

Enantioselective Regulation of Lactate Racemization by LarR in *Lactobacillus plantarum*

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Lactobacillus plantarum is a lactic acid bacterium that produces a racemic mixture of L- and D-lactate from sugar fermentation. The interconversion of lactate isomers is performed by a lactate racemase (Lar) that is transcriptionally controlled by the L-/D-lactate ratio and maximally induced in the presence of L-lactate. We previously reported that the Lar activity depends on the expression of two divergently oriented operons: (i) the *larABCDE* operon encodes the nickel-dependent lactate racemase (LarA), its maturases (LarBCE), and a lactic acid channel (LarD), and (ii) the *larR(MN)QO* operon encodes a transcriptional regulator (LarR) and a four-component ABC-type nickel transporter [Lar(MN), in which the M and N components are fused, LarQ, and LarO]. LarR is a novel regulator of the Crp-Fnr family (PrfA group). Here, the role of LarR was further characterized *in vivo* and *in vitro*. We show that LarR is a positive regulator that is absolutely required for the expression of Lar activity. Using gel retardation experiments, we demonstrate that LarR binds to a 16-bp palindromic sequence (Lar box motif) that is present in the *larR-larA* intergenic region. Mutations in the Lar box strongly affect LarR binding and completely abolish transcription from the *larA* promoter (P_{larA}). Two half-Lar boxes located between the Lar box and the -35 box of P_{larA} promote LarR multimerization on DNA, and point mutations within one or both half-Lar boxes inhibit P_{larA} induction by L-lactate. Gel retardation and footprinting experiments indicate that L-lactate has a positive effect on the binding and multimerization of LarR, while D-lactate antagonizes the positive effect of L-lactate. A possible mechanism of LarR regulation by lactate enantiomers is proposed.

Proteins of the Crp-Fnr family are highly versatile transcriptional regulators that respond to a broad spectrum of signals, such as cyclic AMP (cAMP), anoxia, redox state, oxidative and nitrosative stress, nitric oxide, carbon monoxide, 2-oxoglutarate, or temperature. They are involved in the control of multiple processes, including virulence, stress response, nitrogen fixation, photosynthesis, and various catabolic pathways (1). Members of the Crp-Fnr family are characterized by the presence of two domains: a C-terminally located helix-turn-helix (HTH) DNA binding domain that usually recognizes a 14- to 22-bp palindromic DNA sequence (1) and an N-terminal β -barrel domain that is responsible for dimerization and signal integration (2). Signal integration can involve prosthetic groups attached to the protein (1).

A gene encoding a transcriptional regulator of the Crp-Fnr family, *larR*, has been discovered in the lactate racemization (*lar*) locus of *Lactobacillus plantarum*, a homofermentative lactic acid bacterium that produces lactate (Lac) as the main fermentation product (3). The *lar* locus is composed of two divergent operons: (i) the *larABCDE* (*larA-E*) operon encodes the lactate racemase (LarA), its accessory proteins (LarBCE), and a lactic acid channel (LarD), and (ii) the *larR(MN)QO* (*larR-O*) operon encodes the LarR regulator and an ABC-type nickel transporter composed of a substrate binding protein [Lar(MN), in which the M and N components are fused], a permease (LarQ), and an ATP binding protein (LarO) (Fig. 1A) (3). The lactate racemase is a nickel-dependent enzyme requiring activation by the accessory protein LarE, which itself requires activation by the accessory proteins LarB and LarC and nickel (3). LarD is an aquaglyceroporin that was shown to transport both lactate isomers (4). This lactic acid channel is involved in the racemization of extracellular lactate and lactate metabolism (4). The Lar(MN)QO product was proposed to be a nickel transporter since its inactivation abolished lactate racemization, which could be restored by nickel supplementation in the extracellular medium (3). Concerning LarR, it was suspected to be involved in the transcriptional control of *lar* genes, a function that was not yet investigated (3).

LarR belongs to the PrfA group (see Fig. S1 in the supplemental material), a branch of the Crp-Fnr family mostly linked to pathogenicity (1). The lead member of the group, PrfA from *Listeria monocytogenes*, binds a 14-bp palindromic sequence (5'-TTAAC ANNTGTAA-3'), the PrfA box (5). The effector that activates PrfA is currently unknown but has been proposed to be a host-derived small-molecule second messenger (6). Other members of the PrfA group are the streptococcal regulator of virulence Srv (7), the putative virulence factor Ers of *Enterococcus faecalis* (8), and the acid stress responsive factor RcfB of *Lactococcus lactis* (see Fig. S1) (9). Although the effector of these transcriptional regulators remains unknown, Srv and Ers have been shown to bind DNA *in vitro* (10, 11). The last known member of the PrfA group is the

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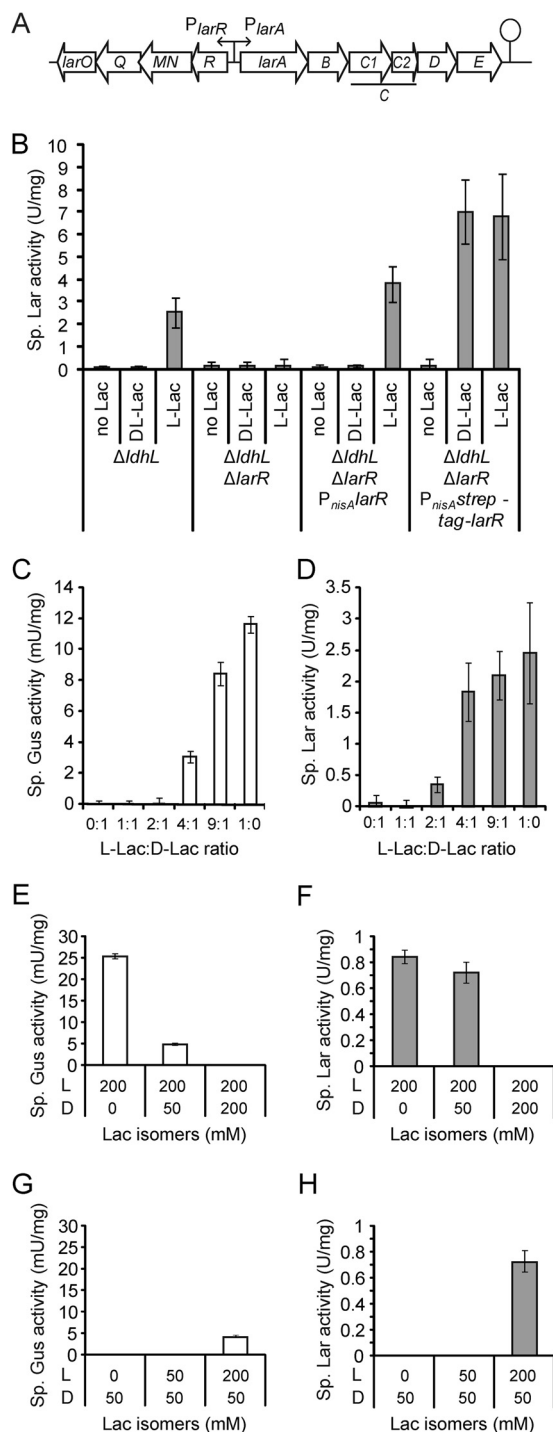


FIG 1 *In vivo* LarR regulation. (A) Schematic representation of the *lar* locus of *Lactobacillus plantarum*, which comprises the *larA-E* operon, responsible for the lactate racemization activity, and the *larR-O* operon, coding for LarR and for an ABC-type nickel transporter. (B) Specific lactate racemase (Lar) activity of the *L. plantarum* Δ *ldhL* mutant (control), the double Δ *larR* Δ *ldhL* mutant, and the Δ *larR* Δ *ldhL* mutant complemented with *larR* (P_{nisA} -*larR*) or StrepII-*larR* (P_{nisA} -strep-tag-*larR*). Complementations were performed in the presence of the nisin inducer. The activity was measured after induction with 200 mM NaCl (no Lac), 200 mM Na-DL-Lac (DL-Lac), or 200 mM Na-L-Lac (L-Lac), as described in Materials and Methods. (C and D) Specific β -glucuronidase (Gus) activities (C) and specific lactate racemase (Lar) activities (D) of the *L. plantarum* Δ *ldhL* mutant expressing the *larA* promoter-*gusA* fusion (P_{larA} -*gusA*) measured after induction with 200 mM Na-Lac at different L-/D-Lac

redox regulator of *Bacillus subtilis*, Fnr_{Bac}, which responds to the oxygen tension (12).

