AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

BIOTECHNOLOGY

Elucidating the Role and Regulation of a Lactate Permease as Lactate Transporter in *Bacillus coagulans* DSM1

Yu Wang,[a,b] Caili Zhang,[a] Guoxia Liu,[a] Jiansong Ju,[b] Bo Yu,[a] Limin Wang[a,c]

[a]CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, People's Republic of China

[b]College of Life Science, Hebei Normal University, Shijiazhuang, People's Republic of China

[c]Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Technology & Business University (BTBU), Beijing, People's Republic of China

ABSTRACT A key feature of *Bacillus coagulans* is its ability to produce L-lactate via homofermentative metabolism. A putative lactate permease-encoding gene (*lutP*) and the gene encoding its regulator (*lutR*) were identified in one operon in *B. coagulans* strains. LutP orthologs are highly conserved and located adjacent to the gene cluster related to lactate utilization in most lactate-utilizing microorganisms. However, no lactate utilization genes were found adjacent to *lutP* in all sequenced *B. coagulans* strains. The stand-alone presence of *lutP* in L-lactate producers indicates that it may have functions in lactate production. In this study, *B. coagulans* DSM1 was used as a representative strain, and the critical roles of LutP and its regulation were described. Transport property assays showed that LutP was essential for lactate uptake. Its regulator LutR directly interacted with the *lutP-lutR* intergenic region, and *lutP* transcription was activated by L-lactate via regulation by LutR. A biolayer interferometry assay further confirmed that LutR bound to an 11-bp inverted repeat in the intergenic region, and *lutP* transcription began when the binding of LutR to the *lutP* upstream sequence was inhibited. We conclusively showed that *lutP* encodes a functional lactate permease in *B. coagulans*.

IMPORTANCE Lactate-utilizing strains require lactate permease (LutP) to transport lactate into cells. *Bacillus coagulans* LutP is a previously uncharacterized lactate permease with no lactate utilization genes situated either adjacent to or remotely from it. In this study, an active lactate permease in an L-lactate producer, *B. coagulans* DSM1, was identified. Lactate supplementation regulated the expression of lactate permease. This study presents physiological evidence of the presence of a lactate transporter in *B. coagulans*. Our findings indicate a potential target for the engineering of strains in order to improve their fermentation characteristics.

KEYWORDS *Bacillus coagulans*, lactate permease, expression regulation, lactate transport

Lactic acid is a hydroxyl acid used primarily in food, pharmaceutical, cosmetic, and chemical industries. It can be produced via microbial fermentation by the reduction of pyruvate (1, 2). As an organic acid, lactic acid may induce acidic conditions that are toxic to most strains (3). However, both yeasts and lactate bacteria are relatively tolerant to lactic acid (4–6). Since the cytosolic pH of most organisms is higher than the pK_a of lactic acid (pK_a of 3.8), intracellular lactic acid predominantly exists in the lactate anion form, which cannot readily diffuse out of cells without the assistance of specific transporters (7). Regulation of lactate transport is a major factor affecting lactic tolerance (5, 6). Most lactate transporters are considered symporters because the lactate anion is transported in the same direction with one or more protons. These lactate-proton symporters are usually indifferent to the isomeric form of the substrate (L-lactate or

Citation Wang Y, Zhang C, Liu G, Ju J, Yu B, Wang L. 2019. Elucidating the role and regulation of a lactate permease as lactate transporter in *Bacillus coagulans* DSM1. Appl Environ Microbiol 85:e00672-19. https://doi.org/10.1128/AEM.00672-19.

Editor Robert M. Kelly, North Carolina State University

Address correspondence to Bo Yu, yub@im.ac.cn, or Limin Wang, wanglimin@im.ac.cn.

Received 20 March 2019
Accepted 7 May 2019
Accepted manuscript posted online 17 May 2019
Published 1 July 2019

D-lactate) and are often capable of also transporting other short-chain monocarboxylates (7). In many organisms, these symporters have a stoichiometry of one proton per anion. The H^+/A^- stoichiometry of one implies that the overall transport process is electroneutral and does not affect intracellular pH homeostasis in lactate-producing cultures (8).

The lactate permease gene, which forms an operon with NAD^+-independent lactate dehydrogenases (iLDH) genes and the putative lactate regulator, is widely distributed in microorganisms that utilize lactate as a carbon and energy source (9, 10). Lactate permease is an influx protein that imports lactate into cells (1). iLDH genes, which are classified into D-iLDH and L-iLDH depending on their substrate specificities, are responsible for the oxidation of lactate into pyruvate (11). The putative lactate regulator senses elevated lactate concentrations and regulates the expression of the lactate utilization operon (12, 13). Because lactate import is crucial for lactate utilization, the lactate permease gene is conserved in most lactate utilization strains (14). Lactate transporters have been characterized in lactate consumers, including bacteria, animal cells, and yeast (9, 15–17). However, studies on lactate transporters in lactate-producing strains are scant. There are some hypotheses of lactate transport in lactate-producing strains, such as ATP-binding-cassette (ABC) transporters for lactate export in homofermentative lactate-producing strains. However, ABC transporters have not yet been experimentally proven to export lactate in lactate producers (8).

Bacillus coagulans is a homofermentative L-lactate producer, with a high optical purity of 99.8% (18). It is able to tolerate high temperatures and metabolize a wide range of sugars, which justifies its potential for low-cost production of lactate from an industrial perspective (19). Thus, lactate production using this species has attracted interest in recent years. The fermentative metabolism of *B. coagulans* is characterized by the glycolytic breakdown of carbohydrates. A late step in this pathway is distinguished by the conversion of pyruvate into lactate, a reaction that oxidizes the NADH formed during glycolysis, thus maintaining cellular redox balance (18). Our previous studies revealed three enzymes responsible for lactate production, NAD^+-dependent L-lactate dehydrogenase (L-nLDH), NAD^+-dependent D-lactate dehydrogenase (D-nLDH), and glycolate oxidase (GOX), which catalyzed the conversion of pyruvate to lactate (20). Analysis of the *B. coagulans* genome sequence annotated a gene encoding lactate permease (LutP), which is conserved in lactate-utilizing-strains. Surprisingly, no homologs of previously characterized lactate-utilizing genes were identified in the genomes of any sequenced *B. coagulans* species. The paucity of information on lactate permease in *B. coagulans* prompted us to investigate its role and regulation mechanisms.

With the advantages of known genetic background and available genetic tools, *B. coagulans* DSM1 was chosen as a representative strain in this study. A gene encoding lactate permease in *B. coagulans* DSM1 was discovered, and its functions and regulation mechanisms were experimentally verified.

RESULTS

LutP is a lactate transporter found in *B. coagulans*, which accumulates L-lactate as the final product of fermentation. A gene annotated as lactate permease (AJH79743.1, *lutP*) is present in the *B. coagulans* DSM1 genome. Lactate permease is an integral membrane protein probably involved in L-lactate transport (https://www.ncbi.nlm.nih.gov/Structure/cdd/pfam02652). To investigate the critical role of LutP in *B. coagulans* DSM1, *lutP* was first deleted and then complemented. The resulting strains were named DSM1 Δ*lutP* and DSM1 *lutP*$^+$, respectively, and cell growth was assayed. Compared to the wild-type strain and DSM1 *lutP*$^+$, DSM1 Δ*lutP* grew poorly during the first 4 h (Fig. 1A). Disruption of *lutP* also impaired glucose consumption (Fig. 1B). Our results indicated that LutP may influence the physiological metabolism of cells, thereby leaving the identification of its role in *B. coagulans* DSM1 in question.

Similarly to the case for all sequenced *B. coagulans* strains in the NCBI database (21–23), no lactate utilization genes were found adjacent to *lutP* in the genome of *B.*

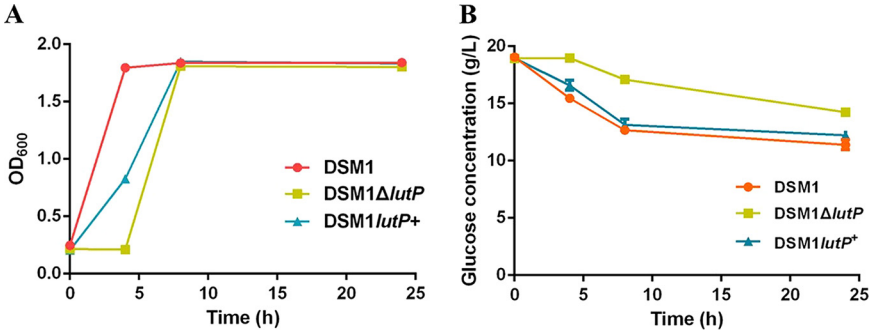

FIG 1 Function analysis of LutP. (A) Effects of LutP on *B. coagulans* DSM1 growth. Cells were incubated in BC medium (10% [vol/vol]) and cell growth was monitored by measuring the optical density at 600 nm. (B) Effects of LutP on glucose consumption. The data points and error bars represent the means and standard deviations from triplicate cultures, respectively.

coagulans DSM1 (NCBI reference sequence NZ_CP009709.1) (see Fig. S1 in the supplemental material). The amino acid sequence identities of LutP from *B. coagulans* DSM1 were compared with those from other strains. LutP from *B. coagulans* has approximately 35% to 67% amino acid sequence identity with those from lactate-utilizing strains and shows 99% amino acid sequence identity with the uncharacterized ones from other *B. coagulans* strains (see Table S1).

