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Dual Role of LldR in Regulation of the *lldPRD* Operon, Involved in L-Lactate Metabolism in *Escherichia coli*▽

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The *lldPRD* operon of *Escherichia coli*, involved in L-lactate metabolism, is induced by growth in this compound. We experimentally identified that this system is transcribed from a single promoter with an initiation site located 110 nucleotides upstream of the ATG start codon. On the basis of computational data, it had been proposed that LldR and its homologue PdhR act as regulators of the *lldPRD* operon. Nevertheless, no experimental data on the function of these regulators have been reported so far. Here we show that induction of an *lldP-lacZ* fusion by L-lactate is lost in an Δ*lldR* mutant, indicating the role of LldR in this induction. Expression analysis of this construct in a *pdhR* mutant ruled out the participation of PdhR in the control of *lldPRD*. Gel shift experiments showed that LldR binds to two operator sites, O1 (positions −105 to −89) and O2 (positions +22 to +38), with O1 being filled at a lower concentration of LldR. L-Lactate induced a conformational change in LldR that did not modify its DNA binding activity. Mutations in O1 and O2 enhanced the basal transcriptional level. However, only mutations in O1 abolished induction by L-lactate. Mutants with a change in helical phasing between O1 and O2 behaved like O2 mutants. These results were consistent with the hypothesis that LldR has a dual role, acting as a repressor or an activator of *lldPRD*. We propose that in the absence of L-lactate, LldR binds to both O1 and O2, probably leading to DNA looping and the repression of transcription. Binding of L-lactate to LldR promotes a conformational change that may disrupt the DNA loop, allowing the formation of the transcription open complex.

The *lldPRD* operon (formerly named *lct*) of *Escherichia coli* is responsible for aerobic L-lactate metabolism. It includes three genes that form a single transcriptional unit inducible by growth in L-lactate. The *lldD* gene encodes the dehydrogenase, *lldP* encodes the permease, and *lldR* encodes a regulatory protein (4). Although L-lactate is also recognized by the permease encoded by *glcA*, the lack of induction of this gene by growth on L-lactate indicates that LldP mediates the uptake of L-lactate in vivo (18, 19).

It has been proposed that the *lldPRD* operon can be transcribed from two promoter sequences (Fig. 1). In this proposal, P1 is responsible for the basal transcription observed under noninducing conditions, and P2 is active only in the presence of L-lactate (13). In addition, the *lldPRD* operon is proposed to be under the control of the global regulator ArcA, which binds to positions −14 to +3 with respect to the transcriptional start site, corresponding to the proposed P2 promoter, and represses the expression of this operon under anaerobic conditions (13).

The LldR protein belongs to the GntR regulator family. This family, named after the repressor of the *Bacillus subtilis* gluconate GntR operon, includes about 270 members, which are distributed among the most diverse bacterial groups and regulate various biological processes (7, 25). The GntR family proteins share amino acid sequence similarities in a 69-residue N-terminal region that determines the DNA binding domain. In contrast, high heterogeneity has been observed among the various C-terminal effector-binding and oligomerization domains. According to structural, phylogenetic, and functional analyses, four subfamilies have been described. LldR belongs to the first subfamily, called FadR, which groups >40% of the GntR regulators (25). Most of the FadR-like proteins are involved in the regulation of oxidized substrates, such as pyruvate (PdhR), gluconate (GntR), glycolate (GlcC), and L-lactate (LldR).

Given the high similarity between the members of the FadR subfamily and the characteristics of their recognition sequences, a model for protein binding has been proposed for this group (25). Members of the FadR subfamily are dimers in solution (16, 24) and bind as dimers to specific palindromic operator sites, with each monomer recognizing a half-site (25, 31). However, at a high protein concentration, GntR of *B. subtilis* is found in a polymerized form (16), which indicates the ability of the GntR-like proteins to oligomerize. In *E. coli*, GntR binds to two operator sites to negatively regulate the transcription of the *gntT* gene. Total repression of *gntT* was suggested to be achieved by DNA looping through interaction between the two GntR molecules (21).

LldR is highly homologous to PdhR (35% identity and 62% similarity overall) in both the amino-terminal and carboxy-terminal domains. PdhR negatively regulates the expression of the *pdhR-aceEF-lpd* operon, involved in the oxidative decarboxylation of pyruvate to acetyl-coenzyme A (22). In the absence of pyruvate, PdhR binds to the palindromic sequence ($^{+11}$AATTGGTaagACCAATT^{+27}) located downstream of the transcriptional start site of the *pdhR* promoter. PdhR repression is antagonized by pyruvate, its effector molecule (23). Recently, *ndh*, encoding NADH dehydrogenase II, and

