Lactate Utilization Is Regulated by the FadR-Type Regulator LldR in *Pseudomonas aeruginosa*

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NAD-independent L-lactate dehydrogenase (L-iLDH) and NAD-independent D-lactate dehydrogenase (D-iLDH) activities are induced coordinately by either enantiomer of lactate in *Pseudomonas* strains. Inspection of the genomic sequences of different *Pseudomonas* strains revealed that the *lldPDE* operon comprises 3 genes, *lldP* (encoding a lactate permease), *lldD* (encoding an L-iLDH), and *lldE* (encoding a D-iLDH). Cotranscription of *lldP*, *lldD*, and *lldE* in *Pseudomonas aeruginosa* strain XMG starts with the base, C, that is located 138 bp upstream of the *lldP* ATG start codon. The *lldPDE* operon is located adjacent to *lldR* (encoding an FadR-type regulator, LldR). The gel mobility shift assays revealed that the purified His-tagged LldR binds to the upstream region of *lldP*. An XMG mutant strain that constitutively expresses D-iLDH and L-iLDH was found to contain a mutation in *lldR* that leads to an Ile23-to-serine substitution in the LldR protein. The mutated protein, LldR[M], lost its DNA-binding activity. A motif with a hyphenated dyad symmetry (TGGTCTTACCA) was identified as essential for the binding of LldR to the upstream region of *lldP* by using site-directed mutagenesis. L-Lactate and D-lactate interfered with the DNA-binding activity of LldR. Thus, L-iLDH and D-iLDH were expressed when the operon was induced in the presence of L-lactate or D-lactate.

Many bacteria can use lactate as the sole carbon and energy source for growth (9, 16, 17, 22, 30). NAD-independent lactate dehydrogenases (iLDHs), which catalyze the oxidation of lactate to pyruvate via a flavin-dependent mechanism, play an essential role in the utilization of lactate in most lactate-utilizing bacteria (8, 15, 16, 17). iLDHs can be classified into 2 subfamilies (D-iLDH and L-iLDH) depending on their substrate specificities (D-lactate versus L-lactate) (14). D-iLDH and L-iLDH have been studied extensively in *Escherichia coli* and *Corynebacterium glutamicum* (10, 11, 28). Whereas D-iLDH is constitutively expressed, L-iLDH is induced only when these strains are grown with L-lactate as the carbon source (10, 11, 28). Regulation of the expression of L-iLDH by the FadR-type regulator LldR in *E. coli* and *C. glutamicum* has been analyzed in detail (1, 13, 15, 28, 31, 33).

Utilization of lactate has also been studied in different *Pseudomonas* strains, such as *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Pseudomonas stutzeri* (5, 12, 21, 22, 27). Unlike the situation in *E. coli* and *C. glutamicum*, D-iLDH and L-iLDH are induced coordinately in these *Pseudomonas* strains (12, 21, 22). Neither of the enzymes is constitutively expressed, and either enantiomer of lactate can induce the expression of both enzymes (12, 21, 22). These results indicate that the iLDH regulatory mechanism in *Pseudomonas* strains may be different from the well-studied mechanism in *E. coli* and *C. glutamicum*.

In a previous report, glycolate was confirmed to inhibit the growth of *P. aeruginosa* in a medium containing lactate as the sole carbon source. This inhibition effect may be due to the fact that glycolate inhibits the induction of iLDH synthesis by lactate. Some spontaneous mutants that are able to grow on lactate medium in the presence of glycolate were isolated. D-iLDH and L-iLDH synthesis in those mutants is constitutive. Based on the phenomenon mentioned above, a hypothetical regulatory protein that controls the transcription of iLDHs was speculated (5). However, to date, no data related to the putative transcription regulator have been disclosed.

In this study, a D-iLDH and L-iLDH constitutive expression mutant of *P. aeruginosa* strain XMG was isolated. The gene encoding the putative transcription regulator LldR was cloned from the parent and mutant strains. It was confirmed that the constitutive expression of D-iLDH and L-iLDH was the result of the mutation of the transcription regulator LldR. Regulation of the *lldPDE* operon, which encodes a lactate permease, an L-iLDH, and a D-iLDH, was also studied in strain XMG.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All the strains and plasmids used in this work are listed in Table 1. *P. aeruginosa* XMG isolated from soil samples was used as the wild type (WT). The strain was deposited at the China Center for Type Culture Collection (CCTCC no. M201024). For the growth experiments, iLDH activity determination, and RNA isolation experiments, strain XMG was cultured in 500-ml baffled shake flasks containing lysogeny broth (LB) medium (3) or minimal salt medium (MSM) (22) at 37°C and 120 rpm. MSM was supplemented with 5.0 g/liter DL-lactate or pyruvate as the sole carbon source.

