Cloning of D-lactate dehydrogenase genes of Lactobacillus delbrueckii subsp. bulgaricus and their roles in D-lactic acid production

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Received: 13 January 2017 / Accepted: 18 April 2017 / © Springer-Verlag GmbH Germany 2017

Abstract Lactobacillus delbrueckii subsp. bulgaricus is a heterogenous lactic acid bacterium that converts pyruvate mainly to D-lactic acid using D-lactate dehydrogenases (D-LDHs), whose functional properties remain poorly characterized. Here, the D-LDHs genes (ldb0101, ldb0813, ldb1010, ldb1147 and ldb2021) were cloned and overexpressed in Escherichia coli JM109 from an inducible pUC18 vector, respectively, and the resulting strains were compared in terms of D-lactic acid production. The strain expressing ldb0101 and ldb1010 gene individually produced more D-lactate than other three strains. Further study revealed that Ldb0101 activity was down-regulated by the oxygen and, therefore, achieved a highest titer of D-lactate (1.94 g/L) under anaerobic condition, and introduction of ldb1010 gene enhanced D-lactate formation (0.94 and 0.85 g/L, respectively) both in aerobic and anaerobic conditions due to a relatively stable qD-lactate. Our results suggested that the enzyme Ldb0101 and Ldb1010 played a role of more importance in D-lactate formation. To the best of our knowledge, we demonstrate for the first time the roles of different D-LDH homologs from L. bulgaricus in D-lactic acid production.

Keywords D-lactate dehydrogenase · D-lactic acid · Gene overexpression · Lactobacillus delbrueckii subsp. bulgaricus · Oxygen

Introduction

The Gram-positive bacterium Lactobacillus delbrueckii subsp. bulgaricus (L. bulgaricus) is widely used as a starter to produce yogurt and other milk-fermented products because of its capacity to produce lactic acid from anaerobic lactose fermentation of milk (Adolfsson et al. 2004). Two isomeric forms of lactate, D (−) and L (+), may be formed via reduction of pyruvate by distinct stereospecific NAD-dependent lactate dehydrogenases (D-LDH or L-LDH) (Garvie 1980). These enzymes belong to either of two evolutionary distinct subfamilies: the D- and L-isomer-specific hydroxyacid dehydrogenases, whose products differ only in their chirality (Sunil et al. 1992b). Many reports have demonstrated that lactic acid bacteria can produce both D- and L-lactate, and the ratio of the two isomers changes with the species and strains throughout the growth cycle (Garmyn et al. 1995), which also have been employed for the classification and identification of lactic acid bacteria (Manome et al. 1998). For example, the ratio of l- and d-lactate produced by the L. helveticus strain was 4:3 at the end of growth (Kylanikkila et al. 2000) and 1:1 by L. plantarum (Garvie 1980). In L. bulgaricus, more than 90% of the pyruvate is converted to D-lactic acid via the glycolysis pathway from the sugars (Razeto et al. 2002). Since D-lactic acid was highly produced in L. bulgaricus, the possibility of higher D-LDH gene expression levels and/or enzyme activities might be existed.

The genome of L. bulgaricus harbors five genes (ldb0101, ldb0813, ldb1010, ldb1147 and ldb2021) that are predicted to encode different D-isomer specific 2-hydroxyacid dehydrogenases (Bernard et al. 1995; Guchte et al. 2006). Recently, ldb0101 and ldb1010 gene products of L. bulgaricus were characterized through gene overexpression in E. coli (Holton et al. 2013; Razeto et al. 2002). The ldhD gene from L. bulgaricus was cloned and over-expressed in...
understand the features of each D-LDH homolog from Yeswanth et al. 2013; Kim et al. 1991; Lie et al. 2012). To identify the characteristics of the enzyme (Bernard et al. 1991; Wang et al. 2015). These five D-LDH genes were cloned in E. coli to unravel the catalytic mechanism (Sunil et al. 1992a; Taguchi and Ohta 1991; Kim et al. 1991). In addition, NAD-dependent d-LDHs from Acetobacter aceti and Acidocella species were cloned and expressed in E. coli as potential candidates for in vitro pyruvate production (Min et al. 2016). Until now, no research on the potential of these homologs from L. bulgaricus in the production of d-lactic acid has been studied.