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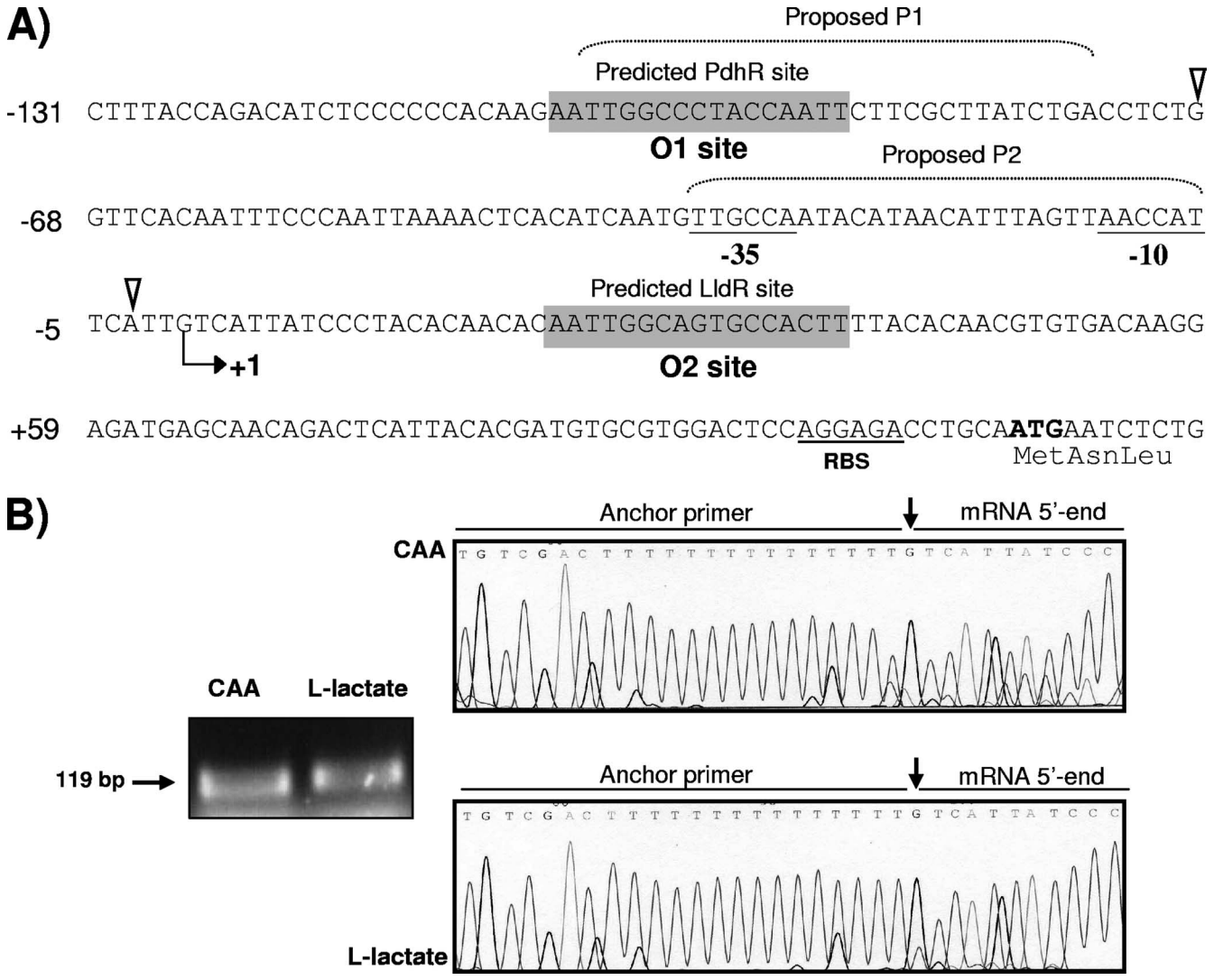


FIG. 1. Organization of transcriptional regulatory elements of the *lldPRD* operon. (A) The *lldPRD* promoter sequence is numbered relative to the 5′ end determined in this study, which is shown by an arrowhead labeled "+1." The −10 and −35 promoter sequences are underlined, and the ribosome binding site (RBS) and predicted ATG start codon (in bold) are indicated. The two previously proposed promoters, i.e., the basal P1 and inducible P2 (13) promoters, and the corresponding transcriptional initiation sites (open triangles) are also indicated above the nucleotide sequence. The predicted PdhR and LldR binding sites (13, 23) are named the O1 and O2 operator sites, respectively. (B) Identification of *lldPRD* 5′ end by sequencing across ligation sites of 5′-RACE products. Chromatograms display the sequences at ligation sites of typical cloned 5′-RACE products derived from transcripts obtained from MC4100 cells grown in CAA or in L-lactate. Arrows indicate the transcription initiation site.

cyoABCDE, encoding the cytochrome *bo* type, were identified as regulation targets of PdhR (20). Comparison of the PdhR binding sites present in different target promoters led to the establishment of ATTGGTNNNACCAT as a consensus sequence for PdhR recognition (20).

On the basis of LldR and PdhR similarity, a computational analysis of potential binding sites in the *lldPRD* promoter was performed by Quail and Guest (23). This analysis identified two sites displaying sequences similar to the PdhR recognition site. Subsequently, Lynch and Lin (13) proposed that the binding site for LldR may be downstream of P1 and P2, while PdhR may interact with the site upstream of these promoters (Fig. 1). According to these locations, PdhR was proposed to be an activator and LldR a repressor of the *lldPRD* operon, although no experimental data were presented by these authors.

