



A novel D(–)-lactic acid-inducible promoter regulated by the GntR-family protein D-LldR of *Pseudomonas fluorescens*

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ABSTRACT

Lactic acid has two stereoisomers of D(–)- and L(+)-forms, both of which are important monomers of biodegradable plastic, poly-lactic acid. In this study, a novel D-lactate inducible system was identified in *Pseudomonas fluorescens* A506, partially characterized and tested as biosensor. The D-lactate catabolic operon (*lldP-dld-II*) was negatively regulated through the inversely transcribed D-*lldR* (encoding a GntR-type regulator), where the repression is relieved by addition of D-lactate. The derepression was specific to D-lactate and marginally affected by L-lactate. The D-LldR-responsive operator, showing dyad symmetry and separated by one base, was located between +11 and +27 from the transcription start site of the *lldP-dld-II* operon. By site-directed mutagenesis, a motif with a dyad symmetry (AATTGGTATACCAATT), present in the upstream region of *lldP*, was identified as essential for the binding of LldR. D-lactate biosensors were developed by connecting the upregulation by D-lactate to a green fluorescent readout. About ~6.0-fold induction by 100 mM D-lactate was observed compared to L-lactate.

1. Introduction

Lactic acid has emerged as an important monomer for the production of renewable biodegradable plastics [1]. It has a chiral center at the second carbon and occurs as D(–)- and L(+)-enantiomers. Enantiomeric purity is important for industrial applications; L-isomer is widely used for the production of bulk biodegradable plastics [2], while D-lactate is mainly used for pharmaceutical purposes as D-lactate is not metabolized in the human body [3]. In most microbial fermentations racemic mixture of D-/L-lactic acid are produced [4], but recent advances in genetic and metabolic engineering approaches enabled the production of optically-pure D- or L-lactic acid [5–8]. Pure enantiomers of lactic acid can also be synthesized by chemical methods, although not preferred [9].

For developing efficient microbial production, various evolutionary strategies such as genome shuffling and random mutagenesis are attempted [10]. In these efforts, availability of high throughput screening (HTS) methods is essential for fast identification and selection of the strain(s) with desired trait(s) from libraries, often composed of over millions of cells [11]. Conventional screenings relying on direct measurements of enzyme activity and/or metabolite concentrations *in vitro*

are laborious and time-consuming, thus impractical for HTS. Moreover, many of these conventional methods have a low detection limit [12]. To address these problems, the transcription factor (TF)-based screening methods have been developed [13]. The TFs are regulatory proteins consisting of two domains, one for the metabolite (inducer molecule) binding and the other for interaction with promoter/operator DNA sequences. Binding of an inducer molecule alters the conformation of DNA-binding domain of a TF and changes its affinity towards the corresponding promoter/operator sequences and/or interaction with the RNA polymerase enzyme. However, most TFs studied thus far respond to relatively large molecules of > C4. Recently, a TF sensing the C3 platform chemical, 3-hydroxypropionic acid, has been identified and successfully developed as biosensor [12]. It was employed in detecting 3-HP *in situ*, engineering core enzymes in the 3-HP biosynthesis pathway or selecting better-performing strains [13]. Transcription factor sensing L-lactic acid has also been reported in *E. coli*, but not developed as biosensor [14]. For D-lactic acid, no such TF has been identified.

The present study aims at screening D-lactate responsive TF and exploring its use as a biosensor. We identified such TF in lactate utilization (*lld*) operon of *P. fluorescens* A506 and performed a genetic and *in*

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vitro analysis to understand the salient features of the lactate-responsive regulator (D-LldR) and its recognition DNA sequences. In addition, D-LldR-based biosensor towards D-lactate was developed and tested. The D-lactate sensor had a dynamic range > 100 mM, with a maximum induction ratio of ~6.0-fold in *Pseudomonas denitrificans* as host.

2. Materials and methods

2.1. Materials

P. denitrificans ATCC 13867 and *P. fluorescens* A506 were purchased from ATCC (USA). The primers were synthesized by Macrogen Co. Ltd. (Seoul, Korea). D/L-lactic acid and all other chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Culture conditions

Unless indicated otherwise, cultivation was carried out with a 20 mL working volume in 250-mL non-baffled Erlenmeyer flasks or with a 400 µL working volume in Corning™ 96-well blocks at 37 °C in an orbital incubator shaker set at 200 rpm. The M9 medium used in this study contained the following components (per liter of deionized water): MgSO₄·7H₂O, 0.25 g; NaCl, 1.0 g; NH₄Cl, 1 g; gluconate, 5 g; and kanamycin, 30 mg. In addition, the medium was supplemented with 100 mM potassium phosphate buffer (pH 7.0). The cells were induced at ~0.4–0.5 OD₆₀₀ with the D/L-lactic acid (in varying concentrations of 0–100 mM) or various chemicals (3-hydroxypropionate, acetic acid, butyric acid; all at one concentration of 25 mM). Afterward, the cells were grown for 4 h and harvested for mRNA or GFP measurements.

