

Characterization and Functional Analysis of the *poxB* Gene, Which Encodes Pyruvate Oxidase in *Lactobacillus plantarum*

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The pyruvate oxidase gene (*poxB*) from *Lactobacillus plantarum* Lp80 was cloned and characterized. Northern blot and primer extension analyses revealed that transcription of *poxB* is monocistronic and under the control of a vegetative promoter. *poxB* mRNA expression was strongly induced by aeration and was repressed by glucose. Moreover, Northern blotting performed at different stages of growth showed that *poxB* expression is maximal in the early stationary phase when glucose is exhausted. Primer extension and in vivo footprint analyses revealed that glucose repression of *poxB* is mediated by CcpA binding to the *cre* site identified in the promoter region. The functional role of the PoxB enzyme was studied by using gene overexpression and knockout in order to evaluate its implications for acetate production. Constitutive overproduction of PoxB in *L. plantarum* revealed the predominant role of pyruvate oxidase in the control of acetate production under aerobic conditions. The Δ *poxB* mutant strain exhibited a moderate (20 to 25%) decrease in acetate production when it was grown on glucose as the carbon source, and residual pyruvate oxidase activity that was between 20 and 85% of the wild-type activity was observed with glucose limitation (0.2% glucose). In contrast, when the organism was grown on maltose, the *poxB* mutation resulted in a large (60 to 80%) decrease in acetate production. In agreement with the latter observation, the level of residual pyruvate oxidase activity with maltose limitation (0.2% maltose) was less than 10% of the wild-type level of activity.

Lactobacillus plantarum is a facultative heterofermentative bacterium (24). Under anaerobic conditions with excess glucose, lactate is the major fermentation end product. Glucose is transported through the membrane by a phosphotransferase system (PTS) and then used by the Embden-Meyerhof-Parnas pathway to produce pyruvate. This pathway generates energy and consumes NAD⁺. Pyruvate is then converted into L- and D-lactate by the stereospecific NAD-dependent lactate dehydrogenases (LDHs), LdhL and LdhD (7), which regenerates NAD⁺ and maintains the redox balance (Fig. 1). However, pyruvate dissipation by different metabolic pathways that result in a mixed acid fermentation has also been observed (7). Acetate is the second major fermentation product in *L. plantarum* (7, 31, 43). Various pathways for acetate production from pyruvate have been identified in this species (Fig. 1). Pyruvate can be metabolized anaerobically into acetate via pyruvate formate lyase, phosphotransacetylase, and acetate kinase (ACK) (Fig. 1) (27). A second possible pathway for acetate production is via the pyruvate dehydrogenase complex (PDH), phosphotransacetylase, and ACK (Fig. 1). This pathway is

thought to be mainly active under aerobic conditions since PDH is strongly inhibited under anaerobic conditions by the relatively high NADH level (3). Although putative *pdh* genes were recently identified in the genome sequence, it was reported that *L. plantarum* had no detectable PDH activity under various growth conditions (6, 18, 25, 30). Previous physiological studies of *L. plantarum* indicated that acetate production is maximal under aerobic conditions with glucose limitation. Under these conditions, acetate originates from lactate utilization (14, 31). The proposed pathway for conversion of lactate to acetate is via two stereospecific lactate oxidases (LoxL and LoxD), pyruvate oxidase (POX), and ACK (14, 31) (Fig. 1). It has been proposed that POX plays a key role in this pathway (43). This enzyme uses oxygen to convert pyruvate into acetylphosphate, which results in the production of CO₂ and H₂O₂ (Fig. 1); H₂O₂ detoxification can subsequently take place via the NADH peroxidase (14, 15).

POX activity in *L. plantarum* is induced by oxygen or hydrogen peroxide and is repressed by glucose (30, 31, 43). These effects have been observed at the enzymatic level, but the regulation mechanisms have not been elucidated. Glucose repression in gram-positive bacteria mainly involves catabolite control protein A (CcpA), whose role in *L. plantarum* was recently established (32). The molecular mechanisms of oxygen regulation in lactic acid bacteria are not known since transcriptional regulators directly involved have not been identified yet.

Acetate production via ACK in *L. plantarum* is accompanied by ATP production. It has been observed that aerobic growth leads to increased production of biomass compared to the

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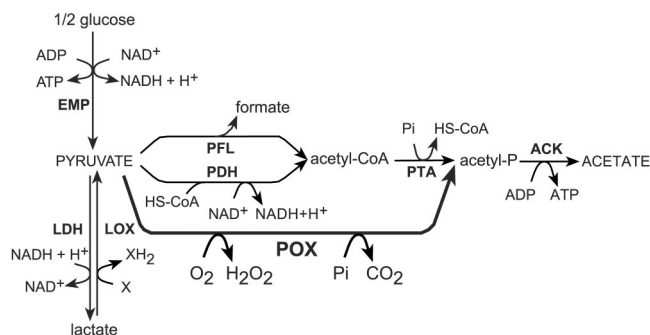


FIG. 1. Lactate and acetate production pathways in *L. plantarum*. EMP, Embden-Meyerhof Parnas pathway; LOX, lactate oxidases; PFL, pyruvate formate lyase; PTA, phosphotransacetylase; CoA, coenzyme A; X, electron acceptor.

production of biomass under anaerobic growth conditions, and it has been hypothesized that the additional ATP produced by ACK contributes to the greater biomass (3, 31, 47). Acetate is an important flavor compound of fermented products in which *L. plantarum* plays a major role (8, 45). At low pH, acetate functions as a membrane-uncoupling agent, inhibiting the

growth of competing microorganisms (2, 9, 40). Due to these properties, a better understanding of the role of the POX involved in acetate production could contribute to improvement of fermentation processes.

An *L. plantarum* POX (PoxB), which is present in the cytoplasm as a homotetramer (262 kDa), has previously been purified and characterized, and its three-dimensional structure has been determined (28, 29, 39, 42). Recent examination of the genome sequence revealed four putative proteins (lp_0849, lp_0852, lp_2629, and lp_3587) that exhibit levels of identity of between 40 and 44% with the PoxB enzyme (25). In this paper we describe the expression pattern of the *poxB* gene with respect to glucose and oxygen regulation. In order to elucidate the functional role of PoxB, the *poxB* gene was constitutively overexpressed, and a stable knockout mutant was constructed. The two mutant strains were analyzed with respect to POX activity and acetate production.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in Luria broth with aeration at 37°C (41). *L. plantarum* was grown in MRS medium (Becton Dickinson, Cockeysville, Md.) at 28°C. Fermentation experiments were