In this paper, we provide evidence that *lldPRD* is not under the control of PdhR but is under the control of LldR, which has a dual regulatory function. This protein can act as a repressor or as an activator, depending on the absence or presence of L-lactate in the medium.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strains and plasmids used in this study are listed in Table 1. Cells were grown on Luria broth (LB) or minimal medium and harvested as described previously (1). Where indicated, carbon sources were added at a 60 mM final carbon concentration. Casein acid hydrolysate (CAA) was used at 0.2%. When required, the following antibiotics were used at the indicated concentrations: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 12.5 μg/ml. To grow strains carrying transcriptional fusions, tryptophan was added at 0.1 mM. 5-Bromo-4-chloro-3-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
E. coli strains		
XL1Blue	*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*]	Stratagene
MC4100	F$^-$ *araD* Δ(*argF-lac*) *rpsL*(Str^r) *relA flhD deoC ptsF rbs*	2
JRG2547	*ace*^{c}816 Δ(*lacIPOZYA*)*X74 rpsL*	8
DY330	W3110 Δ*lacU169 gal490* λ*cI857* Δ(*cro-bioA*)	32
TE2680	F$^-$ λ$^-$ IN(*rrnD-rrnE*) Δ*lacX74 rplS galK2 recD*::Tn*10*d*tet trpDC700*::*putA13033*::(Kan^r Cm^r *lac*)	5
CAG12095	*zab3051*::Tn*10*	17
JA217	MC4100 *ace*^{c}816	This study
JA218	MC4100 Δ*lldR*::*cat*	This study
JA219	MC4100 Δ*lldR*::*cat ace*^{c}816	This study
Plasmids		
pRS550	Ap^r Km^r; contains promoterless *lacZYA*	30
pCAT19	Ap^r Cm^r	6
pMALc2X	Ap^r *lacI*^{q}	New England Biolabs

indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were used at 30 μg/ml and 10 μg/ml, respectively.

Mutant strain construction. Strain JA217 (MC4100 *ace*^{c}816) was obtained in this study. The Tn*10* insertion from strain CAG12095 (zab3051::Tn*10*) (17) was introduced into strain JRG2547 (*ace*^{c}816) by P1 transduction. A tetracycline-resistant transductant that retained the *ace*^{c}816 mutation was selected and used to transduce the two markers into strain MC4100. The presence of the *ace*^{c}816 mutation was confirmed by PCR amplification followed by DNA sequencing. Strains JA218 and JA219, carrying a Δ*lldR*::*cat* mutation, were constructed by gene replacement using targeted homologous recombination, as described by Yu et al. (32). The entire *lldR* gene was replaced with a gene that confers resistance to chloramphenicol (*cat*), which was obtained by PCR from plasmid pCAT19 (6). Linear DNA containing the 50-bp homologous sequences flanking *lldR* in its ends was introduced into strain DY329. After homologous recombination, the Δ*lldR*::*cat* mutation was then introduced by P1 transduction into strain MC4100 to yield strain JA218 or into strain JA217 to yield the double mutant (*lldR*::*cat ace*^{c}816) strain JA219.

Enzyme activities and protein measurements. β-Galactosidase activity was assayed by the hydrolysis of o-nitrophenyl-β-D-galactopyranoside and expressed as Miller units (15). For this purpose, cell cultures were grown until mid-log phase (an optical density at 600 nm of 0.5). The data reported are representative of at least four separate experiments performed in triplicate and are expressed as means ± standard deviations (SD). Protein concentration was determined by the method of Lowry et al. (12).

DNA manipulation and site-directed mutagenesis. Bacterial genomic DNA was obtained as described by Silhavy et al. (29). Plasmid DNA was routinely prepared by the boiling method (9). For large-scale preparation, a crude DNA sample was purified on a column (Qiagen). DNA manipulations were performed essentially as described by Sambrook and Russell (26). DNA sequencing (27) was carried out with an automated ABI 377 DNA sequencer. DNA fragments were amplified by PCR, using *E. coli* chromosomal DNA as a template. When necessary, specific restriction sites were incorporated at the 5′ ends of the primers to facilitate the cloning of the fragments into the appropriate vector. PCRs were performed with *Pfu* DNA polymerase under standard conditions. All primers used in this study are available upon request.

Site-directed mutagenesis of *lldR* binding sites in the probes used in the mobility shift assays was performed by PCR, using primers containing the desired mutations. Insertion of 5 bp or 10 bp into the fragment containing the full-length promoter region was done by crossover PCR (11).

Mapping of the 5′ end of the *lldPRD* transcript. The 5′ region of the *lldPRD* transcript was determined by the rapid amplification of cDNA 5′ ends (5′-RACE) (26), using a commercial 5′-RACE kit (Roche Diagnostics, GmbH). Total RNA was isolated from MC4100 cells grown aerobically to an optical density at 600 nm of 0.5 under noninducing conditions (with CAA) or inducing conditions (with L-lactate as the sole carbon source), using a Qiagen RNeasy total RNA kit, and then treated with RNase-free DNase. The cDNA was transcribed from the preparation of RNA with a specific *lldP* antisense oligonucleotide. A homopolymeric dA tail was added (via terminal transferase) to the 3′ terminus of the *lldP* cDNA. Amplification of reverse transcription products was performed with nested *lldP*-specific primers and an oligo(dT) anchor primer. The obtained products were cloned into a pGEM vector for sequencing and subsequent manipulation.

Construction of *lacZ* fusions and deletions of the *lld* promoter region. To create operon fusions, DNA fragments were obtained by PCR and cloned into plasmid pRS550 (30). The pRS plasmids contain a cryptic *lac* operon and genes that confer resistance to both kanamycin and ampicillin. After transformation of strain XL1-Blue, recombinant plasmids were selected as blue colonies on LB plates containing X-Gal, ampicillin, and kanamycin and sequenced using the M13 primer to ensure that no mutation was introduced. Single-copy fusions on the *E. coli* chromosome were obtained as described by Elliot (5), using strain TE2680. The transformants were selected for kanamycin resistance and screened for sensitivity to ampicillin and chloramphenicol. P1 *vir* lysates were made to transduce the fusions into the desired genetic background.

