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Lactate Racemization as a Rescue Pathway for Supplying D-Lactate to the Cell Wall Biosynthesis Machinery in *Lactobacillus plantarum*

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Lactobacillus plantarum **is a lactic acid bacterium that produces D- and L-lactate using stereospecific NAD-dependent lactate dehydrogenases (LdhD and LdhL, respectively). However, reduction of glycolytic pyruvate by LdhD is not the only pathway for D-lactate production since a mutant defective in this activity still produces both lactate isomers (T. Ferain, J. N. Hobbs, Jr., J. Richardson, N. Bernard, D. Garmyn, P. Hols, N. E. Allen, and J. Delcour, J. Bacteriol. 178:5431–5437, 1996). Production of D-lactate in this species has been shown to be connected to cell wall biosynthesis through its incorporation as the last residue of the muramoyl-pentadepsipeptide peptidoglycan precursor. This particular feature leads to natural resistance to high concentrations of vancomycin. In the present study, we show that *L. plantarum* possesses two pathways for D-lactate production: the LdhD enzyme and a lactate racemase, whose expression requires L-lactate. We report the cloning of a six-gene operon, which is involved in lactate racemization activity and is positively regulated by L-lactate. Deletion of this operon in an *L. plantarum* strain that is devoid of LdhD activity leads to the exclusive production of L-lactate. As a consequence, peptidoglycan biosynthesis is affected, and growth of this mutant is D-lactate dependent. We also show that the growth defect can be partially restored by expression of the D-alanyl-D-alanine-forming Ddl ligase from *Lactococcus lactis*, or by supplementation with various D-2-hydroxy acids but not D-2-amino acids, leading to variable vancomycin resistance levels. This suggests that *L. plantarum* is unable to efficiently synthesize peptidoglycan precursors ending in D-alanine and that the cell wall biosynthesis machinery in this species is specifically dedicated to the production of peptidoglycan precursors ending in D-lactate. In this context, the lactate racemase could thus provide the bacterium with a rescue pathway for D-lactate production upon inactivation or inhibition of the LdhD enzyme.**

In lactic acid bacteria (LAB), the pyruvate formed by the Embden-Meyerhof-Parnas pathway is reduced to lactate by NAD-dependent lactate dehydrogenases (Ldh). These enzymes are stereospecific and produce D-lactate (LdhD, EC 1.1.1.28) or L-lactate (LdhL, EC 1.1.1.27). LAB can be classified on the basis of the lactate stereoisomer(s) produced during growth on glucose, which is thought to reflect the type of Ldh(s) present in a species. LAB are usually divided in three groups based on the ratio of isomers produced (21, 31, 39, 45).

Most lactobacilli are DL-lactate producers, but the ratio of the two isomers is highly variable. This has mainly been attributed to different activities of the LdhD and LdhL enzymes (21, 45). Some exceptions among lactobacilli are *Lactobacillus delbrueckii* subsp. *bulgaricus*, which produces mainly D-lactate, in agreement with the absence of LdhL activity, and *Lactobacillus casei*, where L-lactate is the major isomer formed (21, 39). In this species, the pathway of D-lactate production has not been investigated.

The presence of a lactate racemase (EC 5.1.2.1) has been proposed in a few *Lactobacillus* species, namely, *Lactobacillus sakei* (29), *Lactobacillus curvatus* (45), and *Lactobacillus paracasei* subsp. *paracasei* (formerly *L. casei* subsp. *pseudoplantarum* [45]). In these species, only L-lactate is initially produced, which induces lactate racemase activity, permitting the formation of D-lactate. This results ultimately in the production of almost equal amounts of D- and L-lactic acid (21, 45). The existence of a lactate racemase in *L. sakei* 23K has been further confirmed by showing that mutation of the sole *ldhL* gene prevents the production of both D- and L-lactate (38). The existence of a lactate racemization activity has also been demonstrated in cell extracts of *Pediococcus cerevisiae* and *Pediococcus pentosaceus* (24), and of other non-lactic acid bacteria such as *Clostridium beijerinckii* ATCC 14823 (formerly *Clostridium butylicum* [13]), *Clostridium acetobutylicum* (32), *Megasphaera elsdenii* (27), and several halophilic archaea (41).

Very few biochemical studies on lactate racemase have been reported. This is mainly due to the fact that the enzyme seems to be highly sensitive to oxidation (13, 45). The enzymes from *L. sakei* and *C. beijerinckii* have been purified, and basic biochemical properties have been determined (9, 29). A catalytic mechanism has been proposed for the lactate racemase of *C. beijerinckii*, involving an α-carbonyl intermediate covalently bound to a sulfhydryl group of the enzyme (9). However, no

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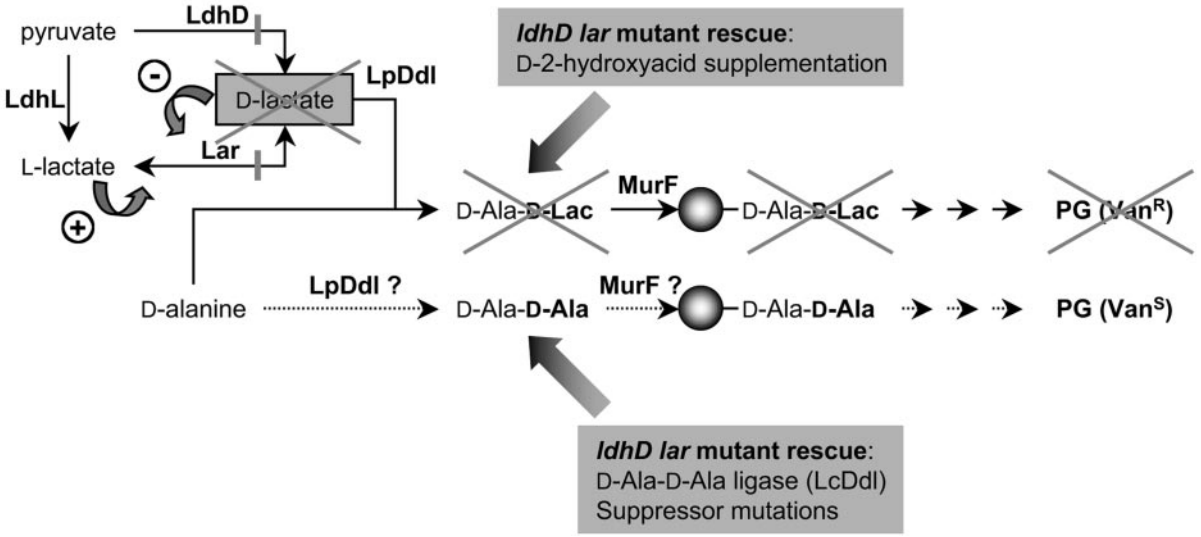

FIG. 1. Schematic representation of the connection between D-lactate production and peptidoglycan biosynthesis in the *L. plantarum* double *ldhD lar* mutant (PG6212). When both D-lactate production pathways (LdhD and Lar) are disrupted (|), production of the D-alanyl-D-lactate depsipeptide is blocked and peptidoglycan synthesis cannot occur (×). The *ldhD lar* mutant can be rescued in the presence of D-lactate or other D-2-hydroxy acids. Alternatively, D-alanine can replace D-lactate by heterologous expression of the D-alanyl-D-alanine ligase from *L. lactis*. Enzymatic reactions potentially involved in the incorporation of D-alanine in the peptidoglycan precursors of the *ldhD lar* suppressor mutants are depicted by dashed arrows. Regulation of the Lar activity by D- and L-lactate is indicated by curved arrows. The UDP-*N*-acetylmuramoyl-L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate moiety of the peptidoglycan precursor is shown as a circle. LdhL, NAD-dependent L-lactate dehydrogenase; LdhD, NAD-dependent D-lactate dehydrogenase; Lar, lactate racemase; LpDdl, *L. plantarum* Ddl ligase; LcDdl, *L. lactis* Ddl ligase; MurF, UDP-*N*-acetylmuramoyl-tripeptide-D-alanyl-D-lactate ligase; PG, peptidoglycan; Van^R, resistant to high concentrations of vancomycin; Van^S, resistant to low concentrations of vancomycin.

N-terminal sequence was determined for the purified enzymes and no gene sequence encoding lactate racemase has been identified in any species to date.

When fermenting glucose, *L. plantarum* produces both D- and L-lactate in a ratio close to 1:1 (20, 45) (Fig. 1). The presence of almost equal levels of both Ldh activities could account for the production of a racemic mixture of lactate, and there is no apparent need to postulate the presence of a lactate racemase (11, 21, 45). Accordingly, an *ldhL* mutant of *L. plantarum* exclusively produces D-lactate (16). However, the finding that an *ldhD* mutant still produces a racemic mixture of lactate led Ferain et al. (17) to suggest the presence of an L-lactate inducible lactate racemase in *L. plantarum*. This observation appears to contradict previously published results describing the lactate racemizing system of *L. plantarum* as a coupled enzymatic system composed of the two stereospecific Ldhs (28).