To investigate the function of the *lutP* gene in *B. coagulans* DSM1, transport properties of LutP were characterized in DSM1 cells with or without *lutP* (Fig. 2A). Cells without *lutP* showed a negligible detection of the radiolabeled L-lactate. Meanwhile, cells with *lutP* accumulated radiolabeled L-lactate and exhibited increased lactate uptake compared to that in DSM1 Δ*lutP*. These results clearly demonstrated that LutP was involved in lactate uptake.

Next, the export ability of LutP was characterized to confirm whether *B. coagulans* LutP is an antiporter for lactate export or a symporter for lactate uptake. As pyruvate was converted to L-lactate via L-nLDH in *B. coagulans* DSM1 (20), the export ability was investigated using [^{13}C]pyruvate as the substrate, and the concentration of extracellular [^{13}C]lactate was detected by a gas chromatography-mass spectrometer (GC-MS). The correlation coefficient (R^2) values of calibration curves of [^{13}C]lactate and unlabeled lactate were 0.9987 and 0.9992, respectively, which were acceptable for the determination of extracellular [^{13}C]lactate concentration (see Fig. S2). As shown in Fig. 2B, cells with *lutP* accumulated less extracellular [^{13}C]lactate than cells lacking the *lutP* gene. The lactate uptake assay revealed that LutP can import lactate into cells (Fig. 2A). So, for the cells with *lutP*, the produced extracellular [^{13}C]lactic acid was imported into cells by LutP, and for the cells lacking *lutP*, the produced [^{13}C]lactic acid accumulated outside

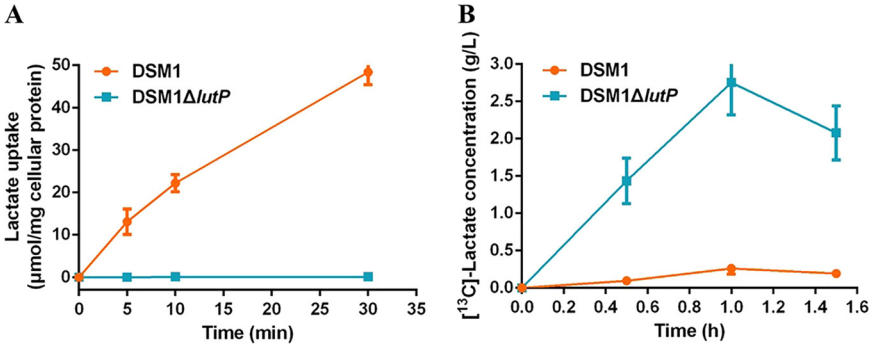

FIG 2 Function analysis of LutP. (A) LutP facilitates L-[^{14}C]lactate uptake. (B) LutP increases the extracellular accumulation of L-[^{13}C]lactate. Error bars represent standard deviations (SDs) calculated from three independent determinations.

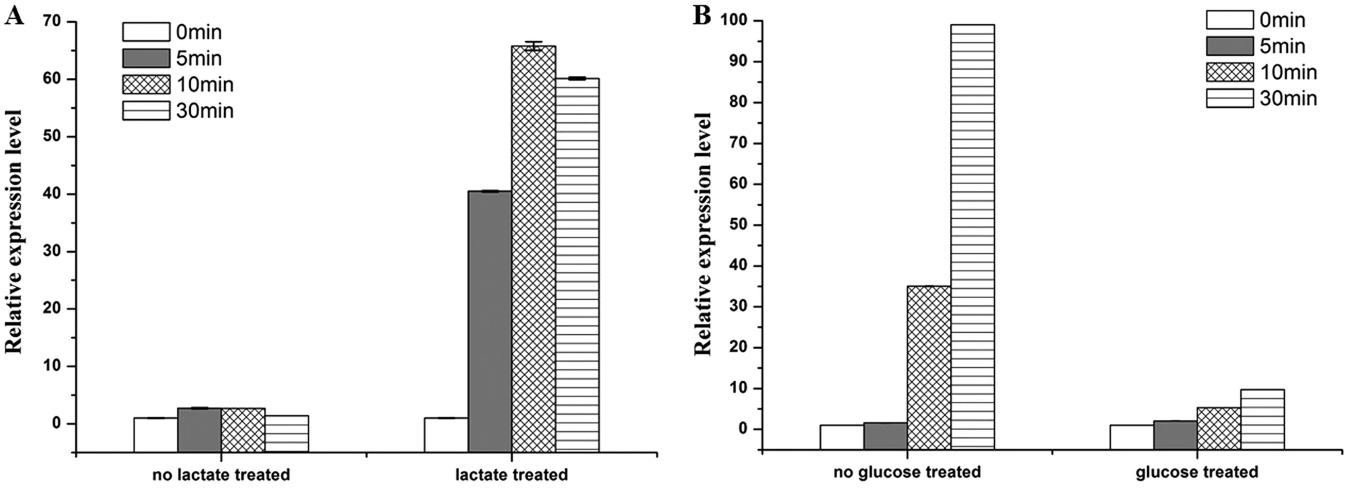

FIG 3 Determination of the relative transcriptional levels of *lutP* gene in BC medium using L-lactate as the stimulator in *B. coagulans* DSM1 Δ*ldhL1* Δ*ldhL2* (A) and glucose as the inhibitor in *B. coagulans* DSM1 (B). The threshold cycles (C_T) for each PCR with different concentrations of cDNA were determined and were compared with that for a standard DNA (the 16S rRNA gene) that was also analyzed at the same time. The *y* axis indicates the transcription ratio of *lutP* at 5 min, 10 min, and 30 min to 0 min. Error bars represent SDs calculated from three independent determinations.

the cells. These results indicated that LutP can import lactate into cells but has no effect on lactate export. We found that [^{13}C]lactate decreased in the *lutP* null strain after a 1-h reaction, and the reason currently remains unknown.

***lutP* gene transcription is activated by lactate and inhibited by glucose.** Sequence alignment with the lactate transporter of *B. subtilis* (LutP, CAB12100.2) showed a 45% identity at the amino acid level (Table S1). The *lutP* gene from *B. subtilis* undergoes lactate-triggered transcription (1, 12). *B. coagulans* DSM1 is an L-lactate producer, and the concentration of L-lactate increased gradually in the fermentation broth (19, 22). To explore whether lactate is an inducible factor for *lutP* transcription in *B. coagulans* DSM1, a *B. coagulans* DSM1 mutant lacking nLDH-encoding genes (DSM1 Δ*ldhL1* Δ*ldhL2*) (19) was used to avoid the influence of lactate produced *in vivo*. Quantitative real-time (RT)-PCR was performed under 5 mM L-lactate treatment for 5, 10, and 30 min (24) (Fig. 3A). The mRNA levels obtained from cells without L-lactate treatment showed that *lutP* transcription ratios of different times (5, 10, and 30 min) to those at 0 min were in the range of 1.39 ± 0.03 to 2.71 ± 0.09. Notably, the transcription ratio increased to 40.50 ± 0.11 when cells were induced using L-lactate for 5 min, and further increased to 65.80 ± 0.75 when induced by L-lactate for 10 min. These results indicated that L-lactate was a stimulator for *lutP* transcription.

Lactate fermentation is a metabolic process by which glucose is converted into pyruvate via the glycolysis pathway and L-lactate is produced gradually by L-nLDH (20). Thus, glucose is dominant during the early stage of fermentation. As shown in Fig. 3B, the mRNA levels obtained from cells with glucose treatment showed that *lutP* transcription ratios of different times (5, 10, and 30 min) to those at 0 min were stable in the range of 2.06 ± 0.03 to 9.71 ± 0.07. However, the transcription ratio increased to 35.02 ± 0.15 in cells without glucose treatment at 10 min and further increased to 99.04 ± 0.06 at 30 min. The RT-PCR analysis showed that glucose inhibited the transcription of *lutP*.

