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Cloning, Expression, and Characterization of the *Lactococcus lactis pfl* Gene, Encoding Pyruvate Formate-Lyase

JOSÉ ARNAU,* FLEMMING JØRGENSEN, SØREN M. MADSEN, ASTRID VRANG, AND HANS ISRAELSEN

Biotechnological Institute, DK-2970 Hørsholm, Denmark

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The *Lactococcus lactis pfl* gene, encoding pyruvate formate-lyase (PFL), has been cloned and characterized. The deduced amino acid sequence of the *L. lactis* PFL protein showed high similarity to those of other bacterial PFL proteins and included the conserved glycine residue involved in posttranslational activation of PFL. The genetic organization of the chromosomal *pfl* region in *L. lactis* showed differences from other characterized *pfl* loci, with an upstream open reading frame independently transcribed in the same orientation as the *pfl* gene. The gene coding for PFL-activase (*act*), normally found downstream of *pfl*, was not identified in *L. lactis*. Analysis of *pfl* expression showed a strong induction under anaerobiosis at the transcriptional level independent of the growth medium used. During growth with galactose, *pfl* showed the highest levels of expression. Constructed *L. lactis pfl* strains were unable to produce formate under anaerobic growth. Higher levels of diacetyl and acetoin were produced anaerobically in the constructed *Lactococcus lactis* subsp. *lactis* biovar diacetylactis *pfl* strain.

Pyruvate has a key role in the metabolic network of *Lactococcus lactis*. In this industrially relevant organism, sugars are metabolized predominantly to generate energy and as a carbon source. The catabolism of sugars leads to pyruvate, whose further metabolism determines the nature of the fermentation (see reference 5 for a review). Lactate is the major end product responsible for acidification, while other minor metabolites, mainly diacetyl, are responsible for aroma in fermented milk products. Several genes involved in pyruvate metabolism have been studied for their role in the production of diacetyl (Fig. 1). Metabolic engineering of pyruvate metabolism in *L. lactis* has focused on the increase of either pyruvate or α-acetolactate levels during growth in the presence of oxygen, mainly via overexpression of α-acetolactate synthase (ALS) genes (*als* or *ilvBN*) and/or inactivation of α-acetolactate decarboxylase encoded by *aldB* (3, 23, 26, 35). A higher level of diacetyl is generally obtained under aerobic growth (12, 35).

During growth in milk, pyruvate formate-lyase (PFL) and lactate dehydrogenase (LDH) are important to sustain growth and for the regeneration of reduced cofactors under anaerobic conditions. Pyruvate is converted into acetyl coenzyme A (CoA) and formate via PFL. The mechanism of this enzyme has been studied in detail only in *Escherichia coli*. The enzyme is activated via free-radical formation and is degraded in the presence of oxygen (9, 19). *pfl* homologs have been characterized in only a few bacteria including *Clostridium pasteurianum*, *Haemophilus influenzae*, and *Streptococcus mutans* (8, 39, 41). Here we describe the cloning, sequence analysis, and characterization of the *L. lactis pfl* gene.

MATERIALS AND METHODS

Bacteria and plasmids. *E. coli* MC1000 (4) and DH5α (Stratagene) were used for cloning. *Lactococcus lactis* subsp. *cremoris* MG1363 (10) and *Lactococcus lactis* subsp. *lactis* biovar diacetylactis DB1341 (kindly provided by Chr. Hansen's A/S, Hørsholm, Denmark) were used in this study. PCR fragments were cloned into pBluescript KS$^+$ (Stratagene) or pSMA500 (20). Plasmid pKmOZ18 was a gift from Y. Sato (Tokyo Dental College, Tokyo, Japan) and includes the *S. mutans pfl* gene (41).

Media and growth conditions. *E. coli* strains were grown in Luria-Bertani medium at 37°C. Erythromycin (250 μg ml^{-1}) or ampicillin (50 μg ml^{-1}) was added as required. *L. lactis* strains were grown in 1.5× M17 (37) supplemented with 0.5% (wt/vol) glucose (GM17), galactose (GalM17), or lactose (LacM17). The media were supplemented with 10 mM citric acid for growth of *L. lactis* subsp. *lactis* biovar diacetylactis DB1341 and transformants derived from this strain. Additionally, a defined medium, SA (16), supplemented with 10-g/liter glucose or lactose, was also used and is referred to as GSA or LSA, respectively. Acetate was omitted, and 100 mM lipoic acid was added to SA for fermentation to allow the synthesis of acetyl-CoA via pyruvate dehydrogenase during aerobic growth (32). Erythromycin (1 μg ml^{-1}) was added as required. Fermentation was carried out with six bench top fermentors (Applikon), each containing 1 liter of medium, set to operate at 30°C. Stirring was applied with a supply of air (aerobic conditions) or nitrogen (anaerobiosis). Oxygen levels were monitored with an Ingold oxygen electrode (Mettler Toledo) and were kept above 20% saturation with air. The fermentors were inoculated with 1 to 10 ml of a fresh overnight culture grown in GM17 or GSA. Growth was monitored by measuring the optical density at 600 nm (OD_{600}), and samples were taken at OD_{600} = 1.0 ± 0.1 for RNA studies and metabolite analysis. Additionally, cultures were grown statically in tightly closed 50-ml tubes containing 50 ml of medium without shaking and were considered anaerobic. Aerobic cultures were grown in 50 ml of medium in 250-ml flasks with shaking (250 rpm). A phenotypic test was conducted among DB1341 and DBKAS7 colonies by replica plating, prior to growth experiments, to ensure their ability to utilize lactose (Lac$^+$) and citrate (Cit$^+$). Only colonies able to grow on LSA plates and that grew as blue colonies on citrate plates (as described elsewhere [18] with Bacto Casamino Acids [Difco] as a substitute for milk protein hydrolysate) were investigated further.

Measurement of β-galactosidase activity in liquid cultures. β-Galactosidase activity was measured for liquid cultures as described elsewhere (15). β-Galactosidase activity in Miller units was calculated as $(522 \times A_{420})/(t \times v \times OD_{600})$, where t is time in minutes and v is the volume of culture used in the assay in milliliters.

Measurement of formate. Formate was assayed enzymatically by using formate dehydrogenase according to the manufacturer's protocol (test combination for determination of formic acid; Boehringer Mannheim).

Measurement of aroma compounds. A colorimetric assay (Voges-Proskauer assay) (40) was used to measure diacetyl and acetoin production. The presence of these metabolites in the samples results in the formation of red color which is monitored by measuring OD_{520}. Overnight cultures of strains DB1341 and DBKAS7 were grown at 30°C anaerobically. Cultures (200 μl) were mixed with 100 μl of 0.3% (wt/vol) creatine–100 μl of 5 M NaOH–50 μl of α-naphthol (freshly made solution in 2.5 M NaOH) in 1.5-ml tubes. The mixtures were incubated at room temperature for 6 min with constant shaking, and the reaction was stopped by adding 1 ml of 4 mM dithiothreitol. Samples were spun down to remove cell debris, and OD_{520} was measured. Acetoin (Sigma) was used as a standard to estimate concentration.