In *L. plantarum*, D-lactate is incorporated as the last residue in the peptidoglycan precursors, where it is involved on conferring resistance to vancomycin (17) (Fig. 1). Substitution of the terminal D-lactate by a D-alanine residue has been observed in an *ldhD ldhL* double mutant, which displays drastically reduced lactate production (17, 18). Nevertheless, D-lactate ending precursors still account for 55% of the total peptidoglycan precursors observed in this strain, illustrating the importance of D-lactate production for cell wall biosynthesis in *L. plantarum* (17). This would be in agreement with the presence of a lactate racemase-dependent rescue pathway that allows production of D-lactate directly from L-lactate in the absence of LdhD activity.

In the present study, the lactate racemase activity was characterized in the *L. plantarum* NCIMB8826 strain and its previously published *ldhD* (17) and *ldhL* (16) mutant derivatives. The L-lactate inducibility of this activity was exploited to identify an operon involved in lactate racemization. A mutant defective for both LdhD and lactate racemase activities was constructed and shown to depend on D-lactate for growth. The growth defect of this mutant was characterized, and it was shown that other D-2-hydroxy acids, but not D-2-amino acids, could efficiently replace D-lactate in the peptidoglycan precursors.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. All of the plasmid constructions were performed in strain TG1 of *Escherichia coli* (22). *L. plantarum* and *L. casei* were grown in DeMan-Rogosa-Sharpe (MRS) broth (Becton Dickinson, Erembodegem-Aalst, Belgium) at 28°C without shaking unless otherwise stated. Cultures in chemically defined medium were carried out in MPL medium (10) with 2% (wt/vol) glucose at 28°C. For induction of lactate racemase activity, L-lactate sodium salt (Fluka, Buchs, Switzerland) was added at a concentration of 200 mM. D-Lactate (20 mM; Fluka) was added for growth of the PG6212 mutant. For supplementation experiments, D-2-amino acids and D-2-hydroxy acids were added at a final concentration of 20 mM; D,L-2-amino acids and D,L-2-hydroxy acids were added at a final concentration of 40 mM. When appropriate, antibiotics were added to the media at the following concentrations: erythromycin, 250 μg/ml for *E. coli* and 10 μg/ml for *L. plantarum* and *L. casei*; chloramphenicol, 10 μg/ml for *L. plantarum*. Nisin A (Sigma, Bornem, Belgium) was used at a concentration of 50 ng/ml for the induction of genes under control of the *nisA* expression signals. The MIC of vancomycin was determined by using the Etest system (AB Biodisk, Solna, Sweden).

DNA techniques and transformation. General molecular biology techniques were performed according to the instructions given by Sambrook et al. (44).

TABLE 1. Bacterial strains plasmids and primers used in this study

Strain, plasmid, or primer	Characteristic(s)[a] or sequence[b]	Source or reference
Strains		
L. plantarum		
NCIMB8826	Wild type	NCIMB[c]
TF101	NCIMB8826 Δ*ldhL*	16
TF102	NCIMB8826 *ldhD*::*cat*	17
TF103	NCIMB8826 Δ*ldhL ldhD*::*cat*	17
PG6351	NCIMB8826 Δ*ldhL larA*::pGIZ635 (P_{ldhL}::*larA-larE*)	This study
PG6210	NCIMB8826 Δ(*larA-larE*)	This study
PG6212	NCIMB8826 *ldhD*::*cat* Δ(*larA-larE*)	This study
PG1174	PG6212 containing pGIM117 plasmid stably integrated at the $tRNA^{Ser}$ locus	This study
PG1104	PG6212 containing pMEC10 plasmid stably integrated at the $tRNA^{Ser}$ locus	This study
L. casei ATCC 393	Wild type	ATCC[d]
L. lactis NZ3900	MG1363 derivative	14
E. coli TG1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	22
Plasmids		
pLAB1301	Em^r Ap^r; *E. coli L. plantarum* shuttle vector	30
pGIZ906	Em^r Ap^r; pLAB1301 with a 0.352-kb insert containing the *ldhL* expression signals of *L. plantarum* NCIMB8826	This study
pGIZ630	Em^r Ap^r; pGIZ906 with a 1.29-kb insert containing the *larA* ORF of *L. plantarum* NCIMB8826 in translational fusion with the *ldhL* expression signals	This study
pGIZ660	Em^r Ap^r; pGIZ906 with a 0.85-kb insert containing the *larB* ORF of *L. plantarum* NCIMB8826 in translational fusion with the *ldhL* expression signals	This study
pGIZ661	Em^r Ap^r; pGIZ906 with a 0.87-kb insert containing the *larC1* ORF of *L. plantarum* NCIMB8826 in transcriptional fusion with the P_{ldhL} promoter	This study
pGIZ662	Em^r Ap^r; pGIZ906 with a 0.54-kb insert containing the *larC2* ORF of *L. plantarum* NCIMB8826 in transcriptional fusion with the P_{ldhL} promoter	This study
pGIZ663	Em^r Ap^r; pGIZ906 with a 0.84-kb insert containing the *glpF1* ORF of *L. plantarum* NCIMB8826 in transcriptional fusion with the P_{ldhL} promoter	This study
pGIZ664	Em^r Ap^r; pGIZ906 with a 1.14-kb insert containing the *larE* ORF of *L. plantarum* NCIMB8826 in transcriptional fusion with the P_{ldhL} promoter	This study
pGIZ665	Em^r Ap^r; pGIZ906 with a 1.37-kb insert containing the *larC1-larC2* ORFs of *L. plantarum* NCIMB8826 in transcriptional fusion with the P_{ldhL} promoter	This study
pGIZ632	Em^r Ap^r; pGIZ906 with a 5.12-kb insert containing the entire *lar* operon of *L. plantarum* NCIMB8826 in translational fusion with the *ldhL* expression signals	This study
pUC18Ery	Em^r Ap^r; pUC18 derivative with a 1.1-kb insert containing the *erm* gene	47
pGIZ907	Em^r Ap^r; pUC18Ery with a 0.352-kb insert containing the *ldhL* expression signals of *L. plantarum* NCIMB8826	This study
pGIZ635	Em^r Ap^r; pGIZ907 with a 1.29-kb insert containing the *larA* ORF of *L. plantarum* NCIMB8826 in translational fusion with the *ldhL* expression signals	This study
pGIZ621	Em^r Ap^r; pUC18Ery with a 3.04-kb insert containing an in-frame fusion between the 5′ region of *larA* and the 3′ region of *larE*	This study
pNZ8048	Cm^r P_{nisA}	35
pMEC10	Em^r Ap^r, integration plasmid, P_{nisA}	43
pGIM117	Em^r Ap^r; pMEC10 with a 1.38-kb insert containing the translational fusion between P_{nisA} and *Lcddl* of *L. lactis*	This study
Primers		
Pldh1	5′-GGGGTACCTATGACGTGTCTGGGCATATTGC-3′	This study
Pldh22	5′-GCTCTAGAGAATGCATAATAAGTCATCCTCTCGTAGTG-3′	This study
104A1	5′-ACGAAGAATTGGTGAATAAGTATGG-3′	This study
104B1	5′-TGGCGTGTTGATTGCTTGGTCCAGG-3′	This study
LP104A1	5′-AAAACTGCAGGCAATTGATTTACCATATGACAAGC-3′	This study
LP104B1	5′-GCTCTAGATTGTAATATTTCTGCTGTGGTTGC-3′	This study
LP105A1	5′-AAAACTGCAGACCACAGCGAGAAATATTACAACAAG-3′	This study
LP105B2	5′-GCTCTAGAAAATCAAGTCCTAAATCGAGTAACG-3′	This study
LP106A1	5′-AAAACTGCAGTAACTGAACAAGGAGAAGATAAATG-3′	This study
LP106B1	5′-GCTCTAGATAGCTTCAATCATTAAGACAGCATC-3′	This study
LP107A1	5′-AAAACTGCAGACCGGACGGCTGATGCTGTCTTAATG-3′	This study
LP107B1	5′-GCTCTAGATTAATACGGAACTACAATGAACACC-3′	This study
LP108A1	5′-AAAACTGCAGTGGCGGTAGATCAATTAGATGAGGAGG-3′	This study
LP108B1	5′-GCTCTAGATCCTTTAATGCTGCTACTAACGTTGC-3′	This study
LP109A1	5′-AAAACTGCAGCAATTGCCGCTTGGTTTATGCATGG-3′	This study
LP109B2	5′-GCTCTAGATCAAGTCGTTGGTAAACTCGTCGTG-3′	This study
LP104KOA1	5′-CCGGAATTCAGTAAGCACCAATTGCGAGCCCCAAC-3′	This study
LP104KOA2	5′-AGCAACTAGCGCCGCCATATTAATG-3′	This study
LP104KOB1	5′-CGGGGTACCTTGCAGCTTGCGACACTAACTTAC-3′	This study
LP109KOA1	5′-CGGGGTACCTAATTGGTGATTTCTTAGTCTTTAACG-3′	This study
LP109KOB2	5′-TCTTGGACTTCAGCTGACATAATTGG-3′	This study