LutR binds to the inverted repeat sequence of the *lutP-lutR* intergenic region in *B. coagulans* DSM1. A helix-turn-helix (HTH)-type transcriptional regulator LutR (AJH80199.1) is located upstream of LutP in *B. coagulans* DSM1 (Fig. 4A). LutR belongs to the GntR regulator family, named after the repressor of the *B. subtilis* gluconate GntR operon (13). This family has been proven to regulate lactate utilization genes via binding with conserved inverted repeat sequences, such as TCATCnGATCA (n denotes any nucleotide) in *Bacillus* strains or AATTGGnnnCCAATT in *Proteobacteria* (Fig. 4B) (1, 11, 13, 14). Although the effectors of various predicted transcription factors for lactate

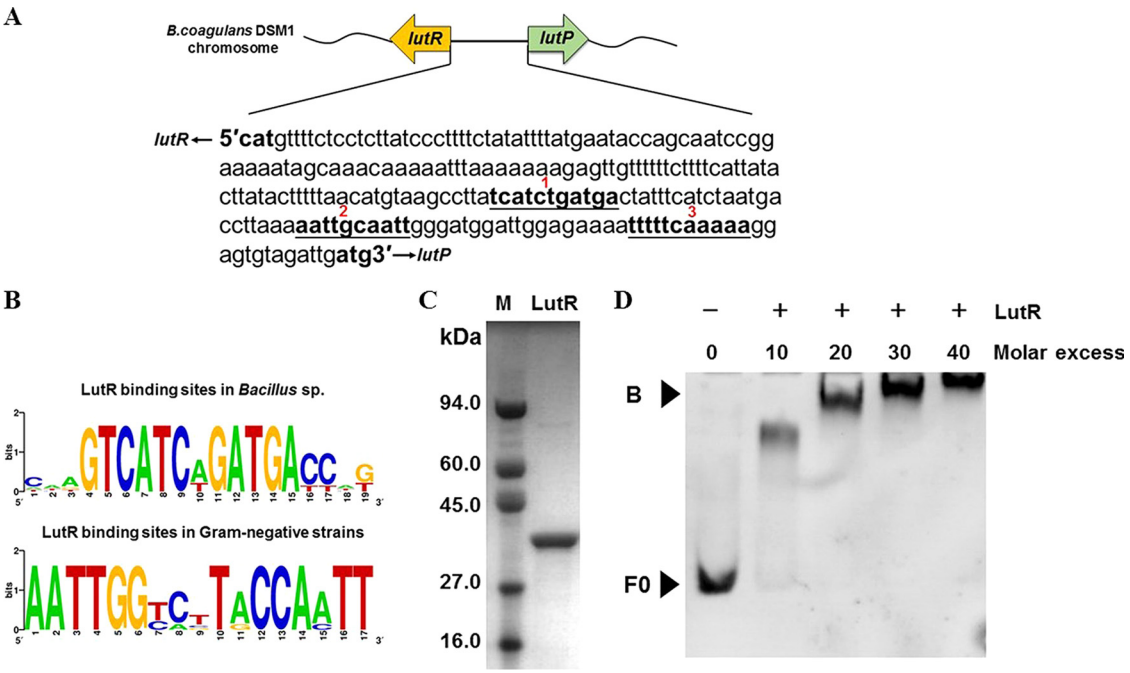

FIG 4 Binding of LutR to the *lutP-lutR* intergenic region. (A) The intergenic region of *lutP-lutR*. Three inverted repeat sequences are labeled as 1, 2, and 3. (B) Predicted binding sites of LutR-binding motifs. The consensus logo was created using WEBLOGO (13). (C) Purified His-tagged LutR protein. (D) EMSAs with the *lutP-lutR* intergenic fragment F0 (43 nM) and purified LutR (0-, 10-, 20-, 30-, and 40-fold molar excess relative to fragment F0). F0 is the *lutP-lutR* intergenic DNA fragment, and B is the LutR/DNA complex.

regulons have not yet been experimentally tested, a genome context analysis of lactate utilization genes across representative members of sequenced strains suggests that LutR may be a regulator for LutP expression in *B. coagulans* DSM1.

To further test this hypothesis, an electrophoretic mobility shift assay (EMSA) and biolayer interferometry (BLI) were used to analyze LutR binding sites. The *lutP-lutR* intergenic region in *B. coagulans* DSM1 contains 218 bases. This region comprises 3 inverted repeat sequences, TCATCTGATGA, AATTGCAATT, and TTTTTCAAAAA (Fig. 4A). Normally, only one consensus sequence-based binding site is present in a lactate-utilizing strain (10, 11). However, both the motif with a hyphenated dyad symmetry, TCATCTGATGA for *Bacillus* strains, and AATTGCAATT for *Proteobacteria* are found in *B. coagulans* DSM1 (Fig. 4B). To determine whether LutR regulates LutP expression by binding to the *lutP-lutR* intergenic region, the LutR protein was first heterologously expressed in *Escherichia coli* and purified to homogeneity using nickel affinity purification and size exclusion chromatography (Fig. 4C). The DNA fragment F0 (the *lutP-lutR* intergenic region) was incubated with increasing amounts of purified LutR and then separated on a native polyacrylamide gel (Fig. 4D). No binding was observed in the absence of LutR. In contrast, an increase in LutR protein in the binding assay resulted in electrophoretic shifts of fragment F0. A 40-fold molar excess of the LutR protein resulted in a complete gel shift. These results indicated that LutR interacted with the *lutP-lutR* intergenic region.

To further determine the binding site of LutR, biolayer interferometry (BLI) was conducted to measure the differences in interference signals with purified LutR and subfragments in F0. Full-length F0 (218 bp) was divided into 3 parts. Fragment F1 (73 bp) did not contain an inverted repeat sequence. Fragment F2 (75 bp) contained the TCATCTGATGA motif and fragment F3 (70 bp) contained two inverted repeat sequences, AATTGCAATT and TTTTTCAAAAA (Fig. 5A). For the BLI assay, biotinylated DNA subfragments F1, F2, and F3 were immobilized on a biosensor at a fixed concentration. Bindings of the purified LutR protein to different subfragments were measured under serial dilutions. The resulting interferograms (Fig. 5B, C, and D) showed that LutR had

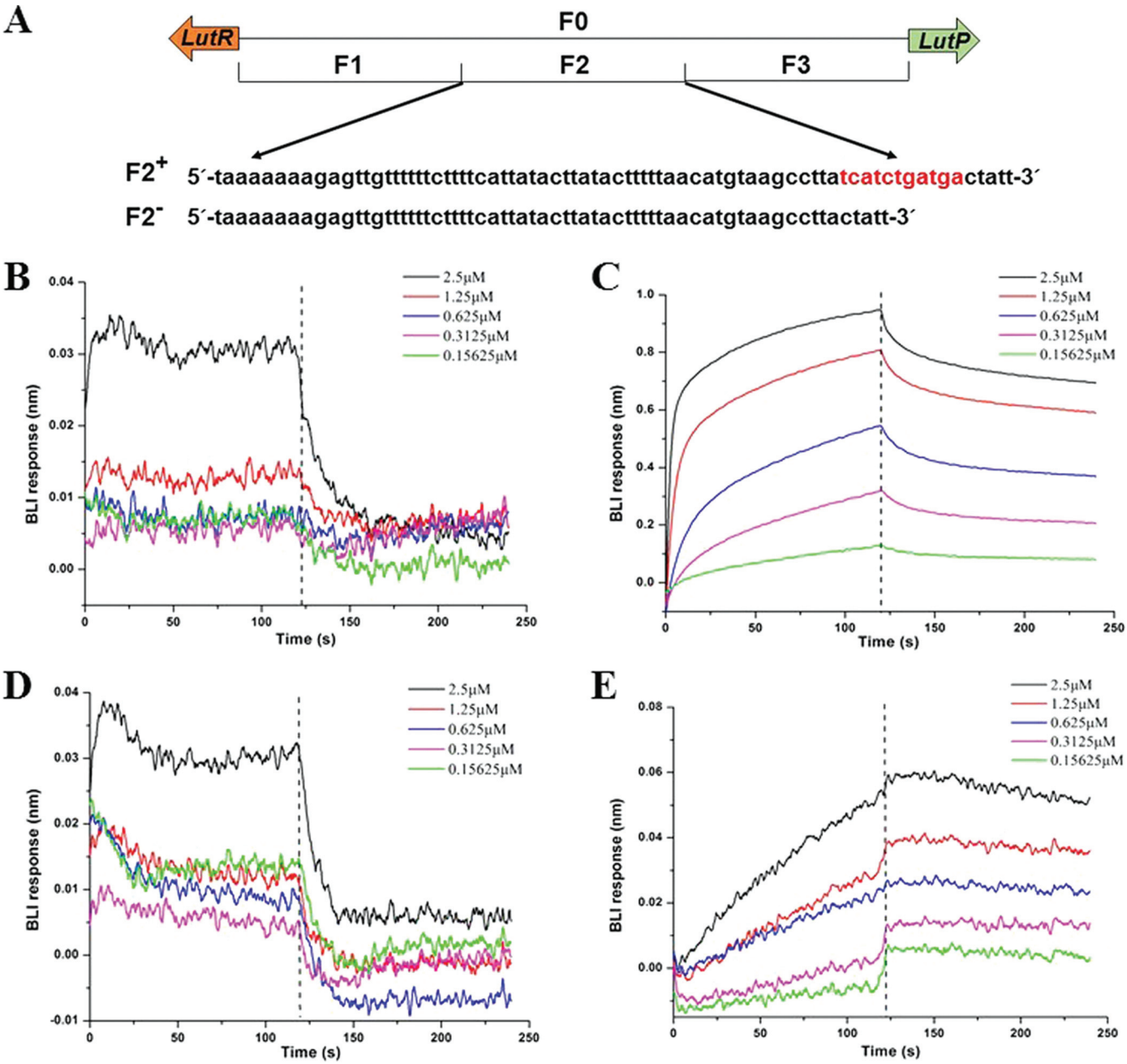

FIG 5 Identification of the binding sites. (A) Nucleotide sequences of the *lutP-lutR* intergenic region of *B. coagulans* DSM1. F0 (218 bp) is the *lutP-lutR* intergenic region, F1 (73 bp) is the fragment that does not contain an inverted repeat sequence. F2 (75 bp) is the fragment with the inverted repeat sequence of TCATCTGATGA. F3 (70 bp) is the fragment with two inverted repeat sequences of AATTGCAATT and TTTTTCAAAAA. $F2^-$ is the fragment with the inverted repeat sequence of F2 deleted. (B) BLI binding assay of LutR to fragment F1. (C) BLI binding assay of LutR to fragment F2. (D) BLI binding assay of LutR to fragment F3. (E) BLI binding assay of LutR to fragment $F2^-$. Different concentrations of 0.15625, 0.3125, 0.625, 1.25, and 2.5 μM purified LutR injected over a Sensor Chip with biotinylated intergenic DNA regions. The vertical dashed lines denote phase I, association with biotinylated intergenic DNA regions, and phase II, dissociation in binding buffer.