Isolation of the iLDH constitutive mutant. After being cultured in LB medium at 37°C for 10 h, cells of *P. aeruginosa* XMG were collected, suspended in sterilized physiological salt solution, spread on a lactate agar plate (containing 5.0 g/liter glycolate), and incubated for 48 h at 37°C (5). A mutant with constitutive D-iLDH and L-iLDH activities was isolated (denoted *P. aeruginosa* CM) and deposited at the CCTCC (no. M2010248).

TABLE 1 Strains and plasmids used in this work

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli DH5α	λ⁻ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r$_K^-$ m$_K^-$) *supE44 thi-1 gyrA relA1*	Invitrogen, America
E. coli BL21(DE3)	F⁻ *ompT hsdS*$_B$(r$_B^-$ m$_B^-$) *gal dcm* (DE3)	Invitrogen, America
P. aeruginosa XMG	Wild-type strain capable of lactate utilization	This study
P. aeruginosa CM	iLDHs constitutive mutant of *P. aeruginosa* XMG	This study
Plasmids		
pEasy-Blunt	Cloning vector; Ap^r and Km^r	Transgene
pEasy-Blunt-lldR	pEASY-Blunt with *lldR* gene of *P. aeruginosa* XMG	This study
pEasy-Blunt-lldR^M	pEASY-Blunt with *lldR* gene of *P. aeruginosa* CM	This study
pETDuet-1	Vector for protein expression; Ap^r	Novagen
pETDuet-lldR	pETDuet-1 with *lldR* gene of *P. aeruginosa* XMG	This study
pETDuet-lldR^M	pETDuet-1 with *lldR* gene of *P. aeruginosa* CM	This study

[a] Km^r and Ap^r indicate resistance to kanamycin and ampicillin, respectively.

Recombinant DNA experiments. The enzymes used for recombinant DNA experiments were obtained from TaKaRa Bio Inc. (China). The oligonucleotides were obtained from Sangon (Shanghai, China). PCR, restriction, and ligation were performed as described by Sambrook and Russell (29). The amplified fragments were purified with a QIAquick PCR purification kit (Qiagen, Germany). The plasmids were isolated from *E. coli* with a QIAprep spin miniprep kit (Qiagen, Germany).

Plasmid construction. *P. aeruginosa* XMG and *P. aeruginosa* CM genomic DNAs were extracted with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The *lldR* genes in *P. aeruginosa* XMG and *P. aeruginosa* CM were amplified by PCR using primer P1 with an NdeI restriction site insertion and primer P2 with an XhoI restriction site insertion (see Table S1 in the supplemental material). The PCR products were ligated first to the pEasy-Blunt vector, and the resulting plasmids were then designated pEasy-Blunt-lldR and pEasy-Blunt-lldR^M. The pEasy-Blunt-lldR and pEasy-Blunt-lldR^M plasmids were then digested with NdeI and XhoI, and the gel-purified fragments were ligated to the pETDuet-1 vector that had been digested with the same restriction enzymes. The resulting plasmids were designated pETDuet-lldR and pET-Duet-lldR^M, respectively. The insertion fragments of pETDuet-lldR and pETDuet-lldR^M were sequenced by Sangon (Shanghai, China). For all the cloning experiments, *E. coli* DH5α was used as the host and was cultivated in LB medium at 37°C. The medium contained 50 μg/ml kanamycin or 100 μg/ml ampicillin when appropriate.

Overproduction and purification of LldR and LldR^M. *E. coli* BL21(DE3) carrying plasmid pETDuet-lldR or pETDuet-lldR^M was grown at 37°C in LB medium with 100 μg/ml ampicillin to an optical density of 0.6 at 600 nm. Then, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to induce the expression of LldR or LldR^M. After cultivation for another 4 h at 37°C, cells were harvested by centrifugation at 12,000 rpm for 5 min at 4°C and washed with 0.85% (wt/vol) sodium chloride solution. The cell pellets were subsequently suspended in the binding buffer (pH 7.4; 20 mM sodium phosphate, 20 mM imidazole, and 500 mM sodium chloride) containing 1 mM phenylmethanesulfonyl fluoride and 10% glycerol. The cells were disrupted by sonication (Sonics 500 W; 20 KHz) for 5 min in an ice bath, and the cell lysate was centrifuged at 12,000 rpm for 20 min at 4°C to remove the debris. The supernatant was loaded onto a HisTrap HP column (5 ml) and eluted with 25% binding buffer and 75% elution buffer (pH 7.4; 20 mM sodium phosphate, 500 mM imidazole, and 500 mM sodium chloride) at a flow rate of 5 ml/min. The fractions containing LldR or LldR^M were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed using 12.5% polyacrylamide gels on a Mini-Protean III system (Bio-Rad).