2.3. Cloning of plasmids and construction of recombinant *P. denitrificans*

The pUCPK' plasmid [15] was used to develop the D-lactic acid biosensor in *P. denitrificans*. DNA amplicons containing the promoters *P_{zwf}*, *P_{lldp}* and *P_{D-LldR}* were PCR amplified from *P. fluorescens* A506 genomic DNA, while the *gfp* gene, which encodes green fluorescence protein (GFP), was amplified from pET-GFP:28 [16]. The combinations of DNA fragments were overlapped and ligated into the vector pUCPK' using traditional cloning methods (Enzymes were purchased from New England Biolabs, USA). The resulting plasmid, pUCPK-D-LldR-*P_{lldp}*-*gfp* was sequence-confirmed at Macrogen Co., Ltd., Korea, and introduced into *P. denitrificans*. The details of the primers used in this study are provided in [Supplementary Table S1](#). The mutant libraries for promoter elements and operator regions were created by using a pair of degenerate primers and tested by the pUCPK-D-LldR-*P_{lldp}*-*gfp* reporter plasmid (Library1~6; depicted in [Fig. 5](#)). Strains and plasmids used in this study are listed in [Table 1](#).

Table 1
Strains and plasmids used in this study.

Strains	Description	Source
Strains		
<i>E. coli</i> TOP10	Cloning host	Invitrogen
<i>E. coli</i> K12 MG1655	Expression host	KCTC
<i>P. denitrificans</i> ATCC 13867	Expression host	ATCC
<i>P. fluorescens</i> A506	Expression host	ATCC
<i>P. fluorescens</i> ΔD-LldR	Mutant strain	This study
Plasmids		
pUCPK'	Shuttle vector (ColE1 and pRO1614 origins)	15
pUCPK'-D-LldR- <i>P_{lldp}</i> - <i>gfp</i>	GFP expression under <i>P_{lldp}</i> promoter	This study
pUCPK'-D-LldR- <i>P_{lldp}</i> - <i>gfp</i>	GFP expression under <i>P_{lldp}</i> - <i>Ln</i> (libraries 1 to 6)	This study
pQSAK'-D-LldR- <i>del</i>	Plasmid used for deletion of D-LldR	This study
pUCPK'- <i>P_{zwf}</i> -D-LldR	D-LldR complementation	This study

The deletions were carried out using a marker-less chromosomal in-frame gene deletion method based on the *sacB* negative counter-selection system. The pQSAK plasmid was used to delete the target gene, putative D-LldR. An engineered fragment containing the ~700 bp upstream and downstream regions of the target gene was generated by PCR using *P. fluorescens* A506 genomic DNA. The resultant engineered fragment was cloned into the pQSAK plasmid (15) to develop the mutant strain by a double recombination. The mutant strains were screened by PCR and further confirmed by sequencing at Macrogen Co., Ltd., Korea.

2.4. RNA extraction and real-time PCR

The *P. denitrificans* strains were grown in M9-minimal medium containing 5 g/l sodium gluconate. The cells were cultivated under aerobic conditions at 37 °C and 200 rpm on an orbital incubator shaker. Each culture was supplemented with 25 mM D-lactic acid at the OD₆₀₀ of ~0.4–0.5. After cultivation for an additional 4 h, approximately 5 × 10⁸ cells were collected and centrifuged at 5000 × g for 10 min (Following manufacturer's recommendations). The cell pellets were immediately suspended in 500 µL of RNA solution (Ambion, UK) and RNA was extracted using a total RNA isolation kit (Macherey-Nagel, Germany). One microgram of total RNA was used for first-strand cDNA synthesis in a 20 µL reaction using a SuperScript III first-strand synthesis system (Invitrogen, USA). A real-time PCR analysis was performed using the SYBR green method in 20 µL reaction volumes using a StepOne Real-time PCR system (Applied Biosystems, USA). The PCR efficiencies of all of the primers were experimentally evaluated and were shown to be suitable for reliable copy number quantification. The mRNA quantity was estimated based on the ΔΔCT method as described previously [17]. The assays were performed in duplicate, and a template-less reaction was used as a negative control. The 5'-RACE system was purchased from Invitrogen (Cat. No: 18374058) and used to determine the transcription start site (+1) following the manufacturer's instruction.

2.5. Molecular modeling and docking of D-LldR with D-/L-lactate

The D-LldR structure was modeled by multiple template threading using Swiss-Model tool [18]. The predicted model was evaluated using the RAMPAGE tool, by calculating the main-chain RMSD (Root Mean Square Deviation) with reference to its template structure (PDB ID: 5KVR and 5TPM) and their amino acid distribution within the allowed regions.

Molecular docking was carried out to examine the binding interaction between the modeled D-LldR protein and D-/L-lactate. The regions in D-LldR for D-/L-lactate binding was predicted by the COACH tool, according to their highest C-scores and cluster sizes. The validated model and predicted active-site residues were used to perform docking studies, with the help of the Maestro program from the SCHRODINGER™v10.1 software package. Briefly, the target protein (D-LldR) and the ligand (D-/L-lactate) were prepared and processed using Protein Preparation Wizard and LigPrep Wizard in the Schrodinger graphical user interface MAESTRO (version 10.1). Bond orders were assigned to the ligand, and hydrogen bonds consistent with the physiological pH (7.0) were added to the receptor. The initial ligand conformations were obtained by a Monte Carlo conformational search. Using the Receptor Grid Generation tool, a receptor grid box (scaling factor: 1.0; partial charge cutoff: 0.25 Å) was generated around the active-site residues predicted by the COACH tool. Ligand docking was performed using XP (extra precision) predefined docking settings and flexible ligand sampling within the grid box. Finally, the docked poses were visualized using the Maestro 10.1 graphical user interface.