TABLE 1. Bacterial strains, plasmids, and oligonucleotides

Strain, plasmid, or oligonucleotide	Relevant characteristics	Reference or source
<i>E. coli</i> strains		
TG1	<i>supE hsdΔ5 thi Δ(lac-proAB) F'[traΔ36 proAB⁺ lacI^a lacZΔM15]</i>	41
EC1000	Km ^r RepA ⁺	26
<i>L. plantarum</i> strains		
Lp80	Wild type, silage strain	23
FL103	Lp80 derivative; <i>poxB</i> ::pGIF010	This study
FL104	Lp80 derivative; Δ <i>poxB</i>	This study
LM3	Wild type	32
LM3-2	LM3 derivative; <i>ccpA1</i>	32
Plasmids		
pJDC9	Em ^r ; <i>lacZ'</i>	1
pGIZ906	Em ^r ; pLAB1301 derivative; <i>L. plantarum</i> overexpression vector containing the strong <i>ldhL</i> expression signals	P. Goffin, laboratory collection
pJIM2374	Em ^r ; Δ <i>repA</i> ; knockout vector replicating exclusively in RepA ⁺ strains (e.g., EC1000)	4
pGIF003	Em ^r ; pJIM2374 with a 600-bp central fragment of the <i>poxB</i> gene	This study
pGIF004	Em ^r ; pJIM2374 with a 918-bp fragment of the upstream region of <i>poxB</i>	This study
pGIF009	Em ^r ; pJIM2374 with a 9-kb fragment of the downstream region of <i>poxB</i>	This study
pGIF010	Em ^r ; pJIM2374 with a 1.2-kb fragment of the <i>poxB</i> gene, bearing a 600-bp in-frame deletion	This study
pGIF101	Em ^r ; pGIZ906 derivative with an ATG translation fusion between the <i>IdhL</i> expression signals and the <i>poxB</i> ORF	This study
Oligonucleotides		
pox1	5'-ATGGTACCGTTATTTGCTTG-3'	This study
pox2	5'-CATGCCATGGTGTGTTATTTGCAATTAG-3' ^a	This study
pox3	5'-GAAGATCTGAGTTTATGACCACTTGAAT-3' ^b	This study
pox4	5'-CCATACAAATGA TCTACTCCCC-3'	This study
pox5	5'-CCTTCTGCTGATAATGCGTCC-3'	This study
pox6	5'-GCCGGTACCCATTGCACCAACTTCTTCAT-3' ^c	This study
pox7	5'-CGTATCCAGTCAAGGGTATTGTCG-3'	This study
pox8	5'-CCAGATTAAACACCTGCCGCTCAG-3'	This study
pox9	5'-CATCAGGTAATTGCTCAATC-3'	This study
pox10	5'-AACTGCAGATGAAACAAACAAAACAAACTAAC-3' ^d	This study
pox11	5'-GCTCTAGATGAATTTAAAACCCACCCTGTCC6-3' ^e	This study

^a The NcoI site is underlined.

^b The BglII site is underlined.

^c The KpnI site is underlined.

^d The PstI site is underlined.

^e The XbaI site is underlined.

performed in modified MRS broth (MRS-CA medium) (1% tryptone, 0.8% beef extract, 0.4% yeast extract, 0.2% K_2HPO_4 , 0.1% Tween 80, 0.041% $MgSO_4 \cdot 7H_2O$, 0.0066% $MnCl_2$) supplemented with 2 or 0.2% (wt/vol) glucose or maltose. Citrate and acetate were omitted from the original medium as both of these compounds inhibit acetate production or POX activity. *L. plantarum* aerated cultures were grown in baffled flasks with shaking, whereas anaerobic cultures were grown in containers by using the GasPak system (Becton Dickinson). Antibiotics were used at the following concentrations: erythromycin, 250 μ g/ml for *E. coli* and 10 μ g/ml for *L. plantarum*; and chloramphenicol, 20 μ g/ml for *E. coli* and 5 μ g/ml for *L. plantarum*.

DNA techniques and transformation. *L. plantarum* chromosomal DNA was prepared as described previously (13). *L. plantarum* was electroporated as reported previously (23). All DNA manipulations were performed by using established procedures (41). The primers used in this study are listed in Table 1. In order to perform an *in vivo* footprint analysis of the *poxB* regulatory region, cultures of strains LM3 and LM3-2 were treated with dimethyl sulfate (Aldrich, Bornem, Belgium) at the onset of the stationary phase. The *in vivo* footprinting experiments were performed as previously described by using primer *pox5* to probe the top strand (32).

Primer extension analysis and RNA hybridization. Total RNA was extracted from *L. plantarum* Lp80 by mechanical lysis with glass beads as described previously (5). Primer extension and sequencing reactions were performed by using nested primers *pox4* and *pox5* as described previously (13). The *pox5* primer was designed to be highly specific for *poxB* since it contained at least eight mismatches in 22 nucleotides (including one mismatch in the last two nucleotides at the 3' end) with the corresponding regions of the four other putative *pox* genes. The DNA template used in the sequencing reaction was plasmid pGIF004 containing the promoter region of *poxB*. Total RNA from *L. plantarum* LM3 and LM3-2 (*ccpA1*) cells grown to the early stationary phase were isolated, and primer extension products of *poxB* transcripts were obtained by using primer *pox5*, as described previously (32). Northern blot experiments were performed as previously described (5). A central fragment of the *poxB* gene was used as a probe for the Northern blot experiments. This fragment was PCR amplified with primers *pox7* and *pox8* and was radiolabeled with [α - ^{32}P]dATP by using a Random Primer labeling kit (Invitrogen Corporation, Carlsbad, Calif.). This probe was specific for *poxB* since it did not exhibit more than 50% identity with the corresponding fragments of the four other putative *pox* genes.

Construction of plasmids for knockout and overexpression of *poxB*. The pJIM2374 plasmid was used as a suicide vector (Δ *repA*) for the *poxB* gene knockout in *L. plantarum*. This plasmid was maintained in *E. coli* EC1000, which contained a functional chromosomal copy of the *repA* gene required for its replication (21). An internal fragment of *poxB* was amplified by PCR with primers *pox7* and *pox8* and cloned into the *Sma*I site of pJDC9 (1). This fragment was recovered from the resulting plasmid as a 0.6-kb *Kpn*I-*Hind*III fragment and was cloned into similarly digested pJIM2374, yielding pGIF003. A second *poxB* fragment was amplified by PCR with primers *pox2* and *pox6* and was cloned into *Nco*I-*Kpn*I-digested pGIF003, yielding pGIF010. In order to overexpress the *poxB* gene in *L. plantarum*, a 1.8-kb *poxB* fragment was amplified by PCR by using primers *pox10* and *pox11* containing a *Pst*I restriction site and an *Xba*I restriction site, respectively. The resulting fragment containing the complete *poxB* open reading frame (ORF) was cloned into *Nsi*I-*Xba*I-digested pGIZ906, yielding pGIF011. In pGIF011, the *poxB* coding region was translationally fused to the expression signals from the *ldhL* gene of *L. plantarum*. This plasmid was then transformed into *L. plantarum* Lp80.

Cloning of the 5' and 3' regions of *poxB*. The suicide plasmid pGIF003, which contained an internal fragment of the *poxB* gene, was integrated by a single crossover into the chromosome of *L. plantarum* Lp80. Chromosomal DNA from one selected integrant was extracted and digested with *Kpn*I and with *Nde*I in order to recover the 5' and 3' regions, respectively. Two plasmids, designated pGIF004 and pGIF009, respectively, were obtained following self-ligation of the restriction products and transformation in *E. coli* EC1000. pGIF004 contained a 0.9-kb fragment of the upstream region of *poxB* (*Kpn*I restriction), and pGIF009 contained a 9.0-kb fragment of the downstream region of *poxB* (*Nde*I restriction). The 0.9-kb fragment and 690 bp of the 9-kb fragment were sequenced on both strands.

Stable knockout of *poxB*. In order to construct a stable *poxB* mutant, the gene was deleted from the *L. plantarum* chromosome by two successive crossover events. The suicide plasmid pGIF010 contained a *poxB* gene fragment with a 0.6-kb in-frame deletion, which removed most of the core domain and one-third of the flavin adenine dinucleotide binding domain (28, 29). Single-crossover pGIF010 integrants were selected on the basis of erythromycin resistance, and the anticipated *poxB* locus genotype was confirmed by PCR by using primers *pox3* and *pox8*, which amplified the complete *poxB* gene, as well as the deleted

version. Excision of the plasmid leading to the mutant genotype was performed by successive culture cycles without antibiotics for 200 generations and subsequent screening for the loss of erythromycin resistance. The anticipated *poxB* deletion in mutant strain FL104 (Δ *poxB*) was confirmed by PCR performed with primers *pox1* and *pox9* (data not shown).