The presence of LarR in the *lar* locus suggests its involvement in the regulation of the lactate racemase (Lar) activity. In *L. plantarum*, the lactate racemase is used as a rescue pathway for the production of D-lactate (D-Lac), which is essential for peptidoglycan biosynthesis (13). The Lar activity of *L. plantarum* has been shown to depend on the L-lactate (L-Lac)/D-Lac ratio: L-Lac induces Lar activity, while the DL-Lac racemic mixture does not, which suggests repression by D-Lac (13). In this study, we investigate the enantioselective regulation of lactate racemization by the LarR regulator *in vivo* using genetic fusions and *in vitro* by analyzing regulator interactions with its DNA targets. We show that LarR is an activator that controls the transcription of Lar-encoding genes by responding differentially to lactate enantiomers: L-Lac acts as a positive effector, while D-Lac antagonizes its effect.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. Plasmid constructions were performed in *Escherichia coli* TOP10 for pUC18Ery (14) and pSIP409 derivatives (15) and in *L. lactis* NZ3900 (16) for pNZ8048 derivatives (17). *E. coli* was grown in Luria broth (LB) at 37°C with aeration, *Lactococcus lactis* was grown in M17 medium (Merck, Germany) supplemented with 0.5% glucose at 28°C at 120 rpm, and *Lactobacillus plantarum* was grown in MRS (De Man-Rogosa-Sharpe) broth (Difco Laboratories, Inc., Detroit, MI) at 28°C without shaking. When appropriate, chloramphenicol and erythromycin were added to the medium at 10 μ g ml⁻¹ for *L. lactis* and *L. plantarum*, and erythromycin and ampicillin were added at 250 μ g ml⁻¹ for *E. coli*. For the induction of genes under the control of the *nisA* expression signals in *L. lactis*, nisin A was added during the early log phase (optical density at 600 nm [OD₆₀₀] of 0.2 to 0.3) at a concentration of 1 μ g liter⁻¹, and the cells were collected 4 h later.

General DNA techniques and transformation. General molecular biology techniques were performed according to the instructions given by Sambrook and Russell (18). Electrotransformation of *E. coli*, *L. plantarum*, and *L. lactis* were performed as described by Dower et al. (19), Lambert et al. (20), and Holo and Nes (21), respectively. PCR amplifications were performed with Phusion high-fidelity DNA polymerase according to the manufacturer's instructions (Finnzymes, Espoo, Finland). The primers used in this study were purchased from Eurogentec (Seraing, Belgium) and are listed in Table S1 in the supplemental material. Primer extension was performed with primer LP104PE4 as previously described (22).

Plasmid and mutant construction. The *larR* deletion vector pGIR002 was constructed by cloning the upstream and downstream regions of the *larR* gene from *L. plantarum* strain NCIMB8826 and the *lox66-P₃₂-cat-lox71* cassette from plasmid pGIZ850 (23) in plasmid pUC18Ery (see Table S2 in the supplemental material). This suicide vector was used to delete *larR* in *L. plantarum* strain TF101 (24) through a two-step homologous recombination, as previously described (20). The excision of the *cat* gene was then performed by transforming plasmid pNZ5348 harboring the *cre* recombinase, as described previously (20). Confirmation of the deletion was performed by PCR amplification with primers LP096UP1 and LP105B1 (see Table S1).

Strain TF101 Δ *larR* was then transformed with ratios, as described in Materials and Methods. (E to H) Specific Gus activities (E and G) and specific Lar activities (F and H) of the *L. plantarum* Δ *ldhL* mutant expressing P_{larA} -*gusA* after induction with 200 mM Na-L-Lac (E and F) or 50 mM Na-D-Lac (G and H) supplemented with 0, 50, or 200 mM Na-D-Lac (E and F) or Na-L-Lac (G and H). The values shown are the mean results of 3 repetitions from 1 significant experiment out of 2 experiments showing similar results. The error bars indicate the confidence intervals at 95% (Student's *t* test).

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s) ^a	Source or reference
<i>L. plantarum</i> strains		
NCIMB8826	Wild type	NCIMB ^b
TF101	NCIMB8826 Δ ldhL	27
LR0002	NCIMB8826 Δ ldhL Δ larR nisRK	This study
<i>L. lactis</i> NZ3900	MG1363 derivative	19
<i>E. coli</i> TOP10	F [−] mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 Δ lacX74 nupG recA1 araD 139 Δ (ara-leu)7697 galE15 galK16 rpsL(Str ^r) endA1 λ [−]	Invitrogen
Plasmids		
pMEC10	Em ^r Amp ^r ; pMC1 derivative for integration of the nisRK genes at tRNA ^{Ser}	28
pUC18Ery	Em ^r Amp ^r ; pUC18 derivative with a 1.1-kb insert containing the erm gene	17
pGIZ850	Em ^r Amp ^r Cm ^r ; pUC18Ery with a 4.215-kb insert containing the loxD gene disrupted by a cat gene under the control of the P32 promoter	26
pNZ5348	Em ^r ; pGID023 derivative containing cre under the control of the lp ₁₁₄₄ promoter	23
pGIR002	Em ^r Amp ^r ; pUC18Ery with a 4.32-kb insert containing the lox66-P32-cat-lox71 cassette from pNZ5319 surrounded by the larR upstream and downstream regions	This study
pBADHisA	Amp ^r P _{araB}	Invitrogen
pGIR991	Amp ^r ; pBADHisA with a 0.82-kb insert containing the P _{araB} -StrepII-larR translational fusion	This study
pNZ8048	Cm ^r P _{nisA}	20
pGIR090	Cm ^r ; pNZ8048 derivative with a 0.78-kb insert containing the P _{nisA} -larR translational fusion	This study
pGIR091	Cm ^r ; pNZ8048 derivative with a 0.82-kb insert containing the P _{nisA} -StrepII-larR translational fusion	This study
pSIP409	Em ^r ; low-copy-no. gusA reporter plasmid	18
pGIR003	Em ^r ; pSIP409 derivative with the P _{larA} -gusA translational fusion	This study
pGIR003B	pGIR003 harboring one point mutation in the Lar box (mutation RRb)	This study
pGIR003C	pGIR003 harboring one point mutation in the half-Lar box 1 (P _{larA} side 1, mutation RRc)	This study
pGIR003D	pGIR003 harboring two point mutations in half-Lar boxes 1 and 2 (P _{larA} side 1 and 2, mutations RRc and RRd)	This study

^a Em^r, Amp^r, and Cm^r indicate resistance to erythromycin, ampicillin, and chloramphenicol, respectively.

^b NCIMB, National Collections of Industrial and Marine Bacteria, Ltd., Aberdeen, Scotland.

pMEC10 in order to integrate the nisRK genes at the tRNA^{Ser} locus (25), generating strain LR0002.

