

Synthesis and Posttranslational Regulation of Pyruvate Formate-Lyase in *Lactococcus lactis*

Claus Rix Melchiorsen, Kirsten Væver Jokumsen, John Villadsen, Mads G. Johnsen, Hans Israelsen and José Arnau
J. Bacteriol. 2000, 182(17):4783. DOI:
10.1128/JB.182.17.4783-4788.2000.

Updated information and services can be found at:
<http://jb.asm.org/content/182/17/4783>

REFERENCES

These include:

This article cites 29 articles, 18 of which can be accessed free
at: <http://jb.asm.org/content/182/17/4783#ref-list-1>

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new
articles cite this article), [more»](#)

Information about commercial reprint orders: <http://journals.asm.org/site/misc/reprints.xhtml>
To subscribe to to another ASM Journal go to: <http://journals.asm.org/site/subscriptions/>

Synthesis and Posttranslational Regulation of Pyruvate Formate-Lyase in *Lactococcus lactis*

CLAUS RIX MELCHIORSEN,^{1*} KIRSTEN VÆVER JOKUMSEN,¹ JOHN VILLADSEN,¹
MADS G. JOHNSEN,² HANS ISRAELSEN,² AND JOSÉ ARNAU²

Department of Biotechnology, Center for Process Biotechnology, Technical University of Denmark,
DK-2800 Lyngby,¹ and Biotechnological Institute, DK-2970 Hørsholm,² Denmark

Received 20 December 1999/Accepted 15 June 2000

The enzyme pyruvate formate-lyase (PFL) from *Lactococcus lactis* was produced in *Escherichia coli* and purified to obtain anti-PFL antibodies that were shown to be specific for *L. lactis* PFL. It was demonstrated that activated *L. lactis* PFL was sensitive to oxygen, as in *E. coli*, resulting in the cleavage of the PFL polypeptide. The PFL protein level and its in vivo activity and regulation were shown by Western blotting, enzyme-linked immunosorbent assay, and metabolite measurement to be dependent on the growth conditions. The PFL level during anaerobic growth on the slowly fermentable sugar galactose was higher than that on glucose. This shows that variation in the PFL protein level may play an important role in the regulation of metabolic shift from homolactic to mixed-acid product formation, observed during growth on glucose and galactose, respectively. During anaerobic growth in defined medium, complete activation of PFL was observed. Strikingly, although no formate was produced during aerobic growth of *L. lactis*, PFL protein was indeed detected under these conditions, in which the enzyme is dispensable due to the irreversible inactivation of PFL by oxygen. In contrast, no oxygenolytic cleavage was detected during aerobic growth in complex medium. This observation may be the result of either an effective PFL deactivase activity or the lack of PFL activation. In *E. coli*, the PFL deactivase activity resides in the multifunctional alcohol dehydrogenase ADHE. It was shown that in *L. lactis*, ADHE does not participate in the protection of PFL against oxygen under the conditions analyzed. Our results provide evidence for major differences in the mechanisms of posttranslational regulation of PFL activity in *E. coli* and *L. lactis*.

The industrially important lactic acid bacterium *Lactococcus lactis* is characterized as an aerotolerant anaerobe. The organism is able to grow in an oxygen-rich environment but is unable to use oxygen for energy generation since it lacks a functional electron transport chain (15). Since energy generation relies solely on substrate-level phosphorylation during both anaerobic and aerobic growth, the biomass yield is roughly independent of the oxygen supply, as opposed to what occurs in organisms capable of oxidative phosphorylation (22). The presence of oxygen is, however, known to affect lactococcal metabolism dramatically, leading to an end product profile different from that produced during anaerobiosis. The formation of diacetyl, an important aroma compound in buttermilk, is favored during aerobic conditions, whereas even low levels of oxygen preclude the formation of formate.

The metabolism of *L. lactis* is normally homofermentative, i.e., lactic acid is produced as the major end product. However, under certain growth conditions, such as growth in chemostat cultures at low dilution rates (27) or on slowly fermentable sugars such as galactose, maltose, and lactose (20, 28), a considerable fraction of the carbon flux is diverted from lactic acid towards the mixed-acid fermentation products formate, acetate, and ethanol. Under anaerobic conditions, the carbon flux from pyruvate is distributed mainly between two competing enzymes: lactate dehydrogenase (LDH) and pyruvate formate-lyase (PFL).

PFL converts pyruvate and coenzyme A to formate and

acetyl coenzyme A and represents the initial step in the formation of mixed-acid end products. When we study the shift from homofermentative to mixed-acid product formation in *L. lactis*, PFL is of particular interest, since its activity is regulated by several different mechanisms. Previous results from our group have shown that transcription of the *pfl* gene, which encodes PFL, is increased during growth on galactose compared to what occurs with glucose and by anaerobiosis (2). It is therefore likely that the PFL enzyme level also depends on the growth conditions. Furthermore, it has been proposed that the glycolytic intermediates glyceraldehyde-3-phosphate and dihydroxyacetone phosphate allosterically inhibit the in vivo activity of PFL in *L. lactis* (9, 10, 28). The inhibitory effect of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate was verified by in vitro characterization of purified PFL from the related organism *Streptococcus mutans* (25). Moreover, at least in *Escherichia coli*, the PFL enzyme is regulated by post-translational modifications that result in the interconversion of the active and inactive forms. These regulatory mechanisms may all participate in the overall regulation of PFL activity in a bacterium growing in a changing environment.