POX assay. Crude cell extracts were prepared from a 50-ml culture pellet. The pellet was washed once with 1 volume of 50 mM sodium phosphate buffer (pH 6.5) and was resuspended in the same buffer. The cells were lysed with glass beads (diameter, 0.17 μ m) by using a Braun homogenizer (Braun, Melsungen, Germany) (5). The lysate was centrifuged, and the POX activity in the freshly prepared supernatant was measured. Pyruvate decarboxylation was determined by oxidative coupling of the reaction product, H_2O_2 , with 4-aminoantipyrine ($\epsilon_{546} = 16.2 \text{ mmol}^{-1} \text{ liter}^{-1} \text{ cm}^{-1}$) in the presence of horseradish peroxidase and sodium 2-hydroxy-3,5-dichlorobenzene sulfonate (39). Production of H_2O_2 in the POX assay was strictly dependent on the presence of pyruvate, but a possible coupling between PDH and NADH oxidase H_2O_2 -forming activities could also result in H_2O_2 production. This interference in the assay was excluded since addition of NAD^+ in the presence of pyruvate to activate a possible PDH activity had no effect on H_2O_2 production. A very low level of NADH oxidase H_2O_2 -forming activity was detected in some samples after addition of NADH alone, but a combination of NADH and pyruvate had no detectable effect on H_2O_2 production. Specific POX activity was expressed in units; 1 U was defined as 1 μ mol of pyruvate consumed per min per mg of protein. The total protein concentration was measured by the Bio-Rad protein assay method (Bio-Rad, Munich, Germany).

LDH assay. Aliquots (1 ml) of a culture were withdrawn at various times, cells were removed by centrifugation at $12,000 \times g$ for 5 min, and the supernatants were stored immediately at -20°C until enzyme assays were performed. The LDH activity in the supernatants was determined by using pyruvate as the substrate as previously reported (13). One unit of activity corresponded to oxidation of 1 μ mol of NADH ($\epsilon_{340} = 6.3 \text{ mmol}^{-1} \text{ liter}^{-1} \text{ cm}^{-1}$) per min.

SDS-PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (20). Proteins were electrophoretically blotted on a Hybond-C nitrocellulose membrane (Amersham, Buckinghamshire, England) by using the Mini transblot system (Bio-Rad, Hercules, Calif.). The subsequent steps were carried out as specified by the supplier of the Western blot AP system (Promega, Madison, Wis.). Immunoblotting was performed with anti-POX polyclonal antibodies (1/100 dilution) as the primary antibodies, which were kindly provided by Lucie Frey (9).

Cell suspensions and fermentation end product analysis. Cell suspensions were prepared as described previously (20). Cells were grown under anaerobic conditions on 2% glucose until the mid-exponential phase (optical density at 600 nm [OD_{600}], 2.5), harvested by centrifugation, washed twice with 1 volume of 100 mM sodium phosphate buffer (pH 6.5), and resuspended in 1/10 the initial culture volume with the same buffer containing 50 mM glucose. Cell suspensions were incubated with aeration at 30°C for 24 h, and supernatant samples were taken at different times during incubation. Glucose, maltose, lactate, and acetate contents were analyzed by high-performance liquid chromatography (HPLC) by using the method of Starrenburg and Hugenoltz (44).

Nucleotide sequence accession number. The nucleotide sequence of the *poxB* gene of *L. plantarum* Lp80 has been deposited in the DDJB, EMBL, and GenBank databases under accession number AY458428.

RESULTS

Characterization of the *poxB* gene from *L. plantarum* Lp80.

The sequence of the *poxB* ORF from *L. plantarum* has been determined previously (G. Schumacher, 2 May 1990, European Patent Office). In order to identify *poxB* transcription signals and to determine the organization of the locus, the 5' and 3' regions surrounding the *poxB* ORF were cloned. The nucleotide sequence of the *poxB* coding sequence from Lp80 was 99% identical to the previously reported *poxB* sequence (EMBL accession no. A07753). Fifteen substitutions were identified; one of these substitutions led to an amino acid modification (Ala247 $\rightarrow$ Val247) in the PoxB protein sequence from Lp80. This residue has not been reported to be involved in catalysis, cofactor binding, or subunit association (28, 29, 46). The complete nucleotide sequence of the *poxB* locus (2,880 bp) from