DNA manipulations and transformation. Chromosomal DNA was isolated from *L. lactis* as previously described (1). Plasmid DNA was isolated from *E. coli*

* Corresponding author. Mailing address: Biotechnological Institute, Koglevej 2, DK-2970 Hørsholm, Denmark. Phone: 45 45 16 04 44. Fax: 45 45 16 04 55. E-mail: arnau@biobase.dk.

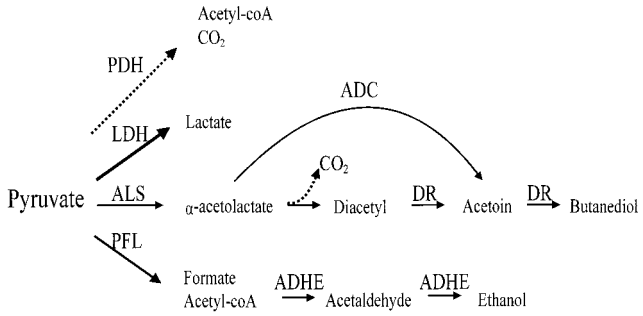

FIG. 1. Metabolic pathways from pyruvate in *L. lactis*. During anaerobic growth, pyruvate is converted into (i) lactate via LDH, (ii) α-acetolactate via ALS, and (iii) formate and acetyl-CoA via PFL. Conversion of pyruvate into acetyl-CoA and CO_2 via pyruvate dehydrogenase (PDH) occurs mainly in aerobiosis (broken line) (5, 32). α-Acetolactate is further metabolized into acetoin via α-acetolactate decarboxylase (ADC) or spontaneously decarboxylated (only in aerobiosis), yielding diacetyl. Diacetyl is the main aroma compound, and it is metabolized into acetoin and butanediol by the action of a proposed diacetyl reductase (DR) (5). Acetyl-CoA is further converted into acetaldehyde and ethanol via the multifunctional enzyme ADHE.

by using Jet Prep kits (Genomed). Plasmid isolation from *L. lactis* was performed as described elsewhere (24). DNA restriction, dephosphorylation, and cloning were performed according to standard procedures (28). DNA fragments were purified from agarose gels by using Jet Sorb kits (Genomed). Probe labelling was carried out by using the Ready-To-Go kit (Pharmacia). Unincorporated nucleotides were removed by using Nick columns (Pharmacia). DNA sequencing was carried out by using T3 and T7 primers (on clone pfl1) and subsequently primers derived from the obtained sequences. Sequencing primers were Cy-5 labelled (Pharmacia), and reactions were run on an ALF Express (Pharmacia). *E. coli* was transformed by electroporation as described elsewhere (28). *L. lactis* strains were transformed essentially as described elsewhere (14).

Construction and screening of an *L. lactis* genomic library with an *S. mutans pfl* probe. A genomic library was constructed by using partially *Sau*3AI-digested DNA of *L. lactis* subsp. *lactis* biovar diacetylactis DB1341 and cloning it into a *Bam*HI-digested λZAP Express vector, according to the manufacturer's instructions (Stratagene). Library clones showed an average insert size of 3 kb. A 1-kb *Eco*RI fragment from the *S. mutants pfl* gene, encompassing positions 1190 to 2213 of the published sequence (codons 298 to 639 of the *pfl* gene), was used to screen the genomic library (approximately 2×10^5 PFU [28]). Filters were washed at low stringency (twice for 30 min at room temperature in 5 × SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and then once for 30 min, 65°C, in 3× SSC–0.1% sodium dodecyl sulfate), and one positive clone, named pfl1, was identified. Following in vivo excision (Stratagene) and plasmid DNA isolation, sequence analysis (ALF Express; Pharmacia) was carried out.

PCR amplifications. By using the *pfl* sequence of strain DB1341 obtained from clone pfl1, a *pfl* DNA fragment was amplified with primers pfl1-20 (5′-GAAG AAGGACGTCATAACCTCC-3′; positions +1311 to +1333 in the MG1363 *pfl* gene [Fig. 3]) and pfl1-1066 (5′-GCATTACTTCTTCATCATCG-3′; positions +2382 to +2363 in the MG1363 *pfl* gene [Fig. 3]), from *L. lactis* MG1363. The fragment was sequenced by using the relevant primers derived from the sequence of the DB1341 *pfl* fragment. Subsequently, inverse PCR was carried out on *Eco*RI-digested and religated chromosomal DNA to clone the upstream region of the *pfl* gene from both strains (Fig. 2). A second inverse PCR was also carried out with *Hha*I-digested DNA from strain DB1341 to clone the upstream region including *orfA* (Fig. 2), and primers derived from the sequence obtained were used to sequence the corresponding chromosomal region of strain MG1363. PCR amplifications were carried out in 50 μl with a GeneAmp DNA amplification reagent kit from Perkin-Elmer Cetus, with the addition of 0.5% Tween 20.

DNA sequence analysis. Analysis of the DNA sequence data and homology searches were performed with the Genetics Computer Group (GCG) program package, version 8 (GCG, Madison, Wis.).

Nucleotide sequence accession numbers. The sequences of the *pfl* regions of strain MG1363 (4,191 bp) and DB1341 (4,977 bp) have been deposited in the EMBL data bank with accession no. AJ000325 and AJ000326, respectively.

Northern blot and primer extension analysis. RNA isolated from exponentially growing cultures (OD_{600} = 1) was separated on 1.2% agarose-formaldehyde gels, and blotting, hybridization, and washes were carried out as described elsewhere (1). Strain-specific *pfl* probes were used for hybridization (see below) (Fig. 2). Autoradiograms were scanned with a Sharp JX-325 scanner, and expression levels were calculated with the Image Master software package (Pharmacia). Primer extension analysis was carried out with 10 μg of total RNA and ^{32}P-end-labelled primer pfl-PE1 (5′-CACCATCATA TGGTTTGTAG TTT-3′, positions +158 to +136), with the avian myeloblastosis virus reverse transcriptase primer extension system (Promega). Products of the primer extension reaction were separated on sequencing gels alongside sequence reactions performed on the corresponding PCR product including the upstream region of the *pfl* gene, with ^{35}S-labelled pfl-PE1. Sequencing reactions were carried out with the Sequenase kit (U.S. Biochemical).

Construction of an *L. lactis pfl* mutant in MG1363 and DB1341. A 460-bp *Sau*3AI internal fragment of the DB1341 *pfl* gene (Fig. 2) was cloned into *Bam*HI-digested pSMA500 and transformed into DB1341, resulting in strain DBKAS7. A 495-bp PCR fragment was amplified from MG1363 with primers pfl-P1MG1363 (5′-GGCCGCTCGAGTTGTGTCTC ACCACTTGAC CC-3′; *Xho*I site underlined) and pfl-P2MG1363 (5′-TAGTAGGATC CCATCATCTT CACCATAACG TGG; *Bam*HI site underlined), cloned into *Xho*I-*Bam*HI-digested pSMA500, and transformed into strain MG1363, resulting in strain MGKAS13. DBKAS7 and MGKAS13 formed blue colonies on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-containing plates. Plasmid integration through homologous recombination was confirmed via PCR in both strains (data not shown).

