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Fine tuning the transcription of *ldhA* for D-lactate production

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Abstract Fine tuning of the key enzymes to moderate rather than high expression levels could overproduce the desired metabolic products without inhibiting cell growth. The aims of this investigation were to regulate rates of lactate production and cell growth in recombinant *Escherichia coli* through promoter engineering and to evaluate the transcriptional function of the upstream region of *ldhA* (encoding fermentative lactate dehydrogenase in *E. coli*). Twelve *ldhA* genes with sequentially shortened chromosomal upstream regions were cloned in an *ldhA* deletion, *E. coli* CICIM B0013-080C (*ack-pta pps pflB dld poxB adhE frdA ldhA*). The varied *ldhA* upstream regions were further analyzed using program NNPP2.2 (Neural Network Promoter Prediction 2.2) to predict the possible promoter regions. Two-phase fermentations (aerobic growth and oxygen-limited production) of these strains showed that shortening the *ldhA* upstream sequence from 291 to 106 bp successively reduced aerobic lactate synthesis and the inhibition effect on cell growth during the first phase. Simultaneously, oxygen-limited lactate productivity was increased during the second phase. The putative promoter downstream of the −96 site of *ldhA* could function as a transcriptional promoter or regulator. B0013-080C/pTH-*rrnB-ldhA*8, with the 72-bp upstream segment of *ldhA*, could be grown at a high rate and achieve a high oxygen-limited lactate productivity of 1.09 g g^{-1} h^{-1}. No transcriptional promoting region was apparent downstream of the −61 site of *ldhA*. We identified the latent transcription regions in the *ldhA* upstream sequence, which will help to understand regulation of *ldhA* expression.

Keywords Promoter engineering · Putative promoter · *Escherichia coli* · D-Lactate fermentation · Cell growth

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Introduction

To overproduce a desired metabolic product, a strategy is applied to overexpress a rate-limiting enzyme by gene manipulation. However, the overexpressed enzyme levels could increase cellular burden, leading to unbalanced metabolism. For instance, overexpression of the *ldhA* gene, encoding the fermentative lactate dehydrogenase (LDH) of *E. coli*, results in very poor growth, because the excess LDH converts the pyruvate pool to lactate, causing a shortage of 3-carbon metabolic intermediates [2]. Increasingly, studies have indicated that moderate rather than high overexpression of the target genes could achieve better results [1, 9, 13]. Accordingly, fine tuning of expression is required.

Gene regulation occurs mainly at the level of transcriptional initiation in *E. coli* and other bacteria. Therefore, manipulation of the transcriptional initiation region is an effective method of regulating gene expression and metabolite formation. Recently, promoter libraries have been formed by applying error prone PCR to mutate the −10 and −35 promoter elements and the intervening nucleotides. After selection, promoters with altered strength and regulation function were obtained [1, 7, 9, 12, 13]. This approach is known as promoter engineering. However, desirable error prone PCR manipulation is difficult to achieve. Expression of *ldhA* is regulated by various factors, such as the gene products of *arcB*, *mlc*, *csrA*, *csrB*, and *glgA* [2, 8]. For that reason, simply shortening the *ldhA* upstream sequence could eliminate regulation at the transcriptional level and alter fermentation performance.

Bioinformatic analysis could help to understand the mechanism of transcriptional regulation. Neural networks (NNs) have been used extensively in promoter prediction with high accuracy. In particular, they have been applied successfully to the *E. coli* genome [4] and other bacteria [10] recognizing the degenerate patterns that characterize promoter motifs [3]. The program NNPP2.2 (Neural Network Promoter Prediction, http://www.fruitfly.org/seq_tools/promoter.html) has also been applied to the *E. coli* genome. The positional accuracy of promoter prediction is ±3 bp. Although a high incidence of false positive is produced, the accuracy of NNPP2.2 in predicting promoter elements is comparable to several other freely available techniques [11].

Previously, we reported on a D-lactate-producing strain, *E. coli* CICIM B0013-070 (*ack-pta pps pflB dld poxB adhE frdA*), that produces a high level of lactate with low levels of by-products [16]. In the present study, the upstream region of *ldhA* gene was shortened sequentially, and the resulting fragments were cloned into an *ldhA* deleted mutant of B0013-070, namely B0013-080C (B0013-070 *ldhA*), to test the effects of these varied lengths of transcriptional regions on lactate fermentation. NNPP2.2 was also used to identify the possible promoting functions in these regions.

Materials and methods

Strains

The genotypes of the *E. coli* strains and plasmids used in the present study are summarized in Table 1. A deletion of *ldhA* in B0013-070 resulted in B0013-080C (Δ*ack-pta*::*dif* Δ*pps*::*dif* Δ*pflB*::*dif* Δ*dld*::*dif* Δ*poxB*::*FRT* Δ*adhE*::*dif* Δ*frdA*::*dif* Δ*ldhA*::*dif*). B0013 and its derivatives are kept in the Culture and Information Centre of Industrial Microorganisms of China Universities (http://cicim-cu.jiangnan.edu.cn/). Details of the primers used in this study are given in Table 2. The *rrnB* transcription terminators were PCR amplified from pKK223-3 using primers RrnB-1 and RrnB-2. After digestion by *Hin*dIII and *Pst*I, this fragment was cloned into the same sites of pTH18kr [5] to produce pTH-*rrnB*. In this plasmid, read-through transcription initiated from the promoter of *kan* gene in pTH18kr could be inhibited. Subsequently, *ldhA′* genes PCR amplified using forward primers Ec-lA3-4 and Ec-PlA1 to Ec-PlA11, respectively, and reverse primer Ec-lA2-2 were digested by *Bam*HI and *Eco*RI and cloned into the same sites in pTH-*rrnB* to create pTH-*rrnB-ldhA*0 to pTH-*rrnB-ldhA*11 (Supplementary Fig. S1). In these plasmids, transcription of *ldhA*' and PO_{lac} are oriented in the opposite direction. Thereafter, plasmids pTH-*rrnB-ldhA*0 to pTH-*rrnB-ldhA*11 were electroporated into B0013-080C to generate strains B0013-080C/pTH-*rrnB-ldhA*0 to B0013-080C/pTH-*rrnB-ldhA*11.