Plasmid pGIR090 (P_{nisA}-larR fusion) was constructed by cloning the larR gene from *L. plantarum* NCIMB8826, which was amplified by PCR with the primers Lp1031 and Lp1032 (see Table S1 in the supplemental material), digested with NcoI and KpnI, and ligated into a similarly digested pNZ8048. The resulting plasmid was transformed into *L. lactis* strain NZ3900. Plasmid pGIR991 (P_{araB}-StrepII-larR fusion) was constructed by cloning the larR gene from *L. plantarum* NCIMB8826 fused to the genetic material encoding the 8-residue minimal peptide sequence (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) called Strep-tag II (referred to herein as StrepII) at the N terminus, which was amplified by PCR with primers LP103OXA1 and LP103OXB1 (see Table S1), digested with NcoI and EcoRI, and ligated into a similarly digested pBADHisA. The resulting plasmid was transformed into *E. coli* TOP10. The plasmid pGIR091 (P_{nisA}-StrepII-larR fusion) was constructed by the insertion of the StrepII-larR fusion from pGIR991 between the NcoI and HindIII restriction sites of pNZ8048. *E. coli* TOP10 containing plasmid pGIR991 was used for StrepII-tagged LarR (referred to herein as rLarR) purification. Plasmids pGIR090 (P_{nisA}-larR fusion) and pGIR091 (P_{nisA}-StrepII-larR fusion) were electrotransformed into strain LR002 for complementation studies. *L. lactis* NZ3900 harboring plasmid pGIR090 (P_{nisA}-larR fusion) was used to prepare total cell extracts containing the untagged version of LarR for electrophoretic mobility shift assays. The plasmids were confirmed by sequencing the P_{nisA}-larR and P_{nisA}-StrepII-larR fusions with primer UP_PNZ8048 (see Table S1).

Plasmid pSIP409 with gusA as a reporter gene was used as a backbone for promoter regulation studies. Plasmid pGIR003 (P_{larA}-gusA fusion) was constructed by cloning the larR-larA intergenic region from *L. plantarum* NCIMB8826, which was amplified with primers PSIP103-104A1/

PSIP103-104B2 (see Table S1 in the supplemental material), digested with NcoI and BamHI, and ligated into a pSIP409 plasmid digested with NcoI and BglII. The plasmids were then purified from *E. coli* TOP10 and electrotransformed into *L. plantarum* TF101 (Δ ldhL mutant, exclusive D-Lac producer). Mutations of the larR-larA intergenic region were performed with the QuikChange protocol on plasmid pGIR003 (26), generating plasmids pGIR003B, pGIR003C, and pGIR003D. Mutations were confirmed by sequencing P_{larA}-gusA fusions with primers CAT1, GUSA2, and GUSB2 (see Table S1).

Complementation studies. Cells from a 10-ml culture of *L. plantarum* TF101 (Δ ldhL), *L. plantarum* LR0002 (Δ ldhL Δ larR) harboring pNZ8048 (empty vector), *L. plantarum* LR0002 harboring pGIR090 (P_{nisA}-larR), or *L. plantarum* LR0002 harboring pGIR091 (P_{nisA}-StrepII-larR) were grown until reaching an OD₆₀₀ of 0.5. For larR induction, nisin A (Sigma-Aldrich, Belgium) was added at a final concentration of 50 ng ml^{−1}. After 1 h of incubation in the presence of nisin, NaCl (control), L-Lac, or D-Lac (lactate sodium salts; Sigma-Aldrich, Belgium) was added to a final concentration of 200 mM. After 4 h of additional incubation, cells were collected by centrifugation at 5,000 × g for 10 min and washed twice with 10 ml of 60 mM Tris-maleate buffer, pH 6.0 (TM buffer), using the same centrifugal conditions.

Electrophoretic mobility shift assays (EMSA). ³²P-radiolabeled primers were obtained by incubating 0.25 nmol of oligonucleotide with 0.4 nmol of [γ -³²P]ATP and 20 units of T4 polynucleotide kinase (Roche Diagnostics, Belgium) in 1 × T4 polynucleotide kinase buffer for 1 h at 37°C. The reaction was stopped by an incubation of 20 min at 70°C, and primers were purified from the excess [γ -³²P]ATP with MicroSpin G-25 columns (GE Healthcare, France). Short DNA probes (30 and 56 bp) were constructed by annealing complementary pairs of radiolabeled oligonucleotides as describe by Sambrook and Russell (18). Longer DNA probes

(127 or 256 bp) were obtained by PCR amplification using ^{32}P -radiolabeled primers and genomic DNA from *L. plantarum* NCIMB8826 as a template (see Table S1 in the supplemental material). Mapping of the different probes on the DNA sequence of the *larR-larA* intergenic region is shown in Fig. S2 in the supplemental material. PCR products were purified using the QIAquick PCR purification kit (Qiagen, France). Binding reactions were conducted at room temperature for 15 min in TE buffer (100 mM Tris-HCl, pH 7.5, and 1 mM EDTA) in the presence of various amounts of rLarR or untagged LarR and the radiolabeled probe (~ 30 pmol). Nonspecific DNA [sonicated salmon sperm DNA or poly(dI-dC)] was not incorporated in the binding reaction mixtures since it has no impact on the formation of rLarR-DNA complexes. For the specificity experiments, 15 ng of rLarR were incubated with a ^{32}P -radiolabeled probe (127 bp) of the *larR-larA* intergenic region of *L. plantarum* mixed with either the same cold probe (specific DNA) or a 137-bp probe of the *larA-larB* intergenic region of *L. plantarum* (nonspecific DNA), obtained by PCR amplification using oligonucleotides LarA-A_A and LarB-B_B (see Table S1). Bound products were separated from free DNA on 6% native polyacrylamide gel (40% acrylamide-Bis-acrylamide 19:1 solution; Bio-Rad) in $1\times$ Tris-borate-EDTA (TBE) buffer. Gels were run at 200 V for 2 to 3 h at 4°C. The gels were revealed with an Imaging Screen-K (Bio-Rad, Belgium) and read on a Pharos FX Plus (Bio-Rad, Belgium). The quantification of the complexes was performed by densitometry using Quantity One software (Bio-Rad, Belgium).

DNase I footprinting assays. Binding reactions were performed at room temperature for 15 min in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 25 mM MgCl_2 buffer. Three units of DNase I (Sigma-Aldrich, Belgium) were added, and the binding reaction mixtures were incubated for 5 min at 30°C. To stop the reaction, 1.5 volumes of stop buffer (20 mM EDTA, 1% SDS, 200 mM NaCl) was added to the reaction mixture. The DNA was precipitated with ethanol, and the pellet was washed three times with 70% ethanol and resuspended in 5 μl loading buffer (80% formamide, 5 M urea, 0.1% bromophenol blue, 0.1% xylene cyanol). The reaction mixtures were heated for 10 min at 100°C, loaded on a 6% denaturing sequencing gel containing 5 M urea, and separated at 75 W for 2 h at room temperature. Marker lanes were loaded with Maxam and Gilbert sequencing reaction mixtures, prepared from the DNA probe as described by Sambrook and Russell (18). The gel was revealed with an Imaging Screen-K (Bio-Rad, Belgium) and read on a Pharos FX Plus (Bio-Rad, Belgium).

Lactate racemase specific activity. Cells were resuspended in 0.5 ml of TM buffer (60 mM Tris-maleate buffer, pH 6.0) and lysed with 0.17- to 0.18-mm glass beads (Sartorius Mechatronics, Belgium) in a FastPrep-24 (MP, Belgium), using 2 runs of 1 min at 6.5 m/s. After lysis, the supernatant was collected by centrifugation at $13,000\times g$ at 4°C for 15 min.

The Lar activity was assayed by incubating cell extracts diluted 50-fold with 20 mM D- or L-Lac (sodium salts) in 60 mM morpholineethanesulfonic acid (MES) buffer, pH 6, at 35°C for 10 min. The reaction was stopped by incubating the mixture for 10 min at 90°C. Lactate conversion was measured using the D-lactic acid/L-lactic acid UV test (R-Biopharm, Germany). The protocol was adapted to 100- μl reaction mixture volumes. The lactate conversion was monitored in 96-well, half-area microplates (Greiner, Alphen a/d Rijn, the Netherlands) by reading the absorbance at 340 nm with a Varioskan Flash multimode reader (ThermoScientific). The protein concentrations were measured with a commercial Bradford protein assay (Bio-Rad, Norway). One unit of Lar activity is defined as 1 μmol of lactate converted in 1 min.