Continued on facing page

TABLE 1—*Continued*

Strain, plasmid, or primer	Characteristic(s)[a] or sequence[b]	Source or reference
LP109KOB3	5′-GGACTAGTAATTCTCCTCCTCATTGACCGTCAC-3′	This study
LLDDLXP2	5′-TTGCTGCAGTTATAAAAGATGATTTTCCCG-3′	This study
LLCpBAD3	5′-TTTGGATCCCCATGGCAAAAGAAACATTAATTTTG-3′	This study

[a] Em^r, Ap^r, and Cm^r indicate resistance to erythromycin, ampicillin, and chloramphenicol, respectively.
[b] EcoRI, KpnI, XbaI, SpeI, NsiI, NcoI, and PstI restriction sites introduced in the primers are underlined in primer sequences.
[c] NCIMB, The National Collections of INdustrial and Marine Bacteria, Ltd., Aberdeen, Scotland.
[d] ATCC, American Type Culture Collection, Rockville, Md.

Electrotransformation of *E. coli* and *L. plantarum* was performed as described by Dower et al. (15) and Aukrust et al. (4), respectively. *L. plantarum* chromosomal DNA was prepared as previously described (17). PCRs were performed with *Taq* DNA polymerase (Promega, Madison, Wis.) in a GeneAmp PCR System 2400 (Applied Biosystems, Lennik, Belgium). The primers used in the present study are listed in Table 1.

Construction of overexpression vectors for genes of the *lar operon*. The P_{ldhL} promoter and *ldhL* translational signals of *L. plantarum* NCIMB8826 were amplified by PCR with primers Pldh1 and Pldh22. This 352-bp fragment was then digested with KpnI and XbaI and cloned into similarly digested pLAB1301 (30). The resulting plasmid was called pGIZ906. A fragment spanning the entire *lar* operon (5.12 kb; see Fig. 4) was amplified by PCR with primers LP104A1 and LP109B2, digested with PstI and XbaI, and cloned under transcriptional and translational control of the *ldhL* expression signals into NsiI/XbaI-digested pGIZ906. The resulting plasmid was designated pGIZ632. Similarly, overexpression vectors for every single gene of the *lar* operon were constructed based on plasmid pGIZ906. Fragments spanning the entire open reading frame (ORF) of *larA* (LP104A1/LP104B1), *larB* (LP105A1/LP105B2), *larC1* (LP106A1/LP106B1), *larC2* (LP107A1/LP107B1), *glpF1* (LP108A1/LP108B1), and *larE* (LP109A1/LP109B2) were obtained, digested with PstI and XbaI, and cloned into NsiI/XbaI-digested pGIZ906, generating plasmids pGIZ630, pGIZ660, pGIZ661, pGIZ662, pGIZ663, and pGIZ664, respectively. The same procedure was repeated with a PCR fragment containing the *larC1* and *larC2* genes as organized in the *L. plantarum* chromosome (LP106A1/LP107B1), generating plasmid pGIZ665. The *larA* and *larB* genes are under the transcriptional and translational control of the *ldhL* expression signals (plasmids pGIZ630 and pGIZ660, respectively), whereas plasmids pGIZ661, pGIZ662, pGIZ663, pGIZ664, and pGIZ665 contain transcriptional fusions between the P_{ldhL} promoter and the *larC1*, *larC2*, *glpF1*, *larE*, and *larC1* genes, respectively.

Integration of the *ldhL* expression signals in front of *larA*. The PCR fragment (Pldh1/Pldh22) containing the P_{ldhL} promoter and *ldhL* translation signals of *L. plantarum* NCIMB8826 was digested with KpnI and XbaI and cloned into similarly digested pUC18Ery (47), generating plasmid pGIZ907. Subsequently, a PCR fragment containing the entire *larA* (LP104A1/LP104B1) gene was obtained, digested with PstI and XbaI, and cloned into NsiI/XbaI-digested pGIZ907. In this plasmid (pGIZ635), the *larA* gene is under the transcriptional and translational control of the *ldhL* expression signals. This vector was introduced by electrotransformation into *L. plantarum* TF101 (Δ*ldhL*), and clones having integrated the vector in the chromosome were selected on MRS containing erythromycin. Integration at the desired locus was confirmed by PCR with primers Pldh1 and LP105B2. The resulting strain was called PG6351 (P_{ldhL}::*larA-larE*).

Deletion of the *lar* operon. The *lar* deletion vector pGIZ621 was constructed in two steps. Initially, a 1.46-kb fragment located upstream of *larA* was amplified by PCR with primers LP104KOA1 and LP104KOB1, digested with EcoRI and KpnI, and cloned into similarly digested pUC18Ery. Subsequently, a 1.58-kb fragment located downstream of *larE* was amplified by PCR with primers LP109KOA1 and LP109KOB3, digested with KpnI and SpeI, and inserted between the KpnI and XbaI sites of the plasmid obtained in the first step. This plasmid, pGIZ621, harbors an in-frame fusion between the 5′ end of *larA* and the 3′ end of *larE*. This suicide vector was used to delete the *lar* operon through a two-step homologous recombination process, as previously described (16). The Δ*lar* genotype was confirmed by PCR with primers LP104KOA2 and LP109KOB2, located upstream and downstream of the recombination regions, respectively. Deletion was carried out in *L. plantarum* NCIMB8826 (wild type) and TF102 (*ldhD*::*cat*), generating strains PG6210 and PG6212, respectively.

Integration of a P_{nisA}::*Lcddl* fusion in PG6212. The *Lcddl* ORF from *Lactococcus lactis* NZ3900 (14) was amplified by PCR with primers LLCpBAD3 and LLDDLXP2. The resulting amplicon (1.06 kb) was digested with NcoI and PstI and cloned in translational fusion with the *nisA* expression signals of similarly digested pNZ8048 (35). The P_{nisA}::*Lcddl* fusion was transferred as a 1.38-kb StuI/KpnI fragment into SfoI/KpnI-digested plasmid pMEC10 (43), generating plasmid pGIM117. This integration plasmid was introduced into *L. plantarum* PG6212 (*ldhD*::*cat* Δ*lar*), and clones having integrated the plasmid in the chromosome were selected on MRS containing erythromycin. Integration at the $tRNA^{Ser}$ locus was confirmed by PCR. The resulting strain was designated PG1174. Similarly, plasmid pMEC10 was introduced in the *L. plantarum* PG6212 mutant, giving the control strain PG1104.

RNA extraction and hybridization techniques. L-Lactate (0.2 M) was added to a *L. plantarum* TF101 culture at an optical density at 600 nm (OD_{600}) of 0.75. At different times after L-lactate addition, 50-ml aliquots were centrifuged at 5,000 × *g* for 10 min, and total RNA was extracted by the method of Anba et al. (1). Northern blot experiments were carried out as described by Lorquet et al. (36), using a 710-bp probe that hybridizes with the *larA* ORF. This probe was obtained by PCR amplification with primers 104A1 and 104B1.

DNA microarray analysis. The DNA microarrays applied in the present study have previously been described and are clone-based arrays that cover more than 85% of the *L. plantarum* WCFS1 genome (40). Differential transcript levels were determined by two-color (Cy5 and Cy3) fluorescent hybridizations of the corresponding cDNAs. The RNA samples from noninduced and L-lactate-induced (90 min) cultures of *L. plantarum* TF101 were labeled during reverse transcription using random hexamers primers and either Cy5- or Cy3-labeled dUTP (Amersham Biosciences, Roosendaal, The Netherlands). Unincorporated dyes were removed from the synthesized cDNA by using Autoseq G50 columns (Amersham Biosciences). The arrays were prehybridized for 45 min at 42°C in prehybridization solution (1% bovine serum albumin, 5× SSC [1× SSC is 0.15 M NaCl plus 0.017 M sodium citrate, pH 7.2], and 0.1% sodium dodecyl sulfate [SDS]). Cy5- and Cy3-labeled samples were mixed before hybridizations. Hybridizations were performed overnight at 42°C in Easyhyb buffer (Roche, Basel, Switzerland) according to the manufacturer's protocol. The slides were subsequently washed twice in 1× SSC and 0.2% SDS, once in 0.5× SSC, and twice in 0.2× SSC at 37°C. Slides were dried and scanned with a ScanArray Express 4000 scanner for both dyes (Perkin-Elmer, Boston, Ma.). Images were analyzed by using ImaGene 4.2 software and applying standard setting for spot flagging (BioDiscovery, El Segundo, Calif.). ImaGene output files were processed by removal of flagged spots, and remaining, high-quality spots were normalized and further interpreted (40). Since on these arrays many genes are represented by more than a single clone, primary selection of regulated genes was based on consistency of direction and magnitude of regulation of expression of clones corresponding to the gene. Subsequently, manual analysis of the regulatory factors obtained for flanking clones was used to determine the predicted boundaries of the regulated chromosomal locus.