Fermentation experiments

Strains (stored as glycerol stocks at −80 °C) were first grown on Luria–Bertani (LB) plates for about 24 h and subsequently colonies were transferred to 50 ml of LB medium containing kanamycin (35 mg/l) as required in a 250-ml flask. After 10 h growth with shaking, cells were harvested by centrifugation and resuspended in modified M9 medium [16]. This suspension was used to inoculate 150 ml fresh modified M9 medium containing 5 g/l glucose (in a 500-ml flask) at a concentration of 0.1 g/l dry cell weight (DCW). After incubation for 9 h with shaking at 200 rpm, this seed culture was used to inoculate a 7-l bioreactor (Bioflow110; New Brunswick Scientific Co., Edison, NJ, USA) containing 3 l modified M9 medium with 30 g/l glucose at the concentration of 0.06 g/l DCW as reported previously [16]. Growth was initiated by sparging air into the bioreactor at 1–7 l/min and the dissolved oxygen concentration maintained above 40 % saturation by agitation at 200–1,000 rpm. The pH was controlled at 7 by automatic feeding of concentrated NH_4OH and 10 % (v/v) H_2SO_4. The oxygen-limited phase was started when the cell concentration reached an optical density at 600 nm (OD_{600}) of ca. 30 (ca. 11.4 g/l DCW). During this second phase, 210 g glucose was added at the start, air sparging was stopped (without sparging nitrogen to simplify the fermentation process), the agitation was slowed to 100 rpm, and the pH was controlled at 7 using 25 % (w/v) $Ca(OH)_2$. The fermentation was stopped when the glucose was exhausted or after 48 h. All cultivations were conducted at 37 °C.

Table 1 *E. coli* strains and plasmids used in this study

	Relevant characteristics	Source or reference
Strains		
CICIM B0013	Wild type	CICIM-CU
CICIM B0013-070	B0013, Δ*ack-pta*::*dif* Δ*pps*::*dif* Δ*pflB*::*dif* Δ*dld*::*dif* Δ*poxB*::*FRT* Δ*adhE*::*dif* Δ*frdA*::*dif*	CICIM-CU; [16]
CICIM B0013-080C	B0013, Δ*ack-pta*::*dif* Δ*pps*::*dif* Δ*pflB*::*dif* Δ*dld*::*dif* Δ*poxB*::*FRT* Δ*adhE*::*dif* Δ*frdA*::*dif* Δ*ldhA*::*dif*	CICIM-CU
Plasmids		
pKK223-3	*bla*, P_{tac}, β-lactamase, *rrnB*	CICIM-CU
pTH18kr	*kan*, P_{lac}, *lacZ*', low copy number cloning vector	CICIM-CU; [5]
pTH-*rrnB*	*kan*, *rrnB*; *rrnB* (PCR using RrnB-1/RrnB-2) from pKK223-3 cloned into *Hin*dIII-*Pst*I sites of pTH18kr	This study
pTH-*rrnB-ldhA*0	*kan*, *rrnB*, *ldhA*0; *ldhA*0 (PCR using Ec-lA3-4/Ec-lA2-2) from B0013 cloned into *Bam*HI-*Eco*RI sites of pTH-*rrnB*	This study
pTH-*rrnB-ldhA*1	*kan*, *rrnB*, *ldhA*1; *ldhA*1 (PCR using Ec-PlA1/Ec-lA2-2) from B0013 cloned into *Bam*HI-*Eco*RI sites of pTH-*rrnB*	This study
pTH-*rrnB-ldhA*2	*kan*, *rrnB*, *ldhA*2; *ldhA*2 (PCR using Ec-PlA2/Ec-lA2-2) from B0013 cloned into *Bam*HI-*Eco*RI sites of pTH-*rrnB*	This study
pTH-*rrnB-ldhA*3	*kan*, *rrnB*, *ldhA*3; *ldhA*3 (PCR using Ec-PlA3/Ec-lA2-2) from B0013 cloned into *Bam*HI-*Eco*RI sites of pTH-*rrnB*	This study
pTH-*rrnB-ldhA*4	*kan*, *rrnB*, *ldhA*4; *ldhA*4 (PCR using Ec-PlA4/Ec-lA2-2) from B0013 cloned into *Bam*HI-*Eco*RI sites of pTH-*rrnB*	This study
pTH-*rrnB-ldhA*5	*kan*, *rrnB*, *ldhA*5; *ldhA*5 (PCR using Ec-PlA5/Ec-lA2-2) from B0013 cloned into *Bam*HI-*Eco*RI sites of pTH-*rrnB*	This study
pTH-*rrnB-ldhA*6	*kan*, *rrnB*, *ldhA*6; *ldhA*6 (PCR using Ec-PlA6/Ec-lA2-2) from B0013 cloned into *Bam*HI-*Eco*RI sites of pTH-*rrnB*	This study
pTH-*rrnB-ldhA*7	*kan*, *rrnB*, *ldhA*7; *ldhA*7 (PCR using Ec-PlA7/Ec-lA2-2) from B0013 cloned into *Bam*HI-*Eco*RI sites of pTH-*rrnB*	This study
pTH-*rrnB-ldhA*8	*kan*, *rrnB*, *ldhA*8; *ldhA*8 (PCR using Ec-PlA8/Ec-lA2-2) from B0013 cloned into *Bam*HI-*Eco*RI sites of pTH-*rrnB*	This study
pTH-*rrnB-ldhA*9	*kan*, *rrnB*, *ldhA*9; *ldhA*9 (PCR using Ec-PlA9/Ec-lA2-2) from B0013 cloned into *Bam*HI-*Eco*RI sites of pTH-*rrnB*	This study
pTH-*rrnB-ldhA*10	*kan*, *rrnB*, *ldhA*10; *ldhA*10 (PCR using Ec-PlA10/Ec-lA2-2) from B0013 cloned into *Bam*HI-*Eco*RI sites of pTH-*rrnB*	This study
pTH-*rrnB-ldhA*11	*kan*, *rrnB*, *ldhA*11; *ldhA*11 (PCR using Ec-PlA11/Ec-lA2-2) from B0013 cloned into *Bam*HI-*Eco*RI sites of pTH-*rrnB*	This study