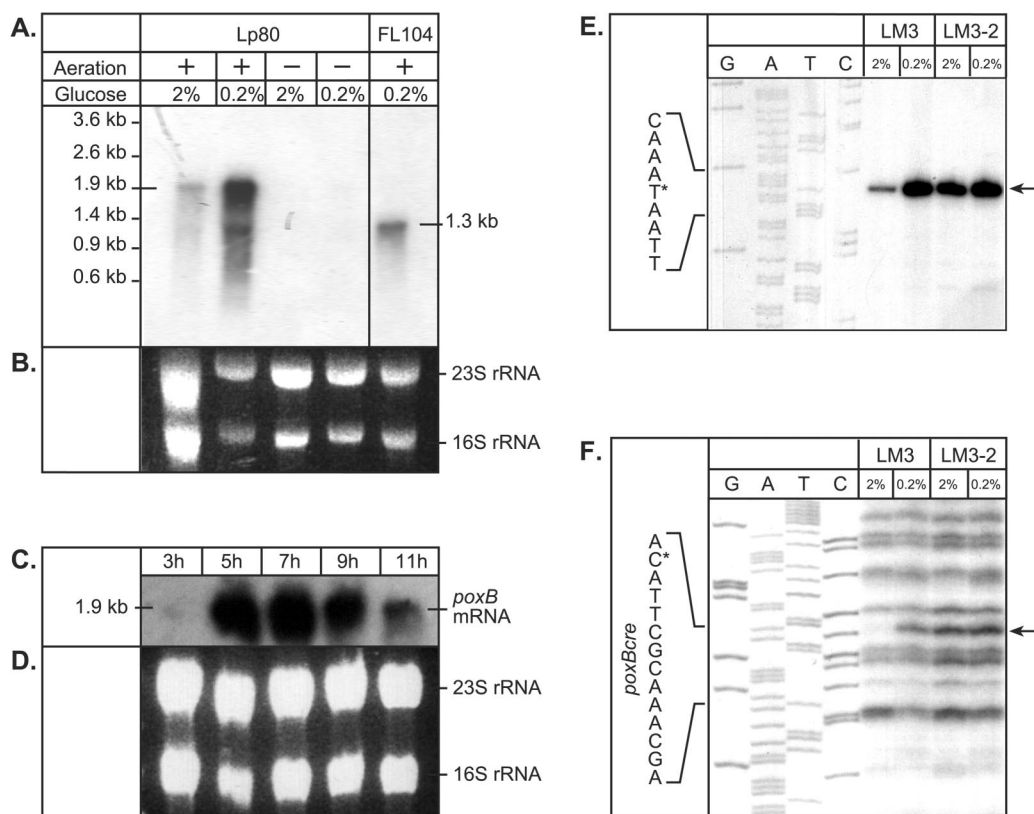


FIG. 2. Transcriptional regulation of *L. plantarum poxB*. (A) Northern blot analysis of *poxB* in Lp80 and FL104 total RNA performed with an internal *poxB* fragment as the probe. Cells were grown under anaerobic conditions (–) or under aerobic conditions (+) on 2% glucose or 0.2% glucose and were harvested in the early stationary phase. (B) RNA electrophoresis of the Northern blot in panel A. (C) Northern blot analysis of *poxB* in Lp80 RNA prepared at different growth stages. Aerated cells grown on 0.2% glucose were harvested, and the total RNA was extracted at various times (as indicated at the top). (D) RNA electrophoresis of the Northern blot in panel C. (E) Primer extension analysis of *poxB* mRNA. Primer extension products were obtained by using primer *pox5* and total RNA extracted from LM3 and LM3-2 cells grown with aeration on 2 or 0.2% glucose. The transcription start is indicated by an asterisk. As a reference, sequencing reactions were performed with plasmid pGIF004 and the same primer. (F) In vivo footprinting analysis of the *poxB* regulatory region. The analysis was performed with methylated DNA extracted from LM3 and LM3-2 cells grown on 2 or 0.2% glucose. G, A, T, and C indicate the nucleotide sequencing reactions for the top strand. G residues in the methylated strand correspond to C residues in the sequencing lane. The arrow indicates the G residue protected from dimethyl sulfate attack, and the asterisk indicates the relative position of this residue in the *cre* sequence.

Lp80 exhibited 98% identity (29 substitutions) to the corresponding locus in the genome sequence of *L. plantarum* WCFS1 (25).

The transcription initiation site of the *poxB* gene was mapped by primer extension analysis in Lp80 (data not shown) and LM3 (Fig. 2E). The 5' regions of *poxB* from the two strains were identical up to 100 bp upstream of the ATG start codon (data not shown). The nucleotide at position 1 was located 46 nucleotides upstream of the ATG start codon in both *L. plantarum* strains (Fig. 3A). A putative σ^A vegetative promoter was identified, and this promoter was composed of a potential –35 box (TTGAAT) and an extended –10 box (TGNTAATAT) (conserved nucleotides are underlined) (16). A potential Shine-Dalgarno sequence was also identified, and it had a free energy (ΔG_f) of –11.8 kcal/mol, which is similar to values obtained for *L. plantarum* (19). A putative hairpin (ΔG_f = –16.4 kcal/mol) that may act as a transcription terminator was identified downstream of the *poxB* ORF.

In order to identify putative oxygen-dependent (or oxygen derivative-dependent) and glucose-dependent regulator bind-

ing sites, the 5' region of the *poxB* ORF was examined. Between the –35 and –10 boxes, a *cre*-like sequence element was identified (21), which may bind the CcpA protein involved in carbon catabolite repression in gram-positive bacteria (Fig. 3). A set of direct repeats was found between the –10 box and the Shine-Dalgarno sequence, and they exhibited a high level of identity to the operator site for the *Bacillus subtilis* OhrR regulator (20 of 30 nucleotides were conserved) (Fig. 3), which represses the *ohrA* gene involved in peroxide detoxification (10, 11). This finding suggests that an OhrR-like regulator is involved in the reported induction of PoxB activity by H_2O_2 (30).

Northern blot analyses were performed with total RNA isolated from *L. plantarum* Lp80 in the early stationary phase by using an internal *poxB* fragment as the probe. A major band corresponding to a 1.9-kb transcript was observed, showing that *poxB* is monocistronic (Fig. 2A). The additional low-intensity bands below the 1.9-kb mRNA band most probably represent degradation products of the *poxB* transcript that comigrated with the rRNA (12). A similar analysis of RNA

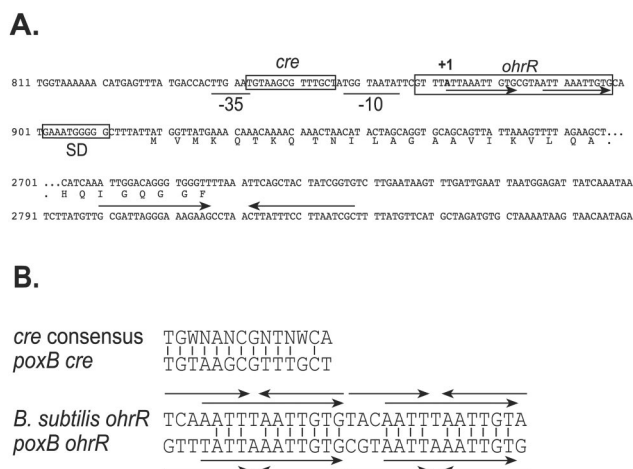


FIG. 3. Genetic characteristics of the *poxB* gene. (A) Nucleotide sequence of the 5' and 3' regions of the *poxB* gene. The facing arrows indicate the putative transcription terminator. -35 and -10, vegetative promoters; +1, transcription start site mapped by primer extension; *cre*, CcpA binding site identified by in vivo footprinting; *ohfR*, putative OhfR binding site including direct repeats (arrows); SD, Shine-Dalgarno sequence. (B) Alignment of the *cre* site from *poxB* with the consensus sequence for CcpA binding (20) and alignment of the putative OhfR binding site with the *B. subtilis* OhfR binding site found in the promoter region of *ohfA* (10, 11).

extracted from a *poxB* mutant (strain FL104) resulted in detection of a single 1.3-kb band whose size corresponded to the anticipated size of the deleted *poxB* transcript, which confirmed both the mutant genotype of FL104 and identification of the *poxB* mRNA.

Transcriptional regulation of *poxB*. Oxygen and glucose regulation of *poxB* at the transcriptional level was then investigated, as were the regulatory mechanisms involved. Total RNA was extracted from strain Lp80 grown under aerobic or anaerobic conditions and with excess glucose (2% glucose) or a limiting concentration of glucose (0.2% glucose). Northern blot analysis revealed that the *poxB* transcript was hardly detected under anaerobic conditions and that the amount of *poxB* mRNA increased substantially under aerobic conditions and with glucose exhaustion (Fig. 2A). In order to examine the kinetics of *poxB* expression during growth, Northern blot analyses were performed with RNA extracted at various times during growth (3, 5, 7, 9, and 11 h) under aerobic conditions with 0.2% glucose (Fig. 2C). During the exponential phase (3 h), the *poxB* transcript could not be detected. At the end of exponential growth (5 h), corresponding to complete exhaustion of glucose (Fig. 4B), a strong 1.9-kb band corresponding to the *poxB* mRNA was observed. Subsequently, the *poxB* transcript appeared to occur at the same level during the stationary phase for up to 7 h before a sharp decrease was observed after 11 h of growth (Fig. 2C).

Since it was observed that the amount of the *poxB* mRNA decreased at a high glucose concentration (Fig. 2A) and a *cre*-like element overlapping the -35 sequence was found by sequence analysis (Fig. 3), the role of CcpA in mediating glucose repression of the *poxB* gene was investigated. Primer extension and in vivo footprinting experiments were performed with the *L. plantarum* LM3-2 strain carrying a null mutation in

ccpA and with the isogenic wild-type strain LM3 grown under aerobic conditions and with glucose exhaustion or excess glucose. Primer extension analysis demonstrated that *poxB* transcription was repressed approximately 20-fold in LM3 cells grown with excess glucose compared with the transcription in cells grown with glucose exhaustion, while no significant difference in the levels of *poxB* transcription was found for the *ccpA* mutant strain grown under the two conditions (Fig. 2E). In order to further examine whether the *cre*-like sequence overlapping the -35 sequence is involved in CcpA-mediated regulation of *poxB* expression, an in vivo footprinting analysis was performed for this region. Chromosomal DNA of LM3 and LM3-2 cells grown on 2% glucose or on 0.2% glucose were methylated with dimethyl sulfate at the onset of the stationary phase. Figure 2F (lanes LM3) shows that protection of the G residue of the top strand in the presence of 2% glucose corresponds to position 2 of the 14-bp *cre* sequence. Protection of this G residue was observed in LM3 cells grown on 2% glucose but not in LM3 cells grown on 0.2% glucose, while no protection was observed in LM3-2 cells grown with excess glucose.