L. bulgaricus is a homofermentative bacterium that produces d-lactic acid during growth and is widely used as a starter culture in yoghurt and fermented milk. It is well known that d-form of lactate causes a metabolic stress upon excessive dietary intake. To minimize the harmful effect of d-lactate during the sauerkraut fermentation, Jin et al. expressed the ldhL gene from L. plantarum in a starter culture Leuconostoc mesenteroides and decreased the d-h-lactate ratio (Jin et al. 2016). Therefore, the functional verification of different d-LDHs of L. bulgaricus was significant for strain improvement and the further use of L. bulgaricus. Metabolic engineering of E. coli is the most frequently used method to identify the characteristics of the enzyme (Bernard et al. 1991; Yeswanth et al. 2013; Kim et al. 1991; Li et al. 2012). To understand the features of each d-LDH homolog from L. bulgaricus in d-lactic acid production, pUC18 was chosen as the expression vector in this study based on its good performances in exogenous gene expressions (Huang et al. 2012; Wang et al. 2015). These five d-LDH genes were cloned separately into pUC18 under the control of lac promoter, and were heterologously expressed in E. coli, respectively. The performances of the resulting strains on lactic acid synthesis were investigated in shake flask culture under anaerobic conditions, and good candidates were selected to further study the regulation mechanism of oxygen thereof. The aim of this study was to provide some clues of the contribution of each D-LDH homolog to d-lactic acid synthesis in L. bulgaricus.

Materials and methods

Strains and plasmids

E. coli JM109 was employed as the host to exclusively identify the roles of the d-LDH homologs of L. bulgaricus in d-lactic acid production with pUC18 as the expression vector. The L. bulgaricus ATCC11842 strain for gene clones of d-LDHs was purchased from China center of industrial culture collection. The PCR reagents and restriction enzymes were purchased from Takara Bio Inc. (Dalian, China).

DNA manipulations and cloning procedure

The L. bulgaricus ATCC11842 was cultured at 37 °C in MRS broth (Merck, Darmstadt, Germany), and the genomic DNA was extracted using the TIANamp Bacteria DNA Kit (TianGen, Beijing, China) according to the manufacturer’s protocol. Based on the sequence of the L. bulgaricus ATCC11842 chromosome in NCBI GenBank (Accession number: NC_008504), the coding sequences of ldb0101, ldb0813, ldb1010, ldb1147 and ldb2021 genes were amplified by PCR with the primers shown in Table 1. The PCR products were purified and ligated into pMD18-T vector (Takara, Dalian, China), respectively. After sequencing, the correct DNA fragments were digested with the corresponding restriction enzymes and ligated to pUC18 digested with the same enzymes to produce the expression plasmids. The resultant plasmids were designated as pUC18ldb0101 to pUC18ldb2021, respectively.

Heterologous expression

The recombinant strains of E. coli JM109 carrying different genes of d-LDH homologs were cultured at 37 °C overnight with shaking at 200 rpm in 3 mL of LB medium containing 1% of tryptone (Oxoid, Hampshire, England), 0.5% yeast extract (Oxoid, Hampshire, England) and 1% NaCl with 100 µg/mL ampicillin. Afterwards, the cultures were inoculated into fresh medium and cultivated until the OD600 reached 0.5–0.6. Isopropyl-d-thiogalactopyranoside (IPTG) was added to the cultures at a final concentration of 0.5 mM; the cultures were incubated at 37 °C for 6 h. After induction, the cells were harvested by centrifugation (10,000×g, 4 °C, 5 min), resuspended with 75 µL Tris–HCl buffer (20 mM, pH 8.0), 20 µL loading buffer and 5 µL mercaptoethanol. After boiling and centrifugation, 10 µL of the supernatants was applied to SDS-PAGE, which was conducted on a 12% polyacrylamide gel using the method of Sambrook and Russell (Sambrook and Russell 2001) with a Protein Electrophoresis System (Bio-Rad, California, USA). The marker proteins (Tiangen, Beijing, China) with molecular weights ranging from 14.4 to 94.0 kDa were used.