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Analytical methods

Cell mass was estimated by measuring the OD_{600} (if $Ca(OH)_2$ had been added during fermentation, samples were pretreated with 1 M HCl at 20-fold of the sample volume to remove the suspended substances) and related to DCW according to a standard curve (1 OD_{600} = 0.38 g/l DCW) [16]. Glucose concentration was estimated with a glucose biosensor [16]. Samples were pretreated with H_2SO_4 (at 5 % of the sample volume) to release organic acids precipitated with $Ca(OH)_2$ during fermentation. Organic acid concentrations were measured by HPLC [16]. Possible promoter regions were predicted using NNPP2.2 (Neural Network Promoter Prediction 2.2, http://www.fruitfly.org/seq_tools/promoter.html) with a default threshold score of 0.8.

Results and discussion

Cloning of *ldhA* from *E. coli* CICIM B0013

ldhA is a recently identified gene in the σ^{32} regulon, whose transcriptional level increases considerably after the induction of σ^{32} [15]. The corresponding promoter (*ldhA*p) recognized by σ^{32} RNA polymerase holoenzyme ($RNAP\sigma^{32}$) has been analyzed using the EcoCyc (http://ecocyc.org/) and Regulon DB (http://regulondb.ccg.unam.mx/index.jsp) databases. Their results show the −35 (TTGTTGA) and −10 (CGCCAT) promoter elements and predict the transcriptional start site "C" at a distance of 6 nucleotides downstream of the −10 hexamer (Fig. 1). However there is no experimental evidence currently

Table 2 Primers used in this study

Primers	Sequence (5'-3')	Corresponding restriction enzyme
RrnB-1	TCC*AAGCTT*CTGTTTTGGCGGATGAGAG	*Hin*dIII
RrnB-2	TTA*CTGCAG*AAGAGTTTGTAGAAACGCAAAAAGGCC	*Pst*I
Ec-lA3-4	TCA*GGATCC*CAGCCCGAGCGTCATCAG	*Bam*HI
Ec-PlA1	CTT*GGATCC*TGCGCCTACACTAAGCATAGTTGTT	*Bam*HI
Ec-PlA2	CTT*GGATCC*CTAAGCATAGTTGTTGATGAATTTTTCAAT	*Bam*HI
Ec-PlA3	CTT*GGATCC*TTGTTGATGAATTTTTCAATATCGCC	*Bam*HI
Ec-PlA4	CTT*GGATCC*ATTTTTCAATATCGCCATAGCTTTCA	*Bam*HI
Ec-PlA5	CTT*GGATCC*ATCGCCATAGCTTTCAATTAAATTTG	*Bam*HI
Ec-PlA6	CTT*GGATCC*AGCTTTCAATTAAATTTGAAATTTTGTAAA	*Bam*HI
Ec-PlA7	CTT*GGATCC*ATGAAACTCGCCGTTTATAGCACA	*Bam*HI
Ec-PlA8	TTA*GGATCC*AATTATATTTGAAATTTTGTAAAATATTTTTAG	*Bam*HI
Ec-PlA9	TTA*GGATCC*GAAATTTTGTAAAATATTTTTAGTAGCTT	*Bam*HI
Ec-PlA10	TTA*GGATCC*AAAATATTTTTAGTAGCTTAAATGTGATTC	*Bam*HI
Ec-PlA11	TTA*GGATCC*TAGTAGCTTAAATGTGATTCAACATCACTG	*Bam*HI
Ec-lA2-2	CTT*GAATTC*GCTGCCGGAAATCATCATTTTTT	*Eco*RI

Restriction sites are italic/underlined

available to confirm the length of the promoter region. On the basis of the characteristics of the Shine–Dalgarno sequence (SD sequence), we assigned a putative SD sequence (GGAG) at the −12 to −9 sites upstream of the initiation codon (Fig. 1).

The sequence of *ldhA* from *E. coli* CICIM B0013 has been analyzed and deposited in the GenBank database (accession no. JQ231279). Compared with *ldhA* from *E. coli* K12 MG1655, one nucleotide difference (from A to T) in the promoter region was observed in B0013 resulting in the replacement of glutamic acid (E) with aspartic acid (D) in the fermentative lactate dehydrogenase structure. However, the other six nucleotide changes in the structural gene do not result in alterations in the amino acid residues.