In *E. coli*, the PFL protein exists in three different forms that may be interconverted as illustrated in Fig. 1 (see reference 19 for a review). PFL is a homodimeric enzyme complex that is synthesized in an inactive form. The enzyme is activated post-translationally via an iron-dependent activating enzyme that introduces a glycyl radical to the one monomer in the enzyme complex (30). The presence of the radical causes extreme sensitivity of active PFL to molecular oxygen, with a half-life of approximately 10 s in an air-saturated buffer at 0°C (18). Exposure to oxygen results in irreversible inactivation due to peptide bond cleavage near the C terminus of the subunit

* Corresponding author. Mailing address: Center for Process Biotechnology, Department of Biotechnology, Technical University of Denmark, Building 223, DK-2800 Lyngby, Denmark. Phone: (45) 45252669. Fax: (45) 45884148. E-mail: CRM@ibt.dtu.dk.

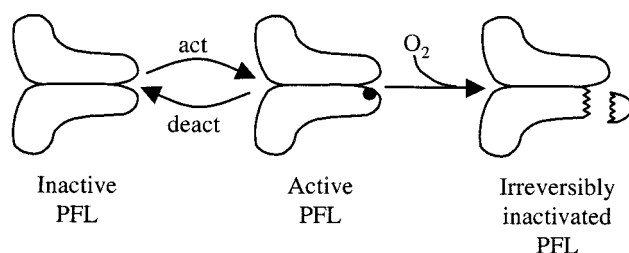


FIG. 1. Interconversion of different forms of PFL in *E. coli* (19). act, PFL activase; deact, PFL deactivase; ●, the free radical of active PFL.

containing the radical. In *E. coli*, active PFL may be reversibly inactivated by removal of the radical. This reaction is catalyzed by PFL deactivase, and a multifunctional alcohol dehydrogenase, ADHE, has been identified to harbor this activity (16). PFL deactivase activity has also been recognized in the oral bacterium *Streptococcus sanguis* but is absent in *S. mutans* (1, 26, 33). It has until now been unknown whether *L. lactis* is capable of protecting its PFL via a deactivase, although the *adhE* gene in *L. lactis* has recently been cloned by our group and displays significant homology to its *E. coli* counterpart (3).

The shift from homofermentative to mixed-acid product formation in lactic acid bacteria has been studied intensively. Regulation of the shift has been associated primarily with the influence of allosteric effectors acting on the LDH and PFL enzymes (1, 10, 28, 32). The regulatory significance of the PFL enzyme level has not yet been studied in detail. This fact may be due to the oxygen-sensitive nature of the enzyme, which severely complicates application of in vitro techniques for measuring enzyme activities, although methods to circumvent these problems have been reported (25, 31). In this study, recombinant *L. lactis* PFL enzyme was purified and polyclonal antibodies were produced to develop immunochemical techniques allowing measurement of PFL in cell extracts of *L. lactis*. Enzyme-linked immunosorbent assay (ELISA) was used for measuring the total PFL protein levels under different growth conditions. These results showed that regulated expression of *pfl* may play an important role in the regulation of anaerobic pyruvate metabolism in *L. lactis*. Additionally, posttranslational modifications of lactococcal PFL were examined by Western blot analysis, and it was demonstrated that activation of PFL in *L. lactis* depends on the growth conditions.

MATERIALS AND METHODS

Bacteria and plasmids. Recombinant protein was produced in *E. coli* M15 (Qiagen) carrying the low-copy-number pREP4 plasmid, which confers kanamycin resistance and mediates constitutive expression of the Lac repressor protein encoded by the *lac I* gene. The pQE30 plasmid (Qiagen) was used for expressing recombinant His-tagged *L. lactis* PFL in M15 by selection for ampicillin resistance. *L. lactis* subsp. *cremoris* MG1363 (11) was used throughout this study for examining PFL expression. The *L. lactis* *pfl* mutant strain MGKAS13 (2) was used to test the specificities of the anti-PFL antibodies produced. The *L. lactis* *adhE* mutant strain MGKAS15 (3) was used to analyze posttranslational modifications of PFL in *L. lactis*.

Media and culturing conditions. *E. coli* M15 was grown in Luria-Bertani broth or agar at 37°C. Kanamycin (25 µg ml⁻¹) and ampicillin (50 µg ml⁻¹) were added as required. Protein production in *E. coli* was initiated by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). *L. lactis* was grown at 30°C in M17 broth or agar (Oxoid) supplemented with 0.5% (wt/vol) galactose or glucose. To enable measurement of end products by high-performance liquid chromatography (HPLC), *L. lactis* was also grown in the defined medium MS10 (7), supplemented with 1% (wt/vol) glucose or galactose. Erythromycin (1 µg ml⁻¹) was added to MGKAS13 and MGKAS15 cultures to retain mutations. Anaerobic *L. lactis* cultures were grown statically in shake flasks in an anaerobic work station (Don Whitley) operating with a gas mixture consisting of 10% H₂, 10% CO₂ and 80% N₂. Aerobic *L. lactis* cultures were grown with shaking (200 rpm) in 500-ml baffled shake flasks containing 100 ml of medium. Samples for

protein analysis and measurement of end products were taken at an optical density at 600 nm of 1.2 ± 0.1.

PCR amplifications. PCR primers were designed to amplify a DNA fragment containing the *pfl* gene flanked by *Bam*HI and *Pst*I restriction sites (underlined) in the 5' and 3' termini, respectively (BamHI-pfl-5'-5'-TATGCGGATCCATG AAAACCGAAGTTACGGAAAAT-3', and PstI-pfl-3'-5'-TATGCCTGCACT TAGATATTGTAAGTGTGCATTACTTCTT-3'). PCR amplification was carried out using a GeneAmp DNA amplification kit from Perkin-Elmer Cetus. Chromosomal DNA from *L. lactis* subsp. *cremoris* MG1363 was isolated as described previously (14) and used as the template for the reaction.

Recombinant-DNA techniques. DNA modifications were performed according to standard procedures (24). DNA fragments were isolated from agarose gels and purified using Jet Sorb kits (Genomed, Bad Oeynhausen, Germany). Preparation of competent *E. coli* M15 cells and transformation were carried out as previously described (13). Plasmids were purified using Jetstar kits (Genomed). DNA sequencing was performed using Cy-5-labeled primers, and reactions were analyzed using an ALF Express apparatus (Pharmacia Biotech, Uppsala, Sweden).

