities of lactate dehydrogenase, acetate kinase, and phosphotransacetylase after growth on pyruvate or glucose; only lactate dehydrogenase activity was diminished after growth on pyruvate. Acetate kinase showed the lowest activities. *L. mesenteroides* generally has higher growth rates than *O. oeni* and contains higher activities, and the activities were slightly decreased after growth on pyruvate.

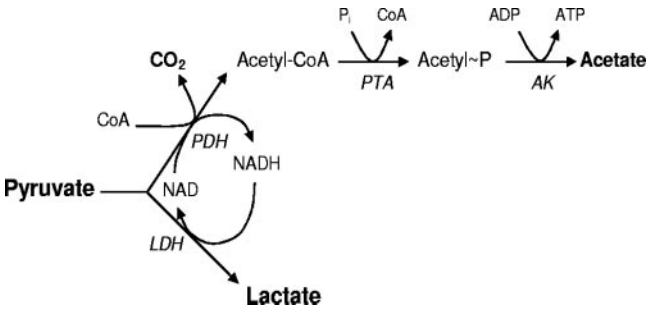

FIG. 2. Scheme showing the intermediates in the pyruvate fermentation pathway of *O. oeni* and *L. mesenteroides*. The scheme shows the intermediates, the enzymes, and the supposed genes in *O. oeni*. LDH, D-lactate dehydrogenase; PTA, phosphotransacetylase; AK, acetate kinase.

Pyruvate dehydrogenase was tested in cell extracts in a coupled assay by the formation of acetyl phosphate, since measurement of NADH formation is susceptible to interference in cell homogenates due to the presence of NADH-oxidizing enzymes. *L. mesenteroides* contained much higher (8.5-fold) PDH activity in pyruvate-grown than in glucose-grown bacteria. In the same assay, *O. oeni* showed no activity of acetyl phosphate (or acetyl-CoA, respectively) formation. However, when pyruvate dehydrogenase was assayed under the same conditions by the decrease of pyruvate, the enzyme was found with substantial activity in pyruvate-grown (but not in glucose-grown) *O. oeni*. *L. mesenteroides* showed the same type of activity, which was again higher in the pyruvate-grown bacteria.

Pyruvate oxidase activity was determined as the O_2-dependent conversion of pyruvate to acetyl phosphate. *O. oeni* contained no activity of this type, even after microoxic growth of the bacteria on pyruvate. *L. mesenteroides*, on the other hand, contained enzyme activity after microoxic, but not after anaerobic, growth on pyruvate, suggesting that this enzyme, which requires O_2 for function, is formed or active only after (micro)aerobic growth.

Pyruvate-cleaving enzymes in LAB: different functions for pyruvate oxidase, pyruvate formate lyase, and pyruvate dehydrogenase. LAB and the related bifidobacteria were screened for genes encoding enzymes for pyruvate cleavage. The genes were identified by a BLAST search and confirmed by the presence of the functional domains. Genes or gene clusters encoding PDH (*pdhABCD* genes), Pox (*poxB* gene),PFL (*pflB* or *pflD* gene), and PFOR (*por* gene, encoding the NifJ type of PFOR) were identified (Table 5). None of the bacteria contained genes for pyruvate decarboxylase or a pyruvate oxidase

TABLE 4. Activities of enzymes related to pyruvate fermentation of *O. oeni* and *L. mesenteroides*[a]

Enzyme and growth condition	Activity (μmol of substrate/g of protein/min) in:	
	O. oeni	*L. mesenteroides*
Lactate dehydrogenase		
Pyruvate	2,300	19,940
Glucose	10,400	35,080
Acetate kinase		
Pyruvate	240	415
Glucose	194	910
Phosphotransacetylase		
Pyruvate	5,810	13,150
Glucose	6,100	18,000
Pyruvate dehydrogenase (acetyl phosphate formation)		
Pyruvate	≤6	102
Glucose	≤6	12
Pyruvate dehydrogenase (pyruvate degradation)		
Pyruvate	208	425
Glucose	<1	286
Pyruvate oxidase		
Pyruvate	<1	<1
Pyruvate + O_2	<1	98

[a] Enzymes were measured in cell homogenates obtained from the bacteria after anoxic growth in MLD medium with pyruvate or glucose as the substrate.