RESULTS

Cloning of the *L. lactis pfl* gene. The *pfl* gene of *S. mutans* has recently been cloned (41). Due to the close taxonomical position of *S. mutans* and *L. lactis*, we decided to use the cloned gene to identify the lactococcal counterpart. A 1-kb *Eco*RI fragment from the *S. mutans pfl* gene, encompassing positions 1190 to 2213 of the published sequence (codons 298 to 639 of the *pfl* gene), was used to screen a genomic library of *L. lactis* subsp. *lactis* biovar diacetylactis DB1341. One positive clone, pfl1, was isolated. Sequence analysis of this clone identified a truncated, uninterrupted open reading frame (ORF) spanning a 1.1-kb fragment that showed homology to other *pfl* genes, at both the DNA and the protein level. Sequence comparisons indicated that the fragment included in clone pfl1 encompassed 367 amino acids of the C-terminal region of the *L. lactis pfl* gene (Fig. 2). Therefore, about 1.2 kb of the 5′ end of the *pfl* gene was missing. A 600-bp *Pst*I-*Eco*RI fragment from clone pfl1, spanning from the polylinker region to the *Eco*RI site in the *pfl* gene of strain DB1341 (Fig. 2), was used to screen the genomic library. Several positive library clones did not yield a plasmid upon excision. The clones isolated included DNA inserts that started at the same *Sau*3AI site as the insert in clone pfl1 (Fig. 2), indicating that a full-length *L. lactis pfl* gene from strain DB1341 in a high-copy-number vector may be toxic in *E. coli*. Thus, cloning of the upstream region of the *L. lactis pfl* gene was carried out by inverse PCR. The *L. lactis* DB1341 *pfl* gene consists of 2,363 bp and encodes a 787-amino-acid PFL protein with a predicted molecular mass of 89.120 kDa.

The *pfl* sequence was also obtained from the well-characterized *L. lactis* subsp. *cremoris* strain MG1363 and used in further sequence analysis. The 787-amino-acid PFL of MG1363 had a predicted molecular mass of 89.105 kDa.

Sequence analysis of the *L. lactis pfl* gene. Homology searches with the deduced *L. lactis* PFL detected a 79% overall protein sequence identity with the *S. mutans* PFL and more than 40% identity with the *E. coli*, *C. pasteurianum*, and *H. influenzae* PFLs (Table 1). In the promoter region of the *pfl* gene, a canonical lactococcal ribosome binding site (AAAGGAG, positions +21 to +27 [Fig. 3]) and the −35 and −10 promoter regions (TTGCTA and TATAAT, at positions −35 to −30 and −13 to −8, respectively) were found. A putative rho-independent transcription terminator with an estimated ΔG_f of −11.4 kcal/mol (6) was located 24 bp downstream of the *pfl* stop codon (positions 2432 to 2445 [Fig. 3]).

In *E. coli*, the expression of *pfl* is regulated by the global transcription factor FNR, via two recognition sequences (FNR boxes; consensus sequence TTGAT-N_4-ATCAA) located in the promoter region of this gene (29). Recently, the isolation of two *fnr*-like genes from *L. lactis* has been described (13), although no detailed characterization of the corresponding

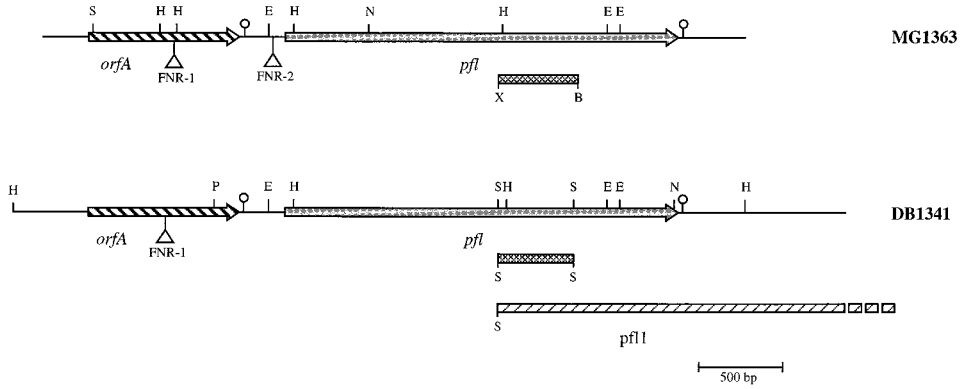

FIG. 2. Genetic map of the *pfl* chromosomal region in *L. lactis* MG1363 (top) and DB1341 (bottom). Only relevant restriction sites are shown; B, *Bam*HI; E, *Eco*RI; H, *Hha*I; P, *Pst*I; N, *Nsi*I; S, *Sau*3AI; X, *Xho*I. Genes are shown as arrows, thickly striped for *orfA* and shaded for *pfl*. Putative transcriptional terminators for *orfA* and *pfl* are shown as open circles. The positions of FNR boxes are indicated by open triangles (FNR-1 and FNR-2). The DNA fragments used as probes and for insertional inactivation are depicted as patterned boxes (see Materials and Methods). The region included in the genomic library clone pfl1 is shown as a thinly striped box.

recognition sequences was reported. Since a *Lactobacillus casei fnr* gene is unable to complement an *fnr* deletion mutant of *E. coli* (11), it is likely that some sequence variation at DNA recognition sites occurs between FNRs from different organisms. Such variation has also been described for FNR recognition sites in other organisms and is detected mainly in the TTGAT motif (33). Interestingly, two sequences (FNR-1 and FNR-2) with significant homology to the *E. coli* consensus were identified in *L. lactis* MG1363 (Fig. 3). FNR-1 (GGAGT-N_4-ATCAA) was also present in strain DB1341 (GAACA-N_4-ATCAA) and was located at positions −692 to −679. FNR-2 (TTTGC-N_4-ATCAA; positions −36 to −23) overlaps with the −35 hexamer of the promoter region of the *pfl* gene (Fig. 3). Sequence divergence in the ATCAA motif was observed at this position in strain DB1341 (TTTGC-N_4-CTCAA). Interestingly, two related sequences are located at similar positions in the promoter region of the *S. mutans pfl* gene (41). One of them (TTGTA-N_4-ATTAA) was located just downstream of the −10 region of the promoter and may be the FNR-2 equivalent in this organism. Another box (GGCAT-N_4-ATCAA) is located 280 bp upstream of the *pfl* start codon and may correspond to the FNR-1 found in *L. lactis*. In either case, 60% conserved positions were observed. However, experimental evidence is needed to support the role of these sequences in regulation of *pfl* expression.