Lactate racemization assays. Assays were performed on crude cell extracts obtained by mechanically breaking the cells from a 50-ml culture with a bead beater, as previously described (36). Lactate racemization activity was assayed as described by Dennis (12), except that Tris-maleate buffer (50 mM [pH 6.0]) was used. The D- or L-lactate initial concentration was 20 mM. Then, 0.1% (wt/vol) yeast extract (Oxoid, Basingstoke, United Kingdom), 2 mM $MgSO_4$, 2 mM $MnSO_4$, and 2 mM ascorbic acid were added since these compounds have been shown to enhance the in vitro lactate racemase activity of *Clostridium acetobutylicum* (32) and *Clostridium beijerinckii* (13). Samples were taken at time zero and after 1 h of incubation at 28°C, and boiled for 10 min to inactivate the enzyme. Concentrations of D- and L-lactate were measured enzymatically (kit no. 1112821; Boehringer Gmbh, Mannheim, Germany). The amounts of total protein in the cell extracts were measured according to the method of Bradford (7), using the Bio-Rad Laboratories (Munich, Germany) protein assay (catalog no. 500-0006). Lactate racemization specific activity is expressed as μmol of D- or L-lactate produced per min per mg of total protein.

Preparation and analysis of the peptidoglycan nucleotide precursor pools. *L. plantarum* cells were grown in MRS or MPL broth to an OD_{600} of 0.7 and treated

with ramoplanin (10 μg/ml; kindly provided by Lepetit, Milan, Italy) for 120 min, as previously described (2). Peptidoglycan precursors were extracted with 20% trichloroacetic acid and analyzed by reversed-phase high-pressure liquid chromatography with a μBondapack C_{18} column (7.8 by 300 nm; Waters, St-Quentin-en-Yvelines, France) at a flow rate of 2 ml min^{-1} with 50 mM ammonium formiate (pH 5.0) (37). Products were detected by measuring the absorbance at 262 nm. The UDP-MurNAc structures were purified by reversed-phase high-pressure liquid chromatography, lyophilized, dissolved in H_2O, and analyzed by mass spectrometry as previously described (6).

RESULTS

Lactate racemase activity in *L. plantarum*. Lactate racemase (Lar) activity was assayed in cell extracts of the wild-type and of the two *ldh* mutants at different stages of growth (Fig. 2A). Significant Lar activity was observed in the TF102 mutant (*ldhD*::*cat*), while TF101 (Δ*ldhL*) and NCIMB8826 (wild type) showed no or close-to-background activities. No significant changes in Lar activity were observed at different growth stages. The Lar activity was assayed in the D- to L-lactate and L- to D-lactate directions, and similar results were obtained, as shown for the *ldhD* mutant in Fig. 2B. The L-lactate/D-lactate ratios in the culture supernatants of theses strains could be correlated with their Lar and Ldh activities. In the wild-type strain, both LdhD and LdhL are present, and both lactate isomers are thus produced (Table 2). In the *ldhL* mutant (TF101), only LdhD activity is present, leading to the exclusive production of D-lactate (Table 2). Lar activity is not detected in this strain, explaining the absence of L-lactate production. In the *ldhD* mutant (TF102), L-lactate is formed through the LdhL activity, while D-lactate could be produced by the Lar activity, thus leading to the formation of a racemic mixture of both isomers (Table 2).

The absence of Lar activity in the *ldhL* mutant and its presence in the *ldhD* mutant were in agreement with the previously proposed hypothesis for the existence of an L-lactate inducible lactate racemase (17). However, the absence of activity in the wild-type strain suggested that some additional factor could regulate lactate racemization in *L. plantarum*. Previous studies on the lactate racemase of *L. sakei* reported a positive effect of L-lactate and a negative effect of D-lactate on the Lar activity (33). The effect of D-lactate was tested by adding different L-lactate/D-lactate ratios to an exponentially growing culture of the *L. plantarum ldhD* mutant (TF102). The cultures were allowed to grow for 2 h, and the cells were collected. When D-lactate (0.2 M) was added, the Lar activity decreased about twofold compared to untreated cultures (Fig. 2B). The same decrease was observed when a mixture of D- and L-lactate (0.1 M each) was used, whereas L-lactate alone (0.2 M) increased the activity by about 7 times. Thus, Lar activity in *L. plantarum* is induced by L-lactate and repressed by D-lactate, and the positive effect of L-lactate is counteracted by the presence of D-lactate, indicating that the Lar activity is regulated by the L-lactate/D-lactate ratio.

The induction of Lar activity by L-lactate (0.2 M, compared to 15 mM D-lactate already produced by the strain, see below) was also observed in the *ldhL* mutant, with an induced specific activity similar to that of the *ldhD* mutant (Fig. 2C). In the wild-type strain, the induced Lar activity was ~13-fold lower than in the two *ldh* mutants (Fig. 2C). This could be explained by the ability of the LdhL enzyme to oxidize L-lactate to pyruvate (23), which can then be reduced to D-lactate through the LdhD activity. This would quickly reduce the L-lactate excess, thus preventing induction of the racemase. In the two *ldh* mutants, however, this Ldh-dependent racemization pathway could not take place.

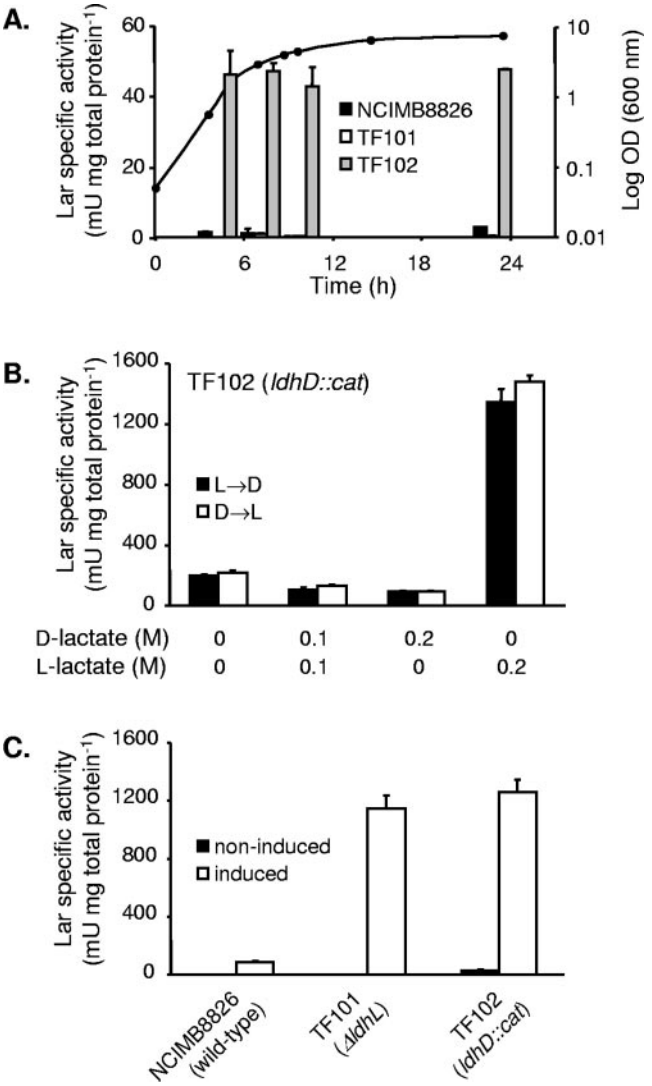

FIG. 2. Lactate racemization (Lar) activity in *L. plantarum.* (A) Lar activity of *L. plantarum* NCIMB8826 (wild-type, black bars), TF101 (Δ*ldhL*, white bars), and TF102 (*ldhD*::*cat*, gray bars) at different growth stages, assayed with L-lactate as a substrate. Growth of the wild type in MRS broth at 28°C is shown as the log of the OD_{600}. Growth of the TF101 and TF102 mutants were comparable to that of the wild-type strain. (B) Influence of D- and L-lactate on the Lar activity of *L. plantarum* TF102. Cells were grown in MRS broth to an OD_{600} of 0.75. Then, D- and/or L-lactate was added to the culture as indicated (bottom of panel B), and the cells were allowed to grow for 2 h. Cells were collected, and Lar activities were measured with L-lactate (■) or D-lactate (□) as a substrate. (C) Lar activity of *L. plantarum* NCIMB8826, TF101, and TF102, assayed with L-lactate as a substrate. The activities were measured with (□) or without (■) induction by L-lactate (0.2 M for 2 h at 28°C). The data shown are from one representative experiment from at least three independent repetitions.