TABLE 5. Pyruvate-cleaving enzymes in lactic acid and related bacteria as deduced from genome sequences[a]

Bacterium[b]	Ortholog[c] of:				Homo- or heterofermentative
	PDH	Pox	PFL	PFOR	
O. oeni	PDH	Pox1 to 5	NP	NP	Heterofermentative
L. mesenteroides	PDH	Pox	NP	NP	Heterofermentative
Lactobacillus brevis	PDH	NP	NP	NP	Heterofermentative
Lactobacillus plantarum	PDH	Pox1 to 5	PflB1 and 2	NP	Facultatively heterofermentative
Lactobacillus casei	PDH	Pox1 to 3	NP	NP	Facultatively heterofermentative
Lactobacillus gasseri	NP	Pox	NP	NP	Homofermentative
Lactobacillus delbrueckii subsp. *bulgaricus*	NP	NP	NP	NP	Homofermentative
Lactobacillus johnsonii	NP	Pox	NP	NP	Homofermentative
Lactococcus lactis	PDH	Pox	Pfl	PFOR	Homofermentative
Lactococcus cremoris	PDH	Pox	Pfl	NP	Homofermentative
Pediococcus pentosaceus	PDH	Pox1 and 2	NP	NP	Homofermentative
Bifidobacterium longum	NP	NP	Pfl	NP	

[a] The presence of PDH, pyruvate oxidase PoxB from *Leuconostoc* (forming acetyl phosphate + H_2O_2), PFL, and PFOR (*por* gene) orthologs is indicated. The positives were found by BLAST search and contain the functional domains characteristic for each enzyme.
[b] Fully sequenced genomes are underlined; the other genomes are draft versions.
[c] NP, no homologous genes present.

of the Pqo type. Most of the bacteria contained more than one gene or gene cluster encoding the PDH, Pox, PFL, and PFOR enzymes (Table 5). The most frequent enzyme was Pox, followed by PDH and PFL. The roles of the PDH, Pox, and PFL enzymes in the metabolism of the LAB are known at least in part, as discussed below, whereas for PFOR of *Lactococcus lactis* no function can be assigned.

(i) Pyruvate oxidase. Pox of the LAB catalyzes formation of acetyl phosphate (pyruvate + P_i + O_2 → acetyl phosphate + CO_2 + H_2O_2) (29) and was found in most of the homo- and heterofermentative bacteria. The enzyme is advantageous in (micro)aerobic growth, resulting in increased acetate production and higher ATP or cell yields, in particular under glucose limitation (15, 19, 20, 24, 30). Bacteria of the *Lactobacillales* group with respiratory capacity (*Streptococcus* strains) do not need the Pox enzyme for this purpose and lack the enzyme (not shown). By combined action of Pox and of a (nonrespiratory) NADH oxidase (12, 16), glucose is oxidized to acetate and CO_2 (idealized reaction: glucose + 4 O_2 → 2 acetate + 2 CO_2 + 4 H_2O_2). This reaction yields 4 and 3 mol of ATP/mol of glucose for homo- and heterolactic bacteria, respectively, compared to 2 and 1 mol of ATP/mol of glucose in (anaerobic) homo- and heterolactic glucose fermentation.

(ii) Pyruvate formate lyase. PFL is the key enzyme for mixed acid fermentation, with acetate, ethanol, and formate as the products in addition to lactate. PFL is found only in LAB capable of homofermentative growth (Table 5), which use this enzyme to shift to mixed acid formation under glucose limitation to increase the ATP yields (3 compared to 2 mol of ATP/mol of glucose in homolactic fermentation) (7, 17, 33). The restriction of PFL to the homofermentative LAB suggests that this is the major or only role for the enzyme in LAB.