The coding sequence of the MG1363 *pfl* gene showed 102 base changes, compared to the corresponding sequence of strain DB1341, and resulted in only four amino acid changes in the PFL primary structure. The lactococcal PFL includes the conserved Gly residue at position 749, flanked by Ser and Tyr residues, which is involved in activation and deactivation of the enzyme in *E. coli* via free-radical formation. This region is present in all PFL proteins characterized to date (Fig. 4) (39). The *L. lactis* sequence ISCCVSP (residues 414 to 420 [Fig. 4]) is highly conserved and includes two adjacent Cys residues that have been implicated in the cleavage of the C-C bond of pyruvate and hydrogen exchange of the glycyl radical of the enzyme (25).

Genetic organization of the *pfl* locus in *L. lactis*. In *E. coli*, *C. pasteurianum*, and *H. influenzae*, the *act* gene, coding for PFL-activase, is located downstream of *pfl* (Fig. 5) (8, 27, 39). A different genetic organization was found in the *S. mutans pfl* region, where two ORFs without known homology flank the *pfl* gene (41). We sequenced the *pfl* chromosomal region to attempt the identification of the *L. lactis act* gene. No potential ORF was identified in the 1-kb region downstream of *pfl* characterized. However, an ORF, *orfA*, just upstream of *pfl* in the same transcriptional orientation (positions −1142 to −225 [Fig. 3]), which may encode a 37-kDa protein, was identified (Fig. 2). A short ribosome binding site (AGG) and a −10 promoter region (positions −1150 to −1148 and −1174 to −1169, respectively) but not a −35 region were found upstream from the *orfA* start codon (Fig. 3). These features are consistent with the low level of expression observed for this gene (not shown). A putative transcriptional terminator (ΔG_f of −4.8 kcal/mol) was found just downstream of *orfA* (positions −222 to −208), suggesting that *pfl* and *orfA* are independently transcribed in *L. lactis* (Fig. 2 and 3). In *E. coli* and *H. influenzae*, *pfl* is part of an operon, together with *focA*, that codes for a putative formate channel protein (Fig. 5) (8, 34). Database searches did not allow the identification of homology between the *L. lactis orfA* and the two *focA* genes identified to date. An *L. lactis* gene, *orfc3*, coding for a hypothetical 37-kDa protein (7), showed 23% protein identity with ORFA. The genetic organization of the *L. lactis pfl* region showed remarkable differences with respect to the corresponding *S. mutans* chromosomal locus (Fig. 5), where a divergently transcribed ORF, *orf1*, is found just upstream of *pfl* and another ORF, *orf2*, located downstream and transcribed independently, showed no homology to any known gene. Neither *orf1* nor *orf2* showed homology to the *L. lactis orfA* or to *focA* (41). Thus, the genetic organization of the *pfl* locus in these closely related organisms showed remarkable differences not found in other bacteria.

TABLE 1. Homology of the *L. lactis* MG1363 PFL to other PFL proteins in databases[a]

Organism[b]	% Identity				
	L. lactis	*S. mutans*	*H. influenzae*	*E. coli*	*C. pasteurianum*
L. lactis	100	79.2	42.2	40.3	38.8
S. mutans (D50491)		100	46.7	43.7	47.3
H. influenzae (U32703)			100	85.2	59.4
E. coli (X08035)				100	61.5
C. pasteurianum (X93463)					100

[a] Sequence comparison was performed with the programs TFASTA and FASTA from the GCG program package, version 8 (GCG).
[b] Accession number shown in parentheses.

Analysis of *pfl* gene expression in *L. lactis* MG1363. The expression of *pfl* is induced under anaerobiosis in *E. coli* (30). Physiological studies have shown that pyruvate is converted mainly to lactate when *L. lactis* is growing in glucose. Formate, ethanol, and acetate are produced as a consequence of PFL activity during anaerobic growth in less favorable carbon sources such as galactose (5).

L. lactis subsp. *lactis* biovar diacetylactis strains can metabolize citrate and lactose, due to the presence of plasmid-encoded *cit* and *lac* genes, and show elevated diacetyl production in citrate-containing media (31). We observed severe alterations in the plasmid profile of strain DB1341 upon transformation (see below), leading to the loss of one or several endogenous plasmids among the transformants. Moreover, novel plasmid bands were also detected in most transformants analyzed (data not shown). This genetic instability complicated the correlation between DB1341 wild-type and mutant strains. Therefore, genetic studies were carried out with the plasmid-free laboratory strain MG1363. The expression of the *L. lactis pfl* gene was studied in exponentially growing cultures of strain MG1363 in M17 medium, containing either glucose or galactose, under anaerobic or aerobic conditions in a fermentor. Northern blot analysis showed a low level of expression under aerobiosis and a 15-fold induction under anaerobiosis during growth in glucose (Fig. 6A, lanes 1 and 2). In medium containing galactose, a much higher expression level was observed in aerobiosis (Fig. 6A, lane 3), and a fivefold induction was observed in the corresponding anaerobic conditions (Fig. 6A, lane 4). Low levels of expression were detected during growth in defined GSA medium, although anaerobic induction was also observed (Fig. 6A, lanes 5 and 6). The size of the detected transcript (2.4 kb) corresponded to the size of the *pfl* gene, confirming that the gene is transcribed independently of *orfA* (Fig. 6A). Primer extension studies located the transcription start site (*tss*) 35 bp upstream of the *pfl* start codon (Fig. 3 and 6B). No alternative *tss* was detected, in contrast to the situation reported for *E. coli*, in which seven promoter regions have been implicated in regulation and expression of *pfl* (29).

Construction and analysis of *L. lactis pfl* mutant strains. Gene disruption of the *pfl* gene was carried out by insertional inactivation in MG1363 and DB1341 (see Materials and Methods for details). One transformant obtained from MG1363, MGKAS13, was selected for further analysis. Transformants obtained from DB1341 showed an altered plasmid complement compared to strain DB1341 (data not shown), and considerable variation in the ability to utilize lactose and citrate was observed. However, instability of the plasmid complement was also observed in DB1341 in the media used in this study. One transformant that showed a Lac^+ Cit^+ phenotype, DBKAS7, was selected for further analysis. To allow comparison of aroma compounds produced, a phenotypic test was carried out before and after growth experiments on both DB1341 and DBKAS7 to ensure that these strains remained Cit^+ and Lac^+ (see above).

Inactivation of the *pfl* gene leads to a transcriptional fusion of the *lacLM* reporter gene (20). β-Galactosidase levels were measured in overnight cultures of strain MGKAS13 grown in M17 with either glucose or galactose (Table 2). In GM17, anaerobic induction was observed, approximately 15-fold, consistent with the induction observed at the RNA level. High levels of β-galactosidase under aerobic conditions were observed for growth with galactose, and a sixfold induction was observed under anaerobiosis in this medium (Table 2), in agreement with the RNA studies (Fig. 6A).