Cloning of an operon involved in lactate racemization in *L. plantarum*. Since previous studies suggested that L-lactate in-

TABLE 2. D- and L-lactate production by *L. plantarum* NCIMB8826 derivatives[a]

Strain	Genotype	Lactate production (concn [mM])	
		D-Lactate	L-Lactate[b]
NCIMB8826	Wild type	126	54
TF101	Δ*ldhL*	180	0
PG6351	Δ*ldhL* P_{ldhL}::*larA-larE*	85	73
TF102	*ldhD*::*cat*	81	82
PG6210	Δ(*larA-larE*)	112	49
PG6212	*ldhD*::*cat* Δ(*larA-larE*)	19[c]	172

[a] The strains were grown for 36 h in MRS broth, and D- and L-lactate concentrations were determined in the supernatant. The data shown are from one of at least three independent experiments that gave essentially the same results.

[b] The L-lactate concentrations are given as the L-lactate concentration in the supernatant of cultures minus the initial concentration of the MRS broth.

[c] The PG6212 mutant was grown in MRS broth containing 20 mM added D-lactate.

duction of the Lar activity occurs at the genetic level (45), the L-lactate inducibility of the Lar activity was used to identify genes that could potentially be involved in lactic acid racemization. For this reason, initial experiments were focused on a more detailed analysis of the L-lactate-mediated induction of Lar activity. The *ldhL* mutant (TF101) was used since it provided the highest induction factor in Lar activity (Fig. 2C). Before addition of L-lactate to an exponentially growing culture of the *ldhL* mutant, the D-lactate concentration was 15 mM. After addition of L-lactate (0.2 M), the L-lactate/D-lactate ratio was thus 13:1, and the Lar activity started to be induced. During the first 2 h, the Lar activity increased gradually and L-lactate was converted to D-lactate by the lactate racemase (Fig. 3A). Consequently, the L-lactate/D-lactate ratio decreased until it reached equilibrium and the Lar activity reached a plateau (120 min after induction; Fig. 3A). When D- and L-lactate concentrations were equivalent, the Lar activity was no longer induced and started to decrease (data not shown).

In order to identify genes whose transcription is induced by L-lactate, the RNAs from induced and noninduced cultures of the *ldhL* mutant were compared by using DNA microarrays. The array analyses were used as a lead-generating approach using induced versus noninduced conditions. Arrays were applied and analyzed with immediate focus of attention toward those genes that displayed the major changes in transcription between the two conditions. The highest induction factor after L-lactate addition (18-fold) was observed for several clones (spots) corresponding to a six-gene operon (lp_0104 to lp_0109) (34). The L-lactate induction of this operon was confirmed by Northern blotting using a probe hybridizing with the first gene (Fig. 3B), revealing a 5.4-kb transcript that would be in agreement with a mRNA encompassing the six genes of the operon. The additional low-intensity bands most probably represent degradation products of the transcript that comigrated with the rRNA (19). The mRNA could not be detected prior to L-lactate induction, suggesting a very low basal level of expression. When L-lactate was added to the culture, the amount of mRNA started to increase and reached its maximal level after 1 h, followed by a decrease to undetectable levels after 2.5 h (Fig. 3B). The time course of the mRNA induction preceded

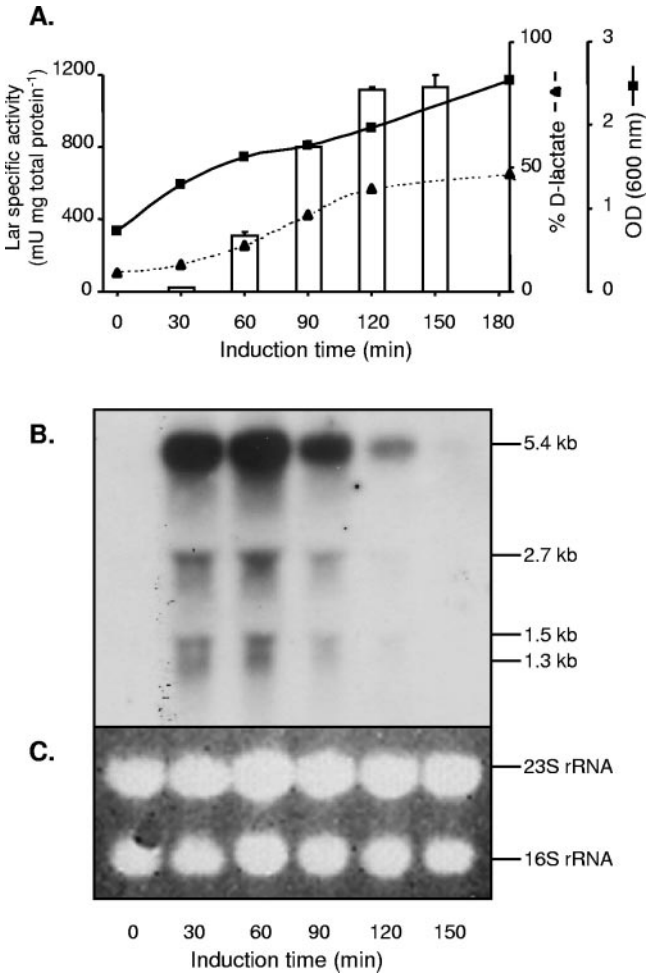

FIG. 3. Time course of L-lactate induction of the lactate racemase activity in *L. plantarum* TF101 (Δ*ldhL*). L-lactate (0.2 M) was added to a TF101 culture at an OD_{600} of 0.75. Samples were taken every 30 min for lactate racemization assay (A, white bars) and Northern blot analysis (B). The OD (A, solid line, squares) and percentage of D-lactate (A, dashed line, triangles) were monitored during induction. The Lar activity was assayed with L-lactate as a substrate. The data presented are from one of three independent experiments that gave essentially the same results. ND, Lar activity not determined. The Northern blot was carried out with a *larA* probe. (C) RNA electrophoresis of the Northern blot in panel B. Equivalent amounts of total RNA (8 μg) were loaded in each lane.

that of the Lar activity, and the Lar activity was maintained for longer than the mRNA (Fig. 3A).

In order to establish a possible involvement of this operon in the lactate racemization activity of *L. plantarum*, a stable operon mutant was constructed by deletion of a 4.65-kb region spanning the 3′ half of the first gene to the 5′ half of the last gene. No Lar activity could be detected in this strain (PG6210) with or without induction by L-lactate. This operon was thus designated *lar* (for lactate racemization), and the six genes were named *larA*, *larB*, *larC1*, *larC2*, *glpF1*, and *larE*, respectively (Fig. 4).

In silico analysis of the *lar* operon. Sequence analysis of the first protein, encoded by *larA*, shows similarity to uncharacterized conserved proteins (COG3875). The *larB* gene product is