D-iLDH and L-iLDH activity assays. Cells of *P. aeruginosa* XMG and *P. aeruginosa* CM were harvested; washed; resuspended in 67 mM phosphate buffer (pH 7.4) containing 20 mM KCl, 5 mM MgSO$_4$, and 1 mM EDTA; and disrupted by sonication in an ice bath. The disrupted cells were centrifuged for 10 min at 10,000 × *g*, and the supernatant was used as the crude cell extract. The activities of D-iLDH and L-iLDH were determined at 30°C in 1 ml of 50 mM Tris-HCl (pH 7.5), 0.1 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and the crude cell extract. The reaction was started by the addition of 50 mM L-lactate or D-lactate, and the rate of MTT reduction was determined by measuring the absorbance changes at 570 nm (24). One unit was defined as the amount of enzyme that reduced 1.0 μmol of MTT per minute under the test conditions. The protein concentrations were determined by a modified Lowry method using bovine serum albumin (BSA) as a standard (23).

RNA isolation, cDNA generation, and RT-PCR. Total RNA was isolated from the *P. aeruginosa* cells grown to an optical density at 600 nm of 0.5 under inducing conditions (with DL-lactate as the sole carbon source), using a Qiagen RNeasy total RNA kit, and then treated with RNase-free DNase I (Qiagen, Germany). The integrity of the RNA was assessed by electrophoresis of 10 μg of total RNA in a 1.5% agarose gel with Tris-borate-EDTA buffer. cDNA was generated by using Superscript II Reverse Transcriptase (Invitrogen, America) and the reagents supplied by the manufacturer (42°C; 50 min). The reaction was inactivated by incubation at 70°C for 15 min. Reverse transcription-PCR (RT-PCR) was performed in accordance with the standard procedures using 1 μM each specific primer (see Table S1 in the supplemental material). The genomic DNA of *P. aeruginosa* XMG was used as a positive control.

Determination of the transcriptional start site. The transcriptional start site of the *lldPDE* operon was determined by random amplification of cDNA ends (RACE)-PCR using a 5′/3′ second-generation RACE kit (Roche, Mannheim, Germany) as recommended by the manufacturer. Amplification of the reverse transcription products was performed with nested *lldP*-specific primers and an oligo(dT) anchor primer. The primers used were Pt1 for the first PCR, Pt2 for the second PCR, and Pt3 for the third PCR (see Table S1 in the supplemental material). The products obtained were cloned into a pEasy-Blunt vector for sequencing.

DNA-binding study. The purified LldR or LldR^M (2 μM) was mixed with the 328-bp *lldR*-*lldP* intergenic region (F0) or HinfI- and AluI-digested DNA fragments (F1, F2, and F3) in a 20-μl mixture. The mixture contained 50 mM Tris-HCl, 10% glycerol, 50 mM KCl, 10 mM MgCl$_2$, and 0.5 mM EDTA (pH 7.5). F0 was obtained by performing PCR with the primers F0F and F0R (listed in Table S1 in the supplemental material). After incubation for 30 min at 30°C, the samples were separated on a 9% native polyacrylamide gel at room temperature and 170 V (constant voltage) using Tris-borate-EDTA (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3) as the electrophoresis buffer. The gels were subsequently stained with SYBR green I according to the instructions of the supplier (Sigma, Rödermark, Germany). To test the effects of L-lactate and D-lactate, the protein was incubated with 40 mM L-lactate or D-lactate in the
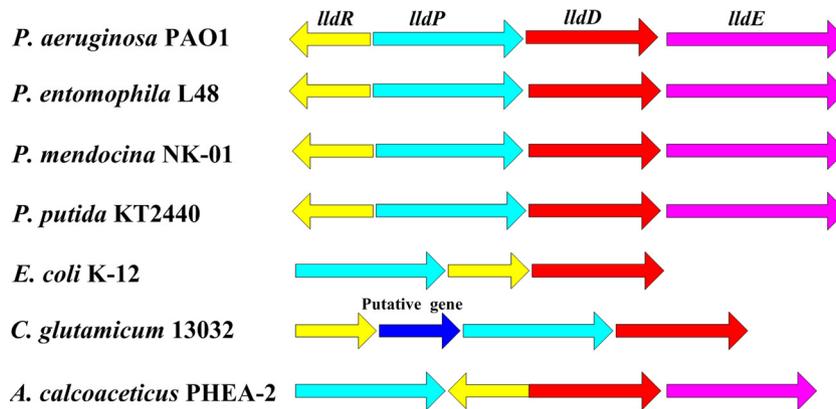
FIG 1 Organizations of lactate utilization genes in different species. Arrows indicate the direction of gene translation.