In order to fine tune *ldhA* expression for lactate production, the *ldhA* genes with 0–291 bp sequences of the

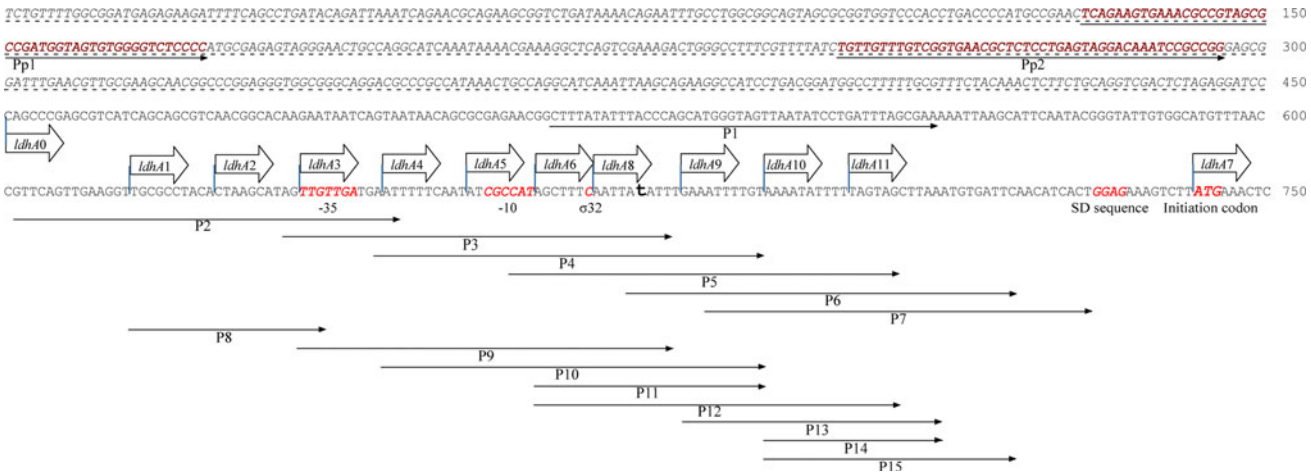

Fig. 1 Partial gene sequence of the *rrnB-ldhA*0 fragment. The *italic* and *underlined* sequence is the *rrnB* terminator (where two putative promoters Pp1 and Pp2 are indicated by *solid arrows*). Sequence not underlined is the upstream sequence of *ldhA* gene. The −10 and −35 elements and transcriptional start site (directed by σ^{32} and provided by analysis of the EcoCyc [http://ecocyc.org/] and Regulon DB [http://regulondb.ccg.unam.mx/index.jsp] databases) as well as the predicted SD sequence and the initiation codon are in *italic* and marked in *red*. The lowercase letter "t" represents the variation from A to T in B0013 compared to *E. coli* K12 MG1655. The *open arrows* indicate the initiation sites of the *ldhA* genes (*ldhA*0 to *ldhA*11) cloned in the present investigation. Putative promoters analyzed using NNPP2.2 (http://www.fruitfly.org/seq_tools/promoter.html) are indicated by *solid arrows*. The P1 to P7 putative promoters exist in the *rrnB-ldhA*0 fragment. Other *solid arrows* indicate the partial sequence of P8 to P15 putative promoters in *rrnB-ldhA*1 to *rrnB-ldhA*10 fragments

upstream region of the initiation codon (Fig. 1) were cloned into the modified low copy number plasmid pTH-*rrnB* (5 copies per chromosome at 30 °C using pTH18kr [5]). The read-through transcription initiated from the promoter of *kan* gene could be inhibited by *rrnB* transcription terminator. Subsequently, the recombinant plasmids were transferred into *E. coli* B0013-080C (*ldhA* deleted mutant in lactate producer *E. coli* B0013-070 [16]). The resulting strains were cultivated under fermentation conditions to test the regulation effects of the *ldhA* upstream region.

Shortening *ldhA* upstream region from 291 to 106 bp reduced cell growth inhibition and improved oxygen-limited lactate productivity

Large differences in the lactate fermentation properties of B0013-070, B0013-080C/pTH-*rrnB-ldhA*0 through B0013-080C/pTH-*rrnB-ldhA*11, and B0013-080C were observed when cultivated in a 7-l bioreactor under aerobic and oxygen-limited conditions (Table 3; Supplementary Fig. S2). Upstream regions of *ldhA* in *rrnB-ldhA*0 through *rrnB-ldhA*11 fragments were analyzed using NNPP2.2 promoter predictor. The predicted promoters are shown in Table 4, the score of which represents the probability of being a promoter [3]. The *ldhA*p identified by EcoCyc and Regulon DB databases was highly coincident with the putative promoter region of P3 (the score is 1.0, Table 4; Fig. 1), indicating the reliability of the present analytical approach. There were two putative promoters in the *rrnB* region in every plasmid (Fig. 1). Besides Pp1 and Pp2, several putative promoters were characterized in *rrnB-ldhA*0 through *rrnB-ldhA*10. However, no additional predicted promoter could be located in *rrnB-ldhA*11. We do not know if the initiation of transcriptions was due to binding at one or several of these sites. Nevertheless, these possible promoter regions provided the primary interaction with holoenzyme, and their alterations further explained the fermentation variations among strains.