a higher binding affinity to fragment F2, with an equilibrium dissociation constant (K_D) value of 4.44×10^{-8} M and K_D error of 1.14×10^{-9} (Fig. 5C). The fractional saturation, $\Theta = R_{eq}/R_{max}$, at equilibrium was calculated, and the Hill function, $\log(\Theta/[1 - \Theta])$, was plotted as a function of log(LutR) (25). The analysis of the linear part of the Hill function provided a linear regression R^2 of 0.9998 and a Hill coefficient greater than 1 (see Fig. S3), indicating a cooperative binding of LutR to fragment F2 (26). Conversely, no binding was detected in fragments F1 and F3 (Fig. 5B and D), indicating that AATTG CAATT and/or TTTTTCAAAAA are poor binders of LutR in *B. coagulans* DSM1.

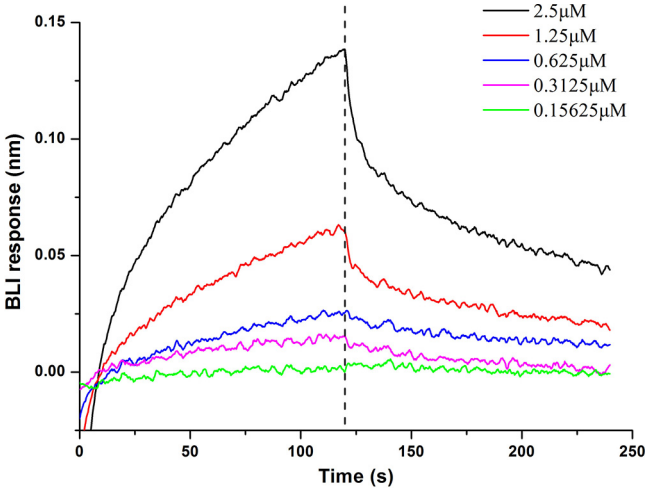

FIG 6 Effects of L-lactate on the DNA-binding activity of LutR. The purified LutR protein was incubated with 5 mM L-lactate in sodium phosphate buffer (20 mM, pH 8.0) for 20 min before BLI binding assay of fragment F2. Different concentrations of 0.15625, 0.3125, 0.625, 1.25, and 2.5 μM purified LutR injected over a Sensor Chip with biotinylated intergenic fragment F2. The vertical dashed line denotes phase I, association with biotinylated fragment F2, and phase II, dissociation in binding buffer.

To validate whether fragment F2 plays a role in the binding of LutR, a BLI assay was conducted with fragment F2$^-$ (the inverted repeat sequence TCATCTGATGA was deleted) (Fig. 5A). Deletion of the consensus sequence TCATCTGATGA abolished the binding of LutR (Fig. 5E). This finding supported that LutR bound to the motif TCATC TGATGA in the upstream region of *lutP* in *B. coagulans* DSM1.

The binding of LutR to the *lutP-lutR* intergenic region is important for *lutP* transcription. The RT-PCR analysis revealed that *lutP* gene expression was affected by L-lactate (Fig. 3). Further BLI analysis suggested that LutR regulated LutP expression by binding to the *lutP-lutR* intergenic region (Fig. 5). To explore the mechanisms underlying the upregulation of the *lutP* gene induced by L-lactate, the binding of purified LutR protein, incubated with 5 mM L-lactate, to subfragment F2 in serial dilution was determined by BLI analysis. The calculated K_D (4.43×10^{-6} M) of lactate-treated LutR was much higher than that of untreated LutR (4.44×10^{-8} M), indicating that L-lactate addition decreased the binding of LutR to F2 (Fig. 6). These results suggest that the presence of L-lactate prevents the binding of LutR to fragment F2. Thus, L-lactate may function as an effector of the LutR protein in *B. coagulans* DSM1. Furthermore, as glucose inhibited *lutP* transcription (Fig. 3B), the binding of purified LutR protein incubated with 120 mM glucose was determined by BLI analysis. Glucose addition did not affect the binding of LutR to fragment F2 (see Fig. S5). Taken together, these results indicate that the binding of LutR to fragment F2 is important for *lutP* transcription.

DISCUSSION

Transport of monocarboxylates, such as lactate, pyruvate, and acetate, is essential for the metabolism and homeostasis of most cells (15). The function and regulation of the L-lactate utilization operon in lactate-utilizing strains have been analyzed in detail. Conversely, studies on lactate transporters in lactate producers are limited. Because lactate transport across the plasma membrane is the first step in lactate metabolism, lactate permease is a conserved component among a wide variety of lactate-utilizing strains (11, 14). The lactate utilization operon is widely present in lactate-utilizing strains, including *Pseudomonas* strains, *Escherichia coli*, and *Bacillus* strains (1, 11, 13). It is composed of a lactate permease-encoding gene, lactate utilization genes (*lldD*, *lldE*, and *lutABC*), and an operon regulator-encoding gene (12). Only the *lutP* gene is present in all sequenced *B. coagulans* strains that do not carry lactate utilization genes adjacent to it (see Fig. S1 in the supplemental material). In this study, *B. coagulans* DSM1 was

used as a representative strain, and the function of LutP in a lactate-producer *B. coagulans* DSM1 was elucidated. The first permease reported to be related to lactate production was Jen1 from *Saccharomyces cerevisiae* (27). Wild-type *S. cerevisiae* does not have the ability to produce lactate (5). Although metabolic pathway manipulation for improving the properties and productivity of microorganisms has become a well-established concept, the export of organic acids is still one of the least understood steps of an integral part of this metabolic process (7, 8). Studies indicated that strengthening lactate dehydrogenase with deletion of lactate permease created a "trapping point" that enabled the irreversible transport of lactate out of the cell (28). Therefore, elucidation of the role and regulation of lactate permease in *B. coagulans* DSM1 may provide a novel lactate transporter for metabolic engineering.

Both biochemical assays and transport activity characterization of the wild-type and *lutP* knockout strains confirmed that LutP contributed to the ability of *B. coagulans* DSM1 to import lactate (Fig. 2) and that *lutP* transcription was induced by lactate (Fig. 3A). This indicates that LutP plays a general role as a lactate transporter in *B. coagulans* strains. Although it was possible to definitively determine the transport role of LutP in *B. coagulans* DSM1, important mechanistic details, including its regulation and interactions with lactate production, are yet to be elucidated. In lactate-utilizing strains, the transcription of the lactate utilization operon was modulated by a regulator, LutR. Most bacterial transcriptional regulators consist of a signal-receiving domain and a DNA-binding domain. Structural analyses have revealed that the HTH is the most widely used DNA-binding motif in bacteria (29). The HTH GntR family has approximately 720 members distributed among diverse bacterial groups, which regulate a wide variety of biological processes. Forty percent of the GntR family is involved in the regulation of enzymes with roles in substrate oxidization (30). In lactate-utilizing strains, members of the GntR family regulators modulate the lactate utilization operon by binding to the HTH motif (31). In this study, we determined that LutR also contains a conserved HTH domain (see Fig. S4), which is typical and conserved in the GntR regulator family (29). This motif with an inverted repeat was reported to be the consensus binding site for LutR (12, 26). For example, LutR in *B. subtilis* binds to the TCATC-N-GATGA motif in the promoter region of the lactate utilization operon (1). We noticed that in *B. coagulans* DSM1, there are 3 inverted repeat sequences in the intergenic region of *lutR-lutP* (Fig. 4A) and that LutR binds to the *lutR-lutP* intergenic region in EMSAs (Fig. 4D). This agrees with the results of the BLI assays, which indicate that LutR binds to the site of TCATCTGATGA in fragment F2 (Fig. 5B). Deletion of the inverted repeat sequence completely blocked LutR's binding to the intergenic region (Fig. 5D).

Homolactic fermentation is a metabolic process by which glucose is converted into lactate (20, 32). During this process, the lactate concentration increases with fermentation time and glucose concentration decreases accordingly (19). It has been reported that intracellular L-lactate affects the function of the regulator LldR in glucose-grown *C. glutamicum* cells (33). The transcription of a number of lactate permeases, such as LldP in *E. coli* (34), LutP in *B. subtilis* (35), LldP in *P. aeruginosa* (11), and Jen1 in yeast (36), is regulated by lactate and/or glucose. It has been shown that binding of the effector molecules to GntR family proteins promotes a conformational change that abolishes DNA-protein interaction (31). In *B. coagulans* DSM1, transcription of *lutP* is induced by L-lactate (Fig. 3A) and inhibited by glucose (Fig. 3B). A BLI assay was conducted to confirm the *in vitro* interaction between LutR and fragment F2, indicating that the DNA-binding affinity was decreased by the addition of L-lactate to the binding solution (Fig. 6). Meanwhile, glucose addition did not modify LutR binding (Fig. S5). These results suggested that effectors, such as L-lactate or glucose, may alter the binding affinity between LutR and fragment F2, and thereby regulate transcriptional levels of LutP.