binding buffer for 15 min before the DNA fragment F4 was added and the mixture was incubated for an additional 30 min. Fragment F4 was obtained by performing PCR with primers F4F and F0R (listed in Table S1 in the supplemental material). All the PCR products used in the gel shift assays were purified with a QIAquick PCR purification kit (Qiagen, Germany) and eluted in distilled deionized water.

Nucleotide sequence accession numbers. The nucleotide sequences of *lldR* in *P. aeruginosa* XMG and *P. aeruginosa* CM have been deposited in GenBank under accession no. JN853772 and JN853771, respectively.

RESULTS

Comparative genomics revealed that the *lldPDE* operon is found in most *Pseudomonas* strains. In a previous work, *P. stutzeri* SDM, a typical strain for studying the molecular mechanism of lactate utilization, was sequenced (19). The L-iLDH-encoding gene in *P. stutzeri* SDM was cloned and identified (unpublished data). A BLAST search of the genome sequence of *P. aeruginosa* PAO1 with the L-iLDH-encoding gene of *P. stutzeri* SDM as the probe revealed a sequence encoding a protein with strikingly high homology to L-iLDH. The AAG08157.1 gene encodes a 381-amino-acid protein that exhibits 88% identity with L-iLDH of *P. stutzeri* SDM. A comparison of the genes in the vicinity of the L-iLDH-encoding gene in *P. aeruginosa* PAO1 with those in the corresponding region of the genome of the closely related bacteria *P. putida* KT2440, *Pseudomonas entomophila* L48, and *Pseudomonas mendocina* NK-01 revealed a high degree of synteny (18, 25, 32, 35). That is, in most of the *Pseudomonas* strains, *lldD*, encoding L-iLDH, is adjacent to *lldP*, which is predicted to encode a lactate permease, and is followed by *lldE*, which is predicted to encode D-iLDH (Fig. 1) (18, 25, 26, 32, 35). It was also noticed that *lldP*, *lldD*, and *lldE* are located adjacent to the homolog of the *lldR* genes in *C. glutamicum* and *E. coli* (8, 15). Thus, it is hypothesized that *lldP*, *lldD*, and *lldE* might comprise the *lldPDE* operon. The operon might be regulated by LldR in different *Pseudomonas* strains.

RT-PCR demonstrated the cotranscription of 3 structural genes in the *lldPDE* operon. The *lldPDE* operon comprises 3 structural genes, which are separated by short intergenic spacers. The possible cotranscription of 3 structural genes in the *lldPDE* operon was assessed by an RT-PCR approach. The cDNA products generated from total RNA extracted from cells induced by DL-lactate were used as templates in RT-PCR to examine the amplification of overlapping regions spanning *lldP-lldD* and *lldD-lldE*. Only a single PCR product was obtained from each amplifi-

cation reaction using the PLF-PLR or LDF-LDR primer pair (Fig. 2). These results were confirmed in 3 replicated experiments using independent RNA samples, and they are consistent with the notion that the *lldPDE* genes are cotranscribed as an operon.

Determination of the *lldPDE* transcription start site. The DNA sequence of the *lldR-lldP* intergenic region in *P. aeruginosa* XMG is presented in Fig. 3. The 5′ ends of the *lldPDE* transcripts were identified by RACE-PCR using total RNA extracted from *P. aeruginosa* XMG grown in minimal medium in the presence of DL-lactate (Fig. 3). Transcription was initiated at a single C located 138 bp upstream of the translational start point of LldP. The promoter contains a TAACCT motif (from position −11 to position −6) and a TTGACA motif (from position −35 to position −30) (Fig. 3), which might be the −10 and −35 regions of the promoter.