Expression and purification of recombinant *L. lactis* PFL. *E. coli* transformants were screened by colony PCR using the BamHI-pfl-5' and PstI-pfl-3' primers. The plasmid was purified from one of these positive clones, and sequencing confirmed an open reading frame encoding a His-tagged *L. lactis* PFL protein (His-PFL). The 12-amino-acid His tag was fused to the N terminus of the native PFL protein containing 787 amino acids (2). Expression of His-PFL was carried out essentially as described in the Qiagen handbook (23), and protein extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% Tris-glycine gels (Novex, San Diego, Calif.) according to the supplier's protocol. After Coomassie blue staining, we observed a major 90-kDa band corresponding to PFL from *L. lactis* subsp. *cremoris* MG1363, which has a predicted molecular mass of 89.1 kDa (2). His-PFL protein was produced in *E. coli*, and affinity purification was carried out under denaturing conditions as described in reference 23. To ensure high purity of His-PFL, a second purification step was applied using preparative SDS-PAGE. After Coomassie blue staining, the His-PFL protein band was isolated from the gels and the protein was subsequently electroeluted using a Little Blue Tank (Isco Inc., Lincoln, Neb.). The recovered protein was freeze-dried and used for production of rabbit polyclonal antibodies (DAKO A/S, Glostrup, Denmark).

Antibody purification and conjugation. The immunoglobulin G (IgG) fraction of the rabbit serum was purified on a protein A-agarose column (Kem-En-Tec, Copenhagen, Denmark) essentially as described in reference 12. The method of Nakane and Kawaoi (21) was used to conjugate horseradish peroxidase (Boehringer Mannheim, Mannheim, Germany) with purified IgG.

Preparation of crude extracts. Cells were harvested by centrifugation (4,000 × g, 10 min). After the cells were washed in 0.3% (wt/vol) KCl and centrifuged (4,000 × g, 10 min, 4°C), the cell pellet was resuspended in 250 µl of extraction buffer (50 mM Tris-HCl, pH 8.0) and transferred to a 2-ml Fastprep tube containing 500 µl of glass beads (diameter, 106 µm; Sigma). Cells were disrupted three times in a Fast Prep 120 apparatus (Bio 101, La Jolla, Calif.) with cooling on ice in between. The lysate was cleared by centrifugation (10,000 × g, 10 min, 4°C). The protein concentration was quantified using the Bradford assay (6) with bovine serum albumin as the protein standard. Anaerobic protein extraction was carried out in an anaerobic workstation essentially as described above except for the disruption of cells, which was accomplished by Whirley mixing (30 s), repeated eight times with cooling on ice in between.

Western blot analysis. SDS-PAGE was carried out using 10% Tris-glycine gels (Novex) as recommended by the supplier. Protein was transferred to nitrocellulose membranes (Novex) using a Novex X Cell Blot module. The membrane was blocked using 3% (wt/vol) skim milk (Difco, Detroit, Mich.) in Tris-buffered saline buffer (150 mM NaCl, 50 mM Tris [pH 7.5]). Incubation with purified anti-PFL IgG and subsequently with alkaline phosphatase-conjugated goat anti-rabbit IgG (DAKO A/S) was carried out in 1.5% (wt/vol) skim milk in Tris-buffered saline. Blots were developed in 5-bromo-4-chloro-3-indolylphosphate (BCIP)-4-nitroblue tetrazolium chloride according to the protocol of the supplier (Boehringer Mannheim).

ELISA measurement of PFL. Total PFL was measured by sandwich ELISA essentially as described in reference 12. ELISA plates (Nunc, Roskilde, Denmark) were coated by overnight incubation at 4°C with 5 µg of purified anti-PFL IgG per ml in phosphate-buffered saline (PBS) (0.9% NaCl, 10 mM PO₄³⁻ [pH 7.2]). The plates were washed and blocked with PBS-Tween (PBS supplemented with 0.1% [vol/vol] Tween 20) before a series of sample dilutions (20 to 200 µg of protein per ml) were applied in doublets. The plates were washed and incubated with 3 µg of horseradish peroxidase-conjugated anti-PFL IgG per ml in PBS-Tween. After a subsequent wash, 100 µl of the substrate (5 µl of 30% H₂O₂ and 8 mg of *o*-phenylenediamine dihydrochloride per 12 ml of substrate buffer [35 mM citrate, 67 mM PO₄³⁻, pH 5.0]) was added to each well and the plates were incubated in the dark for 15 min at room temperature. The reaction was stopped by addition of 150 µl of 1 M H₂SO₄. The absorbance was measured at 492 nm, and the slope of a plot of the A₄₉₂ values of the protein was used as a measure of the total PFL level per mg of total protein.

Analysis of extracellular metabolites. For determination of extracellular metabolites, 1 ml of sample was filtered through a 0.45-µm-pore-size cellulose acetate filter (Sartorius AG, Goettingen, Germany). The filtrate was stored at

TABLE 1. End product formation in *L. lactis* MG1363

Conditions	Yield of indicated end product (mol/mol of sugar) ^a			
	Lactate	Formate	Acetate	Ethanol
O ₂ , glucose	1.63	ND	0.08	0.02
N ₂ , glucose	1.85	0.05	0.04	0.03
O ₂ , galactose	0.86	ND	0.79	0.03
N ₂ , galactose	1.39	0.64	0.31	0.36

^a Yields of end products during exponential growth in shake flasks containing defined MS10 medium supplemented with glucose or galactose. Growth took place in an O₂ or N₂ atmosphere. Samples were taken in the mid-exponential phase (optical density at 600 nm, 1.2 ± 0.1). The relative standard deviation on HPLC measurements was less than 5%. ND, not detectable.