The concentration of L-lactate may accumulate up to ~2 M, and the productivity may reach 2.4 g/liter/h in the fermentation by *B. coagulans* strains (18, 20, 22, 37). L-nLDH is the key enzyme during L-lactate production. Disruption of its encoding genes (Δ*ldhL1* Δ*ldhL2*) completely blocked L-lactate production and redirected pyruvate to other organic acids (18). In this study, we found that LutP has the ability to import

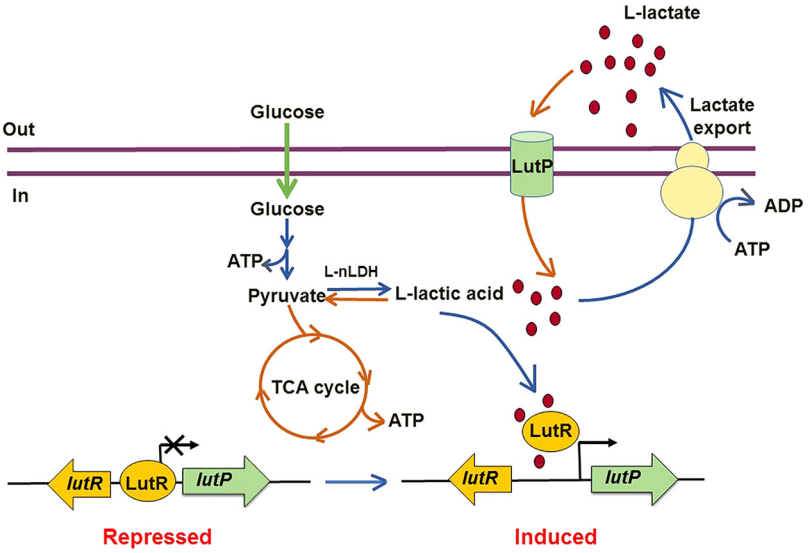

FIG 7 Proposed mechanism of LutR and LutP system for lactate transport in *B. coagulans* DSM1.

lactate into cells but it cannot transport lactate out of cells (Fig. 2). ABC transporters have been found for the export of a wide variety of substrates, ranging from weak organic acids to proteins (8, 38). Although ABC transporters have been theoretically predicted to be responsible for lactate export in homofermentative lactate-producing microorganisms (8), the efflux mechanisms of lactate remain unknown. There are approximately 72 ABC transporter family proteins in *B. coagulans* DSM1, and identification of the transporters responsible for lactate export is under investigation in our laboratory.

During homolactic fermentation, glucose is converted into pyruvate via the glycolysis pathway and L-lactate is produced gradually by L-nLDH (20). ATP is formed during this process, which is required for both cell growth and lactate export. Because improved growth may accelerate lactate production (39), more ATP is required for the high yield of lactate. It is generally accepted that monocarboxylate export is a process of ATP consumption (15). Since the reductive branch of the tricarboxylic acid (TCA) cycle under the strict anaerobic condition does not form ATP (40), ATP will not be sufficient for maintaining cell growth and lactate export at the late stage, especially under the condition of glucose shortage. Based on the data in this study, we proposed a model for LutP function in the L-lactate producer *B. coagulans* DSM1 (Fig. 7). At the early stage of L-lactate fermentation, glucose is dominant in the medium. LutR binds specifically to the invert repeat sequence in LutP, and *in vivo* transcription of *lutP* is maintained at a stable level. With the extension of time, glucose concentrations decrease and L-lactate concentrations increase. The produced L-lactate inhibits LutR binding, thereby promoting *lutP* transcription (Fig. 3). Some of the produced L-lactate is transported into the cells by LutP and reoxidized to pyruvate by L-nLDH (20). Then, pyruvate is channeled into the TCA cycle to generate more ATP under microaerobic conditions to maintain cell growth and lactate export. Our results could help explain why low-speed agitation is fruitful for lactate production in *B. coagulans* strains (22).

In summary, the functions of an uncharacterized lactate permease were demonstrated in an L-lactate producer. Although no lactate oxidase gene is located adjacent to the *lutP* gene, LutP from *B. coagulans* DSM1 transports L-lactate into cells to produce more metabolic energy to maintain cell growth under the condition of glucose shortage. The regulator, LutR, is a repressor of *lutP* transcription, and the transcription inhibition is relieved when LutR interacts with lactate.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains, plasmids, and primers are listed in Table 1. *B. coagulans* DSM1 was purchased from Deutsche Sammlung von Mikroorganismen und

TABLE 1 Strains, plasmids and primers

Strain, plasmid, or primer	Feature(s)[a] or sequence (5′–3′)[b]	Source, reference, or purpose
Strains		
Bacillus coagulans		
DSM1	Wide-type strain capable of L-lactic acid production	DSMZ, Germany
DSM1 Δ*ldhL1* Δ*ldhL2*	*ldhL1* and *ldhL2* null mutant	18
DSM1 Δ*lutP*	The *lutP* disruption mutant	This study
DSM1 *lutP*$^+$	Complementation of DSM1 Δ*lutP* with *lutP*	This study
Lactococcus lactis MG1363	Host for gene cloning	37
Escherichia coli BL21(DE3)	Host for protein expression	Tiangen Co., China
Plasmids		
pMH77	pSH71 replication containing temperature sensitive vector, Cm^r	37
pNW33n	*E. coli-Bacillus* shuttle vector, cloning vector, Cm^r	BGSC, USA
pMH77-Δ*lutP*	*lutP* gene deletion vector, Cm^r	This study
pNW33n-*lutP*	*lutP* gene complementation vector, pNW33n harboring *lutP* gene with *ldhL1* promoter, Cm^r	This study
pETDuet	Protein expression vector, Amp^r	Merck Co., Germany
pETDuet-lutR	N-terminal His-tagged *lutR* in pETDuet, Amp^r	This study
Primers		
Pup-F	CCGGAATTCTGATGGATGGCCGCCACCATCTGAATATC	*lutP* upstream (1,200 bp)
Pup-R	CAAAAAGGAGTGTAGATTGTCAGGAACCCCGCTGCGGAC	
Pdown-F	GTCCGCAGCGGGGTTCCTGACAATCTACACTCCTTTTTG	*lutP* downstream (1,200 bp)
Pdown-R	CCGCTCGAGAAGAATACAAGACCACCCAGGCCGGATA	
P-For	CGCCTTTCATATCCTCTTATTGGTG	Δ*lutP* verification
P-Rev	GCGGAGGAAAGTGACATAAAAAATG	
L1P-L1F	CGGGGTACCAGCCTCATCGCCGGTTTCCCTC	Amplification of *ldhL1* promoter for complementation
L1P-L1R	AAATTTTGTACGTACTCCATATATAATCTTCCTCCCCATC	
L1P-PF	GATGGGGAGGAAGATTATATATGGAGTACGTACAAAATTT	Amplification of *lutP* for complementation
L1P-PR	CCGAAGCTTTTAAGGCACCATCCAGGAAAGG	
LutR-F	CCGAATTCGATGAAGTATAAAAAAATTAAA	Amplification of *lutR* for purification
LutR-R	CAGACTCGAGTTAACGCAGAAAATCCTGCAGGCTG	
F0-F	GTTTTCTCCTCTTATCCCTTTTCTATATTT	Amplification of F0, EMSA
F0-R	CAATCTACACTCCTTTTTGAAAAATTTTCT	
F1-F	GTTTTCTCCTCTTATCCCTTTTCTATATTTTATGAATACCAGCAATCCGG AAAAATAGCAAACAAAAATT	Amplification of F1, EMSA
F1-R	AATTTTTGTTTGCTATTTTTCCGGATTGCTGGTATTCATAAAATATAGAAA AGGGATAAGAGGAGAAAAC	
F2-F	TAAAAAAAGAGTTGTTTTTTCTTTTCATTATACTTATACTTTTTAACATG TAAGCCTTATCATCTGATGACTATT	Amplification of F2, EMSA
F2-R	AATAGTCATCAGATGATAAGGCTTACATGTTAAAAAGTATAAGTATA ATGAAAAGAAAAAACAACTCTTTTTTTA	
F3-F	TCATCTAATGACCTTAAAAATTGCAATTGGGATGGATTGGAGAAAAT TTTTCAAAAAGGAGTGTAGATTG	Amplification of F3, EMSA
F3-R	CAATCTACACTCCTTTTTGAAAAATTTTCTCCAATCCATCCCAATTGC AATTTTTAAGGTCATTAGATGA	
q-16s-F	AGAGTTTGATCMTGGCTCAG	Amplification of reference gene (16S rRNA gene), RT-PCR
q-16s-R	TACGGYTACCTTGTTACGACTT	
LutP-F	TTGACACAGTTCTTCGCTT	Amplification of *lutP*, RT-PCR
LutP-R	GGCAGCAGCCTCTTCAT	

[a]Cm^r, chloramphenicol resistant; Amp^r, ampicillin resistant.
[b]Restriction sites in the primer sequences are underlined.