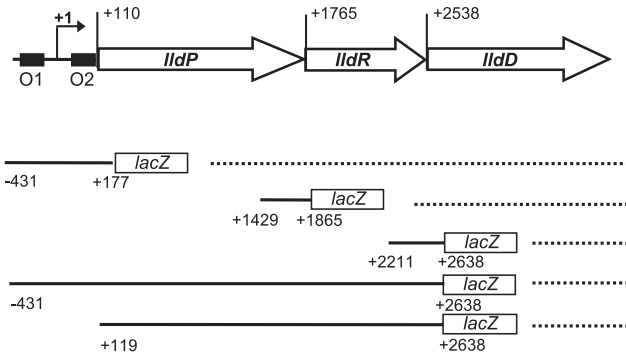
Expression and purification of LldR and PdhR. To overexpress and purify the LldR and PdhR proteins, the corresponding coding regions were amplified by PCR and cloned in-frame into pMAL-c2X (New England BioLabs), resulting in the expression of the protein of interest fused to maltose binding protein (MBP).

The recombinant enzyme was overexpressed in *E. coli* XL1-Blue in the presence of 0.3 mM IPTG. Purification of the MBP fusion proteins was carried out according to an established protocol (New England BioLabs). Purified LldR or PdhR was separated from MBP after proteolytic treatment with factor Xa. MBP-LldR folding made difficult the cleavage of the fusion protein in solution. However, digestion was complete when this proteolytic treatment was applied to the fusion protein bound to the affinity chromatography column. Purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, performed according to a standard procedure (10).

DNA binding studies. A nonradioactive digoxigenin (DIG) gel shift kit for 3′-end labeling of DNA fragments (Roche Diagnostics, GmbH) was used for protein-DNA binding assays. The fragments obtained by PCR were labeled at the 3′ end with terminal transferase and DIG-ddUTP and used in gel shift reaction mixtures according to the manufacturer's instructions. Electrophoretic mobility shift assays were performed with purified proteins. Polyacrylamide gels containing 10% glycerol were run at 4°C using Tris-borate-EDTA buffer. Protein samples were mixed with the labeled probes in a 20-μl reaction volume containing 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 10% glycerol, and 2 mM dithiothreitol. Poly(dI-dC) was used as a nonspecific competitor. Incubation was performed for 15 min at 30°C. Where indicated, L-lactate was added to binding reaction mixtures at increasing concentrations up to 100 mM. Following electrophoretic separation, the oligonucleotide-protein complexes were blotted onto nylon membranes. Chemiluminescence detection of DIG-labeled DNA-protein complexes on the nylon membranes was obtained by exposure to X-ray film.

RESULTS AND DISCUSSION

Promoters in the *lldPRD* genetic system. To determine the functional promoters in the *lld* genetic system, five transcriptional fusions to *lacZ* were constructed (Fig. 2) and transferred into the genetic background of strain MC4100. β-Galactosi-



β-galactosidase activity (Miller units)	
CAA	L-lactate
270±53	2439±238
<10	<10
<10	<10
194±46	1743±186
<10	<10

FIG. 2. Analysis of transcriptional fusions to identify functional promoters in the *lldPRD* genetic system. The extension and direction of the *lld* genes, *lldP* (encoding L-lactate permease), *lldR* (encoding the regulatory protein), and *lldD* (encoding L-lactate dehydrogenase), are indicated by open arrows. The transcriptional start site (position +1) and the two putative operator sites, O1 and O2, are indicated upstream from these genes. Fragments fused to *lacZ* for testing of promoter function are shown below and are numbered relative to the transcriptional start site. These fragments were fused to *lacZ* and introduced as single-copy fusions in the genetic background of strain MC4100. The values for β-galactosidase activity under the different growth conditions are indicated in the table on the right and expressed as means ± SD.

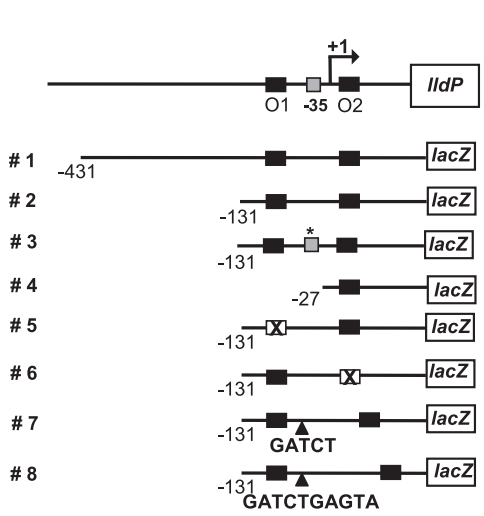
dase activities were measured in cells grown aerobically in CAA or in L-lactate as the sole carbon source. Only the two transcriptional fusions bearing the 5′ upstream region of *lldP* displayed a basal level of expression, of between 194 and 270 Miller units. This level of expression increased around ninefold in cells grown in L-lactate. The three additional constructs displayed negligible β-galactosidase activities under both growth conditions. These results indicate that only the promoter sequences located at the 5′ end of *lldP* are functional and that the three genes of the *lld* system constitute an operon which is inducible by L-lactate. Thus, the transcriptional fusion ϕ(*lldP*::*lacZ*), which contains the −431-to-+177 promoter region, was used for further studies.

Expression of this transcriptional fusion was also analyzed by growth of strain MC4100 in other carbon sources. Basal levels of *lldPRD* expression similar to those obtained with CAA were achieved by growth on D-xylose (205 ± 34 Miller units) or glycolate (271 ± 45 Miller units), while expression of the *lldP*::*lacZ* fusion was repressed by glucose (24 ± 4 Miller units). When L-lactate was added to CAA medium, induction of this fusion was also observed, although β-galactosidase levels (1,200 ± 98 Miller units) were somewhat lower than those obtained with L-lactate as the sole carbon source. This effect, probably due to differences in growth rate, was also seen in other genetic systems when the inducing carbon source (glycolate or L-ascorbate) was used in the presence of CAA (unpublished results).