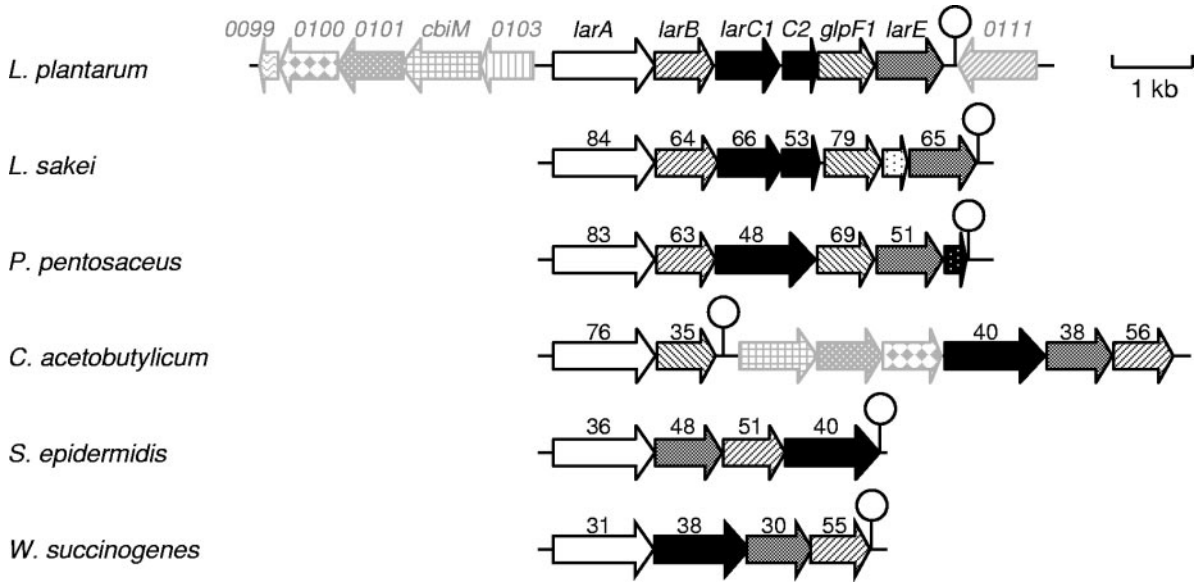

FIG. 4. Structural organization of the *lar* operon in *L. plantarum* and other bacteria. Homologous genes in the different loci are colored the same. The numbers above the genes indicate the percentage of identity between the encoded protein and the corresponding protein of *L. plantarum*. Except for *L. sakei*, the LarC1-LarC2 protein homologues were compared to the complete LarC protein from *L. plantarum*, obtained by artificially adding an A in the frameshift region. Strains: *L. plantarum*, strain WCFS1 (AL935263); *L. sakei*, strain 23K (AY849556), *P. pentosaceus*, strain ATCC 25745 (AAEV000000000); *C. acetobutylicum*, strain ATCC 824 (AE001437); *S. epidermidis*, strain ATCC 12228 (AE015929); *W. succinogenes*, strain DSM1740 (BX571656).

similar to NCAIR mutase (PurE)-related proteins (COG1691). The third and fourth gene products (*larC1* and *larC2*) were found to be similar to the N- and C-terminal parts of uncharacterized conserved proteins (COG1641), respectively. The complete *larC* ORF (*larC1-larC2*) of the *L. plantarum* genome sequence appears to have been disrupted by a −1 frameshift mutation. Indeed, no putative ribosomal binding site is present in front of the *larC2* ATG codon, and the *larC1* stop codon is preceded by a stretch of 10 A's, which was confirmed by resequencing of the *larC1-larC2* region (data not shown). The protein encoded by the *glpF1* gene is annotated as a glycerol uptake facilitator protein, which belongs to the major intrinsic protein family. Finally, the *larE* gene product belongs to a group of putative ATP-utilizing enzymes of the PP-loop superfamily (COG1606).

Although its structure is not entirely conserved, the *lar* operon is found in all sequenced organisms where lactate racemization has been observed, i.e., *L. sakei*, *P. pentosaceus*, and *C. acetobutylicum* (Fig. 4). The organization of the operon is very well conserved between *L. plantarum*, *L. sakei*, and *P. pentosaceus*. The *lar* locus of *C. acetobutylicum* is split in two parts that are interrupted by another putative operon composed of three genes and showing homology to an operon located upstream of the *lar* operon in *L. plantarum* (Fig. 4). The *lar* operon is also found in other sequenced bacteria where no data are available about a possible lactate racemization activity. The best-conserved operons are found in *Staphylococcus epidermidis* and *Wolinella succinogenes*. Although the gene order appears to be variable, these operons contain homologues of *larA*, *larB*, *larC1-larC2* (both as a single ORF), and *larE* (Fig. 4).

Involvement of the *lar* operon in lactate racemization activity. To further establish the role of the *lar* operon in the Lar activity, the full operon was overexpressed under control of the strong constitutive expression signals of the *ldhL* gene from *L. plantarum* using plasmid pGIZ906. The overexpression vector was transformed into *L. plantarum* PG6210 [Δ(*larA-larE*)] and *L. casei* ATCC 393. The latter was selected for heterologous expression since it has no Lar activity and it is phylogenetically close to *L. plantarum*. This strain produces mainly L-lactate, together with 5 to 10% of D-lactate (39; data not shown).

Overexpression of the complete *lar* operon (plasmid pGIZ632) failed to confer Lar activity to *L. casei*, as assayed by either Lar activity measurement in cell extracts or D-lactate production by growing cells (data not shown). *L. plantarum* could not be transformed with this plasmid, suggesting that overexpression of the *lar* operon was toxic in this background. Further analysis of the *lar* operon by overexpression of every single *lar* gene under the control of the *ldhL* expression signals (plasmids pGIZ630, pGIZ660, pGIZ661, pGIZ662, pGIZ663, pGIZ664, and pGIZ665) revealed that toxicity in *L. plantarum* was due to overexpression of either *larB* or *larC1* (data not shown). Importantly, none of the other overexpression plasmids restored Lar activity in the PG6210 [Δ(*larA-larE*)] mutant (data not shown).

Since multicopy constitutive overexpression of the entire *lar* operon was toxic in *L. plantarum*, a monocopy constitutive overexpressing strain (PG6351) was constructed by inserting the *ldhL* expression signals in front of the operon in the chromosome of the TF101 (*ldhL*) mutant. The PG6351 strain was found to produce a racemic mixture of D- and L-lactate (Table 2), a finding which is in agreement with a level of Lar activity

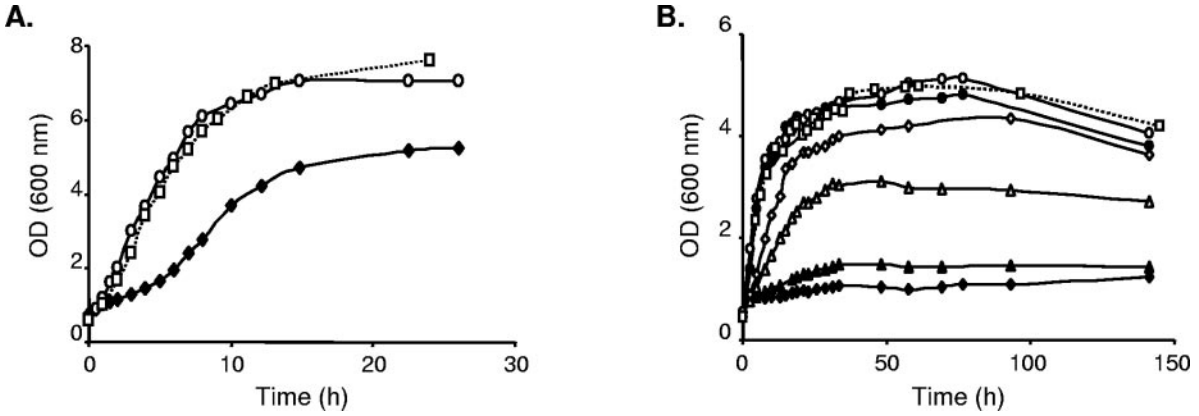

FIG. 5. Growth of the *L. plantarum ldhD lar* mutant (PG6212) in MRS (A) or MPL (B) broth after D-lactate starvation. The mutant was grown to an OD_{600} of 0.5 in the presence of 20 mM D-lactate, washed twice in MRS (A) or MPL (B) broth, and resuspended in fresh MRS (A) or MPL (B) medium with D-lactate concentrations of 0 (◆), 0.01 (▲), 0.05 (△), 0.1 (◇), 0.5 (●), or 20 (○) mM. The DL-lactate-producing wild-type NCIMB8826 strain was introduced as a control (□ [dashed line]). The data presented are from one representative experiment from three independent repetitions.

equivalent to that observed in the *ldhD* mutant when cultivated in the absence of added L-lactate (data not shown). These results clearly show that the *lar* operon is involved in the Lar activity of *L. plantarum* and that regulation of the Lar activity by L-lactate is a direct consequence of the transcriptional regulation of the *lar* operon expression. However, there is no evidence that the *lar*-encoded proteins are directly involved in this process or that the whole *lar* operon is required for Lar activity.

D-Lactate requirement of the *ldhD lar* double mutant. The Δ(*larA-larE*) mutant (PG6210) did not show any difference with the wild-type strain when lactate production (Table 2), growth, or viability (data not shown) were compared. This was expected since the *lar* operon is not expressed in the wild-type background (see Fig. 2A).

In order to evaluate the importance of D-lactate for the physiology of *L. plantarum*, the *lar* operon was deleted in the *ldhD* background (TF102). The *ldhD lar* double mutant (PG6212) grew very poorly in MRS broth containing no added D-lactate (Fig. 5A). Therefore, production of lactate was measured in a culture of PG6212 grown in MRS broth with 20 mM D-lactate. As expected, L-lactate was the only isomer produced, whereas the D-lactate concentration (20 mM, added at the start of the culture) did not change significantly during growth (Table 2). This phenotype confirmed that D-lactate production in the single *ldhD* mutant (TF102) was due to lactate racemization. In analogy, no Lar activity could be detected in the double *ldhD lar* mutant, with or without induction by L-lactate.