–20°C until analysis. Glucose, galactose, lactate, formate, acetate, and ethanol were separated on an Aminex HPX-87H column (Bio-Rad, Hercules, Calif.) using 0.6 ml of 5 mM H₂SO₄ per min as the mobile phase. Glucose, galactose, and ethanol were measured refractometrically using a Waters 410 differential refractometer detector (Millipore Corp., Bedford, Mass.). Lactate, formate, and acetate were detected using a Waters 486 tunable absorbance detector at 210 nm. As indicated in Table 1, no formate was detected in the samples from aerobically growing *L. lactis* cells, i.e., the formate concentration was below the detection limit of 0.2 mM.

RESULTS

Production and characterization of specific anti-PFL antibodies. The PFL enzyme from *L. lactis* was produced recombinantly in *E. coli*. The *pfl* gene from *L. lactis* was amplified by PCR, cloned into the pQE30 vector, and transformed into *E. coli*. The genetic construct hereby obtained was analyzed by sequencing, which confirmed an open reading frame encoding a His-tagged lactococcal PFL protein. His-PFL was produced and purified by affinity chromatography using a Ni-nitrilotriacetic acid column and further purified by preparative SDS-PAGE. Polyclonal rabbit antibodies were produced and purified.

The specificities of the antibodies produced were tested by Western blot analysis using protein extracts of *L. lactis* MG1363 and the *pfl* mutant strain MGKAS13. *L. lactis* MGKAS13 contains an interrupted *pfl* gene encoding a truncated PFL protein. Two bands were observed for strain MG1363 (Fig. 2A, lane 1) corresponding to the full-length (89-kDa) and the cleaved (85-kDa) PFL subunit produced after exposure of active PFL to oxygen, e.g., during protein extraction. These two bands were absent for strain MGKAS13 (Fig. 2A, lane 2), with which only a single band was observed. The molecular mass of this protein (65 kDa) corresponded with the expected molecular mass of a truncated PFL protein (2). Thus, the antibodies were specific towards PFL and therefore suitable for measuring the PFL level in physiological experiments with *L. lactis* by Western blotting and ELISA. The *L. lactis* PFL sequence displays a high homology (40.3% identity) to the *E. coli* PFL protein, but the antibodies obtained did not recognize *E. coli* PFL in Western blot analysis (data not shown).

Oxygen is required for cleavage of *L. lactis* PFL activated during anaerobic growth. As mentioned previously, *E. coli* PFL is activated by introduction of a free radical to a glycine residue (30) in one of the subunits of a PFL homodimer (29). The PFL subunit containing the free radical is cleaved when it is exposed to oxygen. Thus, when cells growing in an anaerobic environment are subjected to oxygen, the PFL activity decreases due to oxygenolytic cleavage of active PFL protein (18).

Assuming full inactivation of active PFL during the aerobic protein extraction procedure, the relative intensities of the two

PFL bands in a Western blot reflect the fraction of PFL that is present in its active form in anaerobically growing cells. Equal levels of intensity of the two bands therefore indicate that the PFL protein pool is fully activated, if we ignore the small difference in molecular mass between cleaved and full-length PFL subunits of 85 and 89 kDa, respectively.

The effect of oxygen on *L. lactis* PFL was examined by Western blot analysis. *L. lactis* cells were grown anaerobically, and protein extraction and SDS-PAGE were carried out under strict anaerobiosis. Only one distinct band corresponding to the full-length PFL subunit was detected in Western blot analysis (Fig. 2B, lane 1), demonstrating that cleavage of *L. lactis* PFL did not occur in the absence of oxygen. When the anaerobically prepared extract was subjected to air for 5 min, PFL was partially cleaved (Fig. 2B, lane 2), confirming that active PFL is sensitive to oxygen. The oxygenolytic cleavage of PFL was even more apparent from the analysis of an aerobically prepared extract of anaerobically grown *L. lactis* cells. In the Western blot (Fig. 2B, lane 3), two distinct bands were observed, demonstrating that extensive cleavage of active PFL took place during the aerobic protein extraction procedure.

The PFL level and product formation in *L. lactis* depend on growth conditions. It has previously been demonstrated that *pfl* transcription in *L. lactis* is strongly induced (6- to 15-fold) under anaerobiosis and that growth on a less favorable carbon source such as galactose results in a higher *pfl* mRNA level than on glucose under both aerobic and anaerobic conditions (2). Physiological experiments have shown that formation of the mixed-acid end products formate, acetate, and ethanol is enhanced during anaerobic growth on galactose compared to that during growth on glucose as a consequence of a higher PFL in vivo activity (10). Using the developed tools, we investigated whether a correlation exists between the PFL protein level and in vivo activity. In this way, it could be assessed whether the PFL protein level plays a role in regulation of the anaerobic pyruvate metabolism of *L. lactis* besides allosteric

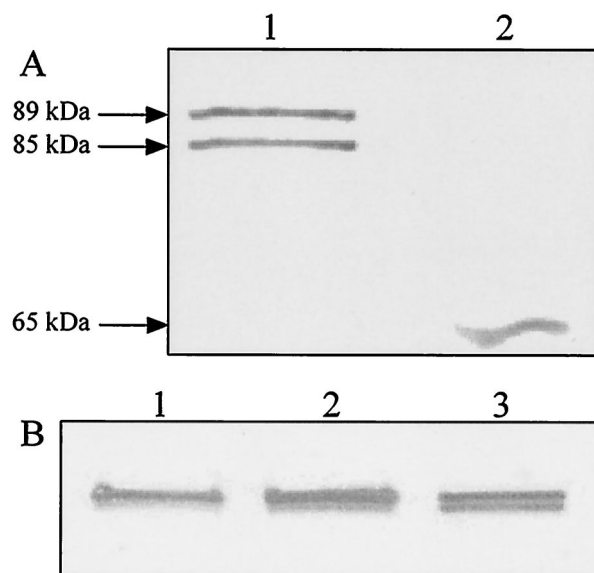


FIG. 2. Specificities of anti-PFL antibodies and analysis of PFL cleavage by Western blotting. (A) Western blot analysis of *L. lactis* MG1363 (lane 1) and the *pfl* mutant strain MGKAS13 (lane 2). (B) Western blot analysis of extracts of MG1363. Lane 1, anaerobic growth and extraction; lane 2, anaerobic growth and extraction with subsequent exposure to air for 5 min; lane 3, anaerobic growth and aerobic extraction.