β -Glucuronidase activity. Cells from 10-ml cultures of *L. plantarum* TF101 carrying pGIR003 ($P_{\text{larA-gusA}}$) were grown until reaching an OD_{600} of 0.7. The cells were then collected by centrifugation at $5,000\times g$ for 10 min and resuspended in fresh MRS medium containing 200 mM lactate at different L-/D-Lac ratios. After 4 h of additional incubation, the culture was split in two, and half of the cells were collected by centrifugation at $5,000\times g$ for 10 min and washed twice with 10 ml of TM buffer using the same centrifugal conditions. These cells were assayed for their

Lar activity, as described above. The other half of the cells were collected by centrifugation at $5,000\times g$ for 10 min and washed twice with 10 ml of 50 mM P_i buffer, pH 7.5, containing 10 mM β -mercaptoethanol (PB buffer), using the same centrifugal conditions. Cells were resuspended in 0.5 ml PB buffer and lysed with glass beads as described above. After lysis, the supernatant was collected by centrifugation at $13,000\times g$ at 4°C for 15 min. The β -glucuronidase (Gus) specific activity was measured by incubation of *L. plantarum* cell extracts diluted 2-fold in Gus buffer (2 mM *p*-nitrophenyl- β -D-glucuronide [Sigma-Aldrich, Belgium] in PB buffer) (27). The reaction was monitored by reading the absorbance at 405 nm every 5 min for 50 min with a Varioskan Flash multimode reader. The protein concentration was measured as described above. One unit of Gus activity is defined as 1 μmol of *p*-nitrophenyl- β -D-glucuronide hydrolyzed in 1 min.

Purification of StrepII-LarR. One liter of *E. coli* TOP10 cells expressing the StrepII-*larR* fusion were induced by adding L-arabinose at a final concentration of 0.2% at the early log phase (OD_{600} of 0.3 to 0.4). Cells were collected 3 h later by centrifugation at $5,000\times g$ for 10 min and washed twice with wash buffer (150 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 1 mM EDTA). Cells were then resuspended in 9 ml wash buffer containing 0.1 mg ml^{-1} lysozyme, incubated for 30 min at 37°C, and lysed by sonication 6 times for 10 s, with a 50-s rest between each run, in a Bioruptor (Diagenode, Belgium). Cell extracts were loaded on a Strep-Tactin high-affinity column (IBA, Germany), and the purification of StrepII-LarR (rLarR) was performed as described previously (28). Purified rLarR fractions contained a major form and a minor form of higher electrophoretic mobility (see Fig. S3 in the supplemental material). Both forms were identified as LarR by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis (see Table S2), suggesting that the minor form resulted from proteolytic cleavage of rLarR.

Bioinformatics analyses. LarR homology searches were performed with BLASTP against the nonredundant protein sequence database from NCBI (release 193) using default parameters. A cutoff E value of 10^{-20} was used in order to only select sequences most similar to LarR of *L. plantarum*. When LarR homologues were found in different strains within one species, only one representative strain was retained. The adjacent genes were then searched for homologous *lar* genes using BLASTP. The most probable LarR candidates controlling lactate racemization were selected based on their high similarity with LarR ($>40\%$ sequence identity) and on their synteny with the *larA* gene, encoding the lactate racemase. Data referring to LarR homologues and their genetic analyses are summarized in Table S3 in the supplemental material.

For the *in silico* analysis of LarR binding sites, *larR-larA* intergenic regions were retrieved from the genome sequences of the 20 identified species where *larR* and *larA* genes are adjacent. Sequences similar to the PrfA box (5) were searched visually and are referred to as Lar boxes (see Fig. S4 in the supplemental material). The sequences of the 20 identified Lar boxes were used to define a consensus Lar box sequence, which was then used to identify half-Lar boxes in the regions adjacent to the initial Lar box (see Fig. S4). Logos were generated with WebLogo 3 (<http://weblogo.berkeley.edu/>) using the 20 Lar boxes identified previously, the 7 PrfA boxes of the core PrfA regulon (29), and 4 ACiD boxes (RcfB binding sites), two previously reported (9) and two identified in *Lactococcus garvieae* (see Table S3).

The genome of *L. plantarum* was searched for instances of the Lar box using the Patser software (http://rsat.ulb.ac.be/patser_form.cgi) (30). The position-specific scoring matrix used as the query was the frequency matrix constructed with the 20 identified Lar boxes. Only putative Lar boxes located in intergenic regions with scores higher than $10[\ln(P) < -12]$, where P is the probability value] were considered.

RESULTS

LarR is a positive regulator of the lactate racemase activity in *L. plantarum*. Since previous transcriptome analyses showed that

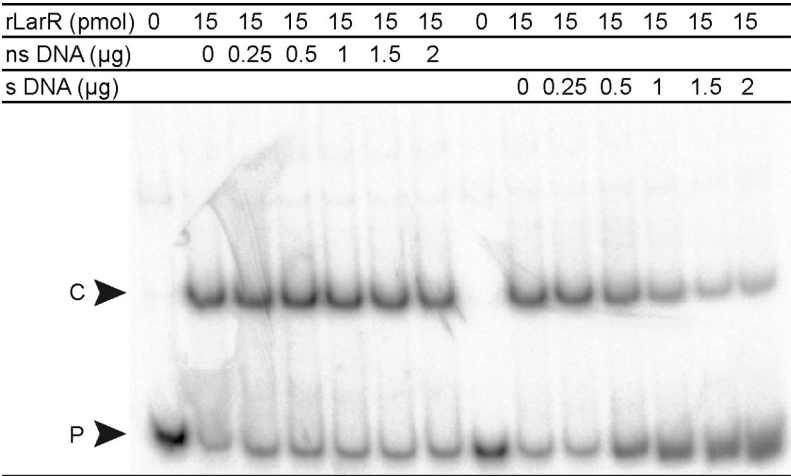


FIG 3 Specificity of rLarR interaction with the *larR-larA* intergenic region. Electrophoretic mobility shift assay (EMSA) of a 127-bp ³²P-radiolabeled probe of the *larR-larA* intergenic region of *L. plantarum* in the presence of purified rLarR and increasing amounts of nonspecific DNA (ns DNA; *larA-larB* intergenic region, 137 bp) or specific DNA corresponding to the same unlabeled probe (s DNA). The LarR-DNA complex (C) and unbound probe (P) are indicated.

quence of the Lar box is almost identical to the consensus sequences of the PrfA box (5) and the ACiD box that is recognized by RcfB, another member of the PrfA group (Fig. 2A) (9).

To investigate the capacity of LarR to bind the Lar Box, an N-terminally StrepII-tagged LarR variant (referred to herein as rLarR) was constructed and purified to homogeneity by affinity chromatography (see Fig. S3 in the supplemental material) (28). To assess the effect of the StrepII tag on LarR activity, the ability of rLarR to complement the Δ *larR* mutant phenotype was evaluated as reported above for the wild-type LarR. StrepII-tagged LarR was able to restore L-Lac induction of the Lar activity in the LarR-deficient background. However, repression by D-Lac was lost (Fig. 1B). This shows that the StrepII tag at the N terminus of LarR impairs D-Lac inhibition without affecting the ability of LarR to respond to L-Lac. In an attempt to circumvent this issue, the StrepII tag was fused to the C terminus of LarR, but the complementation of the Δ *larR* mutant resulted in constitutive Lar activity, suggesting that both L-Lac activation and D-Lac repression were impaired (data not shown).

To examine rLarR's binding capacity, we performed electrophoretic mobility shift assays (EMSA) with radiolabeled probes of 30 bp containing the Lar box of *L. plantarum* (5'-GTATACATTTGTAAAC-3'), the consensus Lar box (5'-GTAAACATTTGTAAAC-3'), or a randomly shuffled Lar box sequence (5'-GTTCTGATTTATAAAC-3') (Fig. 2B). The presence of rLarR was able to slow the migration of both the Lar box of *L. plantarum* and the consensus Lar box but not that of the randomly shuffled sequence (Fig. 2B). To further confirm the identity of the Lar box, EMSA were performed with probes containing mutated *L. plantarum* Lar boxes. Four positions [G→C(LRa)TATAC→G(LRb)ATTTG→C(RRb)TTAAC→G(RRa)] were either mutated individually (LRa and LRb, left repeat mutants, and RRb and RRa, right repeat mutants) or pairwise (LRa+RRa and LRb+RRb) (see Fig. S5 in the supplemental material). All mutations significantly reduced rLarR binding to the Lar box (2- to 5-fold) compared to its binding to the nonmutated probe (see Fig. S5). Finally, the specificity of rLarR binding to the *larR-larA* intergenic region of *L. plantarum* was assessed by adding increasing amounts of specific DNA

cold probe and nonspecific DNA probe to the binding reaction mixtures (Fig. 3). As expected for a specific interaction between LarR and the *larR-larA* region, the formation of the LarR-DNA complex was negatively affected by unlabeled DNA carrying the *larR-larA* intergenic region, whereas nonspecific DNA had no effect (Fig. 3). Altogether, these results show that rLarR specifically binds to the Lar box motif, which consists of a palindromic sequence of 16 bp.