Constitutive overexpression of *poxB* in *L. plantarum*. The *poxB* gene was constitutively overexpressed in order to evaluate its potential control of acetate production in *L. plantarum*. A translational fusion between the strong expression signals from the *ldhL* gene and the *poxB* coding region was constructed (pGIF101) and introduced into *L. plantarum* Lp80. Under aerobic conditions, growth of the recombinant strain, strain Lp80(pGIF101), appeared to be severely inhibited, which could have been due to production of a high level of hydrogen peroxide by the PoxB enzyme (data not shown). In Lp80(pGIF101) grown anaerobically with excess glucose (2% glucose), PoxB overproduction was verified by SDS-PAGE (data not shown). Moreover, the level of POX activity in crude extracts of these cells was 2.1 U/mg, while the control strain [Lp80(pGIZ906)] had no detectable POX activity under these conditions. Finally, this POX activity was 10-fold higher than the maximum activity (0.23 U/mg) observed with Lp80 grown with maltose limitation and under aerobic conditions (Fig. 4D), thereby establishing the functional overproduction of PoxB in *L. plantarum* Lp80.

The impact of PoxB overproduction on fermentation products was also studied. Since the recombinant strain could not grow under aerobic conditions, the fermentation profile was examined with aerated cell suspensions. Cell biomass was prepared by using the conditions described above in order to avoid any POX activity in the wild-type control. Cells were resuspended in phosphate buffer containing glucose and then incubated with a high level of aeration for 24 h. The amounts of glucose, lactate, and acetate in the supernatant were determined by HPLC (Fig. 5). During the first phase of fermentation, both the control [Lp80(pGIZ906)] and the overproducing strain [Lp80(pGIF101)] displayed rapid conversion of glucose to lactate exclusively. Following this first phase, a major difference in the metabolic profiles of the two strains was observed. The lactate concentration in the cell suspension of the control remained stable between 4 and 24 h, while lactate was rapidly converted to acetate by the overproducing strain and acetate eventually became the only fermentation end product (Fig. 5). Since acetate could not be separated easily from acetyl-phosphate by HPLC, ³¹P nuclear magnetic resonance

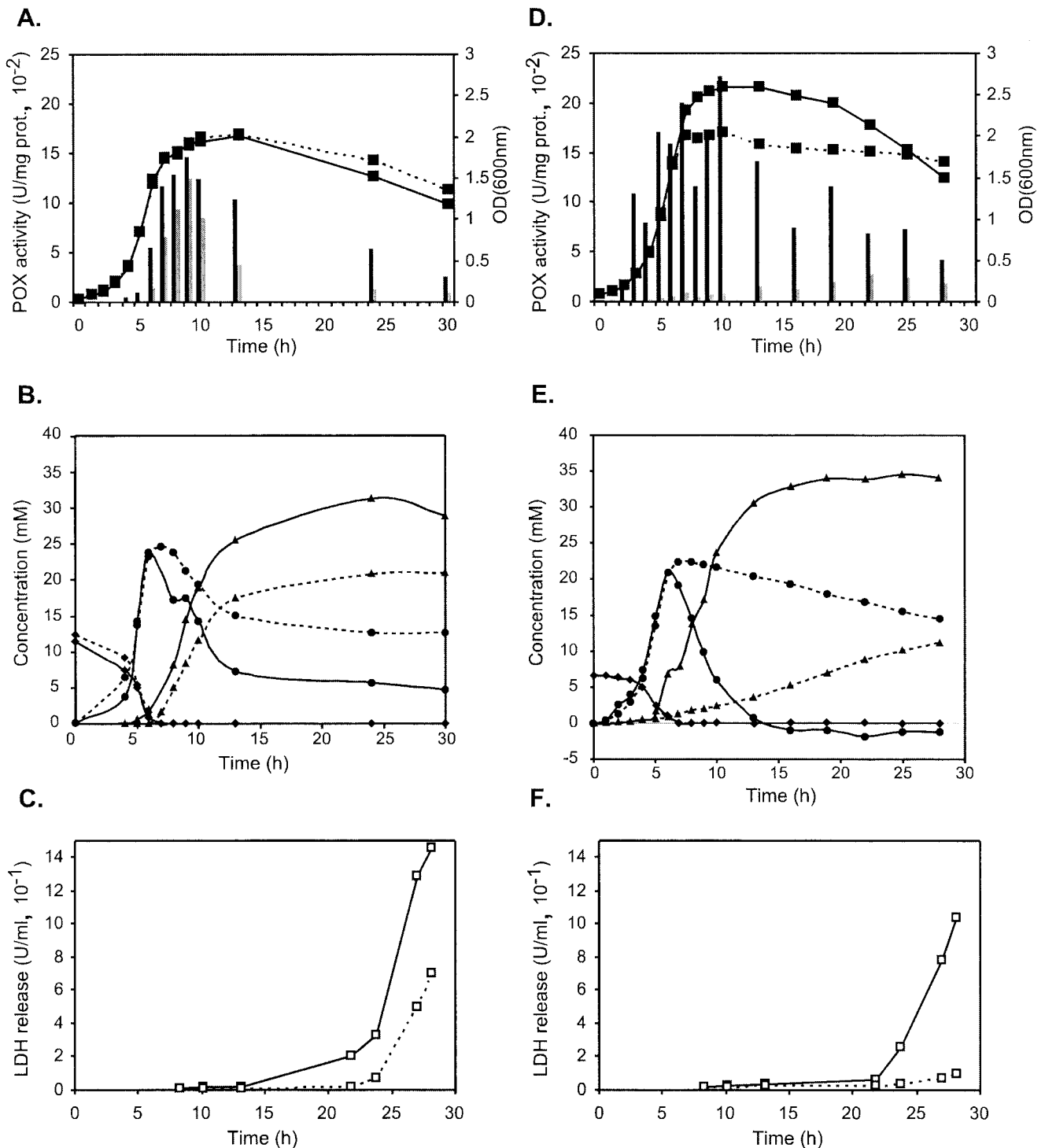


FIG. 4. Growth curves, POX activities, fermentation profiles, and LDH release for strains Lp80 and FL104 grown on MRS-CA medium supplemented with 0.2% glucose or 0.2% maltose. Aerated cultures were harvested at various times during growth. POX specific activity was measured by using crude extracts. LDH activity was measured by using culture supernatants. Glucose, maltose, acetate, and lactate concentrations were determined by HPLC by using culture supernatants. The values are final concentrations from which the initial concentrations measured in MRS-CA medium were subtracted. (A) Growth and POX activity on 0.2% glucose. (B) Fermentation end products on 0.2% glucose. (C) LDH release into culture supernatants on 0.2% glucose. (D) Growth and POX activity on 0.2% maltose. (E) Fermentation end products on 0.2% maltose. (F) LDH release into culture supernatants on 0.2% maltose. Black bars, Lp80 POX activity; gray bars, FL104 POX activity; solid line, Lp80; dotted line, FL104; ■, growth (OD₆₀₀); □, LDH activity; ◆, sugar (glucose or maltose); ●, lactate; ▲, acetate. prot., protein.