TABLE 2. Total PFL protein level during exponential growth of *L. lactis* MG1363

Broth	Relative total PFL level ^a with:			
	Glucose		Galactose	
	O ₂	N ₂	O ₂	N ₂
MS10	1 ^b	1.5	3.2	7.3
M17	1.0	3.0	5.4	13.0

^a ELISA measurements of the total PFL protein level in extracts of *L. lactis* MG1363 under different growth conditions in shake flasks. The relative standard deviation of measurements was less than 10%.

^b All values were normalized with respect to the ELISA signal obtained for aerobically grown cells in glucose-MS10 broth.

modulation of PFL activity, which is generally considered to control the distribution of carbon at the pyruvate branch point (10, 28).

We analyzed the PFL protein level in exponentially growing *L. lactis* cells by Western blotting and by ELISA. Experiments were carried out with both complex M17 medium and defined MS10 medium, the latter allowing measurement of end products by HPLC. Use of defined medium enabled us to compare the PFL in vivo activity to the cellular content of PFL protein. The influence of aeration and the nature of the sugar fermented were examined.

During anaerobic growth on glucose, lactate was the major end product formed, corresponding to 96% {100 × [1.85 mol of lactate/(1.85 mol of lactate + 0.04 mol of acetate + 0.03 mol of ethanol)]} of the carbon flux from pyruvate (Table 1), whereas the remaining 4% was recovered in the mixed-acid end products formate, acetate, and ethanol. During anaerobic growth on galactose, the formation of mixed-acid products was 33% of the total carbon flux from pyruvate. ELISA measurements showed that the total level of PFL was induced fivefold (7.3 versus 1.5) on galactose compared to the level observed on glucose during anaerobic growth in defined medium (Table 2). The observed correlation between the cellular PFL content and the end product profile shows that regulation of *pfl* expression may represent an important mechanism in regulating the shift from homolactic to mixed-acid product formation in *L. lactis* under anaerobic conditions.

Aerobic growth of *L. lactis* led to no detectable formation of formate either on glucose or on galactose, indicating that the PFL enzyme is not present in its active form under these conditions. Despite there being no PFL activity, 47% of the pyruvate pool was directed towards acetate during growth on galactose (Table 1), indicating that either pyruvate dehydrogenase or pyruvate oxidase is active under these conditions. The measured metabolites cannot account for the consumption of carbohydrates under aerobic conditions, indicating that compounds other than those measured were produced. It may be diacetyl or acetoin that is reported to be produced in significant amounts under aerobic conditions (5).

Remarkably, a significant level of PFL was present during aerobic growth although the enzyme is dispensable under these conditions. ELISA measurements showed that PFL protein synthesis was induced only 1.5- and 2.3-fold (7.3 versus 3.2) by anaerobiosis on glucose and galactose, respectively (Table 2). Maintenance of a certain PFL level may represent an advantage as it ensures a rapid adaptation to fermentative growth upon oxygen depletion. However, this advantage is conditioned by the ability of the organism to protect PFL present during aerobic conditions from oxygenolytic cleavage. The mechanism involved in regulation of PFL activation in *L. lactis* was examined and is described below.

Different PFL levels were observed during growth in complex M17 medium under the same conditions as described above (Table 2), showing that factors other than the carbon source and aeration affect the level of PFL in *L. lactis*. However, the overall induction pattern was the same as with defined medium, suggesting that the major regulation of *pfl* expression depends on the examined physiological conditions.

***L. lactis* regulates the activation of PFL depending on the growth conditions.** Western blot analysis allowed us to study posttranslational regulation of PFL in *L. lactis*. The protein extracts prepared for ELISA measurements were therefore also analyzed by Western blotting.

For growth in defined medium, two bands were observed for all growth conditions (Fig. 3A), showing that active PFL was oxygenolytically cleaved during either growth or protein extraction. For cells grown under aerobic conditions (Fig. 3A, lanes 1 and 3), the upper band, corresponding to full-length PFL, was slightly stronger than the lower, indicating that a small fraction of PFL may exist in the reversibly inactivated form during aerobic growth in the defined medium. This effect is clearly evident during growth in complex medium, where PFL is almost entirely present in the deactivated form, as demonstrated by the strong upper band for extracts of cells grown aerobically (Fig. 3B, lanes 1 and 3). These results provide evidence that *L. lactis* is capable of regulating the activation of PFL, depending on the growth conditions. The mechanism for this regulation is unknown but is assumed to be mediated either by a controlled PFL activase or by PFL deactivase activity. The protection mechanism is favored in a complex medium, whereas its capacity is insufficient to fully protect PFL in defined medium.