Culture media and growth conditions

The medium for preculture was LB, and the fermentation medium was M9 containing (per liter): Glucose 9 g,
Table 1  Gene used, primers for PCR and the restriction enzyme of each primer underlined

| Gene name | Primers (5’→ 3’) | Restriction enzyme |
|-----------|------------------|-------------------|
| ldb0101   | F: GCGGGATCCGATGACTAAAAATTTTGCT | BamHI |
|           | R: GCGGTGTCGACTTAGCCAACCTAAACTGG | Sall |
| ldb0813   | F: CTGGGATCCGTGAGGGAGATGCTTAAG   | BamHI |
|           | R: TCCGAAGCTTTAAGTTTGAACGCCCCTGAC | HindIII |
| ldb1010   | F: GCGGGATCCGATGACTAAAAATTTTGCT | BamHI |
|           | R: CCGCAAGCTTTTACGGTAAAGCATGCT  | HindIII |
| ldb1147   | F: CCGGAATTCGATGAGATGTGTATGCTTGAGC | EcoRI |
|           | R: TTCCGTGCCTCTACGATGTTACGCCCAGTCT  | Sall |
| ldb2021   | F: CTGGGATCCGATGAGATGTGTATGCTTGAGC | BamHI |
|           | R: CACGAAGCTTTAAGCCGCACTTCTTC | HindIII |

Na$_2$HPO$_4$.12H$_2$O 15.12 g, KH$_2$PO$_4$ 3.0 g, NaCl 0.5 g, NH$_4$Cl 3.0 g, 1 M MgSO$_4$ 2 mL, 1 M CaCl$_2$ 0.1 mL, 1% (w/v) vitamin B1 0.2 mL, and trace elements solution 0.1 mL. The stock solution of trace elements contained the following in 3 M HCl: AlCl$_3$.6H$_2$O 10 g, FeSO$_4$.7H$_2$O 80 g, CuCl$_2$.2H$_2$O 1.0 g, ZnSO$_4$.7H$_2$O 2.0 g, MnSO$_4$.H$_2$O 10 g, NaMoO$_4$.2H$_2$O 2.0 g, CoCl$_2$.4H$_2$O 4.0 g, and H$_3$BO$_4$ 0.5 g. When appropriate, 100 µg/mL of ampicillin was used for recombinant E. coli.

The seed culture was prepared by the transfer of 1 mL of glycerol stock of recombinant E. coli strains to 30 mL of LB medium in a 250-mL flask, which were aerobically incubated for 8 h at 37 °C and 220 rpm, respectively. The seed cultures were then inoculated into 30 mL of M9 medium with 0.5 mM IPTG and 100 µg/mL ampicillin in a 50-mL flask at 37 °C for 24 h anaerobically, respectively. After metabolites determination, the good D-lactic acid producers were selected for further study.

Aerobic cultivations of the good D-lactic acid producers were conducted with 50 mL of M9 medium with 0.5 mM IPTG at 37 °C and 200 rpm for 24 h in a 250-mL flask, and the anaerobic fermentations were carried out in a 100-mL flask without agitation in anaerobic incubator (Don Whitley scientific, England). The inocula of seed culture were 2%. Samples were withdrawn periodically to determine the cell growth, glucose consumption and metabolites production. Each experiment was carried out in triplicate and the mean value was calculated.