Mapping of the 5′ end of the *lldPRD* transcript. Two transcriptional start points at the 5′ end of the *lldP* gene have been proposed previously (13) (Fig. 1). In this study, the 5′ end of the *lldPRD* mRNA was determined by the 5′-RACE method (26). Several clones were isolated through 5′-RACE with a nested oligonucleotide cDNA pool derived from MC4100 cells grown in CAA or L-lactate. Analysis of the 5′-RACE products (10 clones for each growing condition) in all cases revealed only one transcriptional initiation site, which was located 110 nucleotides (nt) upstream of the predicted ATG start codon (Fig. 1). Inspection of the DNA sequences upstream of nt +1, the mRNA start site, revealed the presence of the putative −35 and −10 sequences (TTGCCA-17 nt-AACCAT) (Fig. 1), whose location coincided with the predicted P2 inducible promoter (13). Both deletion of the −35 promoter sequence and its site-directed mutagenesis to AAAGGA in the ϕ(*lldP*::*lacZ*) transcriptional fusion totally abolished β-galactosidase expression under both inducing and noninducing conditions (Fig. 3, lines 3 and 4). In contrast, mutation of the −35 sequence of the proposed P1 basal promoter, which overlaps with the predicted PdhR operator, did not abolish *lacZ* expression under either of the two conditions tested (Fig. 3, line 5). These results strongly indicate that transcription of the *lldPRD* operon is directed by the single promoter identified in this study.

LldR and PdhR in the regulation of the *lldPRD* operon. The locations of the putative recognition sites (renamed O1 and O2 in this study) identified by in silico analysis of the *lldPRD* promoter region led Lynch and Lin (13) to propose that the LldR protein acted as a repressor. In order to confirm experimentally whether the LldR protein is an activator or a repressor, we constructed strain JA218, in which the gene encoding LldR is replaced by the *cat* cassette, which confers resistance to chloramphenicol. Induction of ϕ(*lldP*::*lacZ*) expression by L-lactate was abolished in the *lldR* mutant (Fig. 4). These results suggest that the function of LldR in the presence of L-lactate is the transcriptional activation of the *lldPRD* operon. In contrast, the basal level of activity in CAA was twofold higher than that of the parental strain, which suggests that LldR is a repressor in the absence of L-lactate.

O1 is highly similar to the PdhR binding site present in the *pdhR-aceEF-lpd* operon (13, 23). In the present study, the putative role of PdhR in the control of the *lldPRD* operon was analyzed by measuring ϕ(*lldP*::*lacZ*) expression in strain JA217 (MC4100 *ace*^{c}816), which carries a nonfunctional PdhR protein. Regardless of the presence or absence of L-lactate as an inducer, no significant differences in ϕ(*lldP*::*lacZ*) expression were observed between strains JA217 and MC4100 (Fig. 4). This suggests that PdhR is not involved in the in vivo regulation of *lldPRD* expression under the conditions tested. Furthermore, the expression of this transcriptional fusion in these strains was also independent of the presence or absence of pyruvate (not shown), indicating that pyruvate is not an effector molecule for the *lldPRD* operon. Expression of



β-galactosidase (Miller units)	
CAA	L-lactate
270 ±53	2439±238
297 ±55	2551 ±199
<10	<10
<10	<10
829 ±86	845 ±79
984 ±85	2841 ±238
779 ±77	2563 ±235
308 ±40	2920 ±246

FIG. 3. Effects of deletions, mutations, or changes in O1-to-O2 helical phasing on *lldPRD* expression. The two LldR binding sites (O1 and O2 operators) are represented by black boxes in the diagram shown at the top. The gray box corresponds to the −35 promoter sequence identified in this study. The different constructs are shown below the top diagram and numbered at the left side. Mutations in either O1 or O2 are indicated by hatched boxes, and the mutation of the −35 promoter sequence is marked by an asterisk. The 5-bp or 10-bp insertions between both operator sites are indicated at the bottom (lines 7 and 8). These fragments were fused to *lacZ* and introduced as single-copy fusions in the genetic background of strain MC4100. Values for β-galactosidase activity under inducing or noninducing conditions of growth are indicated in the table on the right and expressed as means ± SD.

ϕ(*lldP*::*lacZ*) was also assayed in the *lldR pdhR* double mutant strain JA219. This strain had the same pattern of expression as the *lldR* mutant strain JA218 (Fig. 4). These results confirm the role of LldR as the only regulator mediating L-lactate induction of the *lldPRD* operon and its function as a repressor in the absence of L-lactate.

Analysis of LldR and PdhR binding to O1 and O2. To examine the binding of LldR and PdhR to O1 and O2 (Fig. 1), gel shift experiments with purified LldR and PdhR were performed with fragment P77 (containing O1) and fragment P85 (containing O2). The results presented in Fig. 5B show binding of LldR to both fragments, whereas PdhR was able to bind only to fragment P77, containing O1. However, the affinity of PdhR for O1 was much lower than that of LldR. To assess the function of the PdhR preparation, binding of PdhR to its specific operator was analyzed as a control (Fig. 5B). In this case, the PdhR-DNA complex was formed with smaller amounts of protein. As observed by other authors (20), at a higher protein concentration additional complexes with reduced mobility were observed. The low affinity of PdhR for O1 may not be compatible with an in vivo role of this regulator in the control of the *lldPRD* operon.