Analysis of PFL deactivase activity in *L. lactis*. *E. coli* is able to protect active PFL against oxygen by reversible removal of the free radical via a PFL deactivase activity. The multifunctional alcohol dehydrogenase ADHE harbors this activity in *E. coli* (16). The gene encoding the alcohol dehydrogenase in *L. lactis* has recently been cloned by our group (3), and the lactococcal protein sequence displays high homology to its *E. coli* counterpart (44% identity). The *L. lactis* ADHE may therefore possess a PFL deactivase activity. To investigate this, *L. lactis* MG1363 and the *adhE* mutant strain MGKAS15 (3) were cultivated aerobically in complex medium supplemented with glucose. *L. lactis* MGKAS15 contains a truncated *adhE* gene that results in a 13-kDa ADHE protein, rather than the 98-kDa full-length protein. If ADHE is responsible for the protection of PFL during aerobic growth in complex medium

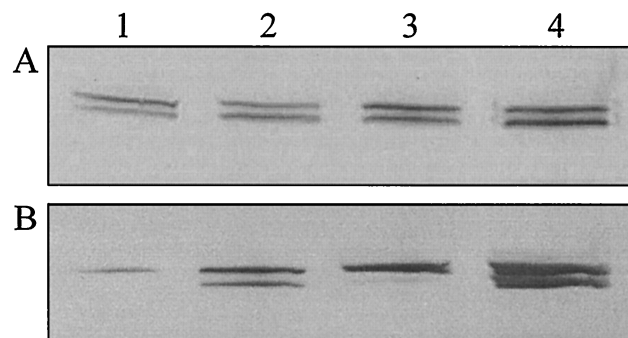


FIG. 3. Western blot of protein extracts from *L. lactis* MG1363 grown in defined MS10 medium (A) and complex M17 broth (B). Aerobic (lane 1) and anaerobic (lane 2) growth on glucose is shown. Aerobic (lane 3) and anaerobic (lane 4) growth on galactose is also shown. Protein extracts were prepared aerobically, which led to oxygenolytic cleavage of activated PFL subunits.

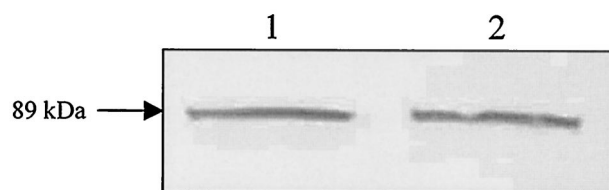


FIG. 4. PFL Western blot analysis of *L. lactis* MG1363 (lane 1) and the *adhE* mutant strain MGKAS15 (lane 2) grown aerobically in complex medium. Protein extraction was carried out aerobically.

as observed in Fig. 3B, two PFL bands should be obtained on Western blotting when protein extracts of strain MGKAS15 grown under these conditions are analyzed. However, only a single PFL band was observed for both strains MGKAS15 and MG1363 (Fig. 4), and hence PFL is present only in its inactive form under these conditions in either of the two strains. Thus, ADHE was not involved in the reversible inactivation of PFL under these conditions. PFL may be deactivated by a different mechanism, or alternatively, PFL might not be activated under aerobic conditions as a consequence of low PFL activase activity.

To investigate whether the observed protection of PFL results from the absence of PFL activase activity or the presence of PFL deactivase activity, *L. lactis* MG1363 and MGKAS15 were grown in complex medium under anaerobic conditions to ensure activation of PFL. Fractions of these cultures were subsequently exposed to aeration by shaking the cultures in baffled shake flasks for 5 and 30 min, and protein extracts of these cells were analyzed by Western blot analysis (data not shown). The aeration did not lead to an altered distribution between the full-length and cleaved PFLs compared to the distribution in anaerobically grown cells, and no significant difference was observed between the two strains. These results show that *L. lactis* ADHE was not responsible for the protection of PFL, in contrast to the situation in *E. coli* (17). Moreover, *L. lactis* was not able to protect activated PFL against oxygen during the transition to aerobiosis by an alternative mechanism. The apparent lack of PFL deactivase activity in *L. lactis* suggests that the protection of PFL observed during aerobic growth in complex medium is brought about by regulated activity of the PFL-activating enzyme.

DISCUSSION

We have purified the PFL protein from *L. lactis* and produced polyclonal antibodies for the analysis of PFL protein level using Western blot analysis and ELISA. These techniques not only provided us with a tool for measuring the cellular content of PFL in physiological experiments with *L. lactis* but also enabled us to investigate the biochemistry of the PFL enzyme in *L. lactis*. The PFL enzyme in *E. coli* has been subjected to considerable fundamental research for decades, but to our knowledge, a detailed analysis of the biochemical nature of the PFL enzyme in *L. lactis* has not been published.

A few reports are available on specific activity measurements of PFL in *L. lactis*. These were determined either for permeabilized cells (28) or as *in vitro* activities in protein extracts (10). These methods are cumbersome, requiring strict anaerobiosis during sample withdrawal and protein extraction, and neither of them allows discrimination between the active and the inactive form of PFL. Thus, immunochemical analysis by Western blotting and ELISA represents an advantageous alternative. ELISA allows a reliable measurement of the total

PFL level, while Western blotting discriminates between the different forms of PFL, thereby providing an indirect measure of the level of active PFL.

In *E. coli*, the half-life of activated PFL has been estimated to about 10 s in an air-saturated buffer at 0°C (18). The amino acid sequence around the activation site is highly conserved in the PFLs of *E. coli* and *L. lactis*, and the lactococcal PFL protein was therefore expected to be equally sensitive to oxygen. By Western blot analysis of an anaerobically prepared protein extract of *L. lactis*, we confirmed that oxygen is required for irreversible inactivation of the *L. lactis* PFL by peptide bond cleavage. A short exposure of the anaerobically prepared protein extract to air resulted in partial cleavage of PFL, and aerobic protein extraction led to extensive cleavage of PFL, confirming the oxygen sensitivity of lactococcal PFL.