Analytical methods

Cell growth was monitored by measurement of the optical density at 600 nm (OD$_{600}$) of appropriately diluted sample with a UV–visible spectroscopy system and converted to dry cell weight (DCW) based on a linear relationship between them.

The formations of D- and L-lactic acid were determined using the D/L-lactic acid assay kit (Megazyme, Ireland). The residual glucose and the extracellular metabolites were determined by a high-performance liquid chromatography (HPLC) system (Shimazu, Kyoto, Japan) equipped with an organic acid analysis column (Aminex HPX-87H, 300 × 7.8 mm, Bio-Rad, USA), a refractive index detector (SPD-20A) and a UV/vis detector (SPD-20AV). The mobile phase was 2.5 mM H$_2$SO$_4$ at the flow rate of 0.5 mL/min, and the column was operated at 50 °C. All samples were filtered through 0.22 µm membrane filters. The standard chemicals were products of Sigma.

Results and discussion

Construction of recombinant strains of E. coli JM109

The chromosomal DNA of L. bulgaricus ATCC11842 was extracted and used as the PCR template. The genes with sizes of 1022 bp for ldb0101, 969 bp for ldb0813, 1002 bp for ldb1010, 957 bp for ldb1147 and 1047 bp for ldb2021 were amplified, and all showed 100% identity with the reported sequences in NCBI GenBank (CAI96942.1, CAI97635.1, CAI97812.1, CAI97949.1 and CAI98759.1), respectively. The amplified D-LDH genes were then inserted into the vector pUC18, respectively, to yield the expression vectors, which were verified by the double digestion (data not shown). The recombinant strains were designated as JM109/pUC18ldb0101 to JM109/pUC18ldb2021, respectively. Details on the construction of recombinant plasmids are shown in Fig. 1.

Expression of D-lactate dehydrogenases in E. coli JM109

As shown in Fig. 2, SDS-PAGE analysis of the constructed cells except JM109/pUC18ldb1147 revealed the presence of the over-expressed proteins which were not present in
wild stain and recombinant *E. coli* harboring pUC18. The recombinant proteins of JM109/pUC18/ldb0101 and JM109/pUC18/ldb1010 exhibited higher expression levels, followed by those of the strain JM109/pUC18/ldb0813 and JM109/pUC18/ldb2021. Bernard et al. suggested that some genes from *Lactobacillus* species were toxic to *E. coli* due to overexpression and/or perturbation of its metabolism because of the wide substrate specificities of the expressed enzymes (Bernard et al. 1994). Since no mutation was detected in PCR-cloned ldb1147 gene compared with the NCBI reported and good performances of other α-LDH genes in *E. coli*, the difficulty encountered in the expression of ldb1147 gene was unlikely associated with toxicity of enzyme activity. In agreement with the research of Lee et al. (1991), this problem may be due to an unidentified restriction modification system in the host, *E. coli*.

**α-Lactic acid production of engineered *E. coli* under anaerobic cultivation**

To evaluate the influences of different α-LDHs on α-lactic acid production, the fermentations of the recombinant *E. coli* strains were conducted in shake flasks under anaerobic condition, with JM109/pUC18 as the control.
The cell growth, pH and lactate production of the engineered strains were determined (shown in Table 2). Compared with the control, the cell growth was inhibited due to the metabolic burden brought by the exogenous protein expressions and the pH decreased as a result of the production of D-lactate. As shown in Fig. 2, the recombinant protein of JM109/pUC18ldb1010 exhibited a higher expression level, and the cell growth was inhibited greatly. Since JM109/pUC18ldb1010 strain only consumed a small amount of glucose compared with other recombinant strains (data not shown), a higher pH value might be correlated with a little organic acid produced in glycolytic pathway.