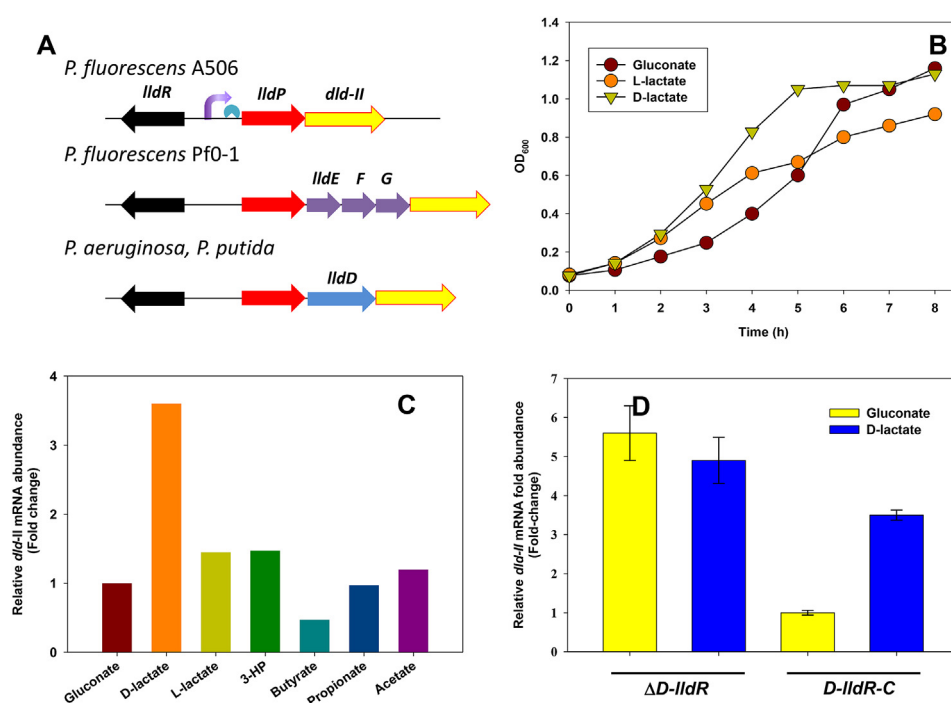


Fig. 1. D-lactate inducible system in *Pseudomonas fluorescens* A506. (A) Gene organization of lactate utilization genes in various *Pseudomonas* strains, Permease (*lldP*), L-LDH (*lldD* or *lldEFG*) and D-LDH (*dld-II*); (B) Time-course profiles of cell growth by wild type *P. fluorescens* A506 on Gluconate, D- and L-lactate, (C) Relative mRNA levels of D-lactate dehydrogenase, *dld-II* gene. The mRNA levels were compared with those of the reference gene, *rpoD*, (D) Deletion and complementation of putative *D-lddR* homologue in *P. fluorescens* A506.

A 1. *Pseudomonas fluorescens* (WP_073523526), *Marinobacter psychrophilus* (WP_048388449), and *Marinospirillum alkaliphilum* (WP_072327194)



2. *Stenotrophomonas maltophilia* (KKD57203)



3. *Streptococcus pneumoniae* (CJL34368), *Halomonas elongata* (WP_013330976), and *Chromohalobacter israelensis* (WP_035414796)



4. *Azotobacter beijerinckii* (SEI81182) and *Alcanivorax* sp. 43B_GOM-46m (WP_026949870)

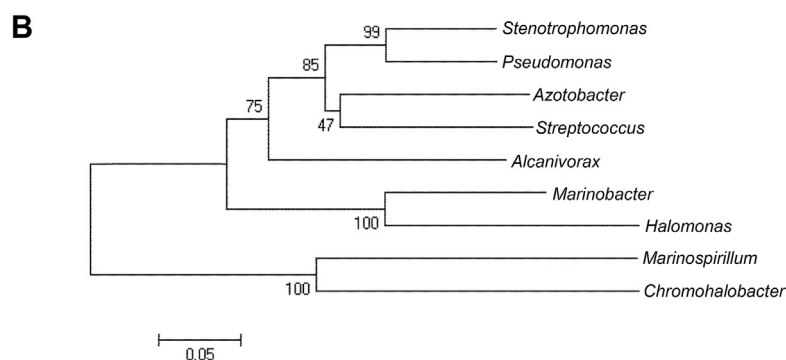


Fig. 2. (A) Gene organization of the *dld* operons involved in D-lactate utilization in various genera. (B) Evolutionary relationship of D-LldR.