Zellkulturen GmbH (DSMZ, Braunschweig, Germany). *B. coagulans* DSM1 and mutant strains DSM1 Δ*ldhL1* Δ*ldhL2* (18), DSM1 Δ*lutP*, and DSM1 *lutP*$^+$ were cultivated in BC medium containing yeast extract (10 g/liter), $(NH_4)_2HPO_4$ (2 g/liter), $(NH_4)_2SO_4$ (3.5 g/liter), bis-Tris (10 g/liter), sucrose (50 g/liter), $CaCl_2$ (3 mg/liter), and $MgCl_2$ (5 mg/liter) at 45°C, 120 rpm (41). Plasmids based on pMH77 were constructed in *Lactococcus lactis* MG1363, which was grown in GM17 medium (5 g/liter soy peptone, 5 g/liter tryptone, 2.5 g/liter yeast extract, 5 g/liter meat extract, 0.5 g/liter ascorbic acid, 0.25 g/liter $MgSO_4{\cdot}7H_2O$, 5 g/liter K_2HPO_4, 5 g/liter glycerol, and 10 g/liter glucose at pH 7.1). *E. coli* was cultured in Luria-Bertani (LB) medium at 37°C, 200 rpm. Ampicillin (Amp) was added to a final concentration of 100 μg/ml for *E. coli* where appropriate. Chloramphenicol (Cm) was added to a final concentration of 25 μg/ml for *E. coli* and 7 μg/ml for *B. coagulans* and *L. lactis* where appropriate.

Construction of the *lutP* disrupted and complemented strains. The *lutP* gene was knocked out using plasmid pMH77 with thermosensitive lactococcal pSH71/pWV01 replicon, and all procedures were conducted according to previously described methods (37, 41). To construct the *B. coagulans* DSM1 (Δ*lutP*) mutant strain, the homologous arms of *lutP*, upstream (1,200 bp) and downstream (1,200 bp),

were amplified using the primers Pup-F/Pup-R and Pdown-F/Pdown-R (Table 1). Following gel purification, the upstream and downstream regions were fused via overlap extension PCR using the primers Pup-F and Pdown-R. The resulting PCR product was gel purified, digested with EcoRI and XhoI, and cloned into pMH77, resulting in plasmid pMH77-Δ*lutP*. The plasmid, pMH77-Δ*lutP*, was first transformed into *L. lactis* MG1363. Next, pMH77-Δ*lutP* was extracted and transformed into *B. coagulans* DSM1 by electroporation. The first single-crossover recombination mutants with integration of the plasmid pMH77-Δ*lutP* into the chromosome were selected on BC plates containing Cm after being cultured overnight at 45°C, followed by a temperature shift to 60°C and further incubation for 12 h. The positive colonies were incubated in BC liquid broth without Cm overnight at 45°C, 120 rpm, and then a dilution series was streaked onto BC plates without Cm and incubated overnight at 45°C. Colonies were sequentially streaked onto BC plates with and without Cm and incubated overnight at 45°C. Colonies that grew on BC plates without Cm, but did not grow on those with Cm, were selected for PCR analysis using primers P-For and P-Rev. All constructed strains were validated using PCR and sequenced.

To complement *lutP*, *lutP* and the *ldhL1* promoter were amplified from *B. coagulans* DSM1 genomic DNA using the primers L1P-PF/L1P-PR and L1P-L1F/L1P-L1R (Table 1). Following gel purification, the *ldhL1* promoter sequence and *lutP* were fused via overlap extension PCR using the primers L1P-L1F and L1P-PR. The resulting PCR product was gel purified, digested with KpnI and HindIII, and cloned into the *E. coli-B. coagulans* shuttle vector pNW33n, resulting in plasmid pNW33n-*lutP*$^+$. Plasmid pNW33n-*lutP*$^+$ was introduced into *B. coagulans* DSM1 Δ*lutP* by electroporation to construct the *lutP*-complemented strain.

RNA isolation, cDNA generation, and RT-PCR. *B. coagulans* DSM1 Δ*ldhL1* Δ*ldhL2* and *B. coagulans* DSM1 were grown in BC medium to the log phase (optical density at 600 nm [OD_{600}] of ~0.6). Then, cells were transferred into BC medium with or without 5 mM L-lactate and 120 mM glucose for 0, 5, 10, and 30 min. Total RNA was extracted using an E.Z.N.A. bacterial RNA kit (Omega). The total RNA concentration was determined via absorbance at 260 nm (NanoVue spectrophotometer; GE). By using random hexamer primers, cDNA copies were synthesized with a Fast Quant RT kit (with gDNase) (Tiangen, China) and amplified with SYBR Premix Ex *Taq* (TaKaRa, China) using the LightCycler 96 RT-PCR detection system (Roche, USA). As recommended by the manufacturer, 2 μl cDNA was used as the template in 20-μl real-time PCR mixtures with 10 pmol of gene-specific primers (24) (Table 1). Threshold cycles (C_T) for each PCR with different cDNA concentrations were determined and compared with that for standard DNA (the 16S rRNA gene) analyzed at the same time (20). The $2^{-\triangle\triangle CT}$ relative quantification method was used to determine mRNA levels (37). The results reported are the averages from at least three experiments with a variability of <15%.

[^{14}C]Lactate uptake. *B. coagulans* DSM1 and its mutant DSM1 Δ*lutP* were grown in BC medium to the exponential growth phase. One milliliter of exponentially growing cells was transferred into BC medium containing 0.2 μCi/ml of L-[^{14}C(U)]lactate (50 μCi; Perkin Elmer) and uptake was stopped at different time points. Cells were harvested, and uptake was stopped by washing twice with ice-cold morpholinepropanesulfonic acid (MOPS; 0.1 M, pH 7.0). Cells were lysed using 250 μl of 0.1 M NaOH. Radioactivity was determined by mixing 200 μl of scintillation liquid with 100 μl cell lysate and counted using a liquid scintillation counter (Beckman Coulter, Fullerton, CA) (42). The protein concentration was determined by the Bradford assay with bovine serum albumin (BSA) as a standard (43) and was used to normalize radioactivity. L-[^{14}C(U)]Lactate uptake was expressed as counts per micromolar per milligram of cellular protein.

Gas chromatography-mass spectrometry analysis. *B. coagulans* DSM1 and mutant strain DSM1 Δ*lutP* were cultured overnight at 45°C. After cultivation, cells were harvested by centrifugation (8,000 × *g*, 10 min at room temperature) and washed once with 0.1 M sodium phosphate buffer (pH 6.8). The catalytic reaction mixture, containing cells (OD_{600} of 30), 50 mM [^{13}C]pyruvate (Sigma-Aldrich), and 100 μM NADH, was incubated at 37°C for different periods of time (30, 60, 90, and 120 min). After centrifugation (8,000 × *g*, 10 min at room temperature), the supernatants (300 μl) were freeze-dried after being frozen in liquid nitrogen and mixed with 500 μl methanol to precipitate the protein. The mixture was shaken for 1 min and centrifuged at 3,000 × *g* for 10 min. The supernatant (300 μl) was transferred to the GC vial and evaporated until it was dry. Chemical derivatization of sample metabolites was performed using a 100-μl aliquot of methoxyamine pyridine solution (15 mg/ml) at 70°C for 1 h. Subsequently, *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (50 μl; Sigma-Aldrich) was added, and the silylation was performed at 70°C for 1 h. After adding 150 μl of *n*-heptane and vortex mixing, the supernatant was transferred to the GC microvial for GC-MS analysis (44, 45).

GC-MS was carried out using a GC-2010 gas chromatograph equipped with a DB-5 capillary column (30 m by 0.15 μm by 15 mm). The initial temperature was maintained at 85°C for 3 min and then raised to 280°C at a rate of 10°C/min. All samples were injected in split mode at 270°C. The mass spectrometer was operated in electron ionization (EI) mode (positive ion, 70 eV), and the quadrupole was 150°C. Mass spectra were acquired in full-scan mode with repetitive scanning from 60 *m/z* to 600 *m/z* for 1 s. The ion source temperature was 230°C and injection volume was 1 ml. GC-MS data were processed by Agilent MSD ChemStation. Raw data were filtered and then subjected to retention time correction and peak alignment. The data matrix was used to normalize to the sum of all peak areas of each sample (46). Under ammonia positive chemical ionization (pressure adjusted to optimize peak areas), ions signals were 262 *m/z* for [^{13}C]lactate and 261 *m/z* for unlabeled lactate. Two calibration curves of [^{13}C]lactate with different concentrations of 0.01, 0.05, 0.10, 0.50, 1.00, 2.50, 5.00, and 10.00 g/liter and unlabeled lactate with different concentrations of 0.01, 0.05, 0.10, 0.50, 1.00, and 10.00 g/liter were used to calculate the concentrations of [^{13}C]lactate and [^{12}C]lactate. The extracellular [^{13}C]lactate concentration was calculated

as follows: [^{13}C]lactate concentration (g/liter) = [^{13}C]lactate concentration (g/liter) − [^{12}C]lactate concentration (g/liter).