LarR multimerizes on the *larR-larA* intergenic region. To characterize the *larA* promoter, the transcriptional start (+1) was mapped by primer extension (data not shown), and the upstream putative -35 and -10 boxes corresponding to a vegetative promoter were localized (Fig. 4A). We also identified two half-Lar boxes in the region between the Lar box and the -35 box of P_{larA} (P_{larA} side 1 and 2). Two additional half-Lar boxes were identified in the presumed *larR* promoter (P_{larR} side 1 and 2) (Fig. 4A and B). Half-sites may have a significant regulatory role, especially for the multimerization of regulators on their target sites (32). In order to identify possible high-molecular-weight complexes (HMWC) which would result from such a multimerization, we performed EMSA with increasing concentrations of purified rLarR and a radiolabeled probe containing the entire *larR-larA* intergenic region (see Fig. S2 in the supplemental material for the 256-bp radiolabeled probe). The addition of rLarR at low concentrations retarded the electrophoretic mobility of the DNA probe as a single band (from 0 to 15 pmol) (Fig. 4C), but multiple discrete bands of lower electrophoretic mobility gradually appeared as the amount of rLarR was increased (from 22.5 to 45 pmol of LarR) (Fig. 4C). The specificity of these HMWC was confirmed by a competition experiment with increasing amounts of a specific DNA cold probe in the binding reaction mixtures (data not shown).

To evaluate whether these HMWC may result from rLarR binding to the half-Lar boxes within the DNA probe, a mutagenesis study of the *larR-larA* intergenic region was performed (Fig. 5A). The effects of point mutations on the formation of the different LarR-DNA complexes were studied using EMSA (Fig. 5B and C). Whereas a point mutation in a highly conserved residue of the Lar box itself (Fig. 5A, RRb mutant) strongly decreased the for-

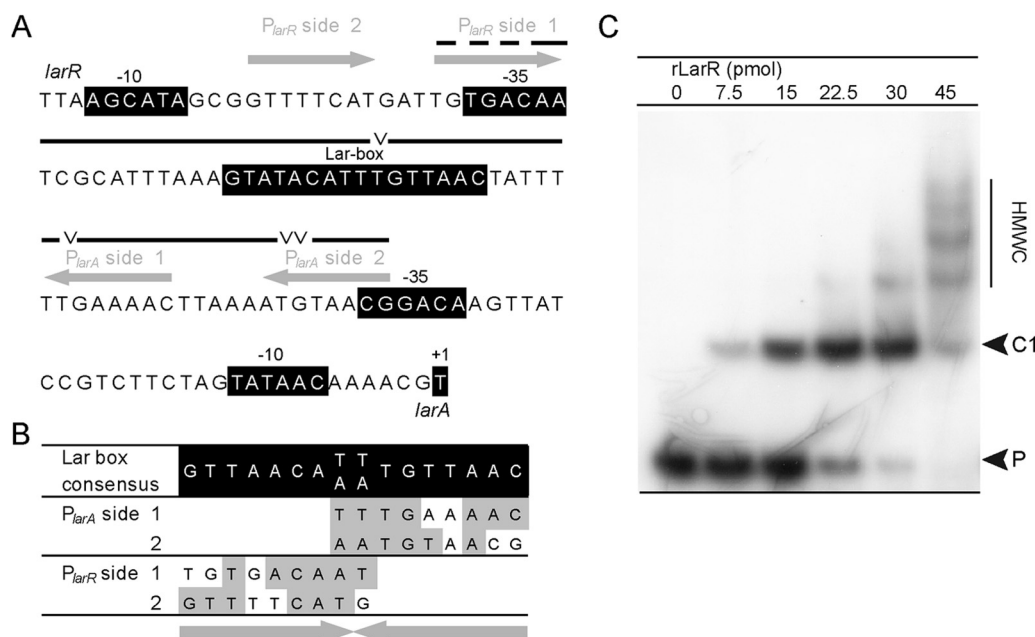


FIG 4 LarR multimerization. (A) DNA sequence of the central part of the *L. plantarum* *larR-larA* intergenic region. The putative -35 and -10 boxes of P_{larR} and P_{larA} , the Lar box, and the mapped transcriptional start (+1) of *larA* are highlighted in black. Protected regions in footprinting assays (lines) and sites displaying enhanced sensitivity to DNase I upon binding of LarR (V), such as are displayed in Fig. 6C, are indicated above the sequence. The half-Lar boxes surrounding the central Lar box are shown by arrows above the sequence. (B) Comparison of the half-Lar box sequences with the consensus Lar box sequence. (C) EMSA of a 256-bp 32 P-radiolabeled probe of the *larR-larA* intergenic region of *L. plantarum* in the presence of increasing amounts of purified rLarR. The lower-molecular-weight rLarR-DNA complex (C1), unbound probe (P), and high-molecular-weight complexes (HMWC) are indicated.

mation of all complexes compared to the results for the native DNA probe (wild type [WT]) (Fig. 5B and C), mutations at the corresponding position in one or both of the half-Lar box motifs located at the *larA* side (RRc and RRc+RRd mutants) (Fig. 5A) only had a minor effect on rLarR binding (Fig. 5B and C). However, a significantly lower abundance of HMWC (complexes 2 and 3, called C2+3) was observed with the mutated probes RRc and RRc+RRd than with the WT (Fig. 5B and C), while the abundance of the main, lower-molecular-weight complex (named C1), was increased (Fig. 5B and C). The ratios between HMWC and complex C1 decreased strongly when half-Lar box motifs were mutated (Fig. 5D). This analysis suggests that complex C1 corresponds to LarR binding to the central Lar box, while more retarded bands arise from occupation of the flanking half-Lar boxes. In addition, these results show that the formation of all rLarR-DNA complexes is primarily dependent on the central Lar box, whereas half-Lar boxes enhance the formation of HMWC but are not required for the formation of the initial complex C1.

To investigate whether multimerization of LarR on the intergenic region is required for the regulation of the *larA-E* operon *in vivo*, mutated promoters were inserted upstream from the *gusA* reporter gene for measurements of transcriptional induction by L-Lac. Mutations of the conserved residue of the Lar box totally suppressed the L-Lac-induced Gus activity, in agreement with a central role for the Lar box in the transcriptional regulation of expression of *lar* genes (Fig. 5E, RRb). A similar effect was observed in the presence of mutations affecting the half-Lar boxes located at the P_{larA} side (Fig. 5E, RRc and RRc+RRd), demonstrating that LarR multimerization via the half-Lar boxes is required for induction by L-Lac.