MGKAS13 should not produce formate under anaerobic conditions as a result of the inactivation of the *pfl* gene in this strain. In strain MG1363, no formate was detected under aerobic conditions in GM17, as expected if the lactococcal PFL is inactivated in the presence of oxygen. Relatively low levels were detected under anaerobiosis. In GalM17, an eightfold

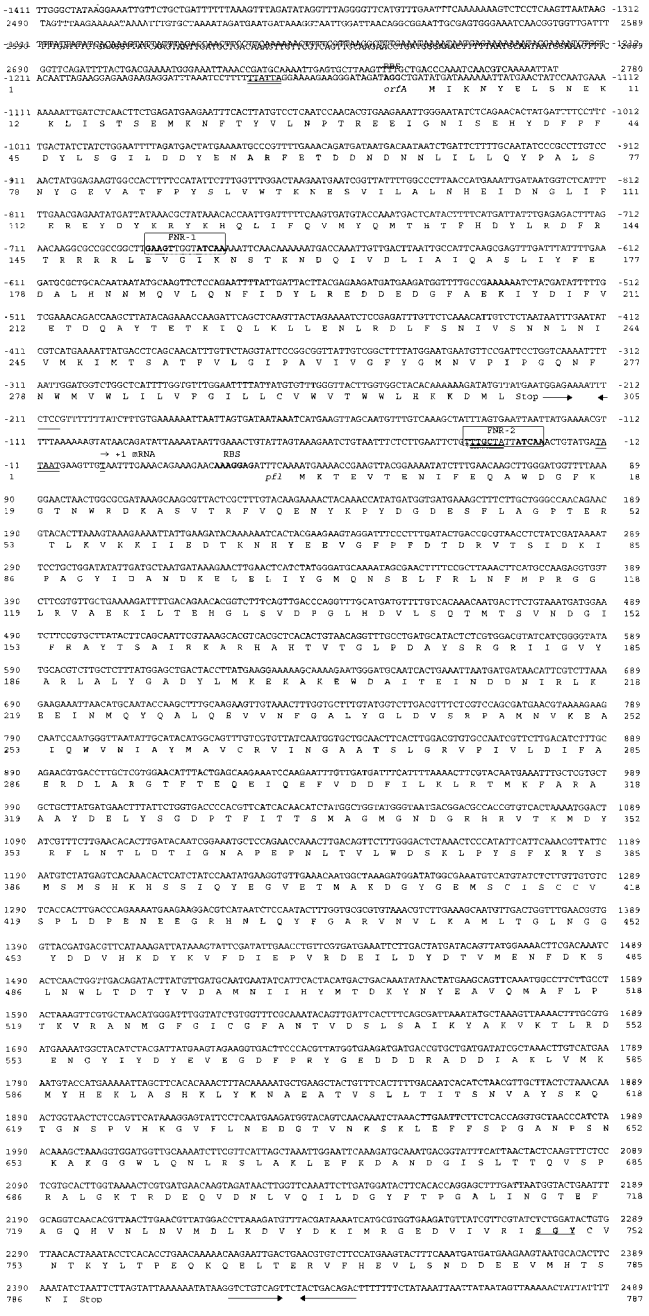

FIG. 3. Sequence of the *L. lactis* MG1363 *pfl* gene. The sequence starts upstream of *orfA* and includes the *pfl* gene and ca. 400 bp downstream of *pfl*. Position +1 (mRNA) refers to the *pfl* transcription start site. Ribosome binding sites for *orfA* and *pfl* are shown in boldface (RBS). Promoter regions, −35 and −10, are shown doubly underlined. Pairs of arrows depict putative transcriptional terminator sequences. FNR-1 and FNR-2 boxes indicate sequences with homology to the consensus recognition sequence of the bacterial FNR transcription factor (33). The deduced protein sequences are shown in single-letter code. The activation site of PFL is single underlined.

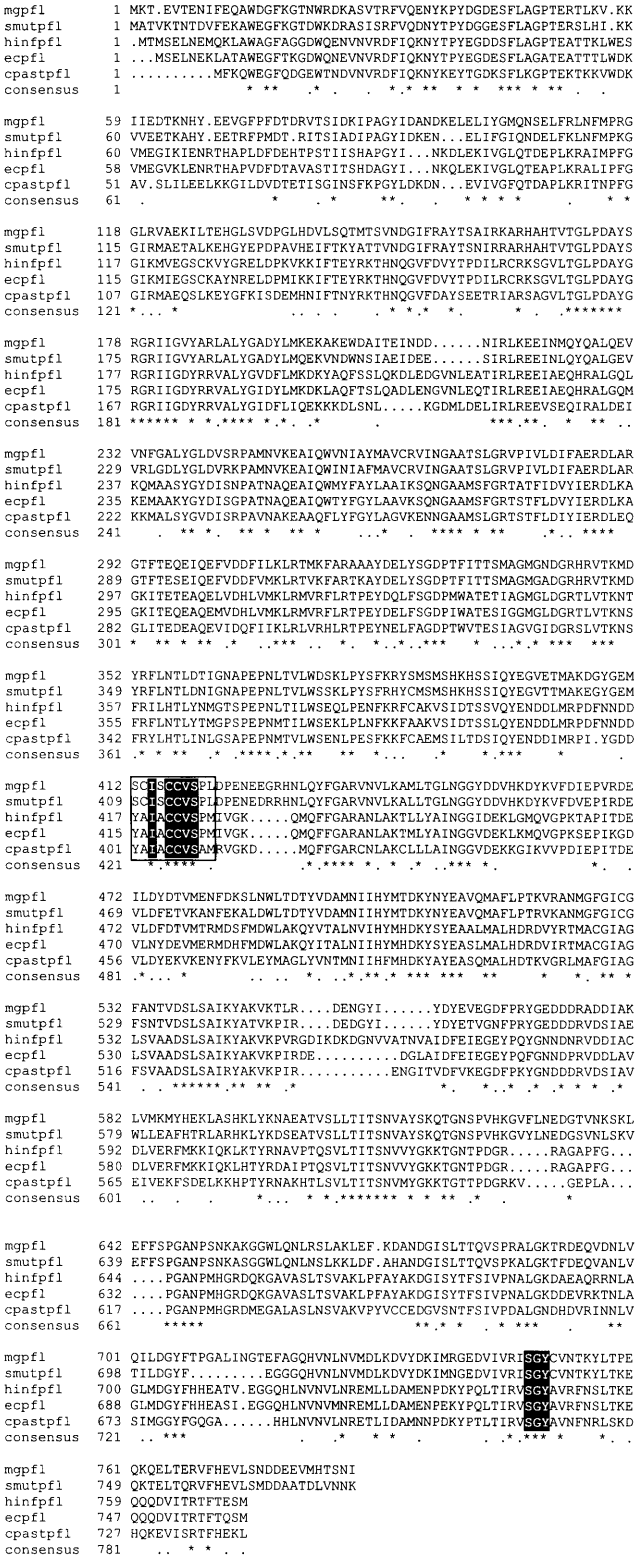

FIG. 4. Multiple alignment of PFL proteins. The lactococcal PFL sequence from strain MG1363 was aligned with all PFL entries available in the databases by using the MULTALIGN and LINEUP functions of the GCG program package, version 8. Asterisks and single dots in the consensus line represent identical and similar amino acid residues, respectively. The catalytic site is shown as a box. Identical residues at the catalytic and activation sites are shown in white-on-black print. Abbreviations: mgpfl, strain MG1363 PFL; smutpfl, *S. mutans* PFL; hinfpfl, *H. influenzae* PFL; ecpfl, *E. coli* PFL; cpastpfl, *C. pasteurianum* PFL.