All the *ldhA* genes cloned in B0013-080C/pTH-*rrnB-ldhA*0 to B0013-080C/pTH-*rrnB-ldhA*3 contained the entire *ldhA*p as revealed by EcoCyc and Regulon DB database analysis (Fig. 1). However, fermentation performances of these strains were different (Table 3). Especially notable was the improved productivity of the B0013-080C/pTH-*rrnB-ldhA*3 strain and the greater lactate yields of the B0013-080C/pTH-*rrnB-ldhA*2 and B0013-080C/pTH-*rrnB-ldhA*3 strains. Therefore, the deletion of sequences may allow different *ldhA* expression. The *ldhA*0 fragment contained a long sequence of 291 bp upstream of the structure gene. The corresponding strain, B0013-080C/pTH-*rrnB-ldhA*0, which had low copies of *ldhA* produced twofold higher lactate yield than B0013-070 (with single copy of *ldhA* gene) during aerobic growth phase. The rapid synthesis of lactate possibly reduced the pyruvate pool for biomass formation [2], resulting in low growth rate and biomass yield. Strains B0013-080C/pTH-*rrnB-ldhA*1 to B0013-080C/pTH-*rrnB-ldhA*3 showed a decrease of aerobic lactate formation per cell compared to B0013-080C/pTH-*rrnB-ldhA*0 (Table 3), whereas the inhibition of biomass formation was reduced, pointing to a possible greater availability of pyruvate. The cell growth rate and biomass yield in B0013-080C/pTH-*rrnB-ldhA*3 was similar to those in the parent strain B0013-070.

Although B0013-080C/pTH-*rrnB-ldhA*0 has several copies of *ldhA*, overexpression of LDH did not increase the oxygen-limited lactate productivity of the strain compared to B0013-070. Oxygen-limited lactate productivities in B0013-080C/pTH-*rrnB-ldhA*1 through B0013-080C/pTH-*rrnB-ldhA*3 were improved significantly compared to B0013-080C/pTH-*rrnB-ldhA*0 and the oxygen-limited lactate productivity in B0013-080C/pTH-*rrnB-ldhA*3 was even higher than B0013-070. Therefore, if sufficient pyruvate remained available at the end of aerobic phase a rapid initiation of lactate production could occur in the oxygen-limited phase. Possibly because of the high lactate production rate, oxygen-limited lactate yields in B0013-080C/pTH-*rrnB-ldhA*2 and B0013-080C/pTH-*rrnB-ldhA*3 were close to the theoretical maximum value of 100 g/100 g glucose. The final by-product levels (acetate, succinate, and pyruvate) were not altered significantly by promoter manipulation among strains.

Computational analysis shows that promoters are located within zones with high densities of coincident putative promoters in *E. coli* [6]. The coincident density is possibly the consequence of a number of promoters evolving from the functional promoters into non-productive ones, or developing as new promoters [14]. Transcriptional regulators as well as other functional promoters may play an important role in keeping these latent signals suppressed [6]. On the other hand, these coincident promoter-like signals might play a negative regulatory role if those sites had a competitive relationship, or a positive regulatory role if they helped in the channeling of the RNA polymerase into the promoter [6]. Other experimental evidence also shows the existence of multiple overlapping or coincident promoter sites that become functional under particular conditions [6]. Sequence analysis of *rrnB-ldhA*0 fragment showed that nine putative promoters were located upstream *ldhA* structural gene (Table 4; Fig. 1), and seven of them (P2 to P7) were coincident. Compared to *rrnB-ldhA*0, *rrnB-ldhA*1 did not have P1 and a complete P2, whereas P8 was produced in the recombinant region. The predicted promoter P8 was further removed in *rrnB-ldhA*2. The *rrnB-ldhA*3 partially lacked P3 (the score is 1.0) which was highly coincident with the *ldhA*p identified by the EcoCyc

Table 3 Comparison of fermentation parameters (mean ± range of duplicate experiments)