Purification of LutR. LutR-encoding gene *lutR* (NCBI Protein accession number AJH80199.1) was amplified using PCR from genomic DNA of *B. coagulans* DSM1 with primers LutR-F and LutR-R (Table 1). The product was digested with EcoRI and XhoI, and inserted into the expression plasmid pETDuet (Merck Co., Germany) to generate pETDuet-lutR, containing a His_6 tag (LEHHHHHH) at the N terminus. The constructed expression vector was transformed into *E. coli* BL21(DE3) and grown at 37°C in LB medium supplemented with 100 μg/ml ampicillin. The protein was expressed by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM at an OD_{600} of 0.6. After induction for 20 h at 20°C, cells were harvested, resuspended in buffer A (pH 7.4; 20 mM sodium phosphate, 20 mM imidazole, and 500 mM sodium chloride), and disrupted by sonication for 10 min in an ice bath. The resulting suspension was centrifuged at 13,000 × *g* at 4°C for 10 min to remove debris. The supernatant was filtered through a 0.45-μm filter, then applied to a HisTrap HP 1 × 5 ml column (GE Healthcare, USA) and eluted with 25% buffer A and 75% buffer B (pH 7.4; 20 mM sodium phosphate, 500 mM imidazole, and 500 mM sodium chloride) at a flow rate of 5 ml/min. Fractions containing the target protein were sequentially purified via chromatography on a HiLoad 10/300 Superdex 200 GL column (GE Healthcare, USA) with buffer C (pH 8.0; 20 mM Na-P_i, 0.2 M NaCl, 0.5 mM EDTA). The fractions containing the appropriate molecular weight of LutR were pooled and detected by SDS-PAGE using 12.5% polyacrylamide gels. The protein concentration was determined by the Bradford assay with BSA as a standard (43).

EMSA. EMSA was performed as previously described (11). For DNA fragments of F1, F2, and F3, synthetic oligodeoxyribonucleotides were used (Table 1). Equimolar amounts of the complementary strands were mixed in annealing buffer C (pH 8.0; 20 mM Na-P_i, 0.2 M NaCl, 0.5 mM EDTA). Double-stranded DNA (dsDNA) was prepared by annealing the DNA mixture, heating the reaction at 90°C for 5 min, and gradually decreasing the temperature to 4°C overnight (30). Purified LutR was mixed with fragments of F0, F1, F2, or 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). Following incubation for 30 min at 30°C, the samples were separated on a 6% native polyacrylamide gel at 4°C and a constant voltage of 80 V 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 Gold nucleic acid gel stain according to the instructions of the supplier (Thermo Fisher Scientific).

Biolayer interferometry. BLI assays were performed using a FortéBio Octet RED 96 system (FortéBio, USA) as previously described (25). All binding studies were carried out at 30°C. The streptavidin (SA) sensor was loaded with biotinylated DNA (1 μg/ml), in a buffer (20 mM Na-P_i [pH 8.0], 0.2 M NaCl, 0.5 mM EDTA) and 0.02% Tween 20 (vol/vol). After reaching baseline in the same buffer, association and dissociation were carried out using purified LutR and the buffer, respectively. Steady-state binding responses were determined by the overall response (nanometers) on each sensor, and the data were analyzed using the system software of Octet RED 96. To determine the Hill coefficient, LutR (0.15625 to 2.5 μM) was used to interact with the immobilized fragments.

Genome and bioinformatics tools. All sequences were downloaded from GenBank. Analysis of the lactate permease gene, the lactate utilization gene distribution, and chromosomal location were performed by SEED (http://theseed.uchicago.edu/FIG/subsys.cgi). BLAST searches were carried out to inspect the sequences encoding the protein with high homology to iLDH, LutR, and lactate permease. The identification of LutR binding sites was performed as previously described (14). An iterative motif detection procedure implemented in the program GeneQuest was used to identify common regulatory DNA motifs in the upstream gene fragments. Sequence logos for the binding sites of LutR were drawn using the WEBLOGO package.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00672-19.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

ACKNOWLEDGMENTS

We thank Ping Xu (Shanghai Jiao Tong University, Shanghai, China) for his critical suggestions and assistance in manuscript preparation. We also thank Yuanyuan Chen and Zhenwei Yang (Institute of Biophysics, Chinese Academy of Sciences) for technical help with Biacore experiments. We thank Editage (Cactus Communications, Inc., Princeton, NJ) for English language editing.

The work was supported by the National Natural Science Foundation of China (31670045) and the STS program of Chinese Academy of Sciences (KFJ-STS-QYZD-094), in part by grants from the Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Technology & Business University (BTBU) (20171013) and the Key Laboratory of Agro-Products Processing, Ministry of Agriculture and Rural Affairs/Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences.

We declare no conflict of interest.