L-Lac enhances binding and multimerization of LarR. We

first investigated the effect of L-Lac alone on the binding activity of rLarR (Fig. 6). EMSA showed that L-Lac stimulates the formation of the lower-molecular-weight complex C1 in the presence of a small amount of rLarR (Fig. 6A and B) and enhances HMWC at a higher rLarR concentration (Fig. 6A). To investigate whether enhanced HMWC formation in the presence of L-Lac results from rLarR binding to different sites along the intergenic region, we performed a DNase I footprinting experiment on a 127-bp radiolabeled PCR product which contains the region between the transcription start sites of P_{larR} and P_{larA} (+1 of P_{larR} was deduced from sequence analysis) (Fig. 6C and D; see also Fig. S2 in the supplemental material). When this probe was incubated in the presence of rLarR, the Lar box region was protected from DNase I degradation. With increasing concentrations of rLarR, the protection extended toward regions adjacent to the Lar box. Although protection was already observed in the absence of L-Lac, the addition of L-Lac to the reaction mixtures resulted in a significant extension of the protected regions, nearly spanning the entire intergenic region (Fig. 6C, 30 pmol LarR plus L-Lac, far right lane). Several positions, indicated by arrowheads in Fig. 6C, exhibited enhanced sensitivity to DNase I in the presence of rLarR, possibly reflecting rLarR-induced changes in the local DNA structure (33). One such sensitive position was found in the Lar box motif, as could be expected from rLarR binding at this position (34). Additional sensitive positions were observed in the adjacent regions (Fig. 6C). The rLarR-protected regions in footprinting assays and the positions of sites of enhanced susceptibility to DNase I treatment correlate well with the positions of the 4 putative half-Lar boxes surrounding the Lar box that were reported above (Fig. 4A and 6C). Together, the results of these *in vitro* experiments show the enhancing effect of L-Lac on the DNA binding activity of rLarR

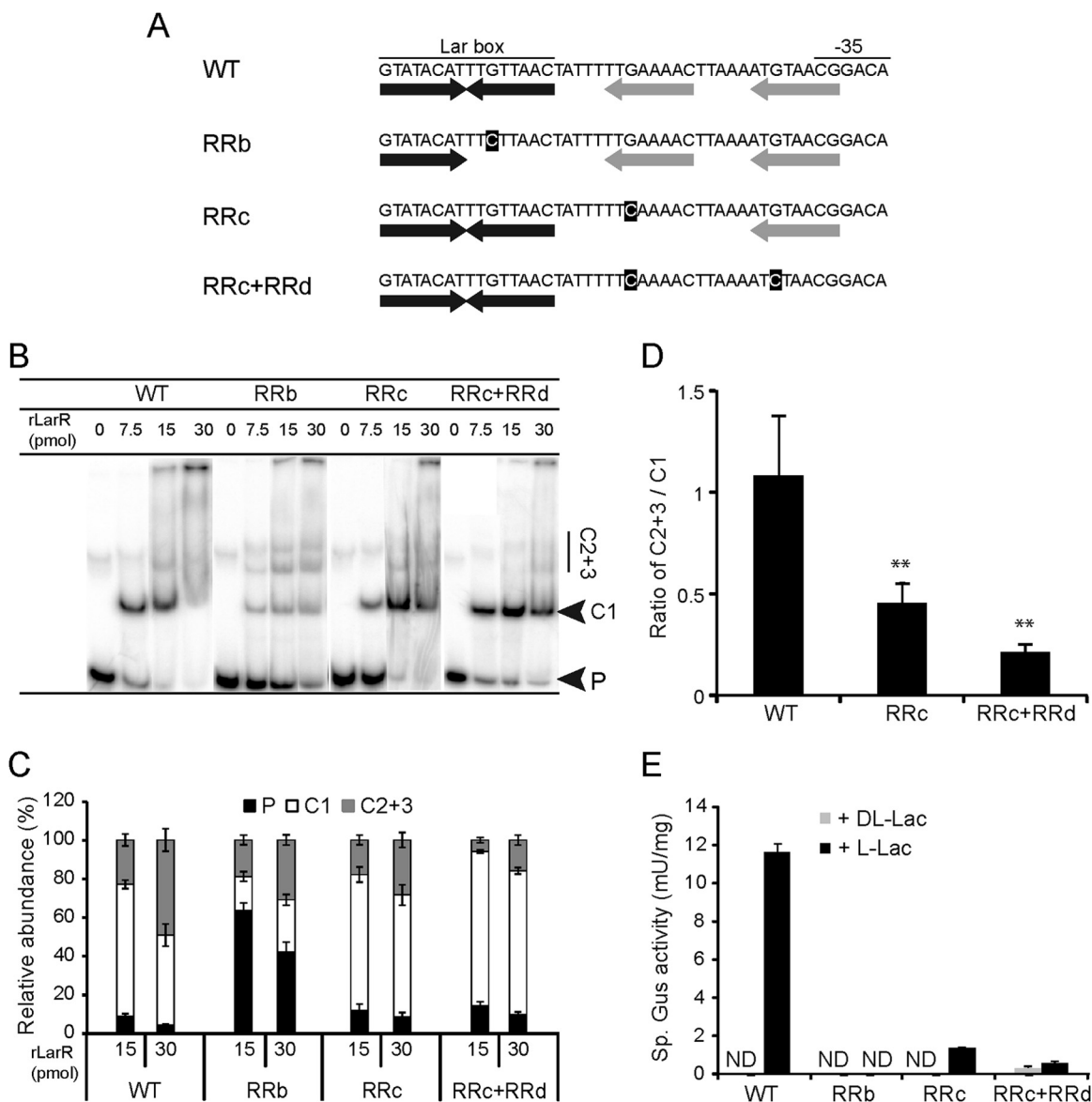


FIG 5 Mutagenesis of the *larR-larA* intergenic region. (A) Native (WT) and mutated DNA sequences (RRb, RRc, and RRc+RRd) of the region, including the Lar box and the -35 box of *P_{larA}*. The inverted repeat of the Lar box (black arrows) and the two half-Lar boxes on the *P_{larA}* side (gray arrows) are shown below the sequence. The mutations in the right repeats are highlighted in black. The arrow corresponding to a mutated right repeat was removed to indicate its alteration. (B) EMSA of 127-bp ³²P-radiolabeled probes of the wild type (WT) and mutated *larR-larA* intergenic regions of *L. plantarum* in the presence of increasing amounts of purified rLarR. The lower-molecular-weight rLarR-DNA complex (C1), unbound probe (P), and high-molecular-weight complexes C2 and C3 (C2+3) are indicated. (C) Relative abundances of P, C1, and C2+3 in EMSA such as that shown in panel B. Data are from 4 replicates run in parallel on the same gel from one representative experiment. The error bars represent the standard deviations. Significance of the results was determined by Student's *t* test: **, *P* < 0.01. (D) Ratios of C2+3 to C1 at 30 pmol of LarR in EMSA such as that shown in panel B. Data are from 4 replicates run in parallel on the same gel from one representative experiment. The error bars represent the standard deviations. Significance of the results was determined by Student's *t* test: **, *P* < 0.01. (E) Gus activities measured with the mutated *larA* promoters after induction by 200 mM DL-Lac or L-Lac. ND, not detected (<0.1 mU). The values shown are the mean results of 3 repetitions from 1 significant experiment out of 2 experiments showing similar results. The error bars give the confidence intervals at 95% (Student's *t* test).

not only on the Lar box itself but also on adjacent regions containing half-Lar boxes, reinforcing the importance of multimerization for LarR activity.

D-Lac antagonizes the L-Lac stimulation effect on LarR binding. Since the D-Lac repression effect was lost with rLarR, we used *Lactococcus lactis* whole-cell extracts expressing the wild-type LarR (no StrepII tag) to investigate the effect of the L-/D-Lac ratio on

LarR binding activity *in vitro* (Fig. 7). At a fixed concentration of 200 mM total lactate (Fig. 7A and B), a positive effect of L-Lac on LarR binding to the *larR-larA* intergenic region was observed, as reported above for rLarR, whereas D-Lac alone and the DL-Lac racemic mixture did not stimulate binding (Fig. 7A and B). EMSA performed at a fixed concentration of L-Lac (200 mM) in the presence of increasing amounts of D-Lac (0, 50, or 200 mM) demon-