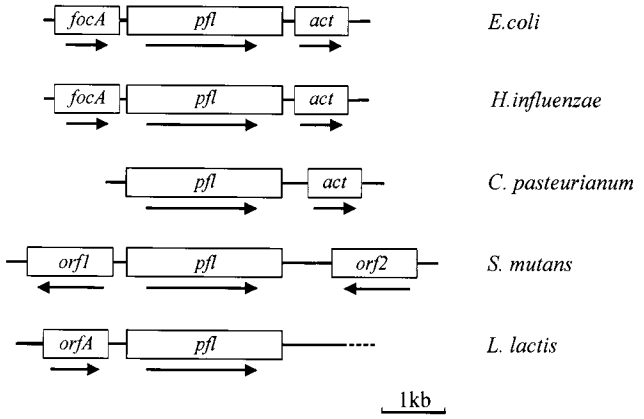

FIG. 5. Genetic organization of the *pfl* locus in bacteria. Comparison of the relative positions of *pfl* and flanking genes in bacteria. The figure is drawn to scale. Open boxes indicate genes; arrows indicate direction of transcription. Accession numbers: X08035 (*E. coli*), U32703 (*H. influenzae*), X93463 (*C. pasteurianum*), and D50491 (*S. mutans*).

higher amount of formate was detected in anaerobiosis (Table 2). No formate was detected in strain MGKAS13 regardless of the medium and conditions used, confirming that this strain carries a *pfl* null mutation. Similar levels of formate were obtained in strain DB1341, compared to MG1363, and no formate was detected in DBKAS7 (data not shown), confirming the *pfl* mutant phenotype in this strain.

L. lactis biovar diacetylactis strains are used in starter cultures due to their ability to produce diacetyl during milk fermentation. A mutation in the *pfl* gene of DB1341 should result in increased pyruvate levels under anaerobic growth. Thus, if excess pyruvate is directed towards the production of diacetyl and acetoin, a higher level of these metabolites would be expected in strain DBKAS7 grown under anaerobiosis. Cell extracts from stationary-phase cultures (OD_{600} of ~3) were assayed by the Voges-Proskauer method as described in reference 4. For strains DB1341 and DBKAS7, diacetyl and acetoin production levels were 0.2 and 1.5 mM in GM17, respectively, and ≤0.05 and 0.2 mM in GalM17, respectively values are the means of two independent experiments. As is evident, a sevenfold increase in the production of aroma compounds was observed in DBKAS7 grown in GM17 and a more than fourfold increase was detected in GalM17 compared to DB1341, demonstrating the effect of a *pfl* mutation in the production of diacetyl and acetoin in a diacetylactis strain. In strain DB1341, very low levels were produced in GalM17, compared to GM17. The high level of *pfl* expression observed in GalM17 might result in a reduction of pyruvate levels through PFL activity and a concomitant reduction in aroma production. The lower level of diacetyl and acetoin produced by DBKAS7 in GalM17 correlated with a higher level of pyruvate observed in this medium (data not shown). Further analysis of metabolite levels in DBKAS7 is required to study the effect of a mutation in the *pfl* gene on the metabolism of pyruvate in this organism.

DISCUSSION

We have cloned the gene coding for PFL, *pfl*, from *L. lactis*. DNA sequence variation was observed between the *pfl* gene of DB1341 (a diacetylactis strain) and MG1363 (*L. lactis* subsp. *cremoris* strain). However, the deduced amino acid sequence showed 99.5% identity between the corresponding PFL proteins. The deduced lactococcal PFL sequence showed high identity to the *S. mutans* PFL and significant homology to all

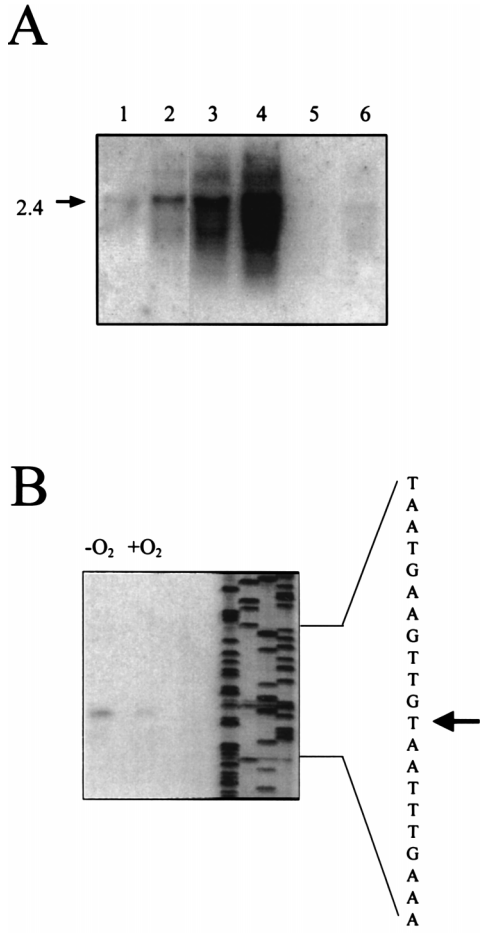

FIG. 6. Northern blot and primer extension analysis of the *L. lactis pfl* gene. (A) Northern blot analysis of total RNA from *L. lactis* MG1363 grown in fermentors. Lane 1, aerobic growth in GM17; lane 2, anaerobic growth in GM17; lane 3, aerobic growth in GalM17; lane 4, anaerobic growth in GalM17; lane 5, aerobic growth in GSA; lane 6, anaerobic growth in GSA. An internal *pfl* DNA fragment from MG1363 was used as a probe (Fig. 2). The size of the *pfl* transcript is shown on the left in kilobases. (B) Primer extension carried out with primer pfl-PE1 on total RNA of strain MG1363 grown anaerobically (leftmost lane) or aerobically on GM17. A sequence ladder was run with the same primer and is shown on the right. The *pfl* transcription start site is indicated with an arrow beside the sequence line.

other PFL proteins present in the databases. The *L. lactis* PFL included the conserved regions necessary for free-radical formation and a catalytic site (Fig. 4) (25, 38).

A large promoter region is involved in the regulation of *pfl* expression in *E. coli* (29). Seven promoters and recognition sites for two major transcription factors, ArcA and FNR, have been identified in this region. FNR boxes have been found in the promoter region of genes involved in anaerobic respiration and pyruvate metabolism in a number of bacteria (33). Analysis of the *L. lactis pfl* promoter region permitted the identification of two putative FNR boxes, FNR-1 and FNR-2, solely on the basis of sequence homology. Remarkably, the location of FNR-2, overlapping with the −35 promoter region of *pfl*, might result in enhanced regulation of expression if the FNR protein facilitates transcription initiation by interaction with the RNA polymerase (17, 33). The upstream region of the *S. mutans pfl* gene also included two putative FNR boxes similar in sequence and location to FNR-1 and FNR-2, although homology to the *L. lactis* and *E. coli* FNR boxes was restricted to the ATCAA motif. Analysis of the promoter region of the *C. pasteurianum pfl* gene did not allow the identification of putative FNR boxes. In this bacterium, PFL has been suggested to play a role in anabolism and to supply the cells with C_1 units, and consequently, regulation of expression may not include FNR (39). The possible role of FNR-1 and FNR-2 in the regulation of *pfl* expression in *L. lactis* is currently being investigated, and the recent isolation of *fnr*-like genes (13) represents an invaluable tool for achieving this goal.