The dependence of the *ldhD lar* mutant toward D-lactate was investigated in a D-lactate-starved culture. Because MRS broth contains very small amounts of D-lactate, the starvation experiment was carried out in a chemically defined medium containing no D-lactate (MPL [10]). The strain was cultivated in MPL broth containing 20 mM D-lactate. At an OD_{600} of 0.5, the cells were collected, washed twice in MPL broth without added D-lactate, and resuspended in the same volume of MPL broth containing D-lactate concentrations ranging from 0 to 20 mM. When no D-lactate was added, growth of the *ldhD lar* mutant was blocked (Fig. 5B). When increasing concentrations of D-lactate were added, the growth defect was gradually relieved. Both the growth rate and maximal OD_{600} reached were dependent on the concentration of added D-lactate. At D-lactate concentrations higher than 0.5 mM, growth of the *ldhD lar* mutant appeared to be comparable with growth of the wild-type NCIMB8826 strain (Fig. 5B).

Supplementation with D-2-amino acids or D-2-hydroxy acids. D-Lactate is a major component of the peptidoglycan precursors in *L. plantarum*, where it is incorporated as the last residue of the pentadepsipeptide (17). Therefore, we suspected that the growth defect of the *ldhD lar* mutant in the absence of added D-lactate could be connected to a defect in cell wall biosynthesis. D-Lactate enters the peptidoglycan biosynthesis pathway at the level of the reaction catalyzed by the Ddl ligase. In the wild-type *L. plantarum* strain, this enzyme (LpDdl) synthesizes the D-alanyL-D-lactate depsipeptide, which is then added to the peptidoglycan precursor by the MurF synthetase (46) (see Fig. 1).

To evaluate the intrinsic flexibility of the cell wall biosynthesis machinery, supplementation of growth of the *ldhD lar* mutant by the addition of precursor substrates other than D-lactate was determined. None of the D-2-amino acids tested could supplement growth of the *ldhD lar* mutant (Ala, Ser, Asn, Gln, Thr, Met, and D-2-aminobutyrate, as well as the achiral amino acid glycine; Table 3). D-Alanine has been shown to supplement an alanine racemase-deficient mutant of *L. plantarum* (42), indicating that *L. plantarum* is able to take up this D-2-amino acid from the medium. Therefore, the lack of supplementation of the *ldhD lar* mutant with D-alanine can be interpreted as a consequence of the inability of the peptidoglycan synthesis machinery to replace D-lactate by D-alanine. As for the other D-2-amino acids, no data are available about the presence of uptake systems in *L. plantarum*.

In contrast, D-lactate, D-2-hydroxybutyrate, D-2-hydroxycaproate, D-2-hydroxyisovalerate, D-2-hydroxyisocaproate, and D-mandelate showed supplementation of the mutant on plates (Table 3). D-2-Hydroxyvalerate, however, was unable to supplement growth of the *ldhD lar* mutant (Table 3).

The presence of D-lactate in the peptidoglycan precursors of

TABLE 3. Supplementation of the *ldhD lar* mutant with D-2-hydroxy acids and D-2-amino acids

Compound	R	Supplementation[a]	Vancomycin MIC (μg/ml)[b]
None		−	(1–4)
Glycine [$RCH(NH_2)COOH$]	H	−	(1–4)
D-2-Amino acids [$RCH(NH_2)COOH$]			
D-Alanine	CH_3	−	(1–4)
D-Serine	CH_2OH	−	(1–4)
D-Asparagine	CH_2CONH_2	−	(1–4)
D-Glutamine	$(CH_2)_2CONH_2$	−	(1–4)
D-Threonine	$CH(OH)CH_3$	−	(1–4)
D-Methionine	$(CH_2)_2SCH_3$	−	(1–4)
D-2-Aminobutyrate	CH_2CH_3	−	(1–4)
D-2-Hydroxy acids [$RCH(OH)COOH$]			
D-Lactate	CH_3	+	>256
D-2-Hydroxybutyrate	CH_2CH_3	+	>256
D-2-Hydroxyvalerate	$(CH_2)_2CH_3$	−	(1–4)
D-2-Hydroxycaproate	$(CH_2)_3CH_3$	+	128
D-2-Hydroxyisovalerate	$CH(CH_3)_2$	+	48
D-2-Hydroxyisocaproate	$CH_2CH(CH_3)_2$	+	128
D-Mandelate	Phenyl	+	64

[a] −, Single colonies able to grow in the absence of any supplementing compound were observed at a frequency of 10^{-6}.
[b] (1–4), the MIC of single colonies growing in the absence of any supplementing compound.

L. plantarum NCIMB8826 is responsible for the high level of vancomycin resistance of this strain (MIC > 256 μg/ml, Table 4) (17). As expected, when the *ldhD lar* mutant was plated on MRS containing 20 mM D-lactate, it was resistant to high concentrations of vancomycin (MIC > 256 μg/ml, Tables 3 and 4). Previous studies on *Enterococcus faecalis* have shown that the replacement of the C-terminal residue of the peptidoglycan precursors by various D-2-hydroxyacids modulates the level of resistance to vancomycin (3, 26). The vancomycin resistance of the *ldhD lar* mutant supplemented with these D-2-hydroxy acids was thus estimated (Table 3). Except for D-2-hydroxybutyrate and D-lactate, which showed MICs greater than 256 μg/ml, the vancomycin resistance of the mutant supplemented with the other D-2-hydroxy acids was significantly lowered, suggesting that these compounds are readily incorporated in the peptidoglycan precursors. Accordingly, peptidoglycan precursor analysis of the *ldhD lar* mutant supplemented with D-lactate and D-2-hydroxybutyrate demonstrated incorporation of these D-2-hydroxyacids at the last position of the pentadepsipeptide (Table 4). As a control, these compounds were added to the growth medium of the *L. plantarum* wild-type strain, but no differences were observed concerning the level of vancomycin resistance (MIC > 256 μg/ml) whatever the D-2-hydroxy-acid used. However, D-2-hydroxycaproate, D-2-hydroxyisocaproate, and D-mandelate had a slightly negative effect on growth.

These data support a strict requirement for D-lactate in the *ldhD lar* mutant, which is correlated to cell wall biosynthesis. Moreover, they illustrate the intrinsic flexibility of the *L. plantarum* cell wall biosynthesis machinery to incorporate alternative D-2-hydroxy acids, whereas D-2-amino acids cannot replace D-lactate.

Suppression of the *ldhD lar* growth defect by expression of the D-alanyl-D-alanine forming Ddl enzyme from *Lactococcus lactis*. It has been shown that overexpression of the D-alanyl-D-alanine forming Ddl ligase from *L. lactis* in *L. plantarum* confers sensitivity to vancomycin through the incorporation of D-alanine instead of D-lactate at the last position of the peptidoglycan precursor (M. Deghorain, unpublished data).

We evaluated the ability of the *L. lactis* Ddl (LcDdl) to suppress the growth defect of the double *ldhD lar* mutant. A translational fusion was created between the *nisA* expression signals and the *Lcddl* gene and integrated as a single copy in the PG6212 chromosome. In this strain (PG1174), expression of the *Lcddl* gene is positively controlled by the presence of the inducer nisin (8). Suppression of the *ldhD lar* mutant growth

TABLE 4. Cytoplasmic cell wall precursors of *L. plantarum* NCIMB8826 and derivatives

Strain	Culture medium	Vancomycin penta(depsi)peptide peptidoglycan	
		MIC (μg/ml)	End of precursor
NCIMB8826 (wild type)	MRS	>256	D-Lactate[a]
TF103 (Δ*ldhL ldhD*::*cat*)	MRS	2	D-Lactate (55%)D-alanine (45%)[a]
PG6212 (*ldhD*::*cat* Δ*lar*)	MPL + D-lactate (20 mM)	>256	D-Lactate[b]
	MPL + D-2-hydroxybutyrate (20 mM)	>256	D-2-Hydroxybutyrate[b]
PG1174	MPL + nisin (50 ng/ml)	1	D-Alanine

[a] Data from reference 17.
[b] Incorporation of these D-2-hydroxy acids into the pentadepsipeptide peptidoglycan precursors was confirmed by mass spectrometry.