2.6. GFP (green fluorescent protein) fluorescence assay

The recombinant strains were inoculated into the modified M9 minimal medium (as described above), and fluorescence and OD₆₀₀ were measured with 3 h intervals after inoculation. Fluorescence was measured by a Synergy H1 microplate reader (BioTek instruments, USA) using 486-nm excitation and 535-nm emission filters. The

measured fluorescence values were normalized to OD₆₀₀ and reported as specific fluorescence.

2.7. SMARTer 5'-RACE

Full length of *lldP* cDNA was obtained by performing 3' and 5' RACE using the SMARTer™ RACE cDNA amplification kit from Clontech

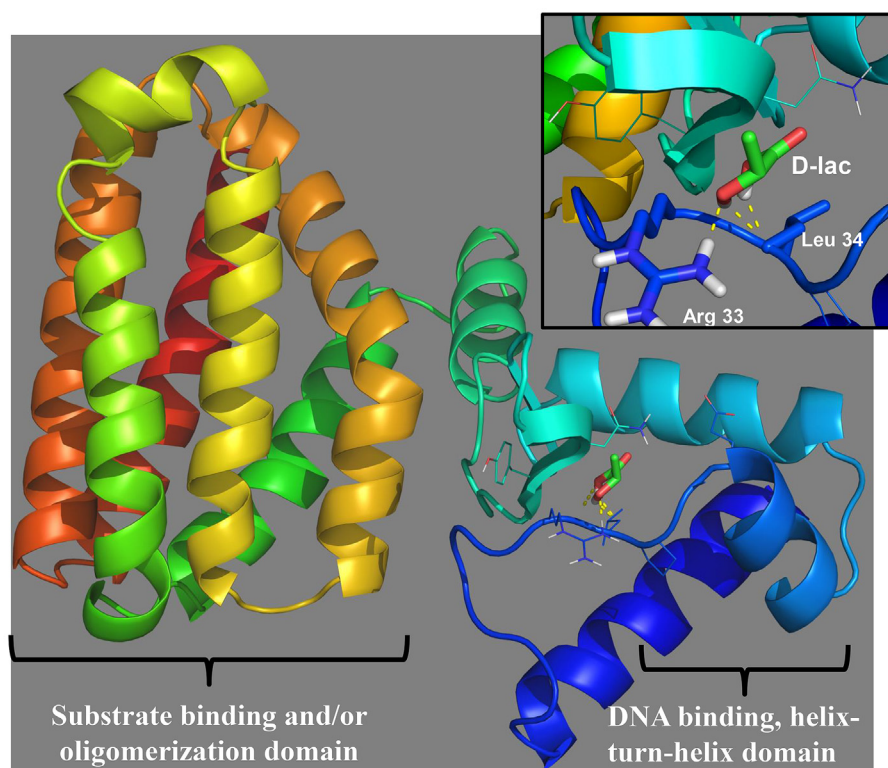


Fig. 3. Predicted three-dimensional (3D) structure and docking analysis of D-LldR from *P. fluorescens* A506. The inset shows amino acid interactions with D-lactate in the active-site pocket. The hydrogen bonds are represented by the yellow dotted lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

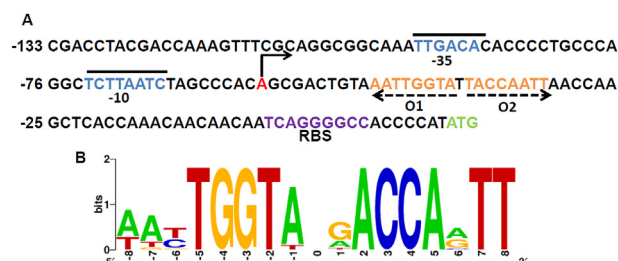


Fig. 4. Organization of transcriptional regulatory elements of the *dld* operon in *P. fluorescens* A506. (A) *lldP/dld-II* promoter sequence is numbered relative to 5' end of translation start. The putative -10 and -35 regions (blue), transcription start site (TSS) (red), operator site for D-LldR binding (orange), RBS (purple), and ATG translation start (green) are indicated. (B) A WebLogo was generated using several predicted and experimentally verified (foot-printing) regulatory binding sites that belong to GntR family. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(According to manufacturer's instructions). Briefly, First-strand cDNA synthesis is primed using a modified oligo (dT) primer. After SMARTScribe Reverse Transcriptase (RT) reaches the end of the mRNA template, several non-templated residues were added. The SMARTer A Oligonucleotide anneals to the tail of the cDNA and serves as an extended template for SMARTScribe RT. For sequencing full-length ORFs, the purified fragments were ligated with pGEM®-T vector. The resulting product was transformed into *E. coli* XL1-Blue.

2.8. Analytical methods

The cell concentration was determined in a 10 mm path length cuvette using a double-beam spectrophotometer (Lambda 20, PerkinElmer, Norwalk, CT).