Strain	Specific growth rate μ (1/h)	Lactate overall volumetric productivity (g l^{-1} h^{-1})[a]	Oxygen-limited lactate volumetric productivity (g l^{-1} h^{-1})[b]	Oxygen-limited lactate specific productivity (g g^{-1} h^{-1})[c]	Oxygen-limited specific glucose consumption rate (g g^{-1} h^{-1})[d]	Oxygen-limited lactate yield (g/100 g glucose)[e]	Aerobic biomass yield (g/100 g glucose)[f]	Aerobic lactate yield (g/100 g glucose)[g]	Yield (g/100 g glucose)[h]				
									Biomass	Lactate	Acetate	Succinate	Pyruvate
B0013-070	0.47 ± 0.01	3.09 ± 0.12	7.56 ± 0.40	0.62 ± 0.03	0.78 ± 0.03	81 0 ± 4.1	34.9 ± 1.7	20.4 ± 1.0	14.1 ± 0.5	63.4 ± 2.6	0.7 ± 0.0	0.9 ± 0.0	0.2 ± 0.0
pTH0[i]	0.21 ± 0.01	1.80 ± 0.09	4.29 ± 0.21	0.46 ± 0.02	0.58 ± 0.01	80.1 ± 3.9	27.2 ± 0.3	40.8 ± 2.0	9.4 ± 0.3	68.5 ± 3.2	0.5 ± 0.0	1.4 ± 0.0	0.1 ± 0.0
pTH1[i]	0.26 ± 0.01	2.18 ± 0.11	6.05 ± 0.35	0.61 ± 0.03	0.78 ± 0.03	79.7 ± 0.4	30.7 ± 1.6	37.9 ± 2.1	11.1 ± 0.9	69.6 ± 3.7	0.5 ± 0.0	0.6 ± 0.0	0.0 ± 0.0
pTH2[i]	0.29 ± 0.01	2.67 ± 0.06	7.41 ± 0.28	0.75 ± 0.02	0.75 ± 0.03	99.9 ± 1.7	38.1 ± 1.0	41.6 ± 1.7	11.6 ± 0.5	82.0 ± 3.9	0.5 ± 0.0	0.9 ± 0.0	0.0 ± 0.0
pTH3[i]	0.49 ± 0.02	3.96 ± 0.20	10.58 ± 0.37	0.86 ± 0.02	0.88 ± 0.01	97.0 ± 2.1	34.8 ± 0.8	29.2 ± 1.2	14.9 ± 0.6	78.2 ± 3.0	0.3 ± 0.0	1.1 ± 0.0	0.0 ± 0.0
pTH4[i]	0.51 ± 0.02	2.91 ± 0.10	5.98 ± 0.30	0.50 ± 0.02	0.68 ± 0.03	73.2 ± 3.6	38.0 ± 0.9	23.6 ± 0.1	13.9 ± 0.4	61.0 ± 2.8	0.2 ± 0.0	0.8 ± 0.0	0.0 ± 0.0
pTH5[i]	0.31 ± 0.02	2.78 ± 0.14	8.58 ± 0.46	0.65 ± 0.03	0.68 ± 0.03	96.9 ± 3.3	38.2 ± 0.5	30.6 ± 1.6	15.1 ± 1.0	80.4 ± 3.5	0.6 ± 0.1	1.1 ± 0.0	0.0 ± 0.0
pTH6[i]	0.49 ± 0.02	3.94 ± 0.08	10.29 ± 0.51	0.85 ± 0.04	0.87 ± 0.04	97.4 ± 4.2	37.3 ± 0.9	28.4 ± 1.1	14.7 ± 0.7	80.4 ± 3.9	0.2 ± 0.0	0.7 ± 0.0	0.0 ± 0.0
pTH8[i]	0.53 ± 0.03	4.37 ± 0.22	11.73 ± 0.59	1.09 ± 0.05	1.09 ± 0.02	99.9 ± 3.9	34.4 ± 1.2	19.1 ± 0.8	13.0 ± 0.6	84.6 ± 4.2	0.4 ± 0.0	1.1 ± 0.0	0.1 ± 0.0
pTH9[i, j]	0.52 ± 0.02	0.18 ± 0.01	0.24 ± 0.01	0.03 ± 0.00	0.12 ± 0.00	27.7 ± 1.3	42.7 ± 2.0	0.0 ± 0.0	14.3 ± 0.7	17.3 ± 0.4	0.5 ± 0.0	20.4 ± 1.0	1.8 ± 0.0
pTH10[i]	0.38 ± 0.02	3.00 ± 0.11	7.38 ± 0.24	0.57 ± 0.01	0.88 ± 0.02	85.9 ± 3.3	43.1 ± 2.1	30.2 ± 1.4	10.3 ± 0.2	72.4 ± 3.2	0.5 ± 0.0	1.2 ± 0.0	0.0 ± 0.0
pTH11[i, j]	0.52 ± 0.02	0.69 ± 0.04	0.80 ± 0.03	0.10 ± 0.00	0.18 ± 0.01	58.9 ± 2.1	51.0 ± 2.5	16.8 ± 0.7	10.9 ± 0.5	47.3 ± 2.3	1.3 ± 0.0	8.2 ± 0.4	1.3 ± 0.1
pTH7[i, j]	0.48 ± 0.03	0.02 ± 0.01	0.03 ± 0.00	<0.01	0.06 ± 0.00	6.7 ± 0.4	36.2 ± 1.9	0.4 ± 0.0	18.1 ± 0.6	2.8 ± 0.2	1.6 ± 0.6	10.2 ± 0.8	2.3 ± 0.1
B0013-080C[j]	0.50 ± 0.02	0.04 ± 0.00	0.04 ± 0.00	<0.01	0.08 ± 0.00	6.1 ± 0.2	38.1 ± 1.2	0.5 ± 0.0	16.8 ± 0.3	3.3 ± 0.1	1.0 ± 0.0	20.4 ± 1.1	2.1 ± 0.1

[a] Average volumetric productivity for lactate during the overall fermentation process

[b] Average volumetric productivity for lactate during the oxygen-limited phase

[c] Average specific lactate productivity during oxygen-limited phase

[d] Average specific glucose consumption rate during the oxygen-limited phase

[e] Average lactate yield during the oxygen-limited phase

[f] Average biomass yield during the aerobic phase

[g] Average lactate yield during the aerobic phase

[h] Average yield during the overall fermentation process

[i] Abbreviation for B0013-080C/pTH-*rrnB-ldh*A0 to B0013-080C/pTH-*rrnB-ldh*A11

[j] Incomplete utilization of glucose substrate

Table 4 Predicted promoters of gene fragments *rrnB-ldhA*0 to *rrnB-ldhA*10[a]