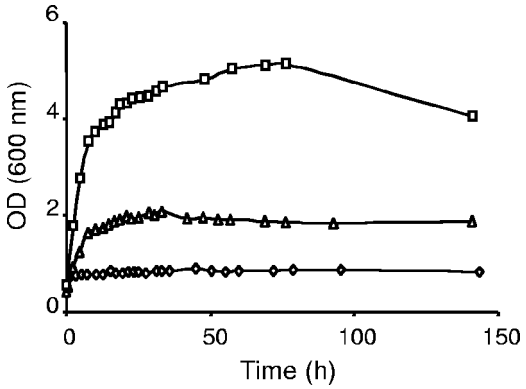

FIG. 6. Suppression of the growth defect of the *L. plantarum ldhD lar* mutant (PG6212) with the D-alanyl-D-alanine forming LcDdl ligase from *L. lactis*. The nisin-controlled expression cassette (P_{nisA}::*Lcddl*) was stably integrated into the PG6212 chromosome (strain PG1174). The PG1174 strain was grown in MPL broth containing nisin (50 ng/ml) and D-lactate (20 mM) to an OD_{600} of 0.5. Cells were then washed twice in MPL broth and resuspended in MPL containing (triangles) or not (diamonds) nisin (50 ng/ml). The parent PG6212 strain supplemented with D-lactate (20 mM) was introduced as a control (squares). The data shown are from one of three independent experiments that gave essentially the same results.

defect by expression of LcDdl was estimated in D-lactate-starved MPL cultures, as described above. In the absence of nisin, growth stopped rapidly after D-lactate starvation (Fig. 6), similar to the blockage of growth of the *ldhD lar* mutant in the absence of D-lactate (see Fig. 5B). In contrast, when nisin was added at a subinhibitory concentration (50 ng/ml) (43) to the D-lactate-starved culture, a partial regain of growth of strain PG1174 was observed. Restoration of growth is directly correlated to heterologous *L. lactis Lcddl* expression since no growth could be observed in a control strain (PG1104) where pMEC10 (43) was integrated in the PG6212 chromosome in the presence or absence of nisin (data not shown). Growth restoration, however, was only partial, compared to supplementation with D-lactate (20 mM; Fig. 6), suggesting either that D-alanine could not efficiently replace D-lactate in the cell wall precursors of *L. plantarum* or that D-lactate was also required for other cellular processes.

Overexpression of the LcDdl enzyme in this mutant rendered the strain sensitive to low concentrations of vancomycin (MIC = 1 μg/ml; Table 4). This phenotype is likely caused by a modified cell wall composition due to the presence of 100% peptidoglycan precursors ending in D-alanine (Table 4). Partial suppression of the *ldhD lar* growth defect with the LcDdl ligase from *L. lactis* univocally demonstrates that this mutant is affected in cell wall biosynthesis due to the inability of the LpDdl enzyme to produce D-alanyl-D-lactate and hence pentadepsipeptide peptidoglycan precursors.

DISCUSSION

The lactate racemization activity of *L. plantarum* NCIMB8826 was characterized. It was shown to be induced by L-lactate and repressed by D-lactate (see Fig. 1), as already observed for the lactate racemase of other organisms (33, 45). The L-lactate inducibility of the Lar activity was used to identify a locus involved in lactate racemization. This locus, named *lar*, is composed of six genes that are organized in an operon: *larA*, *larB*, *larC1*, *larC2*, *glpF1*, and *larE*. Except for the *glpF1* gene, encoding a membrane protein of the aquaglyceroporin family, no clear function can be attributed to any of the genes of the *lar* operon. Notably, no gene of this operon shows similarity to any known racemase.

The involvement of this operon in lactate racemization in *L. plantarum* was clearly established by constitutive overexpression of the full *lar* operon in an *ldhL* strain (TF101 [16]), which led to complete restoration of L-lactate production by this strain. It was further confirmed in the *ldhD* mutant (TF102 [17]), where deletion of the *lar* operon completely abolished D-lactate production (PG6212). However, overexpression of the entire *lar* operon, as well as every single *lar* gene, did not lead to lactate racemase activity in *L. casei*. This was not due to a lack of expression from the P_{ldhL} promoter since overexpressed proteins could be observed by SDS-polyacrylamide gel electrophoresis (data not shown). Therefore, it appears that expression of the *lar* operon, although required for lactate racemization, is not the only determinant of this activity. It cannot be excluded that the lactate racemization process also involves gene(s) located outside the *lar* operon, even if they are not regulated by L-lactate. The *lar* operon could play an indirect role in lactate racemization, such as synthesis of a cofactor required for the lactate racemase enzyme, although the purified lactate racemases of *C. beijerinckii* and *L. sakei* do not seem to require any cofactor (13, 29). Alternatively, it is possible that the lactate racemase consists of more than one polypeptide, some of them being encoded by genes located outside of the *lar* operon. Whether the lactate racemase is a heteromultimer or merely a pathway leading from one lactate stereoisomer to the other would then remain to be answered. Purification of the lactate racemase, as well as determination of the enzymatic function of the *lar*-encoded proteins, should help to clarify these issues.

Since the Lar activity is induced by L-lactate and repressed by D-lactate, its physiological function appears to be the production of D-lactate from L-lactate. The importance of D-lactate for the biosynthesis of the *L. plantarum* cell wall has previously been established (17): D-lactate is incorporated at the last position of the peptidoglycan precursor instead of the usual D-alanine. This feature has also been observed in *P. pentosaceus*, *L. casei*, and *Leuconostoc mesenteroides* (5, 25), where it confers resistance to high levels of vancomycin (5, 17).

In the present study, we observed an absolute D-lactate requirement for the growth of *L. plantarum*, which was shown to be connected to cell wall biosynthesis. Various other D-2-hydroxy acids could efficiently supplement the growth defect of the L-lactate producing *ldhD lar* mutant (see Fig. 1). Supplementation with these D-2-hydroxy acids was found to modify the level of vancomycin resistance of this strain, probably as a consequence of their incorporation in the cell wall precursors. Indeed, the *ldhD lar* mutant cultivated in the presence of D-2-hydroxybutyrate was shown to contain peptidoglycan precursors ending in this hydroxy acid. In constrast, neither D-alanine nor any other D-2-amino acid could supplement the growth defect of the *ldhD lar* mutant. It seems that the LpDdl ligase cannot efficiently synthesize the D-alanyl-D-alanine dipeptide in *L. plantarum* (Fig. 1). This is confirmed by the

observation that expression of the LcDdl ligase from *L. lactis*, a lactic acid bacterium known to produce exclusively peptidoglycan precursors ending in D-alanine, suppresses the growth defect of the *ldhD lar* mutant, leading to vancomycin sensitivity (Fig. 1). Thus, the limiting growth factor of the *ldhD lar* mutant seems to be the inability to synthesize both depsipeptides (due to the lack of D-lactate or other D-2-hydroxy acids) and dipeptides (due to an apparent specificity of the LpDdl ligase toward D-2-hydroxy acids).

Although D-lactate is required for growth of the *ldhD lar* mutant strain, suppressor mutants able to grow in the absence of this compound were observed at a low frequency (10^{-6}) on MRS plates without added D-lactate. These mutants were sensitive to very low levels of vancomycin (MIC = 1 to 4 μg/ml) and were shown to incorporate D-alanine instead of D-lactate in the peptidoglycan precursors (data not shown; see Fig. 1), suggesting that a suppressor mutation could have occurred that would allow synthesis of such modified peptidoglycan precursors. Genetic analysis of these suppressor mutations should provide us with a convenient method to identify key enzymes controlling the nature of the carboxy-terminal residue of the penta(depsi)peptide moiety of the peptidoglycan precursors in *L. plantarum*.

The observation that D-alanine cannot efficiently replace D-lactate at the carboxy terminus of the penta(depsi)peptide in the *ldhD lar* mutant appears to contradict the presence of D-alanine ending peptidoglycan precursors in the double *ldhD ldhL* mutant (Table 4) (17). It should be noted that during the construction of the *ldhD ldhL* (TF103) mutant, only one clone was obtained that could only be isolated on a D-lactate containing medium but was later shown to grow in the absence of added D-lactate (17). The presence of a suppressor mutation similar to that of the *ldhD lar* suppressor clones able to grow in the absence of D-lactate can thus not be ruled out in the *ldhD ldhL* double mutant.

For the first time, D-lactate was shown to be absolutely required for growth of *L. plantarum*. In this respect, the presence of an alternative pathway for D-lactate production, the lactate racemase, could be of importance in order to ensure that sufficient amounts of D-lactate are available even under conditions where the LdhD enzyme cannot function. Although this compound might also be needed for purely metabolic purposes, it seems that its major role in *L. plantarum* is connected to peptidoglycan biosynthesis. D-2-Hydroxy acids would be preferred to D-2-amino acids for incorporation at the last position of the peptidoglycan precursors. D-Lactate being the most abundant D-2-hydroxy acid in *L. plantarum*, it is a substrate of choice for the synthesis of these precursors. It thus appears that the cell wall synthesis machinery of *L. plantarum* has evolved to produce and incorporate exclusively D-lactate ending precursors. However, efficient supplementation of the *ldhD lar* mutant by other D-2-hydroxy acids suggests some flexibility of the cell wall biosynthesis enzymes. This mutant thus provides a unique tool for the study of in vivo modifications of the *L. plantarum* cell wall.

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