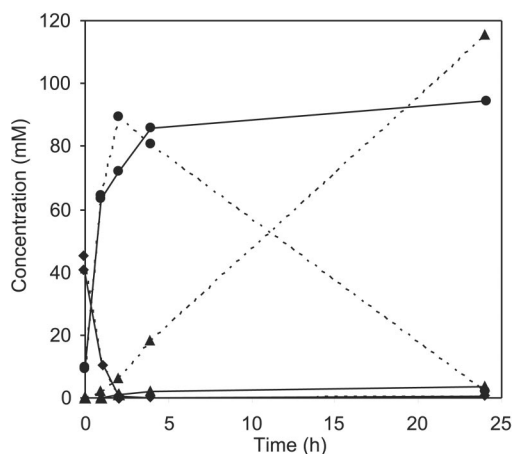


FIG. 5. Fermentation profiles obtained from aerated cell suspensions of Lp80(pGIZ906) (control strain) and Lp80(pGIF101) (PoxB-overproducing strain). Cells were collected in the mid-exponential growth phase from cultures grown under anaerobic conditions and with 2% glucose and were resuspended in phosphate buffer containing glucose (50 mM). Glucose, acetate, and lactate concentrations were determined by HPLC of cell suspension supernatants. Solid line, Lp80(pGIZ906); dotted lines, Lp80(pGIF101); ♦, glucose; ●, lactate; ▲, acetate.

was used to confirm the exclusive production of acetate (data not shown).

Characterization of a stable *poxB* mutant. In order to study the importance of acetate production under aerobic conditions for *L. plantarum* metabolism and physiology, a stable *poxB* mutant (FL104, Δ *poxB*) was constructed. Cells were grown with aeration and glucose limitation in order to maximize *poxB* expression, and the POX activities of Lp80 and FL104 crude extracts were determined (Fig. 4A). In Lp80, the activity increased dramatically at the end of exponential growth and decreased only during later stages of the stationary phase. POX activity was also observed in FL104, although the level was reduced compared to the level observed for the wild-type strain. During the exponential phase, no POX activity was detected in FL104 until after 6 h of growth. At the end of exponential growth, the POX activity in FL104 increased and exhibited the same pattern as the Lp80 POX activity, but the

POX activity always was lower than the activity in Lp80. The POX activity in FL104 was between 20% (exponential phase and late stationary phase) and 85% (early stationary phase) of the activity detected in the wild type. These observations show that one or more additional POXs are active in the early stationary phase. A Western blot experiment was performed by using polyclonal antibodies raised against a purified POX preparation from *L. plantarum*. A band corresponding to the PoxB molecular mass (66 kDa) was observed with Lp80 crude extracts, but a weak band was visible at a similar position with the FL104 extracts (data not shown). The calculated molecular masses (between 63.5 and 64.2 kDa) of the four other putative POXs are close to the apparent molecular mass of the band observed for FL104. These results indicate that one or more POXs coexist in *L. plantarum*.

Effect of glucose limitation on the FL104 fermentation profile. The first analysis of the impact of *poxB* inactivation on fermentation products was performed with batch cultures grown under aerobic conditions and with a low glucose concentration (0.2%) (Fig. 4A and B and Table 2). The amounts of glucose, lactate, and acetate in the supernatant were determined by HPLC. In analogy with the cell suspension experiment, uncoupling between the glucose consumption phase and the conversion of lactate to acetate was observed. The glucose consumption phase resulted in production of lactate as the major fermentation product. This phase took place during exponential growth when the total POX activity was very low (Fig. 4A and B). The conversion of lactate to acetate started at the onset of the stationary phase, when glucose was completely exhausted and the total POX activity reached the maximum value. In the late stationary growth phase, the conversion process slowed down and finally stopped, although lactate was still available. During this phase, decreases in the OD₆₀₀ and POX activity were observed. At the end of the conversion process with 0.2% glucose, acetate accounted for 85 and 60% of total fermentation products in Lp80 and FL104, respectively (Table 2). The limited decrease in acetate production in FL104 (25%) can probably be explained by the high residual POX activity. Surprisingly, the calculated carbon balance for the conversion of lactate to acetate was greater than 1 for both strains (approximately 1.3), suggesting that acetate is produced at the expense of one or more compounds in addition to lactate

TABLE 2. Concentrations of lactate, acetate, glucose, and maltose in culture supernatants of *L. plantarum* Lp80 and FL104 grown in MRS-CA medium under aerobic conditions and with sugar limitation (0.2% [wt/vol] sugar)^a

Carbon source	Strain	Sugar consumption phase				Lactate consumption phase			
		Substrate consumption (mM) ^b		Product formation (mM) ^b		Carbon balance	Lactate consumption (mM) ^b	Acetate formation (mM) ^b	Carbon balance
		Glucose	Maltose	Lactate	Acetate				
Glucose (0.2%)	Lp80	11.7		25.3	1.7	1.15	20.6	26	1.26
	FL104	13.7		29.1	ND ^c	1.06	15.6	20.1	1.29
Maltose (0.2%)	Lp80		6.6	21	3.9	1.06	22.2	27.3	1.23
	FL104		6.6	22.6	0.75	0.88	8.1	9.9	1.22

^a Compounds were quantified by HPLC at the end of the sugar consumption phase (6 h for Lp80 and 7 h for FL104) and at the end of the lactate consumption phase (30 h for glucose and 28 h for maltose).

^b The values are the final concentrations from which the initial concentrations measured in MRS-CA were subtracted. The data are the data from one experiment that was representative of two independent analyses in which similar results were obtained.

^c ND, not detected.

(Table 2). As neither ethanol, acetoin, nor pyruvate was produced during growth, we hypothesized that one or more growth medium compounds could be used by the cells for this lactate-independent acetate production.

Effect of maltose limitation on POX activity and the FL104 fermentation profile. Since glucose repression of *poxB* is due to catabolite repression, maltose was used as an alternative carbon source as it has been reported to be a non-PTS sugar in *Lactobacillus* species closely related to *L. plantarum* (34, 48). Cultures were grown with aeration on 0.2% maltose.

The POX activity in FL104 was remarkably low in the presence of 0.2% maltose compared to the activity in the presence of 0.2% glucose up to 10 h of growth, and the residual activity accounted for 3 to 4% of the total POX activity in Lp80 (Fig. 4D). After this, the POX activities were similar under the two culture conditions. These findings indicate that PoxB contributes significantly to the total POX activity in the presence of 0.2% maltose. In Lp80, in contrast to what was observed in the presence of 0.2% glucose, the total POX activity increased at an earlier stage of exponential growth when maltose was still present, showing that there was partial relief of the glucose repression effect when maltose was a carbon source. In analogy with the experiments performed with cells grown on 0.2% glucose, the total POX activity for maltose-grown Lp80 was maximal in the early stationary phase and then decreased during the late stationary phase.

The fermentation profile in the presence of 0.2% maltose was analyzed (Fig. 4E and Table 2). During the sugar consumption phase, maltose was fermented mainly into lactate. However, concomitant acetate production starting at 5 h was observed for Lp80 with 0.2% maltose (Table 2). At the end of this phase, the acetate production in FL104 was fivefold lower than that in Lp80, suggesting that PoxB made a large contribution to acetate production during growth of the wild type (Table 2). These results contrast with the data obtained with cell suspensions and cells growing on 0.2% glucose, in which a strong uncoupling between glucose consumption and acetate production was observed. Nevertheless, the two strains started converting lactate to acetate at approximately the same time during growth. This conversion represented only a minor pathway in FL104 compared to the pathway in Lp80 and appeared to be reduced compared to the conversion in FL104 cells grown with 0.2% glucose. In the late stationary phase (28 h), acetate accounted for 41% of the total end products in FL104, compared to 100% of the total end products for Lp80. The final lactate concentration for the wild-type strain was even lower than the initial background concentration measured in the growth medium.