(iii) Pyruvate dehydrogenase. The PDH of gram-positive bacteria consists of four subunits (PdhABCD) and is strongly inhibited by NADH (30–32). Since the NADH/NAD^+ ratio is high during glucose fermentation, PDH is supposed to be active mainly under aerobic conditions. Therefore, in (anoxic) glucose fermentation, pyruvate is mainly metabolized by lactate dehydrogenase and PFL (12). In the presence of O_2, on the other hand, pyruvate is metabolized in significant amounts by PDH. The surplus NADH is reoxidized by NADH oxidase. In this way glucose is metabolized to acetate plus CO_2 (1 glucose + 4 O_2 → 2 acetate + 2 CO_2 + 4 H_2O_2), yielding 4 or 3 mol of ATP/mol of glucose, respectively, from the glycolytic or pentose phosphate pathway, compared to 2 or 1 mol of ATP in homo- or heterolactic fermentation. The broad distribution of the PDH enzyme in the LAB and its aerobic expression suggest an important role for PDH (together with NADH oxidase) in pyruvate oxidation under oxic conditions.

The PDH-plus-NADH oxidase-dependent pathway functions alternatively to pyruvate oxidase with the same ATP yields. It is not known to what extent either pathway is used in the presence of O_2 or whether the two pathways are used for different purposes. A similar situation exists in *Escherichia coli*, which contains PDH and pyruvate oxidase PoxB (pyruvate: quinone reductase). These two enzymes function with different energetic efficiencies, and their alternative function is not completely clear (1, 10). In *O. oeni* and other LAB which are limited in cellular HSCoA contents due to pantothenate auxotrophy (26), use of the HSCoA-independent pyruvate oxidase pathway could be advantageous.

Growth of heterolactic acid bacteria by pyruvate fermentation: a new function for pyruvate dehydrogenase. *O. oeni* and *L. mesenteroides* are able to grow with pyruvate under anoxic conditions. The pyruvate is converted to lactate, acetate, and CO_2 by the following pathway (see also Fig. 2): 2 pyruvate → 1 D-lactate + 1 acetate + 1 CO_2. One molecule of pyruvate is decarboxylated to acetyl-CoA, which allows generation of ATP by acetate kinase. The ATP yield (0.5 mol/mol of pyruvate) is in agreement with the growth yields. The pathway was confirmed by the presence of the corresponding genes for the enzymes in the genomes of the bacteria (http://www.jgi.doe.gov/). The key enzyme of the pathway is PDH. The broad distribution of the PDH enzyme within LAB, most of which are not able to ferment pyruvate (Table 5), and its aerobic expression suggest that the principal function of the enzyme is pyruvate oxidation combined with the oxidation of NADH. The function of PDH in pyruvate fermentation presumably represents a "misuse" or adaptation for a pathway that is normally not intended for PDH. Pyruvate oxidase, which is also present in the bacteria, requires O_2 for function and is therefore not suitable for pyruvate fermentation.

From the growth yield and rate, it can be calculated (see reference 26) that *O. oeni* and *L. mesenteroides* require activities of 250 and 1,330 U/g of protein for each of the enzymes of the pyruvate fermentation pathway. In *O. oeni*, only PDH (pyruvate-degrading activity) has slightly lower activity (208 U/g protein). *L. mesenteroides* also contains the enzyme activities, and only acetate kinase and pyruvate dehydrogenase activities are lower than expected. Pyruvate fermentation is not widespread among lactic acid bacteria. Thus, the heterofermentative *L. plantarum* and the homofermentative *Lactobacillus lactis* ferment pyruvate at low rates, but the reaction does not support substantial growth.

Only a few other fermentation reactions are known to use PDH instead of PFOR or PFL for cleavage of pyruvate. Thus, *Bacillus subtilis* ferments glucose in the absence of electron acceptors via pyruvate to lactate, acetate, and some other products. The acetate production depends on PDH, which normally functions in aerobic metabolism (5, 21, 22). *Eubacterium pyruvativorans* is one of the few bacteria growing by fermentation of external pyruvate (35). The fermentation products are short-chain fatty acids, and the fermentation presumably requires PFOR. Thus, *E. pyruvativorans* seems to be the only other bacterium described in addition to *O. oeni* and *L. mesenteroides* that is capable of pyruvate fermentation, but the fermentation reactions are different.

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