An XMG mutant strain that constitutively synthesizes D-iLDH and L-iLDH has a mutation within the *lldR* gene. Glycolate inhibited the growth of *P. aeruginosa* XMG in MSM (22) containing lactate as the sole carbon source (see Fig. S1a in the supplemental material). Inducement of D-iLDH and L-iLDH by lactate in *P. aeruginosa* XMG was inhibited by the addition of glycolate (see Table S2 in the supplemental material). *P. aeruginosa* CM, a mutant strain of *P. aeruginosa* XMG, could grow well in the lactate medium in the presence of glycolate (see Fig. S1b in the supplemental material). Glycolate did not inhibit the expression of D-iLDH and L-iLDH in *P. aeruginosa* CM (see Table S2 in the supplemental material).

Unlike the situation in *P. aeruginosa* XMG, in which D-iLDH and L-iLDH could be induced only by lactate, the expression of
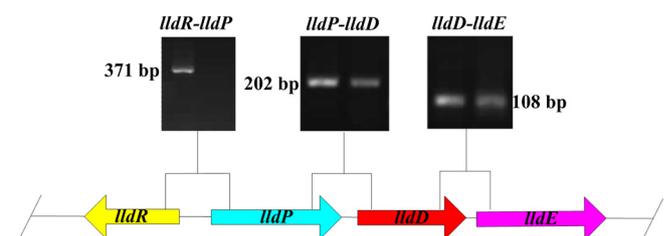

FIG 2 Identification of the cotranscription of 3 structural genes in the *lldPDE* operon by using RT-PCR. Genomic DNA of *P. aeruginosa* XMG was used as a positive control (left lanes). cDNA was isolated and RT-PCR was performed as described in the text (right lanes).
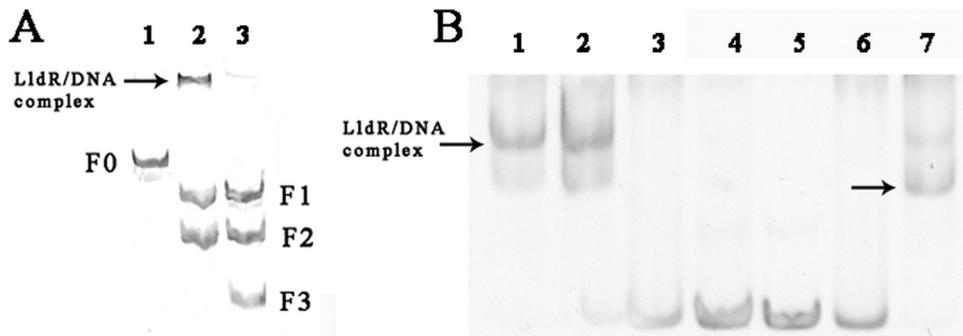
FIG 5 Identification of the binding site of LldR from *P. aeruginosa* XMG by gel shift assays. (A) Binding activities of LldR with F1, F2, and F3. Lane 1, free F0; lane 2, binding of LldR with F1, F2, and F3; lane 3, free F1, F2, and F3. (B) Binding activities of LldR with subfragment F4 and derived fragments with the mutations M1, M2, M3, M4, M5, and M6. Lane 1, wild-type nucleotide sequence (fragment F4); lane 2, nucleotides AAT (underlined with inverted arrows in Fig. 3) were changed to CCG (fragment M1); lane 3, nucleotides TGGT (underlined with inverted arrows in Fig. 3) were changed to GGTG (fragment M2); lane 4, nucleotides AATTGGT (underlined with inverted arrows in Fig. 3) were changed to CCGGGTG (fragment M3); lane 5, nucleotides ACCAATT (underlined with inverted arrows in Fig. 3) were changed to CACGGCC (fragment M4); lane 6, nucleotides ACCA (underlined with inverted arrows in Fig. 3) were changed to CACG (fragment M5); lane 7, nucleotides ATT (underlined with inverted arrows in Fig. 3) were changed to GCC (fragment M6). The oligonucleotides used for amplification of the fragments are listed in Table S1 in the supplemental material.

ing gene is regulated by LldR (4, 8, 15, 20). In *P. aeruginosa* XMG, the expression of L-iLDH and D-iLDH is coordinately induced by lactate. Unlike the situation in *C. glutamicum* and *E. coli*, in most *Pseudomonas* strains, the L-iLDH- and D-iLDH-encoding genes are adjacent to each other (Fig. 1). The RT-PCR analysis further confirmed the cotranscription of the D-iLDH- and L-iLDH-encoding genes in *P. aeruginosa* XMG. Thus, the coordinated expression of L-iLDH and D-iLDH in *Pseudomonas* strains is due to the fact that both the L-iLDH- and D-iLDH-encoding genes are in the same lactate utilization operon and are controlled by the same regulator. In *Acinetobacter calcoaceticus*, the coordination of L-iLDH and D-iLDH activities by lactate was also reported (2). As shown in Fig. 1, the L-iLDH- and D-iLDH-encoding genes are downstream of the LldR-encoding gene and adjacent to each other in *A. calcoaceticus* PHEA-2, implying a similar regulatory mechanism in the newly genome-sequenced strain (36).