Remarkably, a decrease in the OD₆₀₀ of the Lp80 culture grown on 0.2% maltose was observed when acetate production stopped, while during this time the OD₆₀₀ of FL104 remained stable. This decrease in OD₆₀₀ suggested that a lytic process was taking place. In order to confirm this hypothesis, lysis was monitored by determining the release of cytoplasmic LDHs (LDHL and LDHD). The global LDH activities in culture supernatants were measured during growth of Lp80 and FL104 with a low sugar concentration (Fig. 4C and F). The activity in culture supernatants after 28 h of growth was 10-fold higher for Lp80 than for FL104 with 0.2% maltose, while a twofold increase was observed with 0.2% glucose.

Effect of excess glucose or maltose on the FL104 fermentation profile. Lp80 and FL104 were grown under aerobic conditions on 2% glucose and 2% maltose (Fig. 6 and Table 3). Under these conditions, sugar exhaustion did not occur at the onset of the stationary phase. POX activities were measured in the exponential and late stationary growth phases (Fig. 6A and C). In Lp80, the total POX activity was not fully repressed by a high glucose concentration (fivefold decrease compared to the activity with 0.2% glucose), and a higher level of POX activity was observed with 2% maltose than with excess glucose. In FL104, the residual POX activity in the exponential phase was 35 to 40% of the wild-type activity with 2% glucose and 3 to 5% of the wild-type activity with 2% maltose. These results confirmed previous observations that PoxB contributes substantially to the total POX activity on maltose. At a high sugar concentration, the levels of production of lactate and acetate were similar for the two strains (Fig. 6). On 2% glucose, the acetate production in Lp80 was twofold higher than that in FL104 (Fig. 6B and D). In Lp80, acetate accounted for 11% of the fermentation products after 30 h of fermentation, whereas the level was reduced approximately twofold (6%) in FL104 (Table 3). On 2% maltose, acetate production in Lp80 accounted for 24% of the total metabolites, which was twice the level observed with 2% glucose (Fig. 6D and Table 3). Under these conditions, the acetate production observed for FL104 was fivefold lower (5%) than that observed for Lp80. The residual acetate production (5 to 6%) in FL104 suggests that other POX enzymes that are not fully repressed by high sugar concentrations contributed.

DISCUSSION

Here we describe cloning of the *poxB* gene from *L. plantarum* Lp80 and characterization of its transcriptional regulation by glucose repression, growth phase, and aeration. Our results elucidate some of the *poxB* expression regulatory mechanisms. Glucose repression was confirmed at the genetic level in two *L. plantarum* strains (Lp80 and LM3) by Northern blot and primer extension experiments, which demonstrated that *poxB* transcription was repressed by excess glucose. Involvement of the CcpA protein in this regulatory mechanism was demonstrated by the relief of catabolite repression in a strain carrying a *ccpA* null mutation (a LM3 derivative). Moreover, an in vivo footprinting analysis which showed that there was CcpA-dependent protection of a *cre* sequence overlapping the *poxB* -35 sequence corroborated the role of CcpA in *poxB* transcription regulation. Another aspect of *poxB* regulation concerns oxygen and hydrogen peroxide induction. Both of these compounds have been reported previously to induce POX activity in *L. plantarum* (30). The Northern blot experiment demonstrated that oxygen induction takes place at the transcriptional level, but a direct role of oxygen has not been clarified. In silico searches for putative binding sites of regulators (FNR, ResD, PerR, OhrR) known to be involved in oxygen or hydrogen/organic peroxide regulation in gram-positive bacteria revealed the presence of a strongly conserved putative binding site for OhrR (10, 11, 17, 33, 37). The position of this site between the promoter -10 box and the Shine-Dalgarno sequence of *poxB* is consistent with the repressor role of OhrR (10). Furthermore, two OhrR homologues displaying 42 and

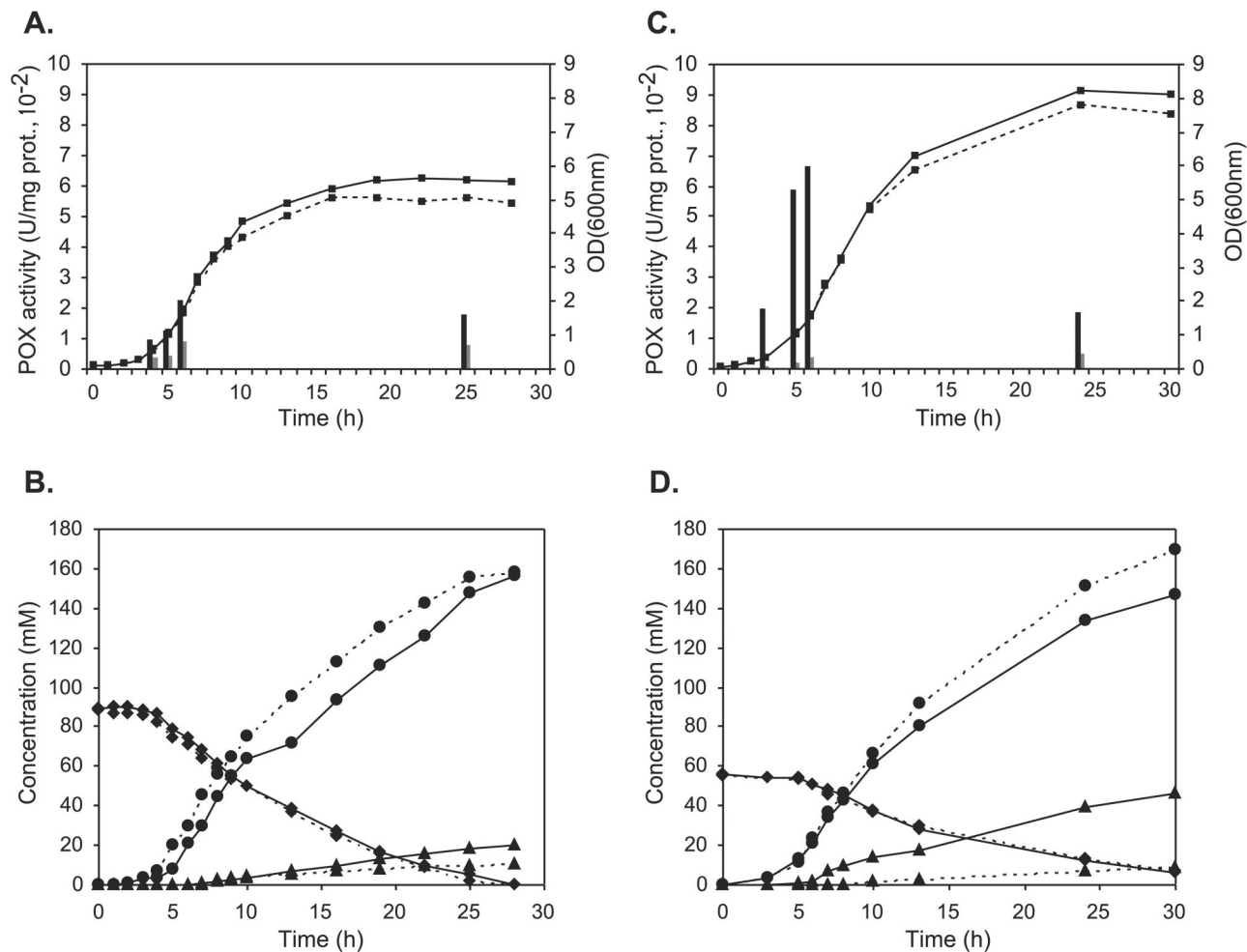


FIG. 6. Growth curves, POX activities, and fermentation profiles for strains Lp80 and FL104 grown on MRS-CA medium supplemented with 2% glucose or 2% maltose. The experimental conditions were the same as those described in the legend to Fig. 4. (A) Growth and POX activity on 2% glucose. (B) Fermentation end products on 2% glucose. (C) Growth and POX activity on 2% maltose. (D) Fermentation end products on 2% maltose. Black bars, Lp80 POX activity; gray bars, FL104 POX activity; solid line, Lp80; dotted line, FL104; ■, growth (OD₆₀₀); ♦, sugar (glucose or maltose); ●, lactate; ▲, acetate. prot., protein.