Gene fragment	Predicted promoter		
	Name	Score[b]	Sequence (5'-3')[c]
*rrnB-ldhA*0	P1	0.87	CTTTATATTTACCCAGCATGGGTAGTTAATATCCTGATTTAGCGAA
	P2	0.91	GTTCAGTTGAAGGTTGCGCCTACACTAAGCATAGTTGTTGATGAAT
	P3	1.00	AGTTGTTGATGAATTTTTCAATATCGCCATAGCTTTCAATTAtATT
	P4	1.00	AATTTTTCAATATCGCCATAGCTTTCAATTAtATTTGAAATTTTGT
	P5	0.93	CATAGCTTTCAATTAtATTTGAAATTTTGTAAAATATTTTTAGTAG
	P6	0.99	AtATTTGAAATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTC
	P7	0.90	ATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTCAACATCACT
*rrnB-ldhA*1	P8	0.89	*TGCAGGTCGACTCTAGAGGATCC*TGCGCCTACACTAAGCATAGTTG
	P3	1.00	AGTTGTTGATGAATTTTTCAATATCGCCATAGCTTTCAATTAtATT
	P4	1.00	AATTTTTCAATATCGCCATAGCTTTCAATTAtATTTGAAATTTTGT
	P5	0.93	CATAGCTTTCAATTAtATTTGAAATTTTGTAAAATATTTTTAGTAG
	P6	0.99	AtATTTGAAATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTC
	P7	0.90	ATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTCAACATCACT
*rrnB-ldhA*2	P3	1.00	AGTTGTTGATGAATTTTTCAATATCGCCATAGCTTTCAATTAtATT
	P4	1.00	AATTTTTCAATATCGCCATAGCTTTCAATTAtATTTGAAATTTTGT
	P5	0.93	CATAGCTTTCAATTAtATTTGAAATTTTGTAAAATATTTTTAGTAG
	P6	0.99	AtATTTGAAATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTC
	P7	0.90	ATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTCAACATCACT
*rrnB-ldhA*3	P9	1.00	*CC*TTGTTGATGAATTTTTCAATATCGCCATAGCTTTCAATTAtATT
	P4	1.00	AATTTTTCAATATCGCCATAGCTTTCAATTAtATTTGAAATTTTGT
	P5	0.93	CATAGCTTTCAATTAtATTTGAAATTTTGTAAAATATTTTTAGTAG
	P6	0.99	AtATTTGAAATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTC
	P7	0.90	ATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTCAACATCACT
*rrnB-ldhA*4	P10	1.00	*C*ATTTTTCAATATCGCCATAGCTTTCAATTAtATTTGAAATTTTGT
	P5	0.93	CATAGCTTTCAATTAtATTTGAAATTTTGTAAAATATTTTTAGTAG
	P6	0.99	AtATTTGAAATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTC
	P7	0.90	ATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTCAACATCACT
*rrnB-ldhA*5	P5	0.93	CATAGCTTTCAATTAtATTTGAAATTTTGTAAAATATTTTTAGTAG
	P6	0.99	AtATTTGAAATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTC
	P7	0.90	ATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTCAACATCACT
*rrnB-ldhA*6	P11	0.81	*GGTCGACTCTAGAGGATCC*AGCTTTCAATTAtATTTGAAATTTTGT
	P12	0.93	*TCC*AGCTTTCAATTAtATTTGAAATTTTGTAAAATATTTTTAGTAG
	P6	0.99	AtATTTGAAATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTC
	P7	0.90	ATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTCAACATCACT
*rrnB-ldhA*8	P6	0.99	AtATTTGAAATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTC
	P7	0.90	ATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTCAACATCACT
*rrnB-ldhA*9	P13	0.84	*GACTCTAGAGGATCC*GAAATTTTGTAAAATATTTTTAGTAGCTTAA
	P7	0.90	ATTTTGTAAAATATTTTTAGTAGCTTAAATGTGATTCAACATCACT
*rrnB-ldhA*10	P14	0.82	*TCTGCAGGTCGACTCTAGAGGATCC*AAAATATTTTTAGTAGCTTAA
	P15	0.84	*CGACTCTAGAGGATCC*AAAATATTTTTAGTAGCTTAAATGTGATTC

[a] The promoter sequences were predicted using NNPP2.2 (http://www.fruitfly.org/seq_tools/promoter.html) with an organism type of prokaryote and a minimum promoter score of 0.8; besides the above promoters, predicted promoters Pp1 (*TCAGAAGTGAAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCC*) and Pp2 (*TGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGG*) were presented in each fragment in the *rrnB* terminator

[b] Score of the predicted promoter using NNPP2.2

[c] Underlined and italic sequences are from *rrnB* terminator, other sequences are from the chromosomal upstream region of *ldhA*; the predicted transcription start sites are shown in boxes; the lowercase letter "t" in bold represents the variation from A to T in B0013 compared to *E. coli* K12 MG1655

and Regulon DB databases and generated P9. Therefore, the formation of these coincident putative promoters or other target sites could change the binding by factors to the transcription initiation site and cause the differences in fermentation performance. As 90 % of the known promoters occur in the 250-bp-long region upstream of the gene initiation codon [6], the putative promoters Pp1 and Pp2 observed in *rrnB* fragment could only produce, if any, a minor effect on LDH expression.

A transcriptional promoting region may occur downstream of the −96 site of *ldhA*

Strains B0013-080C/pTH-*rrnB-ldhA*4 through B0013-080C/pTH-*rrnB-ldhA*6 and B0013-080C/pTH-*rrnB-ldhA*8 were still able to produce lactate with high efficiency in spite of the *ldhA*p being partially or entirely removed up to the σ^{32} site (Table 3). Hence, either the upstream sequence on pTH-*rrnB* vector provided the structural *ldhA* with promoter or some other transcriptional initiation regions existed downstream of the −96 site. However, in these strains, the distances between either Pp1 or Pp2 (the promoter-like region in *rrnB*) and the structural *ldhA* were larger than 250 bp. This observation points to the existence of some other functional transcription initiation regions downstream of the −96 site. The removal of the σ^{32} recognized promoter may have eliminated the repression effect on the putative promoters, and finally transcription could be initiated at these promoter-like regions. Alternatively, the real promoter of *ldhA* existed downstream of the −96 site.