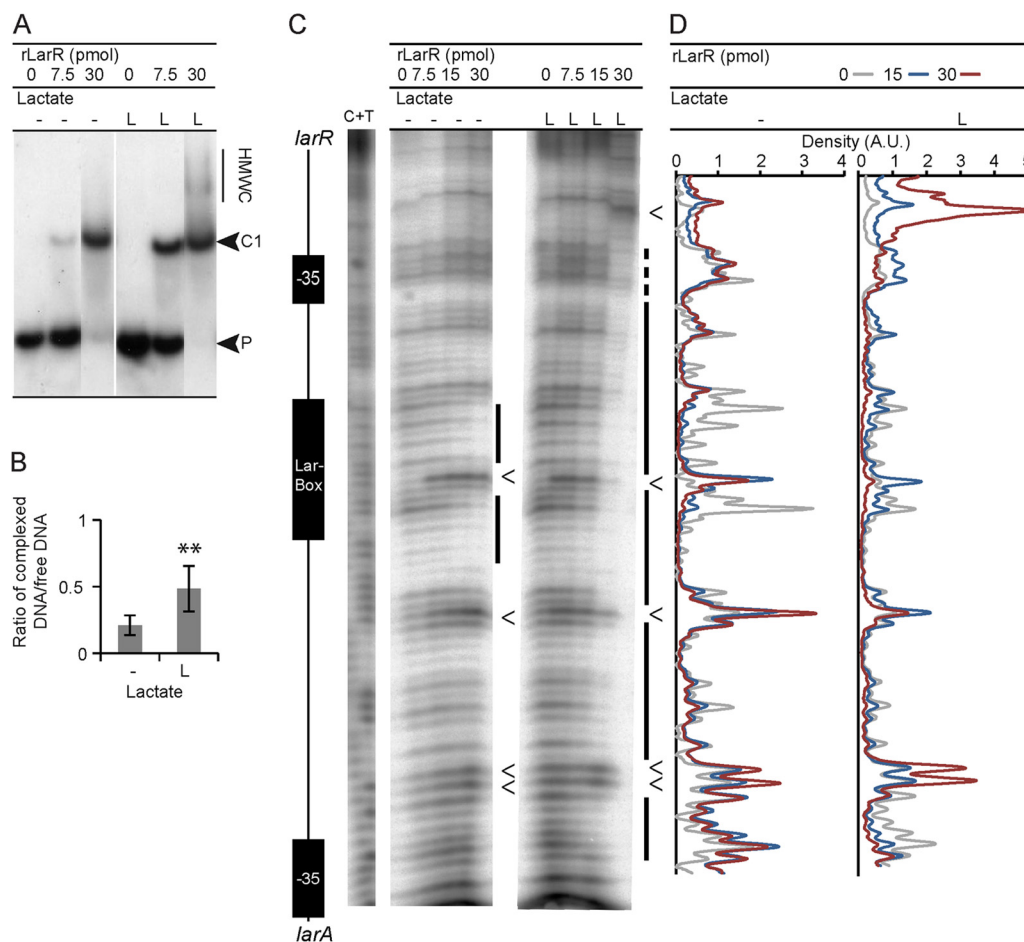


FIG 6 Effect of L-Lac on rLarR DNA binding. (A) EMSA of a 127-bp 32 P-radiolabeled probe of the *larR-larA* intergenic region of *L. plantarum* in the presence of purified rLarR without or with 200 mM L-Lac (L). The lower-molecular-weight LarR-DNA complex (C1), unbound probe (P), and high-molecular-weight complexes (HMWC) are indicated. (B) Mean values of complexed DNA/free DNA ratios from EMSA such as that shown in panel A. Data are from 6 replicates from 1 significant experiment out of 3 experiments showing similar results. The error bars represent the standard deviations. Significance was determined by Student's *t* test: **, $P < 0.01$ compared to the results for the control. (C) DNase I footprint of a 127-bp 32 P-radiolabeled probe of the *larR-larA* intergenic region of *L. plantarum* with increasing amounts of rLarR without or with 200 mM L-Lac (L). The C+T bands correspond to the Maxam and Gilbert sequencing reaction. A schematic representation of the *larR-larA* intergenic region is shown on the left. The lines on the right represent the protected regions, and the arrowheads indicate the enhancements. (D) Density measurements (A.U., arbitrary units) of the DNase I footprint shown in panel C. The areas under the curves of the data series have been standardized in order to be equal. Data are from 1 representative experiment out of 3 experiments showing similar results.

strated that the stimulatory effect of L-Lac on LarR binding is inhibited by D-Lac (Fig. 7C and D).

These *in vitro* assays performed with cell extracts containing the untagged version of LarR suggest that D-Lac may directly or indirectly act as an antagonist of L-Lac in its ability to stimulate LarR binding.

DISCUSSION

Because of the reaction it catalyzes, the lactate racemase of *L. plantarum* only has to be active when there is an imbalance between the two lactate isomers. Accordingly, a previous study demonstrated that Lar activity in *L. plantarum* is only transiently induced upon the addition of exogenous L-Lac and disappears as soon as the excess enantiomer (L-Lac) has been converted into D-Lac. The addition of exogenous D-Lac, however, is unable to induce Lar activity (13). In the present study, we unequivocally demonstrated that the L-/D-Lac ratio is the actual physiological signal that con-

trols Lar expression, as a result of the inducing effect of L-Lac being counteracted by D-Lac (Fig. 1).

The regulation of Lar activity by the D-/L-Lac ratio was shown to occur at the transcriptional level and involves the transcriptional activator LarR, a new member of the PrfA group of regulators belonging to the Crp-Fnr family (see Fig. S1 in the supplemental material). To our knowledge, this is the first report of a member of this family being involved in lactate-dependent regulation. Since the binding of purified LarR was affected by L-Lac *in vitro* without the presence of any additional factor, L-Lac is most probably a direct effector molecule of LarR. Therefore, LarR would be the first regulator of the PrfA group whose effector has been identified. All previously characterized lactate-dependent transcriptional regulators belong either to the GntR (LldR regulators) or LysR (LlpR from *Shewanella oneidensis*) families. Among all lactate-dependent regulators, the regulation pattern of LarR is unique in that one isomer (L-Lac) acts as a positive effector

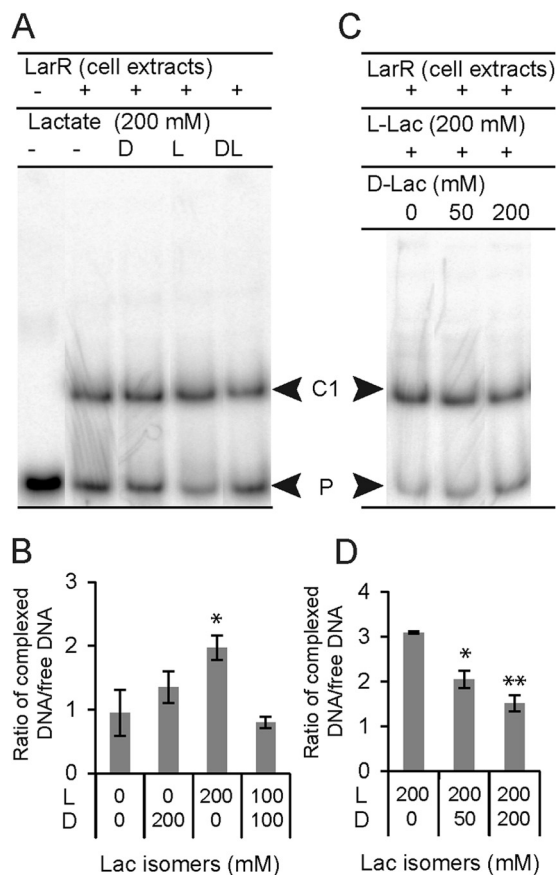


FIG 7 Effects of lactate isomers on untagged LarR DNA binding. (A) EMSA of a 127-bp 32 P-radiolabeled probe of the *larR-larA* intergenic region of *Lactobacillus plantarum* in the presence of *Lactococcus lactis* cellular extracts (2 μ g) containing untagged LarR without or with 200 mM D-Lac (D), L-Lac (L), or DL-Lac (DL, equimolar ratio). The LarR-DNA complex (C) and unbound probe (P) are indicated. (B) Mean values of complexed DNA/free DNA ratios from EMSA such as that shown in panel A. (C) EMSA of a 127-bp 32 P-radiolabeled probe of the *larR-larA* intergenic region of *L. plantarum* in the presence of *L. lactis* cellular extracts (2 μ g) containing untagged LarR with 200 mM Na-L-Lac supplemented with 0, 50, or 200 mM Na-D-Lac. (D) Mean values of complexed DNA/free DNA ratios from EMSA as shown in panel B. Data are from 3 replicates from 1 significant experiment out of 2 experiments showing similar results. The error bars represent the standard deviations. Significance was determined by Student's *t* test: *, $P < 0.05$; **, $P < 0.01$. Effects of lactate isomers are compared to the effect of the control (no lactate).

whereas the other (D-Lac) antagonizes the positive effect of the first. Although some LldR regulators have been described to respond differentially to D- and L-Lac, none were demonstrated to display an antagonistic regulation by the two isomers: in *Escherichia coli* and *Corynebacterium glutamicum*, only L-Lac acts as an inducer of gene expression, whereas D-Lac has no effect (35, 36). Only LlpR from *S. oneidensis* was reported to display a regulation pattern reminiscent of that driven by LarR, with a positive regulation by L-Lac but no induction with DL-Lac; however, the molecular mechanism of this differential regulation was not investigated (37).