38% identity with OhrR of *B. subtilis* (lp_1360 and lp_0889) were identified in the genome sequence of *L. plantarum* WCFS1 (25). The OhrR regulator is involved in oxidative stress in *B. subtilis*, acting as a repressor that is inactivated by organic peroxides. The organic peroxides are produced by hy-

drogen peroxide attack on fatty acids (10). Therefore, an indirect effect of oxygen on *poxB* regulation could be postulated.

The present study demonstrated that PoxB is not the only active POX of *L. plantarum* since the *poxB* knockout did not completely eliminate POX activity. With a high level of aera-

TABLE 3. Concentrations of lactate, acetate, glucose, and maltose in culture supernatants of *L. plantarum* Lp80 and FL104 grown in MRS-CA medium under aerobic conditions and with excess sugar (2% [wt/vol] sugar)^a

Carbon source	Strain	Substrate consumption (mM) ^b		Product formation (mM) ^b		% Acetate	Carbon balance
		Glucose	Maltose	Lactate	Acetate		
Glucose (2%)	Lp80	89.2		156.5	19.9	11	0.99
	FL104	88.2		158.6	10.8	6	0.96
Maltose (2%)	Lp80		49.3	147.1	46.4	24	0.98
	FL104		47.8	169.1	8.7	5	0.93

^a Compounds were quantified by HPLC at the end of the experiment (28 h for glucose and 30 h for maltose).
^b The Values are the final concentrations from which the initial concentrations measured in MRS-CA medium were subtracted. The data are the data from one experiment that was representative of two independent analyses in which similar results were obtained.

tion and glucose limitation, the POX activity in the mutant still was equivalent to 85% of the wild-type activity at the onset of the stationary phase. The comparison of the kinetics of POX induction in the wild type and the mutant suggested that one or more additional POX proteins are regulated by glucose since a sharp peak of activity was observed in the early stationary phase for the mutant strain when glucose was completely exhausted. Surprisingly, an impressive decrease in POX activity (>90%) was observed in the mutant strain when there was excess maltose and when there was maltose limitation. Maltose behaves like a non-PTS sugar since relief of catabolite repression of total POX activity occurs during the exponential growth phase of the wild-type strain. However, there is no obvious hypothesis to explain why maltose seems to repress POX activity in the mutant strain. To our knowledge, no such maltose repression effect has been reported previously.

This study showed that the PoxB enzyme plays a key role in acetate production from maltose in *L. plantarum* under aerobic conditions. With a high level of aeration and excess sugar, acetate and lactate were produced concomitantly during sugar fermentation. Acetate could account for up to 24% of the total end products when there was excess maltose. Inactivation of PoxB reduced the amount of acetate by 20 and 80% in the presence of excess glucose and excess maltose, respectively. This suggests that PoxB competes with LDHs for the pyruvate pool. Previously, it has been shown that the NADH oxidase and the NADH peroxidase of *L. plantarum* are strongly induced by aeration (30). These two enzymes contribute to the NADH/NAD⁺ balance through regeneration of the NAD⁺ necessary for glycolysis (3). The consequence of activation of these enzymes is a reduction in the NADH pool, which results in reorientation of pyruvate towards NADH-independent enzymes, such as PoxB. The results of the cell suspension experiment performed with the PoxB-overproducing strain are consistent with this hypothesis. The cell biomass was prepared from cultures grown under anaerobic conditions and with excess glucose. The fact that anaerobic conditions strongly reduce NADH oxidase and NADH peroxidase activities could explain the absence of acetate in the supernatants of cell suspensions during the glucose consumption phase.

With high levels of aeration and sugar exhaustion, conversion of lactate to acetate takes place. The conversion process is extremely efficient in the wild-type strain and could result in homoacetic fermentation, such as that observed with cells growing with maltose limitation. Under these conditions, the PoxB contribution to acetate production is very important since the PoxB-deficient strain displayed very limited conversion of lactate to acetate and there was a 60% reduction in the final amount of acetate produced. With glucose exhaustion, the conversion of lactate to acetate was slowed down in the *poxB* mutant, and there was a moderate effect on the final amount of acetate produced. Additionally, the data obtained with resting cells of the PoxB-overproducing strain demonstrated that a POX activity was absolutely required in this conversion process. Moreover, if the other enzymes (lactate oxidase, ACK) anticipated to be involved in this metabolic pathway are regulated, they do not strongly control the conversion process.

Although the acetate production in both the wild type and the mutant strain could be explained by the relative POX activity measured under all conditions tested, we cannot ex-

clude the possibility that there is an alternative pathway for acetate production under aerobic conditions. Pyruvate formate lyase cannot be involved since this enzyme is oxygen sensitive and formate production was not detected in our experiments (27). An alternative route for acetate production under aerobic conditions is a coupling between PDH and NADH oxidase. Such a coupling was invoked to explain a high level of acetate production in *Lactococcus lactis* when the NADH oxidase was overexpressed (35). Although an NADH oxidase activity was clearly induced at similar levels in both Lp80 and the PoxB-deficient strain under aerobic conditions, we were unable to detect any PDH activity under the different growth conditions, confirming previous observations that a POX enzyme(s) plays the major role in acetate production under aerobic conditions (6, 18, 30; data not shown).

The energy produced by substrate phosphorylation generated via the acetate kinase during acetate production has been reported to increase biomass production in *L. plantarum* (3, 31, 47). Although this aspect was not examined in detail here, the OD₆₀₀ reached in the stationary phase by the PoxB-deficient strain was clearly reduced with maltose limitation, and acetate production and POX activity were affected most. Interestingly, when the conversion of lactate to acetate stops in the stationary phase, a decrease in the OD₆₀₀ resulting from a lytic process was observed. A possible explanation for these observations involves the coupling between acetate and ATP production; in the absence of ATP production, protons can no longer be extruded by ATPases, which results in dissipation of the proton motive force. Dissipation of the proton motive force has been shown to be one of the mechanisms that trigger autolysis of gram-positive bacteria (22, 38). Altogether, these results suggest that the coupling between acetate and ATP production via the POX enzyme could play an important role in *L. plantarum* survival when sugar is limiting. Similarly, it has been shown recently that the ATP pool is more rapidly dissipated in a POX-deficient strain of *Streptococcus pneumoniae* (36). Although no lysis was reported, the ATP pool depletion was associated with a dramatic decrease in the viability of the mutant strain (36).

In future work we will try to elucidate the molecular mechanisms of oxygen regulation of *poxB*. The possible involvement of the two OhrR homologues identified in the *L. plantarum* genome will be studied by using gene knockouts. In order to clarify the functional role of the four additional *pox* genes identified in the genome sequence, experiments involving single inactivation and multiple inactivation, as well as individual overproduction, are in progress.

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