3. Results and discussion

3.1. Comparative genome analysis and identification of D(-)-lactate-specific LldR

Strains growing on D-lactate as a sole carbon source should have catabolic genes for D-lactate and it is probable that the expression of the catabolic gene is induced by D-lactate. In the case of 3-hydroxypropionic acid, a structural isomer of lactate, the catabolic genes have been reported to be controlled in an inducible manner in various bacterial strains [17]. L-Lactic acid is produced by many bacteria during growth on sugars; however microbial growth on lactates, especially D-lactate, as a sole carbon source, has not been well studied. *Shewanella oneidensis* MR-1, extensively adopted in electrofermentation, is known to grow on both D- and L-lactate and its catabolic operons have already been identified [19; Fig. S1]. Three operons (*lldP_dld-II*, *lldEFG* and *lldR*) contain six genes, which includes lactate permease (*lldP*), D-lactate dehydrogenase (*dld-II*), L-lactate dehydrogenase (*lldEFG*) and a lysR family transcriptional regulator (*lldR*). To study inducibility of the *lldP_dld-II* and *lldEFG* operons, *S. oneidensis* MR-1 was cultured on three different carbon sources, gluconic acid, L-lactic acid, and D-lactic acid, and transcription of these operons were compared by RT-PCR (Fig. S1). The expression of the *lldEFG* operon increased by ~14-17-fold, when the strain was grown on lactate compared to gluconic acid. However, the transcription elevation by L-lactate and D-lactate were almost the same, suggesting that LldR of *S. oneidensis* MR-1 does not discriminate D-lactate from L-lactate. In the case of *lldP* and *dld-II*, no such elevation has been observed, indicating that their expression is not under the control of LldR. Brutinel et al. have reported that D-lactate dehydrogenase (*dld-II*) of *S. oneidensis* MR-1 is expressed constitutively, independent of growth substrates [20,21]. As our target is to identify D-lactate-specific TF, the LldR of *S. oneidensis* MR-1 is not interesting.

Pseudomonas strains are also known to grow well on lactate [22]. Genomes of all *Pseudomonas* strains, sequenced thus far, were examined for their lactate catabolic operons (Fig. 1A). Many *Pseudomonas* strains had the *lldP_lldD/EFG_dld-II* genes, which should encode lactate

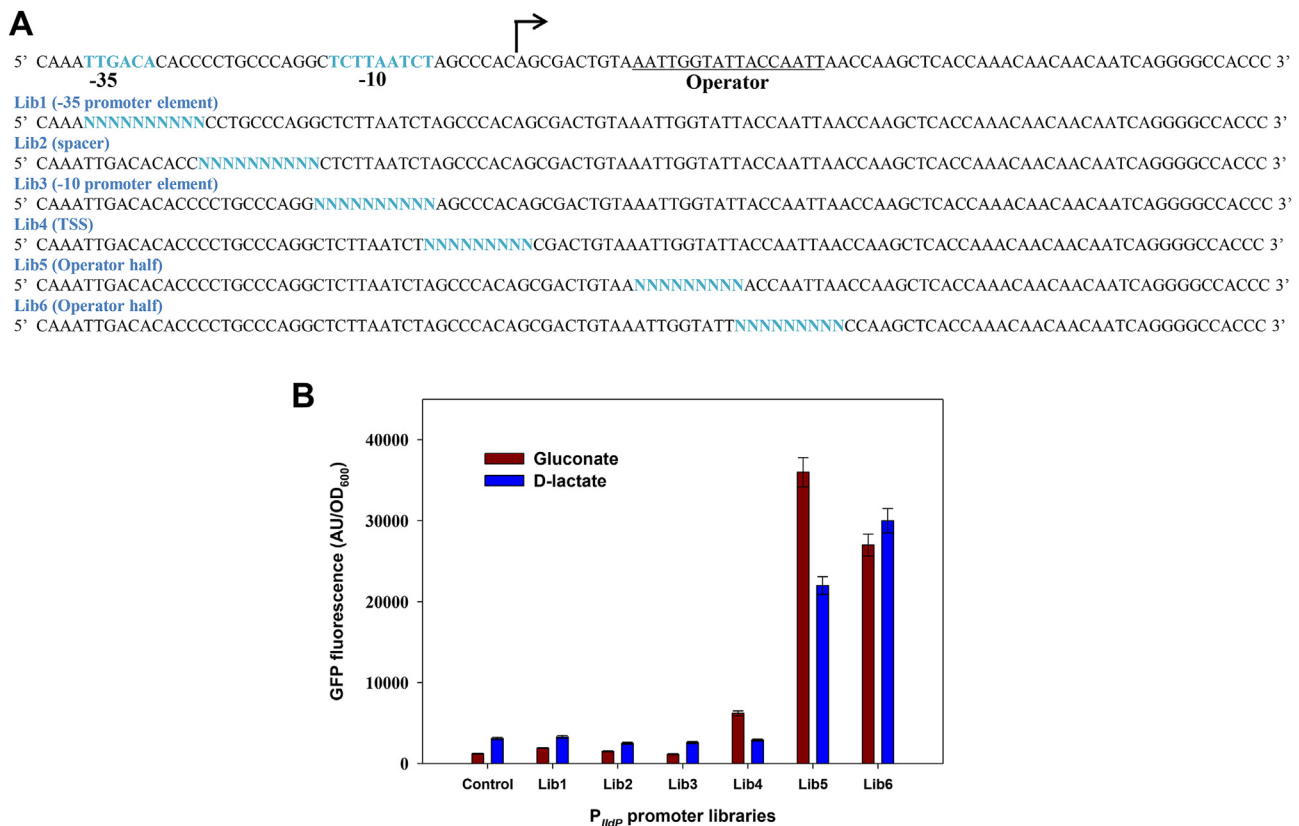


Fig. 5. Characterization of P_{lldP} obtained from promoter library design. (A) The 5' end mapping analysis of regulatory elements (promoter and operator sites) in the intergenic regions of the *dld* operon in *P. fluorescens*. (B) GFP fluorescence response representing 8–15 colonies of the P_{lldP}^* libraries.