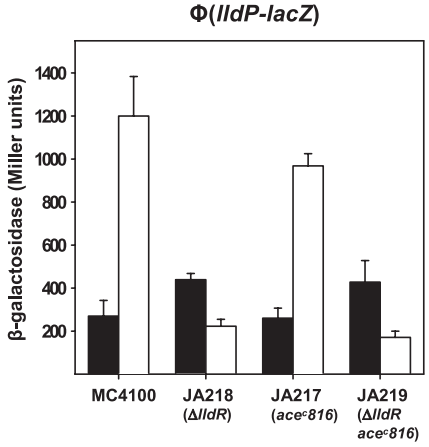


FIG. 4. β-Galactosidase activities of the Φ(*lldP-lacZ*) transcriptional fusion in different genetic backgrounds. Cells were grown aerobically in CAA (black bars) or in CAA plus 20 mM L-lactate (white bars). Activity values are expressed as means ± SD.

Alignment of the O1 and O2 sequences with the PdhR operator site present in the *pdhR* promoter (AATTGGTaagAC CAATT) revealed high identity in the palindromic sequences, except for the nucleotides flanking the spacer of the operator (underlined). Only one of these underlined nucleotides is conserved in O1, and none of them are conserved in O2 (Fig. 5A). Thus, PdhR binding results indicate that these positions of the palindrome are involved in specific recognition by the cognate regulator. This is in agreement with the recent description of the PdhR consensus sequence derived from the *ndh* and *cyoABCDE* operators, where these positions are always conserved (20).

Since the LldR protein bound to O1 and O2, gel shift assays were performed with probe P77 or P85 and increasing amounts of LldR (Fig. 6A). Binding of LldR to probe P77 was observed at a lower concentration of LldR, suggesting that this protein displays a greater affinity for O1 than for O2. As stated above, FadR-like proteins bind as dimers to the specific palindromic operator. Following this model, the P77- and P85-LldR complexes, displaying the same electrophoretic mobility, can be attributed to an LldR dimer bound to O1 and O2, respectively. As in the case of PdhR, at higher LldR concentrations another complex with reduced mobility was observed with probe P77, probably resulting from the interaction between two dimeric LldR molecules, with one of them being tightly bound to O1.

To confirm that LldR binds to the postulated operator se-

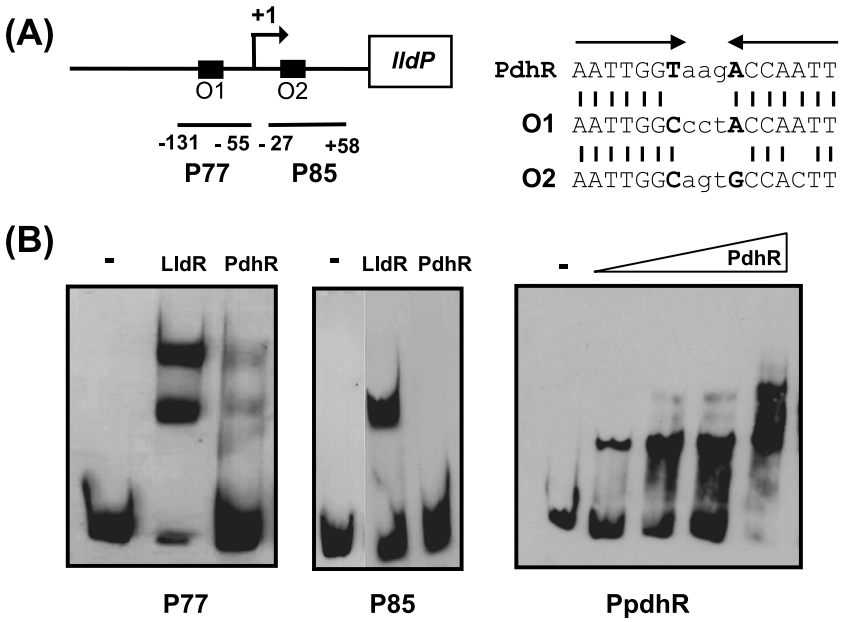


FIG. 5. Binding of LldR and PdhR to promoter fragments containing the O1 or O2 operator site. (A) (Left) Diagram of the *lldP* promoter region with the proposed O1 and O2 sites and the promoter fragments used as probes (P77 and P85). (Right) Sequence alignment of the PdhR operator present in the *pdhR-aceEF-ldp* operon promoter and the operators O1 and O2 in the *lldP* promoter. The arrows indicate the inverted repeat present in the operator sites recognized by GntR-like bacterial proteins. (B) Gel shift assays performed with the indicated DIG-labeled DNA probes. Probes P77 (encompassing O1) and P85 (encompassing O2) were added to binding mixtures containing 15 pmol of either purified LldR or PdhR. The probe encompassing the PdhR operator site was added to binding mixtures containing increasing amounts of PdhR (0.1, 0.4, 0.8, or 2 pmol). Reaction mixtures were incubated at 30°C for 15 min and directly subjected to polyacrylamide gel electrophoresis (PAGE).

quences O1 and O2, site-directed mutagenesis of 3 nt in each half-site of the two palindromic sequences was performed (Fig. 6B). This yielded probes P77*a and P77*b (mutations in O1) and probes P85*a and P85*b (mutations in O2). Gel shift experiments performed with P85*a and P85*b showed that mutations in either of the two half-sites of O2 abolished LldR binding (Fig. 6B). Insertion of 2 bp (TT) between the two symmetry elements of O2 also abolished LldR binding, suggesting that the distance between the two half-sites of the LldR inverted repeat is crucial for binding.