Although lactate permease activity was not assayed in this work, the transcription of the LldP-encoding genes in *P. aeruginosa* XMG is also induced by lactate (see Table S4 in the supplemental material). Mutation of LldR in *P. aeruginosa* XMG would result in enhanced *lldP* transcription. As the LldP- and L-iLDH-

FIG 6 Effects of pyruvate, L-lactate, and D-lactate on the DNA-binding activities of LldR. Line 1, free F0; lane 2, purified His-tagged LldR was used in 10-fold molar excess relative to F0; lane 3, purified His-tagged LldR was used in 10-fold molar excess relative to F0 (40 mM D-lactate was added); lane 4, purified His-tagged LldR was used in 10-fold molar excess relative to F0 (40 mM L-lactate was added); lane 5, purified His-tagged LldR was used in 10-fold molar excess relative to F0 (40 mM pyruvate was added).

encoding genes are also cotranscribed in *P. aeruginosa* XMG and transcription of the *lldPDE* operon starts with a C that is located 138 bp upstream of the ATG start codon of LldP, the expression of LldP should also be regulated by LldR.

There is considerable sequence identity between the LldR protein from *P. aeruginosa* XMG and the LldR proteins from *E. coli* (42% sequence identity; NP_418061.1) and *C. glutamicum* (29% sequence identity; NP_602104.1) (8, 15). All 3 of these regulators belong to the FadR subfamily of the transcription factor family GntR (8, 15, 28). However, LldR from *P. aeruginosa* XMG exhibits only 26% sequence identity with FadR, the acyl-coenzyme A (CoA)-responsive regulator of fatty acid degradation and biosynthesis of *E. coli* (NP_415705.1) (6, 7, 34). On the other hand, unlike FadR, LldR proteins in *P. aeruginosa* XMG, *E. coli*, and *C. glutamicum* contain conserved residues involved in Zn^{2+} binding (Arg104, Asp152, His156, His 205, and His227 in *P. aeruginosa* XMG) (13).

In previous studies, the motif TNGTNNNACNA was reported to be the consensus operator for FadR-type regulators (15, 28). For example, LldR in *C. glutamicum* binds to the motif TGGTCT GACCA in the promoter region of the L-lactate utilization operon (13, 15). In *P. aeruginosa* XMG, a motif with hyphenated dyad symmetry AATTGGTCTTACCAATT is also present in the upstream region of the *lldPDE* operon. The binding site of LldR in *P. aeruginosa* XMG was identified by a gel shift assay. The mutational analysis revealed that the half-sites AAT and ATT were not involved in the binding event. Only the half-sites TGGT and ACCA were essential for binding with LldR in *P. aeruginosa* XMG (Fig. 5).

The crystal structure of LldR in *C. glutamicum* was determined in a previous study. Similar to FadR, LldR in *C. glutamicum* contains an N-terminal DNA-binding domain and a C-terminal ligand-binding/dimerization domain (13). The amino acid residues Lys4, Arg32, Arg42, and Gly63 in the N-terminal domain of LldR in *C. glutamicum* are crucial for DNA binding (13). LldR from *P. aeruginosa* XMG contains 3 crucial amino acid residues (Arg38, Arg48, and Gly69) in the N-terminal DNA-binding domain. For further comparison between the lactate utilization processes in *C. glutamicum* and *P. aeruginosa*, structural analysis of LldR of *P. aeruginosa* XMG should also be conducted in future research.
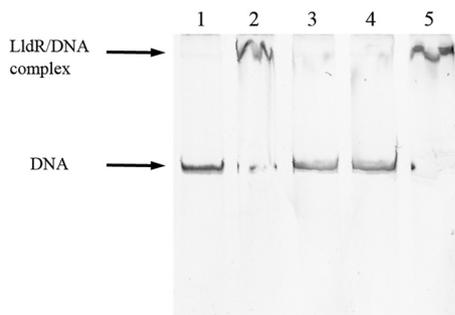
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