As shown in Table 2, the highest D-lactate concentration of 949.6 mg/L was achieved in JM109/pUC18ldb0101, followed by JM109/pUC18ldb1010 (673.9 mg/L). Due to a lower protein expression, only 84.3 and 25.2 mg/L of D-lactate were obtained in E. coli carrying ldb0813 and ldb2021 genes, respectively. Since recombinant protein was hardly detected in JM109/pUC18ldb1147, no D-lactate formed was reasonable. The enhanced D-lactate production in JM109/pUC18ldb0101 and JM109/pUC18ldb1010 could be attributed to the higher protein expression levels, as can be seen in the SDS-PAGE (Fig. 2). Few L-lactate accumulations in each engineered strain might be attributed to a lower gene expression level of t-LDH in E. coli.

Oxygen regulation on lactate production

*L. bulgaricus* is well-known facultatively anaerobic bacteria that can grow in oxygenated environments (Horiuchi and Sasaki 2012). It was reported that the lactate dehydrogenase activity was regulated by the oxygen (Collins and Lascelles 1962). Since JM109/pUC18ldb0101 and JM109/pUC18ldb1010 strains showed a good performance in D-lactate synthesis, the responses of Ldb0101 and Ldb1010 by oxygen were investigated in flasks under anaerobic and aerobic conditions.

The profiles of the cell growth, glucose consumption and D-lactate production of JM109/pUC18ldb0101 and JM109/pUC18ldb1010 are determined and shown in Fig. 3. The specific rates of glucose consumption ($q_{\text{Glu}}$) in culture of JM109 expressing ldb0101 and ldb1010 under anaerobic conditions were somewhat higher than those under aerobic conditions (3.63 and 3.59 mmol/g h under anaerobic culture, 1.97 and 3.09 mmol/g h in aerobic case, respectively). The lower $q_{\text{Glu}}$ (1.97 mmol/g h) in JM109/pUC18ldb0101 might be attributed to a decreased enzyme activity involved in glycolytic pathway under aerobic condition. At the end of the fermentation, the residual glucose was not exhausted yet (data not shown). The aerobic culture enhanced the cell density of both strains, which achieved a highest OD$_{600}$ of 2.59 and 1.54 at 18 h, 56.9 and 57.1% higher than those under anaerobic condition (1.65 and 0.98), respectively. This indicated that aerobicosis was favorable to cell growth, which was related to the sufficient ATP supplied in oxidative phosphorylation (Kihira et al. 2012). The over-expression of ldb1010 gene introduced more metabolic burdens to the cell and resulted in a less biomass compared with that of ldb0101 gene, although their expression level in SDS-PAGE seemed similar.

As shown in Fig. 3, D-lactate productions in JM109/pUC18ldb0101 and JM109/pUC18ldb1010 were improved with the increased biomass, which achieved a highest concentration at 21 h of the fermentation. At the end of the fermentation, 1.94 g/L of D-lactate was achieved with a yield of 0.57 mol/mol glucose under anaerobic condition in JM109/pUC18ldb0101. With the oxygen supplied, the D-lactate production was declined, 0.41 g/L with a yield of 0.14 mol/mol, respectively. On the contrary, a good performance of D-lactate synthesis (0.94 g/L) was observed in JM109/pUC18ldb1010 under aerobic condition, 135% higher than that of JM109/pUC18ldb0101. When the anaerobic fermentation was conducted, 0.85 g/L of D-lactic acid was produced. Although the D-LDH activities were not determined in this study, we used $q_{\text{D-lactate}}$, as the measure of specific activities of D-LDH. 2.07 and 0.28 mmol/g h of $q_{\text{D-lactate}}$ were achieved in JM109/pUC18ldb0101 under the anaerobic and aerobic conditions, respectively, indicating that the Ldb0101 activity was down-regulated by the