For the high-affinity operator O1, mutations at either of the two half-sites (P77*a and P77*b) did not abolish LldR binding but significantly reduced its affinity (Fig. 6B). To analyze the importance of the O1 spacer nucleotides in LldR binding, site-directed mutagenesis was performed to change the spacer O1 sequence into the corresponding O2 sequence (P77*c). Gel shift experiments showed that this mutated O1 construct behaved like O2 and displayed reduced affinity for LldR binding (Fig. 6B). These results indicate that these positions are important for binding and formation of the LldR-DNA complex.

Effect of L-lactate on LldR function. Binding of the effector molecules to the C-terminal domain of the FadR-like proteins promotes a conformational change that often abolishes DNA-protein interaction (3, 23). Nevertheless, the addition of L-lactate (at concentrations up to 100 mM) to binding reaction mixtures did not modify the number or the mobility of the complexes formed between LldR and P77 or P85 (data not shown). Evidence that L-lactate promoted a conformational change in LldR was obtained from experiments performed to improve cleavage of the MBP-LldR fusion protein. In solution, this protein was not cleaved with factor Xa, probably because protein folding made the cleavage site inaccessible. In such cases, addition to the reaction of any molecule able to interact with and change the protein conformation has been reported to improve digestion (pMAL protein fusion and purification system instruction manual, New England BioLabs). Thus, in our case, L-lactate was tested as a putative ligand of LldR. Digestion of the MBP-LldR protein was accomplished in the presence of 10 mM L-lactate (Fig. 7). This result indicates that L-lactate does bind to LldR and promotes a conformational change that, in this case, allows the accessibility of the recognition site of factor Xa. This conformational change did not prevent LldR-DNA binding but may allow interaction of LldR with RNA polymerase or other proteins involved in the formation of the transcription complex.

Function of the two operator sites recognized by LldR in the control of the *lldPRD* operon. To analyze the in vivo participation of O1 and O2 in the regulation of the *lldPRD* operon, single-copy *lacZ* fusions of promoter fragments containing different deletions or mutations were introduced in the genetic background of strain MC4100 (Fig. 3). The fusion containing the full-length promoter (Fig. 3, line 1) was used as an expression reference. Constructions with deletions upstream of position −331 or −231 were tested and yielded the same level of β-galactosidase activity as the full-length promoter (not shown). The large deletion still containing O1 and O2 (Fig. 3, line 2) also maintained the β-galactosidase level of the full-length construct. These results suggest that no other *cis*-acting elements controlling *lldPRD* transcription are present upstream of position −131.

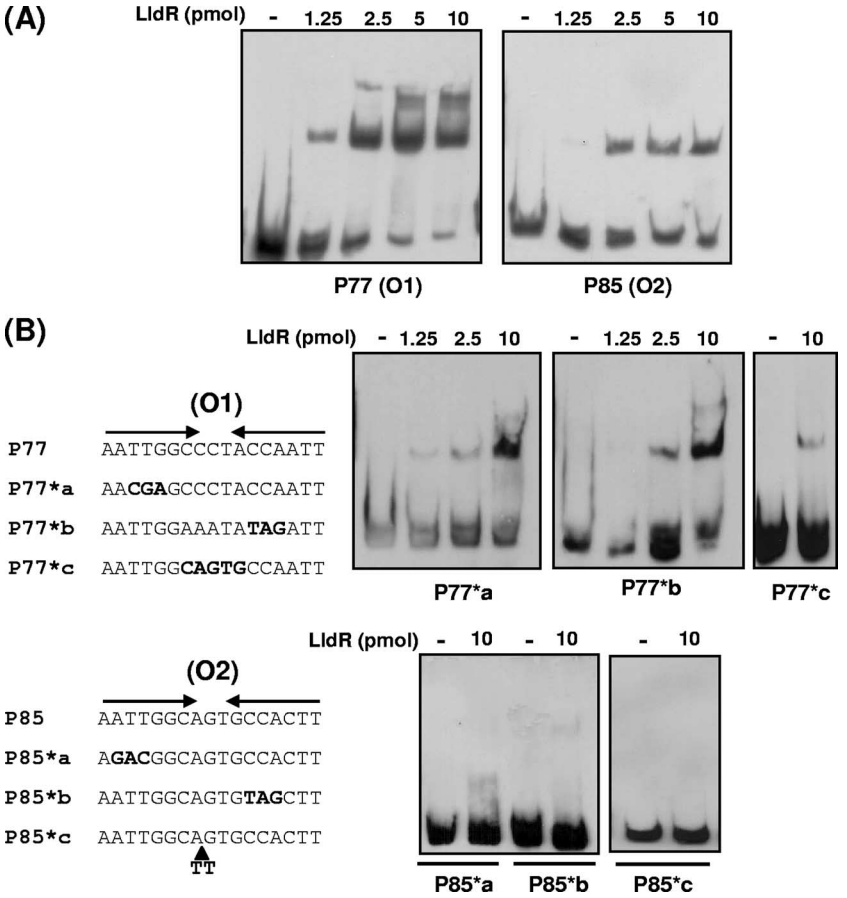


FIG. 6. Characterization of LldR binding to O1 and O2 operators. (A) Electrophoretic mobilities of the LldR-O1 and LldR-O2 complexes formed at increasing concentrations of protein. DIG-labeled DNA probes (for the region of each probe, see Fig. 4) were incubated at 30°C for 15 min with the indicated amounts of LldR and subjected to PAGE. (B) Effects of mutations in O1 and O2 operator sites on LldR binding. Mutations introduced by site-directed mutagenesis into the O1 or O2 palindromic sequence are shown in bold below the corresponding wild-type sequence. The arrows indicate the inverted repeat present in each operator site. The corresponding DIG-labeled fragments were added to binding mixtures containing the indicated amounts of LldR and incubated and processed as described above.