Due to the oxygen sensitivity of the active PFL, it is reasonable to assume that virtually all active PFL is cleaved during an aerobic protein extraction procedure. Therefore, with a known radical stoichiometry of one per homodimer (29), the relative intensities of the two bands reflect the fraction of PFL existing in its active form. A PFL pool consisting of 100% active PFL will after complete irreversible inactivation result in approximately equal intensities for the two bands, if the small difference in the molecular weights of the cleaved and full-length subunits does not affect antibody recognition. The presence of only a full-length PFL band under a given growth condition implies that PFL exists only in its nonradical form and is therefore not catalytically active. The Western blot analysis showed that PFL was fully activated during anaerobic growth in defined medium. Similar findings have been made with *E. coli*, in which PFL is present exclusively in its active form during growth under strictly anaerobic conditions (8).

It was demonstrated that *L. lactis* protects its PFL from oxygenolytic cleavage when it is grown aerobically in a complex medium (Fig. 3B) but does not significantly do so in a simple defined medium (Fig. 3A). During aerobic growth in complex medium, PFL was exclusively present in its nonactivated full-length form. Protection of PFL results either from reversible removal of the radical via PFL deactivase activity or from no or low PFL activase activity, precluding activation of PFL. We investigated whether the multifunctional alcohol dehydrogenase ADHE is responsible for protection of PFL via deactivase activity in *L. lactis*, as in *E. coli*. By comparing the Western blot profiles of PFL in aerobically growing cells of *L. lactis* MG1363 and the *adhE* mutant strain MGKAS15 (3), it was concluded that the lactococcal ADHE protein was not responsible for the observed protection of *L. lactis* PFL during aerobic growth in complex medium. The protection of PFL from oxygenolytic cleavage was therefore assumed to be due to down-regulation of PFL activase activity, conceivably in combination with an alternative mechanism for PFL deactivation. The deactivation may be catalyzed by a different protein. Alternatively, if the PFL turnover in *L. lactis* is very high, the requirement for PFL deactivation would be obviated. The PFL-activating enzyme remains unidentified in *L. lactis*, and the recent release of data on the *L. lactis* genome (4) has not revealed a candidate for a PFL activase.

Since *L. lactis* is able to control the activation of PFL and thereby protects PFL against oxygenolytic cleavage, it is clear that the total PFL level is not proportional to the PFL activity under all growth conditions. However, since PFL seemed fully activated during anaerobic growth in defined medium (Fig. 3A, lanes 2 and 4), ELISA measurements of total PFL were assumed to give a reliable measure of the cellular contents of active PFL under these conditions. A fivefold higher level of PFL was observed during anaerobic growth on galactose than

on glucose, correlating with the previously reported fourfold induction of *pfl* transcription (2). Intracellular concentrations of the glycolytic intermediates glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, acting as allosteric inhibitors of PFL, have been reported to be lower on galactose than on glucose (9, 10). These observations have been used to explain the different product profiles obtained with the two sugars. Even though allosteric modulation of PFL activity may participate in the regulation of the shift from homolactic to mixed-acid product formation, the higher PFL protein level observed during growth on galactose than during growth on glucose clearly shows that regulated *pfl* expression may also play an important role. *L. lactis* strains with constitutive expression of *pfl* are currently being constructed to investigate the role of the PFL protein level in the regulation of the anaerobic pyruvate metabolism of *L. lactis*.

The induction of PFL during anaerobic growth on galactose is significantly higher for *L. lactis* MG1363 than was previously reported for *L. lactis* NCDO 2118, with which the PFL in vitro activity was observed to be twofold lower on glucose than on galactose or lactose (10). This discrepancy may be due to differences in the strains investigated; NCDO 2118 was originally isolated from plants, an atypical habitat for strains of *L. lactis* normally associated with the manufacture of dairy products.

It is notable that a significant level of PFL was produced under aerobic conditions, during which no in vivo activity of PFL was observed. Although PFL appears to be dispensable under these conditions, a certain level of PFL may represent an advantage when it grows in a variable environment. The ability to activate PFL upon oxygen depletion will presumably ensure a more rapid transition to fermentative metabolism than that of organisms restricted to de novo synthesis of the PFL protein.

The methods presented here will be used for measuring the PFL protein level in further physiological studies of *L. lactis*. These experiments aim at quantifying the relative significance of the mechanisms involved in the regulation of PFL in vivo activity, which in our opinion plays a central role in the regulation of the shift from homofermentative to mixed-acid product formation.

ACKNOWLEDGMENTS

This work was financed by the Center for Process Biotechnology at the Technical University of Denmark.

We thank Lars B. Nielsen and Henriette S. Egeblad (Biotechnological Institute) for fruitful discussion and advice concerning antibody purification and labeling.