| Strains        | OD$_{600}$ | pH     | L-lactic acid (mg/L) | D-lactic acid (mg/L) |
|----------------|-----------|--------|----------------------|----------------------|
| JM109/pUC18    | 3.19 ± 0.37 | 5.98 ± 0.08 | 18.6 ± 1.2          | 0                     |
| JM109/pUC18 ldb0101 | 2.03 ± 0.41 | 4.75 ± 0.04 | 19.8 ± 0.83         | 949.6 ± 41.8         |
| JM109/pUC18 ldb0813 | 1.99 ± 0.26 | 5.40 ± 0.06 | 8.2 ± 0.46          | 84.3 ± 9.8           |
| JM109/pUC18 ldb1010 | 0.76 ± 0.02 | 6.07 ± 0.06 | 21.9 ± 1.31         | 673.9 ± 79.3         |
| JM109/pUC18 ldb1147 | 2.75 ± 0.50 | 5.64 ± 0.09 | 6.6 ± 0.75          | 0                     |
| JM109/pUC18 ldb2021 | 2.65 ± 0.35 | 5.79 ± 0.04 | 3.6 ± 0.18          | 25.2 ± 1.49          |
oxygen, in accordance with the research of Collins and Lascelles (1962). Interestingly, regardless of aerobic or anaerobic culture, no obvious differences of \( q_{D-lactate} \) in \( JM109/pUC18\text{-}ldb1010 \) (1.30 and 1.36 mmol/g h, respectively) were observed. This implied that the Ldb1010 activity might be independent of the oxygen, and it was very important for D-lactic acid production when the aerobic cultivation was conducted.

It is well known that both Ldb0101 and Ldb1010 use NAD(+) as cofactor (Dennisi and Kaplan 1960; Holton et al. 2013). Levanon et al. reported that the intracellular NADH/NAD\(^+\) ratio decreased significantly with increasing culture dissolved oxygen levels, since NADH is consumed in the electron transfer chain with NAD\(^+\) regeneration (Levanon et al. 2005). Hence, under the aerobic condition, a reduction in D-lactate formation in \( JM109/pUC18\text{-}ldb0101 \) suggested that the cell partially used oxygen as the electron acceptor through the electron transfer chain. Under anaerobic condition, the cell has a higher intracellular NADH, resulting in lactate accumulation. The different behavior in \( JM109/pUC18\text{-}ldb1010 \) was not clear yet, and the possibility that Ldb1010 enzyme employed a cofactor different from Ldb0101 might be existed.

Furthermore, the L-lactate production was almost undetectable among the two engineered strains under different conditions (data not shown), which indicated the heterogenous overexpression of \( ldb0101 \) and \( ldb1010 \) gene in \( E. coli \) enhanced the metabolic flux from pyruvate to D-lactic acid. Acetate was the main by-product, and the aerobiosis enhanced acetate accumulation in \( JM109/pUC18\text{-}ldb1010 \) and \( JM109/pUC18\text{-}ldb1010 \) due to a higher phosphotransacetylase and acetate kinase (Pta-Ack) activity.

It is well known that \( E. coli \) is usually employed as a host to study the properties of the enzyme through heterogenous gene overexpression. Ren et al. overexpressed four bile salt hydrolases genes in \( E. coli \) BL21, which demonstrated that all 4 BSH proteins were responsible for hydrolyzing activity in \( L. plantarum \) ST-III (Ren et al. 2011). Based on the gene overexpression and flask cultivations, the data indicated that the Ldb0101 and Ldb1010 homologs might act in a different way in catalyzing the pyruvate to D-lactic acid: when the oxygen was sufficient, the enzyme Ldb1010 worked principally, and when the oxygen was exhausted, Ldb0101 played a role of more importance. Further study aimed at D-LDHs gene transcription in \( L. bulgaricus \) will be carried out to verify this hypothesis.

Acknowledgements This work was supported by the National Science and the Technology Pillar Program during the 12th Five-year Plan Period (Grant Nos. 2013BAD18B01 and 2012BAD12B08) and the Program of Shanghai Committee of Science and Technology, China (Grant No. 16DZ2280600).

Compliance with ethical standards

Conflict of interest No conflict of interest was declared.

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