The identification of putative transcriptional terminators upstream and downstream of *pfl*, together with Northern blot analysis, showed that the *L. lactis pfl* gene is transcribed independently, in contrast to *E. coli*, in which *pfl* is part of an operon with *focA*. The genetic organization of the *E. coli pfl* chromosomal region is conserved in *H. influenzae* and *C. pasteurianum*. However, major divergence is observed in the streptococcal and lactococcal *pfl* loci, where neither the *act* gene nor sequence similarities between the ORFs found were detected (Fig. 5).

The regulation of PFL plays a central role in the change of fermentation patterns in *S. mutans*, especially under carbon limitation (41). No molecular study of gene expression of the *S. mutans pfl* gene has been published (36). We have studied expression of the *L. lactis pfl* gene, during exponential growth with glucose or galactose in rich medium and in defined medium. Anaerobic induction of *pfl* expression was observed in all media, although the overall levels were highest with growth in GalM17. Comparison of β-galactosidase levels in the *pfl* mutant strain, MGKAS13, and mRNA levels in MG1363 suggested that PFL is regulated at the transcriptional level in *L. lactis*. The absence of anaerobic formate production in MGKAS13 demonstrated that the gene characterized encodes PFL.

We have shown that anaerobic growth of the diacetylactis *pfl* strain, DBKAS7, resulted in higher levels of diacetyl and acetoin, especially in GM17 medium. Surprisingly, lower levels of these metabolites were produced by DBKAS7 in GalM17, compared to GM17. Preliminary metabolite analysis suggested that pyruvate might be directed mainly towards lactate in DBKAS7 (not shown). Additional experiments are in progress to examine the level of other compounds involved in pyruvate metabolism.

As mentioned above, PFL activity is posttranslationally regulated in *E. coli* via the formation of a free radical by the PFL-activase encoded by the *act* gene (27). The active form of the enzyme is irreversibly inactivated in the presence of oxygen. Alternatively, protection from damage by oxygen is accomplished by removal of the free radical, resulting in an inactive PFL protein that is not affected by oxygen. This deactivation activity resides in the multifunctional alcohol dehydrogenase enzyme (ADHE) (19). The presence of an *adhE* homolog has been questioned for streptococci and other lactic acid bacteria (5). We have cloned and characterized an *adhE*

TABLE 2. Characterization of the *L. lactis* MG1363 *pfl* strain, MGKAS13

Strain	Growth medium	Aerobic		Anaerobic	
		Formate (mM)	β-Gal (U)	Formate (mM)	β-Gal (U)
MG1363	GM17	0		5.3	
	GalM17	0		42	
MGKAS13	GM17	0	9.5	0	150.0
	GalM17	0	94.6	0	600.0

homolog in *L. lactis* by complementation of a strain of *E. coli* unable to grow under anaerobiosis (2), and experiments are currently being carried out to analyze the role of the lactococcal ADHE in regulation of PFL activity.

Metabolic engineering of pyruvate metabolism in *L. lactis* has been carried out via overexpression of ALS genes and/or inactivation of α-acetolactate decarboxylase (21, 22, 35; also, see the introduction). Additionally, an *L. lactis ldh* strain lacking LDH would be expected to direct the pyruvate pools towards α-acetolactate via ALS and yield a higher amount of diacetyl (Fig. 1). However, such a strain might have a reduced growth rate and acidification in fermentation without pH control (26). Since the lactococcal ALS has a low affinity for pyruvate, high pyruvate pools are required to increase diacetyl production (21, 32). Moreover, an anabolic ALS that shows higher affinity for pyruvate, compared to ALS, is encoded by the *L. lactis ilvBN* genes (3).

All above-mentioned approaches have yielded an increase in diacetyl and acetoin formation in aerated cultures. Under these conditions, alteration in the expression of *pfl* would not result in a change in diacetyl levels, due to the PFL requirement for an oxygen-free environment (38). However, fermentation using lactic acid bacteria occurs normally in an anaerobic environment in which PFL activity plays an important role. The results presented in this paper support the hypothesis that anaerobic growth of an *L. lactis pfl* strain should yield higher levels of aroma compounds. Our working model for an engineered strain of *L. lactis* capable of producing higher levels of diacetyl during milk fermentation relies on the regulated expression of *pfl*, together with the characterization and inactivation of the genes involved in further metabolism of diacetyl.