REFERENCES

- Abbe, K., S. Takahashi, and T. Yamada. 1982. Involvement of oxygen-sensitive pyruvate formate-lyase in mixed-acid fermentation by *Streptococcus mutans* under strictly anaerobic conditions. *J. Bacteriol.* **152**:175–182.
- Arnau, J., F. Jorgensen, S. M. Madsen, A. Vrang, and H. Israelsen. 1997. Cloning, expression, and characterization of the *Lactococcus lactis* *pfl* gene, encoding pyruvate formate-lyase. *J. Bacteriol.* **179**:5884–5891.
- Arnau, J., F. Jorgensen, S. M. Madsen, A. Vrang, and H. Israelsen. 1998. Cloning of the *Lactococcus lactis* *adhE* gene, encoding a multifunctional alcohol dehydrogenase, by complementation of a fermentative mutant of *Escherichia coli*. *J. Bacteriol.* **180**:3049–3055.
- Bolotin, A., S. Mauger, K. Malmgren, S. D. Ehrlich, and A. Sorolin. 1999. Low-redundancy sequencing of the entire *Lactococcus lactis* IL1403 genome. *Antonie Leeuwenhoek* **76**:27–76.
- Boumerdassi, H., M. Desmazeaud, C. Monnet, C. Y. Boquien, and C. Corrieu. 1996. Improvement of diacetyl production by *Lactococcus lactis* ssp. *lactis* CNRZ 483 through oxygen control. *J. Dairy Sci.* **79**:775–781.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Cocaign-Bousquet, M., C. Garrigues, L. Novak, N. D. Lindley, and P. Loubiere. 1995. Rational development of a simple synthetic medium for the sustained growth of *Lactococcus lactis*. *J. Appl. Bacteriol.* **79**:108–116.
- Conradt, H., M. Hohmann-Berger, H. P. Hohmann, H. P. Blaschkowski, and J. Knappe. 1984. Pyruvate formate-lyase (inactive form) and pyruvate formate-lyase activating enzyme of *Escherichia coli*: isolation and structural properties. *Arch. Biochem. Biophys.* **228**:133–142.
- Fordyce, A. M., V. L. Crow, and T. D. Thomas. 1984. Regulation of product formation during glucose or lactose limitation in nongrowing cells of *Streptococcus lactis*. *Appl. Environ. Microbiol.* **48**:332–337.
- Garrigues, C., P. Loubiere, N. D. Lindley, and M. Cocaign-Bousquet. 1997. Control of the shift from homolactic acid to mixed-acid fermentation in *Lactococcus lactis*: predominant role of the NADH/NAD⁺ ratio. *J. Bacteriol.* **179**:5282–5287.
- Gasson, M. J. 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* **154**:1–9.
- Harlow, E., and D. Lane. 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Inoue, H., H. Nojima, and H. Okayama. 1990. High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**:23–28.
- Johansen, E., and A. Kibbenich. 1992. Characterization of *Leuconostoc* isolates from commercial mixed strain mesophilic starter cultures. *J. Dairy Sci.* **75**:1186–1191.
- Kaneko T., M. Takahashi, and H. Suzuki. 1990. Acetoin fermentation by citrate-positive *Lactococcus lactis* subsp. *lactis* 3022 grown aerobically in the presence of hemin or Cu²⁺. *Appl. Environ. Microbiol.* **56**:2644–2649.
- Kessler, D., I. Leibrecht, and J. Knappe. 1991. Pyruvate-formate-lyase-deactivase and acetyl-CoA reductase activities of *Escherichia coli* reside on a polymeric protein particle encoded by *adhE*. *FEBS Lett.* **281**:59–63.
- Kessler, D., W. Herth, and J. Knappe. 1992. Ultrastructure and pyruvate formate-lyase radical quenching property of the multimeric AdhE protein of *Escherichia coli*. *J. Biol. Chem.* **267**:18073–18089.
- Knappe, J., F. A. Neugebauer, H. P. Blaschkowski, and M. Ganzler. 1984. Post-translational activation introduces a free radical into pyruvate formate-lyase. *Proc. Natl. Acad. Sci. USA* **81**:1332–1335.
- Knappe, J., and G. Sawers. 1990. A radical-chemical route to acetyl-CoA: the anaerobically induced pyruvate formate-lyase system of *Escherichia coli*. *FEMS Microbiol. Rev.* **75**:383–398.
- Lohmeier-Vogel, E. M., B. Hahn-Hägerdahl, and H. J. Vogel. 1986. Phosphorus-31 NMR studies of maltose and glucose metabolism in *Streptococcus lactis*. *Appl. Microbiol. Biotechnol.* **25**:43–51.
- Nakane, P. K., and A. Kawaoi. 1974. Peroxidase-labeled antibody. A new method of conjugation. *J. Histochem. Cytochem.* **22**:1084–1091.
- Nielsen, J., and J. Villadsen. 1994. Bioreaction engineering principles. Plenum Press, New York, N.Y.
- Qiagen. 1997. The QIAexpressionist, a handbook for high-level expression and purification of 6xHis-tagged proteins, 3rd ed. Qiagen, Hilden, Germany.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Takahashi, S., K. Abbe, and T. Yamada. 1982. Purification of pyruvate formate-lyase from *Streptococcus mutans* and its regulatory properties. *J. Bacteriol.* **149**:1034–1040.
- Takahashi, N., K. Abbe, S. Takahashi-Abbe, and T. Yamada. 1987. Oxygen sensitivity of sugar metabolism and interconversion of pyruvate formate-lyase in intact cells of *Streptococcus mutans* and *Streptococcus sanguis*. *Infect. Immun.* **55**:652–656.
- Thomas, T. D., D. C. Ellwood, and V. M. Longyear. 1979. Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. *J. Bacteriol.* **138**:109–117.
- Thomas, T. D., K. W. Turner, and V. L. Crow. 1980. Galactose fermentation by *Streptococcus lactis* and *Streptococcus cremoris*: pathways, products, and regulation. *J. Bacteriol.* **144**:672–682.
- Unkrig, V., F. A. Neugebauer, and J. Knappe. 1989. The free radical of pyruvate formate-lyase. Characterisation by EPR spectroscopy and involvement in catalysis as studied with the substrate-analogue hypophosphite. *Eur. J. Biochem.* **184**:723–728.
- Wagner A. F. V., M. Frey, F. A. Neugebauer, W. Schäfer, and J. Knappe. 1992. The free radical in pyruvate formate-lyase is located on glycine-734. *Proc. Natl. Acad. Sci. USA* **89**:996–1000.
- Wood, N. P., and D. J. O'Kane. 1964. Formate-pyruvate exchange reaction in *Streptococcus faecalis*. *J. Bacteriol.* **87**:97–103.
- Yamada, T., and J. Carlson. 1975. Regulation of lactate dehydrogenase and change of fermentation products in streptococci. *J. Bacteriol.* **124**:55–61.
- Yamada, T., S. Takahashi-Abbe, and K. Abbe. 1985. Effects of oxygen on pyruvate formate-lyase in situ and sugar metabolism of *Streptococcus mutans* and *Streptococcus sanguis*. *Infect. Immun.* **47**:129–134.