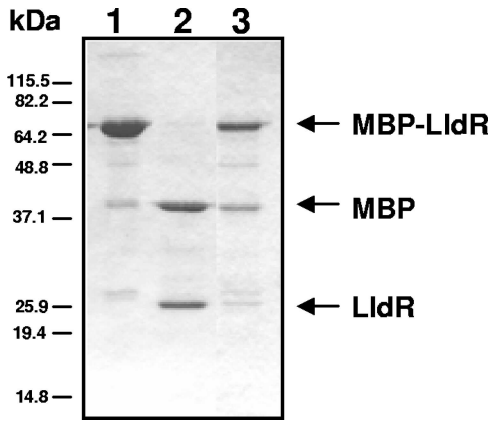


FIG. 7. Effect of L-lactate on cleavage of the fusion protein MBP-LldR by factor Xa. Proteins were separated by sodium dodecyl sulfate-10% PAGE and stained with Coomassie blue. Lane 1, MBP-LldR fusion protein eluted from the amylose column with 10 mM maltose before cleavage with factor Xa; lane 2, MBP-LldR digestion products after an overnight incubation with factor Xa in the presence of 10 mM L-lactate; lane 3, MBP-LldR digestion products after an overnight incubation with factor Xa in the absence of 10 mM L-lactate. Molecular masses of the markers are indicated on the left.

The involvement of O1 and O2 in control of the *lldPRD* operon was assessed by analysis of promoter fusions containing mutations in one of these *cis*-acting elements. The O1 and O2 mutations present in probes P77*a and P85*b, respectively, were introduced into the *lacZ* promoter fusion. Mutations in O1 abolished induction by L-lactate (Fig. 3, line 5). This confirms the role of the O1 site in the activation mediated by LldR in the presence of L-lactate. In addition, mutations in O1 yielded a threefold increase in the basal transcription level, suggesting that O1 may be involved in *lldPRD* operon repression under noninducing conditions. The same results were obtained with the O1 mutation present in P77*b (not shown). In contrast, mutations in O2 did not affect the induction level in the presence of L-lactate (Fig. 3, line 6). Therefore, O2 is not required for activation of *lldPRD* transcription. As in the case of O1, mutations in O2 increased the basal transcription level, reflecting its contribution to the repression of this operon in the absence of L-lactate.

Effect of changing DNA helical phasing between the two LldR operator sites. The results presented above indicate that full repression under noninducing conditions requires the oc-

cupancy of the two operators by LldR, which probably results in DNA looping through interaction between LldR molecules. Repression by means of DNA looping was shown in the regulation of many other operons in *E. coli*, such as the *ara*, *gal*, *lac*, and *deo* operons (14), and it was also proposed for other members of the GntR family (21). In order to test whether repression was affected by the helical phasing of the operators present in the *lldP* promoter region, we changed their angular orientation by inserting 5 bp between O1 and O2 (Fig. 3, line 7). The construct containing the 5-bp insertion exhibited increased basal β-galactosidase activity in CAA, a fact consistent with the abolition of DNA looping mediated by the binding of LldR molecules to O1 and O2 in their normal orientation. However, induction of this transcriptional fusion by L-lactate was not impaired. This result agrees with the previous observation that only mutations in O1 abolished L-lactate induction.

Insertion of 10 bp between O1 and O2 to restore their normal angular orientation did not modify the β-galactosidase expression pattern (Fig. 3, line 8). This result reinforces the hypothesis of DNA looping mediated by interaction between LldR molecules bound to O1 and O2 on the same helical face, which leads to operon repression in the absence of L-lactate.

Alternatively, other mechanisms can be considered to explain the basal repression observed when both O1 and O2 are occupied. For instance, two LldR dimer molecules bound to these operators on the same face of the helix would act synergistically to repress transcription. However, due to the distance between the two end points of O1 and O2 (100 base pairs), other proteins may be required for an effective synergic interaction between the LldR molecules.

Model for the control of the L-lactate operon. In this study, we have provided evidence to support the hypothesis that LldR has a dual function as a repressor and activator of *lldPRD* operon transcription, depending on the presence of L-lactate. From these results, a model for the control of the L-lactate operon by LldR may be derived.

When L-lactate is not present, LldR binds to both O1 (positions −105 to −89) and O2 (positions +22 to +38). On the basis of the different LldR affinities for these operators, LldR tightly bound to O1 may facilitate interaction of a new molecule of LldR with O2. It is widely accepted that in vivo occupancy of the weaker binding site (O2 in this model) is often associated with cooperativity of protein-protein interactions. This mechanism increases the local concentration of the protein, which facilitates its binding to the weaker site (28). This probably leads to DNA looping and to the repression of transcription. Due to the loop size, other factors, such as the architectural proteins HU and Fis, might contribute to stabilizing this loop (28). The contribution of IHF to this mechanism can be ruled out since the expression pattern of the *lldP*::*lacZ* fusion was not modified by mutations in the genes encoding the IHF subunits (*himA*::*cat* and *himD*::*cat* mutants) (not shown).

When L-lactate is present, binding of this effector molecule to the C-terminal domain of LldR promotes a conformational change that may lead to destabilization of the DNA loop in such a way that the transcription open complex is formed. In this situation, LldR bound to O1 may interact with RNA polymerase or other transcriptional regulators to activate *lldPRD* transcription.

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