In comparison to *rrnB-ldhA*3, *rrnB-ldhA*4 lacked the P9 putative promoter, and P4 was altered at one nucleotide to generate P10 (Table 4). Similar to the fermentation results with the B0013-080C/pTH-*rrnB-ldhA*3 strain, the aerobic lactate yield in B0013-080C/pTH-*rrnB-ldhA*4 was held at moderate level. Aerobic biomass yield and growth rate were improved, indicating that the repression effect of lactate synthesis was diminished. However, oxygen-limited lactate productivity and yield were decreased significantly, possibly due to a complete lack of the putative promoter P3. In this case, the P10, P5, P6, or P7 putative promoter would function as promoter. Nonetheless, the resulting LDH was not sufficient or negative regulation of LDH generation was aroused. Compared to *rrnB-ldhA*4, *rrnB-ldhA*5 did not have P10. The *rrnB-ldhA*6 regenerated P11 and P12. The strains B0013-080C/pTH-*rrnB-ldhA*5 and B0013-080C/pTH-*rrnB-ldhA*6 produced higher lactate yields and productivities in contrast to B0013-080C/pTH-*rrnB-ldhA*4. Furthermore, the cell growth rate and lactate productivity in B0013-080C/pTH-*rrnB-ldhA*6 reached the same level as those of B0013-080C/pTH-*rrnB-ldhA*3, implying that the lactate production capability had recovered and that the putative promoter P10 in B0013-080C/pTH-*rrnB-ldhA*4 could play a negative role in lactate production.

The *ldhA*8 fragment initiates transcription from the σ^{32} start site (though *ldhA*8 was cloned without this site, the upstream *Bam*HI site could include the "C" base). The corresponding strain B0013-080C/pTH-*rrnB-ldhA*8 accumulated a slightly lower lactate level during the aerobic growth phase, compared with B0013-070. However, under oxygen-limited conditions, the lactate volumetric (11.7 g l^{-1} h^{-1}) and specific (1.1 g g^{-1} h^{-1}) productivities of B0013-080C/pTH-*rrnB-ldhA*8 were the highest of all the strains (Table 3). Furthermore, the oxygen-limited lactate yield was close to the theoretical value with by-products such as acetate, succinate, and pyruvate held at low levels These levels could be due to an excess pyruvate pool accumulation during the aerobic phase. Consequently, the aim to accumulate biomass with a higher growth rate during aerobic phase and to produce lactate with increased productivity during the oxygen-limited phase was achieved in strain B0013-080C/pTH-*rrnB-ldhA*8. Translation in bacteria begins with the binding of 30S ribosomal subunit on ribosome binding site (SD sequence) on messenger RNA (mRNA). Subsequently, the translation initiation complex is formed surrounding the initiation codon "AUG", and the 50S ribosomal subunit combines with this complex to form the 70S ribosome. The ribosome covers mRNA at a width of about 30 nucleotides. Transcription initiation site in P6 was located at the −27 site. While including the start codon, the length could reach 30 nucleotides, which provided enough binding region for ribosome. Therefore, P6 was the possible promoter for *ldhA*8. On the other hand, the transcription start site in P7 was located at the −18 site, and the resulting mRNA could not provide sufficient binding region for ribosome.

Jiang et al. [8] demonstrate that a 369-bp segment of the *ldhA* upstream is sufficient for normal expression of the *ldhA* gene. Here, the 72-bp fragment upstream *ldhA* could still promote the expression of *ldhA*. The improved aerobic growth rate and oxygen-limited lactate productivity in the B0013-080C/pTH-*rrnB-ldhA*8 strain suggest that a simple transcriptional manipulation strategy could be useful in improving fermentations of other metabolic products.

There could be no transcriptional promoting region downstream of the -61 site of *ldhA*

Further shortening of the *ldhA* upstream region to 61 bp resulted in *ldhA*9, in which P6 was interrupted and P13 was formed (Table 4). As a result, B0013-080C/pTH-*rrnB-ldhA*9 hardly produced lactate aerobically, and a very low yield of lactate was generated during oxygen-limited phase with succinate as the main product (Table 3). Accordingly,

the recombinant P13 could not promote transcription initiation. Interestingly, with even an shorter upstream segment, the B0013-080C/pTH-*rrnB-ldhA*10 strain produced a high yield of lactate, indicating that the recombinant promoter P14 or P15 possibly worked as a real promoter. However, the growth rate, lactate productivity, and yield in B0013-080C/pTH-*rrnB-ldhA*10 did not reach the levels of those in B0013-080C/pTH-*rrnB-ldhA*8. Except for Pp1 and Pp2 in *rrnB*, no predicted promoter could be detected in *rrnB-ldhA*11. Since Pp2 is positioned in the range of 250 bp upstream *ldhA*, it would affect *ldhA* expression slightly. B0013-080C/pTH-*rrnB-ldhA*11 generated a low lactate yield at an extremely low productivity but with a significant succinate yield. The *ldhA*7 fragment initiates from the start codon without SD sequence (Fig. 1). Product yields in B0013-080C/pTH-*rrnB-ldhA*7 and B0013-080C strains were similar except that the succinate accumulation in B0013-080C/pTH-*rrnB-ldhA*7 was lower. Apparently the structure of pTH-*rrnB* did not affect the expression of the cloned *ldhA* segments. As the possible functional promoter in B0013-080C/pTH-*rrnB-ldhA*10 was a recombinant of *rrnB* and *ldhA*10, no functional transcription initiation region appears to exist downstream of the −61 site of *ldhA*.

In conclusion, cell growth and lactate fermentation performance were regulated by shortening the *ldhA* upstream region. The strain B0013-080C/pTH-*rrnB-ldhA*8 produced the highest oxygen-limited lactate productivity and aerobic growth rate, but further studies are still required to identify the length and components of the transcription unit in order to improve lactate production further. To our knowledge, this study represents the first assessment of the application of promoter engineering in adjusting lactate production and cell activity in *E. coli*.

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