All necessary *cis* DNA elements for the LarR-driven regulation of Lar activity were found to be present in the *larA-E* intergenic region. A conserved DNA motif—the Lar box—was identified in this region, which was shown to be essential for the lactate-depen-

dent regulation of P_{larA} via the binding of LarR. The Lar box consensus sequence is nearly identical to those of the PrfA and ACiD (RcfB) boxes (Fig. 2A). As suggested by the palindromic nature of the Lar box, LarR probably binds this motif as a dimer, like most members of the Crp-Fnr family (1). In addition, LarR was also found to bind secondary sites (half-Lar boxes) located between the Lar box and the -35 sequence of the *larA* promoter, resulting in a multimerization of LarR on the promoter region (Fig. 5 and 6). Half-Lar boxes surrounding the Lar box were identified in the *larR-larA* intergenic regions of other species where *larR* and *larA* are syntenic (see Fig. S4 in the supplemental material), suggesting that multimerization may be generalized to other LarR regulators.

The differential roles of the two lactate isomers in Lar activity *in vivo* could be reproduced in our *in vitro* DNA binding experiments: L-Lac stimulated the binding of LarR to the Lar box, whereas D-Lac counteracted this stimulatory effect (Fig. 6 and 7). L-Lac was also demonstrated to stimulate the multimerization of LarR on P_{larA} . Nevertheless, although the L-/D-Lac ratio had a dramatic effect on the Lar activity *in vivo*, the L-Lac-dependent stimulation of LarR binding and multimerization *in vitro* was relatively weak. Moreover, LarR was found to bind the Lar box DNA motif, even in the absence of its effector, and D-Lac did not negatively affect binding. If the only effect of L-Lac was to stimulate the binding and multimerization of LarR, one should expect that the overproduction of LarR alone would activate P_{larA} , even in the absence of L-Lac; however, such an effect was not observed in our complementation experiments, where LarR was overproduced using the nisin-inducible expression system. Altogether, these observations indicate that additional regulatory mechanisms must be involved. This situation is analogous to the behavior of the nitrogen starvation regulator NtcA, a well-characterized Crp-Fnr member that requires 2-oxoglutarate for its activation (38). NtcA binding to its DNA target sequence was previously reported to be only weakly stimulated by 2-oxoglutarate; rather, it was demonstrated that the effector is required for the activation of transcription initiation by NtcA (39). In most cases, regulators of the Crp-Fnr family bind a palindromic motif centered around position -41 relative to the transcriptional start (1), as classically found in class II Crp-dependent promoters (2). Similarly, if both half-Lar boxes at the *larA* side are occupied by LarR, the most downstream LarR dimer would also be centered around position -41 (Fig. 4A). This could allow the direct activation of the RNA polymerase.

Based on our results and on the current knowledge of the activation mechanism of Crp/Fnr members (using NtcA as a reference), we postulate the following model for P_{larA} activation by LarR (Fig. 8). At a high L-/D-Lac ratio, L-Lac would activate LarR through a conformational change, resulting in a stimulation of LarR binding on the Lar box as well as LarR multimerization on the half-Lar boxes. L-Lac would also promote the direct interaction of the proximal LarR dimer with the RNA polymerase, resulting in transcriptional activation. At a low L-/D-Lac ratio, the presence of D-Lac would maintain LarR in an inactive form—for instance, by competing with L-Lac for the same binding site—and, hence, limit LarR binding, multimerization, and interaction with the RNA polymerase.

Regulation of gene expression is one mechanism employed by living organisms to tune energy expenses according to their physiological requirements in changing environmental conditions. Therefore, the regulation pattern of genes associated with a given function can shed light on its physiological role. For *L. plantarum*,

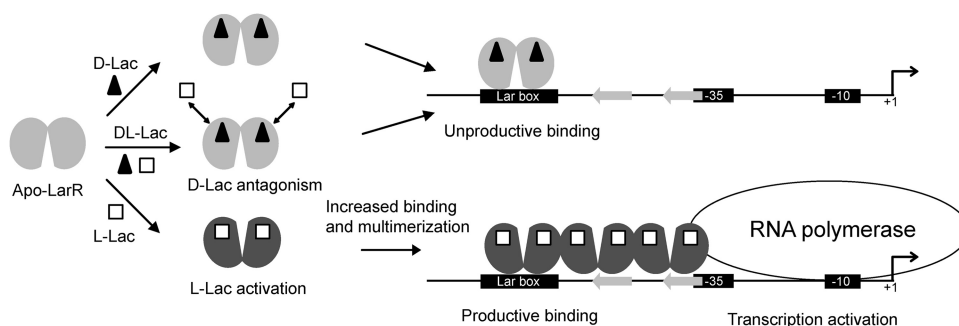


FIG 8 Hypothetical model of P_{larA} regulation by LarR. In the presence of L-Lac, activated LarR binds to the Lar box motif and multimerizes on the half-Lar boxes. This will promote direct interaction of one LarR dimer with the RNA polymerase, resulting in transcriptional activation of the P_{larA} (productive binding). In the presence of D-Lac, D-Lac could block LarR activation, for instance, by impairing L-Lac recognition as illustrated here, which will result in limited LarR binding and multimerization and absence of transcriptional activation (unproductive binding).

we previously published expression data corresponding to the specific regulation pattern performed by LarR (induction by L-Lac but not by DL-Lac) (3). In addition to the *larA-E* and *larR-Q* operons, the *accD2-A2* locus coding for the acetyl-coenzyme A (CoA) carboxylase was found to be similarly regulated (3). We performed an *in silico* search for Lar boxes in the *L. plantarum* genome using the Patser tool (see Materials and Methods), but we failed to identify a LarR binding site upstream from this locus, suggesting that it is not directly under the control of LarR. Five putative Lar boxes were found in intergenic regions outside the *lar* locus (data not shown). However, all of them are located upstream from genes that are unrelated to lactate or carbon metabolism (data not shown), and their LarR binding ability remains to be demonstrated. From these analyses, the function of LarR seems restricted to the induction of Lar in the presence of L-Lac but not in the presence of DL-Lac. Therefore, this unique regulation pattern of Lar implies that only the conversion of L- to D-Lac is physiologically relevant. The role of Lar activity may be linked to the absolute dependency of the peptidoglycan biosynthesis machinery toward D-Lac (13): lactate racemization would ensure D-Lac production even under conditions where the D-Lac dehydrogenase (LdhD) activity is impaired. A possible alternative role of Lar consistent with its regulation pattern would be the substitution of LdhD for the utilization of D-Lac as a carbon and energy source. In agreement with both possible roles, it has been reported that conditions of very slow growth in *L. plantarum* result in a lower abundance of *ldhD* transcripts and higher abundance of *larA-E* transcripts (40).

To conclude, we demonstrated that LarR is a positive regulator that controls Lar expression by sensing the L-/D-Lac ratio. We mapped its DNA binding sites and showed that its multimerization is required for transcription activation. Finally, we showed that both lactate isomers affect its binding, yet in an antagonistic way, which is a unique example of a regulatory circuit where two enantiomers of the same molecule are performing opposite roles on the same regulator. Future work will be dedicated to deciphering the molecular mechanisms involved in the recognition of lactate isomers by LarR.

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