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REFERENCES

1. **Arnau, J., K. I. Sørensen, K. F. Appel, F. K. Vogensen, and K. Hammer.** 1996. Analysis of heat shock gene expression in *Lactococcus lactis* MG1363. Microbiology **142:**1685–1691.
2. **Arnau, J., F. Jørgensen, S. M. Madsen, A. Vrang, and H. Israelsen.** Unpublished data.
3. **Benson, K. H., J. J. Godon, P. Renault, H. G. Griffin, and M. J. Gasson.** 1996. Effect of *ilvBN*-encoded α-acetolactate synthase expression on diacetyl production in *Lactococcus lactis*. Appl. Microbiol. Biotechnol. **45:**107–111.
4. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179–207.
5. **Cocaign-Bousquet, M., C. Garrigues, P. Louibiere, and N. D. Lindley.** 1996. Physiology of pyruvate metabolism in *Lactococcus lactis*. Antonie Leeuwenhoek **70:**253–267.
6. **de Vos, W. M., and G. Simons.** 1994. Gene cloning and expression systems in lactococci, p. 52–105. *In* M. Gasson and W. de Vos (ed.), Genetics and biotechnology of lactic acid bacteria. Chapman and Hall, London, United Kingdom.
7. **Donkersloot, J. A., and J. Thompson.** 1995. Cloning, expression, sequence analysis and site-directed mutagenesis of the Tn*5306*-encoded N^5-(carboxyethyl)ornithine synthase from *Lactococcus lactis* K1. J. Biol. Chem. **270:** 12226–12234.
8. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomp, B. A. Dougherty, J. M. Merrick, et al.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
9. **Frey, M., M. Rothe, A. F. Volker Wagner, and J. Knappe.** 1994. Adenosylmethionine-dependent synthesis of the glycyl radical in pyruvate formate-lyase by abstraction of the glycine C-2 pro-S hydrogen atom. J. Biol. Chem. **269:**12432–12437.
10. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1–9.
11. **Gostik, D. O., A. S. Irvine, J. Green, M. J. Gasson, and J. R. Guest.** 1996. Purification and characterisation of FLP, the FNR-like protein of *Lactobacillus casei*, abstr. H1. *In* 5th Symposium on Lactic Acid Bacteria, Veldhoven, The Netherlands.
12. **Goupil, N., G. Corthier, S. D. Ehrlich, and P. Renault.** 1996. Imbalance of leucine flux in *Lactococcus lactis* and its use in the isolation of diacetyl-overproducing strains. Appl. Environ. Microbiol. **62:**2636–2640.
13. **Griffin, H. G., H. M. Underwood, D. Gostik, J. R. Guest, and M. J. Gasson.** 1996. Analysis of *fnr* genes in *Lactococcus lactis*, abstr. H24. *In* 5th Symposium on Lactic Acid Bacteria, Veldhoven, The Netherlands.
14. **Holo, H., and I. F. Nes.** 1989. High-frequency transformation by electroporation of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. **55:**3119–3123.
15. **Israelsen, H., S. M. Madsen, A. Vrang, E. B. Hansen, and E. Johansen.** 1995. Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn*917-lacZ* integrants with the new promoter-probe vector, pAK80. Appl. Environ. Microbiol. **61:**2540–2547.
16. **Jensen, P. R., and K. Hammer.** 1993. Minimal requirements for exponential growth of *Lactococcus lactis*. Appl. Environ. Microbiol. **59:**4363–4366.
17. **Kaiser, M., and G. Sawers.** 1995. Fnr activates transcription from the P6 promoter of the *pfl* operon *in vitro*. Mol. Microbiol. **18:**331–342.
18. **Kempler, G. M., and L. L. McKay.** 1980. Improved medium for detection of citrate-fermenting *Streptococcus lactis* subsp. *diacetylactis*. Appl. Environ. Microbiol. **39:**926–927.
19. **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.
20. **Madsen, S. M., B. Albrechtsen, E. Hansen, and H. Israelsen.** 1996. Cloning and transcriptional analysis of two threonine biosynthetic genes from *Lactococcus lactis* MG1614. J. Bacteriol. **178:**3689–3694.
21. **Marugg, J. D., D. Goelling, U. Stahl, A. M. Ledeboer, M. Y. Toonen, W. M. Verhue, and C. T. Verrips.** 1994. Identification and characterization of the α-acetolactate synthase gene from *Lactococcus lactis* subsp. *lactis* biovar diacetylactis. Appl. Environ. Microbiol. **60:**1390–1394.
22. **Monnet, C., V. Phalip, P. Schmitt, and C. Diviès.** 1994. Comparison of α-acetolactate synthase and α-acetolactate decarboxylase in *Lactococcus* ssp. and *Leuconostoc* ssp. Biotechnol. Lett. **16:**257–262.
23. **Monnet, C., P. Schmitt, and C. Diviès.** 1994. Diacetyl production in milk by an α-acetolactic acid accumulating strain of *Lactococcus lactis* ssp. *lactis* biovar diacetylactis. J. Dairy Sci. **77:**2916–2924.
24. **O'Sullivan, D. J., and T. R. Klaenhammer.** 1993. Rapid mini-prep isolation of high-quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. Appl. Environ. Microbiol. **59:**2730–2733.
25. **Parast, C. V., K. K. Wong, S. A. Lewisch, and J. W. Kozarich.** 1995. Hydrogen exchange of the glycyl radical of pyruvate formate-lyase is catalyzed by cysteine 419. Biochemistry **34:**2393–2399.
26. **Platteeuw, C., J. Hugenholtz, M. Starrenburg, I. van Alen-Boerrigter, and W. M. de Vos.** 1995. Metabolic engineering of *Lactococcus lactis*: influence of the overproduction of α-acetolactate synthase in strains deficient in lactate dehydrogenase as a function of culture conditions. Appl. Environ. Microbiol. **61:**3967–3971.
27. **Rödel, W., W. Plaga, R. Frank, and J. Knappe.** 1988. Primary structures of *Escherichia coli* pyruvate formate-lyase and pyruvate formate-lyase-activating enzyme deduced from the DNA nucleotide sequences. Eur. J. Biochem. **177:**153–158.
28. **Sambrook, J., E. F. Fritsch, and T. E. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Sawers, G.** 1993. Specific transcriptional requirements for positive regulation of the anaerobically inducible *pfl* operon by ArcA and FNR. Mol. Microbiol. **10:**737–747.
30. **Sawers, G., A. F. V. Wagner, and A. Böck.** 1989. Transcription initiation at multiple promoters of the *pfl* gene by $E\sigma^{70}$-dependent transcription in vitro and heterologous expression in *Pseudomonas putida* in vivo. J. Bacteriol. **171:**4930–4937.
31. **Smith, M. R., J. Hugenholtz, P. Mikoczi, E. de Ree, A. W. Bunch, and J. A. M. de Bont.** 1992. The stability of the lactose and citrate plasmids in *Lactococcus lactis* subsp. *lactis* biovar diacetylactis. FEMS Microbiol. Lett. **96:**7–12.
32. **Snoep, J. L., M. J. T. de Mattos, M. J. C. Starrenburg, and J. Hugenholtz.** 1992. Isolation, characterization, and physiological role of the pyruvate dehydrogenase complex and α-acetolactate synthase of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis. J. Bacteriol. **174:**4838–4841.
33. **Spiro, S.** 1994. The FNR family of transcriptional regulators. Antonie Leeuwenhoek **66:**23–36.
34. **Suppmann, B., and G. Sawers.** 1996. Isolation and characterization of hypophosphite-resistant mutants of *Escherichia coli*: identification of the FocA

protein, encoded by the *pfl* operon, as a putative formate transporter. Mol. Microbiol. **11:**965–982.
35. **Swindell, S. R., K. H. Benson, H. G. Griffin, P. Renault, S. D. Ehrlich, and M. J. Gasson.** 1996. Genetic manipulation of the pathway for diacetyl metabolism in *Lactococcus lactis*. Appl. Environ. Microbiol. **62:**2641–2643.
36. **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.
37. **Terzaghi, B. A., and W. E. Sandine.** 1975. Improved medium for lactic acid streptococci and their bacteriophages. Appl. Environ. Microbiol. **29:**807–813.
38. **Wagner, A., M. Frey, F. Neugebauer, W. Schafer, and J. Knappe.** 1992. The free radical in pyruvate formate-lyase is located on glycine-734. Proc. Natl. Acad. Sci. USA **89:**996–1000.
39. **Weidner, G., and G. Sawers.** 1996. Molecular characterization of the genes encoding formate-lyase and its activating enzyme of *Clostridium pasteurianum*. J. Bacteriol. **178:**2440–2444.
40. **Westerfeld, W. W.** 1945. A colorimetric determination of blood acetoin. J. Biol. Chem. **161:**495–502.
41. **Yamamoto, Y., Y. Sato, S. Takahashi-Abbe, K. Abbe, T. Yamada, and H. Kizaki.** 1996. Cloning and sequence analysis of the *pfl* gene encoding pyruvate formate-lyase from *Streptococcus mutans*. Infect. Immun. **64:** 385–391.