permease (*lldP*) and D-lactate dehydrogenase (*dld-II*). In addition, they had a divergently transcribed operon for the *lldR* gene which should encode a TF controlling the expression of the *lldP*, *lldD*/*EFG*, *dld-II* operon. Interestingly, *P. fluorescens* A506 lacked the *lldD*/*EFG* genes encoding L-lactate dehydrogenase, suggesting that the *lldR* gene product of *P. fluorescens* A506 might exhibit specificity to D-lactate. To answer the hypothesis, growth experiments were conducted using gluconate or D/L-lactate as carbon source (Fig. 1). *P. fluorescens* A506 could grow on both D- and L-lactate as a sole carbon and energy source, but D-lactate (specific growth rate, $\mu = 0.52 \text{ h}^{-1}$) gave a better growth than L-lactate ($\mu = 0.41 \text{ h}^{-1}$). Dld-II can be responsible for the assimilation of both L-lactate and D-lactate, or alternatively, there can exist unknown LldD isomer(s). The transcription of D-lactate dehydrogenase (*dld-II*) was examined in the presence of D- or L-lactate, some organic acids with similar structure as lactic acid, and other intermediates that appear in the central carbon metabolism. Transcription of *dld-II* increased by 3.6-fold in the presence of 25 mM D-lactate, compared to gluconic acid; while with L-lactate or 3-HP (structural isomers of D-lactate), the increase in transcription was marginal as ~1.3-fold (Fig. 1B). With other organic acids such as acetate, propionate and butyrate, no induction was observed. This indicates that LldR of *P. fluorescens* A506 is a D-lactate-specific TF. To the best of our knowledge, this is the first report on the existence of D-lactate responsive gene regulation system in bacteria.

To confirm the physiological role of D-LldR (hereafter, 'D' is added to LldR to emphasize its inducer specificity) as a TF, deletion and subsequent complementation experiments were conducted (Fig. 1D). In the deletion mutant ($\Delta\text{D-lldR}$), transcription of *dld-II* was high for both gluconate and D-lactate, and D-lactate did not improve the transcription of *dld-II*. On the other hand, when D-lldR was re-introduced into the ΔlldR mutant via a plasmid, up-regulation of *dld-II* by D-lactate was fully restored. These results confirm that D-LldR is the responsive TF and it

negatively controls the transcription of *dld-II*.

3.2. Virtual screening of D-lactate inducible expression systems and molecular modeling of D-LldR

With an objective to identify more D-LldR-mediated D-lactate specific inducible gene expression systems, various public and private databases such as NCBI (nr, refseq.protein, and env_nr), MBGD (Microbial Genome Database), and SEED databases were screened using homology searches. The D-LldR gene of *P. fluorescens* A506 was used as primary query and cutoff level was arbitrarily set at E-value of 0.01. The D-LldR homologue of *P. fluorescens* A506 occurred in nine different genera only. All strains had D-lactate dehydrogenase (*dld-II*) only without L-lactate dehydrogenase, except the genera *Stenotrophomonas* which had both D- and L-lactate dehydrogenases (Fig. 2A). These nine genera were arbitrarily divided into four groups based on the gene organization and location on the chromosome. Although not well studied, we expect that these organisms should have a similar promoter system as that of *P. fluorescens* A506, with specificity to D-lactate. It is interesting to know that the D-LldR-specific inducible system is limited to only a few bacterial genus.

A phylogenetic tree was generated to analyze the proximity among the D-LldR variants (Fig. 2B). The D-LldR of *P. fluorescens* A506 was close to the genus *Stenotrophomonas* and divergent from those of *Marinospirillum* and *Chromohalobacter*. Moreover, the amino acid sequences of the enzymes LldP and Dld-II appeared to be highly conserved among these species. The multiple sequence alignment for the D-LldR homologs exhibited a high amino acid sequence homology (Fig. S2).

Amino acid sequences of the D-LldR protein, location of DNA-binding helix-turn-helix (HTH) structure in the protein (see below), the position of operator site (see below) and mode of regulation strongly suggest that D-LldR belongs to the GntR-type TF. The GntR-type TFs

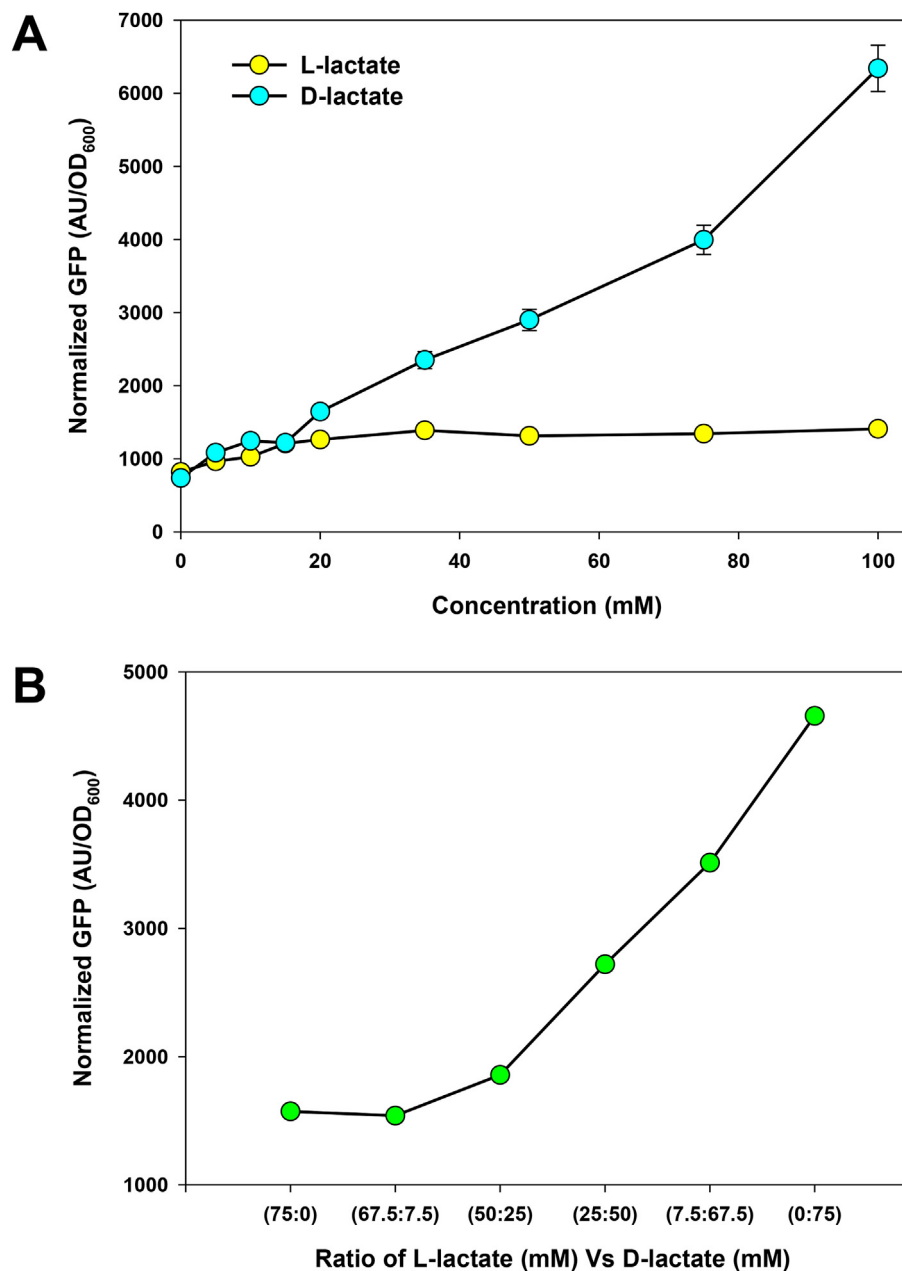


Fig. 6. Transcription factor (D-LldR)-based D-lactate biosensor. (A) Dynamic range. (B) Specificity towards D-lactate in the mixture of D- and L-lactate.

binds to a palindromic operator site, usually present after the transcription start site (TSS), and repress transcription. Upon inducer binding, GntR protein is removed from the operator site and transcription of downstream genes starts [23,24]. For better understanding of the structural and functional characteristics, protein model of D-LldR and its interaction with D-lactate was studied (protein–ligand interactions) and compared with that of L-LldR of *E. coli*. Because the crystal structure of D-LldR is unavailable, multiple template threading was employed to generate the structure [25]. As expected the HTH domain was located towards the N-terminal of both LldR proteins and the effector binding domain towards its C-terminal. This is common in repressible GntR type TFs. Docking of D-/L-lactate to D-LldR was performed to understand the structural feature of binding. The best docking pose gave the low Glide docking score (empirical scoring function) of -4.8 kcal/mol with three hydrogen bonds and two hydrophobic contacts, suggesting high reliability of the simulation. Several intermolecular interactions were identified between D-LldR and the

D-lactate molecule: two amino acid residues (ARG33 and LEU34) present in the loop region of D-LldR protein and hydrophobic contacts with the substrate-binding domain D-lactate (Fig. 3). On the other hand, docking with other isomers such as L-lactate and 3-HP exhibited significantly higher docking scores, -3.1 kcal/mol for L-lactate and -1.8 kcal/mol for 3-HP. These results also support our hypothesis that D-LldR is specific to D-lactate.

3.3. Analysis and characterization of D-lldR intergenic regions

Promoter region controlled by the D-lldR TF in *P. fluorescens* A506 was studied. Analysis of the nucleotide sequence within the intergenic region between the divergently transcribed genes (D-lldR and *lldP_dld-II* operons) and the transcript of *lldP_dld-II*, revealed the presence of putative promoter elements (-10 and -35) and a conserved perfect palindromic operator site located between $+11$ and $+27$ from TSS (Fig. 4A). The TSS was predicted using the NNPP tool [26] and then

confirmed by 5' end mapping (Fig. S3). The half-symmetry palindromic sequences of the operator had eight bases, 'AATTGGTA' with perfect symmetry, and were separated by a single nucleotide, 'T'. The WebLogo construction over 34 known operator sequences [27] (Fig. 4B) exhibited a highly conserved motif (3' WNN**TGGT**W-N**ACCANTT**5'), specific for GntR family members.

Promoter libraries were designed with two motivations; (i) to verify *in silico* predictions of operator region/motif, and (ii) to design a fine-tuned gene expression system using P_{ltd} promoter. Degenerate libraries were constructed for promoter elements (−35, spacer and −10 regions; Library 1–3), TSS (Library 4) and operator (half-site, Library 5–6) (Fig. 5A). To test these libraries without disturbance by synthesis of lactic acid, recombinant *P. denitrificans* that does not naturally produce lactic acid was used as host. Medium-copy plasmids pUCPK' expressing GFP under the control of P_{ltd}^* (* indicates mutation in the promoter region) promoter libraries were constructed and introduced to *P. denitrificans*. The expression of TF D-LldR was under the constitutive P_{zwf} promoter (controlling glucose-6-phosphate dehydrogenase gene in *P. denitrificans*) (Fig. S4). The cultures were supplemented with 50 mM gluconate as carbon source, and 25 mM D-lactate was either added or not added. As many as 95 colonies for each library were screened on deep 96-well blocks (Nunc™ 2.0 mL DeepWell™ Plates).

Fig. 5B represents GFP fluorescence by at least 8–15 of the total colonies screened/tested. As expected, colonies belonging to the Lib. 4–6 that represent randomization at TSS and operator regions, improved their expression levels significantly by ~6 and 10–12-folds in the presence of gluconate and D-lactate, respectively, but lost their inducibility towards D-lactate (Fig. 5). In Lib. 5–6, the symmetry of the GntR binding motif was disturbed. Therefore, binding of LldR to operator region can be either weak or fully abolished, resulting in higher GFP fluorescence. However, no significant difference in the GFP readout, upon randomizing either −35/−10 or spacer regions of the P_{ltd} (in Library 1–3) was noticed. On the other hand, randomization of the TSS region in Lib. 4 enhanced transcription [28]. Although not understood, we believe that upon randomization, the stability ($t^{1/2}$) of the mRNA might vary and enhanced GFP expression. These results indicate or confirm that; (i) P_{ltd} is regulated by 'repression-based' transcriptional control, (ii) LldR strongly binds to the operator region in the absence of D-lactate, and (iii) strength of the core promoter of P_{ltd} itself can be improved by altering the operator site.

According to previous studies, the GntR family is often involved in the expression of multiple sugar transporters [29]. The sugar transporters are membrane proteins and their expression is usually maintained at a low level and tightly controlled. Therefore, it is not surprising that the maximum inducibility in the current D-LldR system is low in the range of 3–6-folds. For biotechnological application, however, better induction system, i.e., high promoter strength and induction fold, is needed. The current study suggests promoter strength can be easily improved, but induction fold might not.

3.4. Performance of D-LldR as biosensor towards D(−)-lactate

The recombinant *P. denitrificans* strain expressing pUCPK-D-LldR- P_{ltd} -gfp was studied as a biosensor to detect or monitor D-lactate. The cells were cultured in the M9 minimal medium in the presence of varying concentrations of pure D/L-lactic acid with gluconate as sole carbon source. When determined at 18 h, the fluorescence signal was enhanced as the concentration of D-lactic acid increased, reaching the maximum at 100 mM D-lactic acid (Fig. 6A). With L-lactic acid, on the contrary, no such increase in GFP was observed with increasing concentration. The dynamic range for D-lactic acid appeared to be 35–100 mM and the maximum inducibility was ~6-fold. The experiments were repeated with the mixtures of D/L-lactic acid at different ratios. Total concentration (sum of D- and L-lactic acid) was fixed at 75 mM and GFP was measured at 18 h of cultivation (Fig. 6B). The biosensor exhibited high specificity towards D-lactic acid even in the

presence of acid mixtures. These results indicate that D-LldR biosensor can be used to detect and monitor D-lactic acid under the condition where both stereoisomers are produced.

In this study, D-Lactate specific TF and its cognate operator sequence were identified and characterized. D-LldR belongs to GntR family and it functions as a transcriptional repressor of lactate catabolic genes. A biosensor was developed based on D-lactate inducibility, and its applicability for monitoring D-lactate was verified. Improvement of this regulatory system for induction fold and sensitivity are under progress.

Conflicts of interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.synbio.2019.08.004>.

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