REFERENCES

1. Chiu KC, Lin CJ, Shaw GC. 2014. Transcriptional regulation of the L-lactate permease gene *lutP* by the LutR repressor of *Bacillus subtilis* RO-NN-1. Microbiology 160:2178–2189. https://doi.org/10.1099/mic.0.079806-0.
2. Bosma EF, van der Oost J, de Vos WM, van Kranenburg R. 2013. Sustainable production of bio-based chemicals by extremophiles. Curr Biotechnol 2:360–379. https://doi.org/10.2174/18722083113076660028.
3. Kawahata M, Masaki K, Fujii T, Iefuji H. 2006. Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. FEMS Yeast Res 6:924–936. https://doi.org/10.1111/j.1567-1364.2006.00089.x.
4. Benthin S, Villadsen J. 1995. Production of optically pure D-lactate by *Lactobacillus bulgaricus* and purification by crystallisation and liquid/liquid extraction. Appl Microbiol Biotechnol 42:826–829. https://doi.org/10.1007/BF00191176.
5. Branduardi P, Sauer M, De Gioia L, Zampella G, Valli M, Mattanovich D, Porro D. 2006. Lactate production yield from engineered yeasts is dependent from the host background, the lactate dehydrogenase source and the lactate export. Microb Cell Fact 5:4. https://doi.org/10.1186/1475-2859-5-4.
6. Wang Y, Li Y, Pei X, Yu L, Feng Y. 2007. Genome-shuffling improved acid tolerance and L-lactic acid volumetric productivity in *Lactobacillus rhamnosus*. J Biotechnol 129:510–515. https://doi.org/10.1016/j.jbiotec.2007.01.011.
7. Skory C-D, Hector R-E, Gorsich S-W, Rich J-O. 2010. Analysis of a functional lactate permease in the fungus *Rhizopus*. Enzyme Microb Tech 46:43–50. https://doi.org/10.1016/j.enzmictec.2009.08.014.
8. van Maris AJ, Konings WN, van Dijken JP, Pronk JT. 2004. Microbial export of lactic and 3-hydroxypropanoic acid: implications for industrial fermentation processes. Metab Eng 6:245–255. https://doi.org/10.1016/j.ymben.2004.05.001.
9. Jiang T, Gao C, Ma C, Xu P. 2014. Microbial lactate utilization: enzymes, pathogenesis, and regulation. Trends Microbiol 22:589–599. https://doi.org/10.1016/j.tim.2014.05.008.
10. Georgi T, Engels V, Wendisch V-F. 2008. Regulation of L-lactate utilization by the FadR-type regulator LldR of *Corynebacterium glutamicum*. J Bacteriol 190:963–971. https://doi.org/10.1128/JB.01147-07.
11. Gao C, Hu C, Zheng Z, Ma C, Jiang T, Dou P, Zhang W, Che B, Wang Y, Lv M, Xu P. 2012. Lactate utilization is regulated by the FadR-type regulator LldR in *Pseudomonas aeruginosa*. J Bacteriol 194:2687–2692. https://doi.org/10.1128/JB.06579-11.
12. Chai Y, Kolter R, Losick R. 2009. A widely conserved gene cluster required for lactate utilization in *Bacillus subtilis* and its involvement in biofilm formation. J Bacteriol 191:2423–2430. https://doi.org/10.1128/JB.01464-08.
13. Aguilera L, Campos E, Giménez R, Badía J, Aguilar J, Baldoma L. 2008. Dual role of LldR in regulation of the *lldPRD* operon, involved in L-lactate metabolism in *Escherichia coli*. J Bacteriol 190:2997–3005. https://doi.org/10.1128/JB.02013-07.
14. Pinchuk GE, Rodionov DA, Yang C, Li X, Osterman AL, Dervyn E, Geydebrekht OV, Reed SB, Romine MF, Collart FR, Scott JH, Fredrickson JK, Beliaev AS. 2009. Genomic reconstruction of *Shewanella oneidensis* MR-1 metabolism reveals a previously uncharacterized machinery for lactate utilization. Proc Natl Acad Sci U S A 106:2874–2879. https://doi.org/10.1073/pnas.0806798106.
15. Soares-Silva I, Sá-Pessoa J, Myrianthopoulos V, Mikros E, Casal M, Diallinas G. 2011. A substrate translocation trajectory in a cytoplasm-facing topological model of the monocarboxylate/H^+ symporter Jen1p. Mol Microbiol 81:805–817. https://doi.org/10.1111/j.1365-2958.2011.07729.x.
16. Casal M, Paiva S, Queirós O, Soares-Silva I. 2008. Transport of carboxylic acids in yeasts. FEMS Microbiol Rev 32:974–994. https://doi.org/10.1111/j.1574-6976.2008.00128.x.
17. Pacheco A, Talaia G, Sá-Pessoa J, Bessa D, Gonçalves MJ, Moreira R, Paiva S, Casal M, Queirós O. 2012. Lactic acid production in *Saccharomyces cerevisiae* is modulated by expression of the monocarboxylate transporters Jen1 and Ady2. FEMS Yeast Res 12:375–381. https://doi.org/10.1111/j.1567-1364.2012.00790.x.
18. Sun L, Zhang C, Lyu P, Wang Y, Wang L, Yu B. 2016. Contributory roles of two L-lactate dehydrogenases for L-lactic acid production in thermotolerant *Bacillus coagulans*. Sci Rep 6:37916. https://doi.org/10.1038/srep37916.
19. Wang LM, Zhao B, Liu B, Yu B, Ma CQ, Yang CY, Su F, Hua DL, Li QG, Ma YH, Xu P. 2010. Efficient production of L-lactic acid from corncob molasses, a waste by-product in xylitol production, by a newly isolated xylose utilizing *Bacillus* sp. strain. Bioresour Technol 101:7908–7915. https://doi.org/10.1016/j.biortech.2010.05.031.
20. Wang L, Cai Y, Zhu L, Guo H, Yu B. 2014. Major role of NAD-dependent lactate dehydrogenases in the production of L-lactic acid with high optical purity by the thermophile *Bacillus coagulans*. Appl Environ Microbiol 80:7134–7141. https://doi.org/10.1128/AEM.01864-14.
21. Su F, Yu B, Sun J, Ou HY, Zhao B, Wang L, Qin J, Tang H, Tao F, Jarek M, Scharfe M, Ma C, Ma Y, Xu P. 2011. Genome sequence of the thermophilic strain *Bacillus coagulans* 2-6, an efficient producer of high-optical-purity L-lactic acid. J Bacteriol 193:4563–4564. https://doi.org/10.1128/JB.05378-11.
22. Peng L, Wang L, Che C, Yang G, Yu B, Ma Y. 2013. *Bacillus* sp. strain P38: an efficient producer of L-lactate from cellulosic hydrolysate, with high tolerance for 2-furfural. Bioresour Technol 149:169–176. https://doi.org/10.1016/j.biortech.2013.09.047.
23. Rhee MS, Moritz BE, Xie G, Glavina Del Rio T, Dalin E, Tice H, Bruce D, Goodwin L, Chertkov O, Brettin T, Han C, Detter C, Pitluck S, Land ML, Patel M, Ou M, Harbrucker R, Ingram LO, Shanmugam KT. 2011. Complete genome sequence of a thermotolerant sporogenic lactic acid bacterium, *Bacillus coagulans* strain 36D1. Stand Genomic Sci 5:331–340. https://doi.org/10.4056/sigs.2365342.
24. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622. https://doi.org/10.1373/clinchem.2008.112797.
25. Wang L, Tang H, Yu H, Yao Y, Xu P. 2014. An unusual repressor controls the expression of a crucial nicotine-degrading gene cluster in *Pseudomonas putida* S16. Mol Microbiol 91:1252–1269. https://doi.org/10.1111/mmi.12533.
26. Engohang-Ndong J, Baillat D, Aumercier M, Bellefontaine F, Besra GS, Locht C, Baulard AR. 2003. EthR, a repressor of the TetR/CamR family implicated in ethionamide resistance in mycobacteria, octamerizes cooperatively on its operator. Mol Microbiol 51:175–188. https://doi.org/10.1046/j.1365-2958.2003.03809.x.
27. Lodi T, Fontanesi F, Guiard B. 2002. Co-ordinate regulation of lactate metabolism genes in yeast: the role of the lactate permease gene *JEN1*. Mol Genet Genomics 266:838–847. https://doi.org/10.1007/s00438-001-0604-y.
28. Li C, Tao F, Xu P. 2016. Carbon flux trapping: highly efficient production of polymer-grade D-lactic acid with a thermophilic D-lactate dehydrogenase. Chembiochem 17:1491–1494. https://doi.org/10.1002/cbic.201600288.
29. Rigali S, Derouaux A, Giannotta F, Dusart J. 2002. Subdivision of the helix-turn-helix GntR family of bacterial regulators in the FadR, HutC, MocR, and YtrA subfamilies. J Biol Chem 277:12507–12515. https://doi.org/10.1074/jbc.M110968200.
30. Gao YG, Suzuki H, Itou H, Zhou Y, Tanaka Y, Wachi M, Watanabe N, Tanaka I, Yao M. 2008. Structural and functional characterization of the LldR from *Corynebacterium glutamicum*: a transcriptional repressor involved in L-lactate and sugar utilization. Nucleic Acids Res 36:7110–7123. https://doi.org/10.1093/nar/gkn827.
31. Quail MA, Guest JR. 1995. Purification, characterization and mode of action of PdhR, the transcriptional repressor of the *pdhR-aceEF-lpd* operon of *Escherichia coli*. Mol Microbiol 15:519–529. https://doi.org/10.1111/j.1365-2958.1995.tb02265.x.
32. Viana R, Yebra MJ, Galán JL, Monedero V, Pérez-Martínez G. 2005. Pleiotropic effects of lactate dehydrogenase in activation in *Lactobacillus casei*. Res Microbiol 156:641–649. https://doi.org/10.1016/j.resmic.2005.02.011.
33. Pequignot C, Dussap CG, Pons A, Gros JB. 1997. Intra- and extracellular concentrations of glutamate, lactate and acetate during growth of *Corynebacterium glutamicum* on different media. J Ind Microbiol Biotech 18:312–318. https://doi.org/10.1038/sj.jim.2900386.
34. Dong JM, Taylor JS, Latour DJ, Iuchi S, Lin EC. 1993. Three overlapping *lct* genes involved in L-lactate utilization by *Escherichia coli*. J Bacteriol 175:6671–6678. https://doi.org/10.1128/jb.175.20.6671-6678.1993.
35. Irigül-Sönmez Ö, Köroğlu TE, Öztürk B, Kovács ÁT, Kuipers OP, Yazgan

KA. 2014. In *Bacillus subtilis* LutR is part of the global complex regulatory network governing the adaptation to the transition from exponential growth to stationary phase. Microbiology 160:243–260. https://doi.org/10.1099/mic.0.064675-0.
36. Dulermo R, Gamboa-Meléndez H, Michely S, Thevenieau F, Neuvéglise C, Nicaud JM. 2015. The evolution of Jen3 proteins and their role in dicarboxylic acid transport in *Yarrowia*. Microbiologyopen 4:100–120. https://doi.org/10.1002/mbo3.225.
37. Zhang C, Zhou C, Assavasirijinda N, Yu B, Wang L, Ma Y. 2017. Non-sterilized fermentation of high optically pure D-lactic acid by a genetically modified thermophilic *Bacillus coagulans* strain. Microb Cell Fact 16:213. https://doi.org/10.1186/s12934-017-0827-1.
38. Poelarends GJ, Mazurkiewicz P, Putman M, Cool RH, Veen HW, Konings WN. 2000. An ABC-type multidrug transporter of *Lactococcus lactis* possesses an exceptionally broad substrate specificity. Drug Resist Updat 3:330–334. https://doi.org/10.1054/drup.2000.0173.
39. Zhao B, Wang L, Ma C, Yang C, Xu P, Ma Y. 2010. Repeated open fermentative production of optically pure L-lactic acid using a thermophilic *Bacillus* sp. strain. Bioresour Technol 101:6494–6498. https://doi.org/10.1016/j.biortech.2010.03.051.
40. Vuoristo KS, Mars AE, Sanders JPM, Eggink G, Weusthuis RA. 2016. Metabolic engineering of TCA cycle for production of chemicals. Trends Biotechnol 34:191–197. https://doi.org/10.1016/j.tibtech.2015.11.002.
41. Kovács ÁT, van Hartskamp M, Kuipers OP, van Kranenburg R. 2010. Genetic tool development for a new host for biotechnology, the thermotolerant bacterium *Bacillus coagulans*. Appl Environ Microbiol 76:4085–4088. https://doi.org/10.1128/AEM.03060-09.
42. Le Floch R, Chiche J, Marchiq I, Naiken T, Ilc K, Murray CM, Critchlow SE, Roux D, Simon M-P, Pouyssegur J. 2011. CD147 subunit of lactate/H^+ symporters MCT1 and hypoxia-inducible MCT4 is critical for energetics and growth of glycolytic tumors. Proc Natl Acad Sci U S A 108:16663–16668. https://doi.org/10.1073/pnas.1106123108.
43. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. https://doi.org/10.1016/0003-2697(76)90527-3.
44. Zhang WQ, Hua YL, Zhang M, Ji P, Li JX, Zhang L, Li PL, Wei YM. 2015. Metabonomic analysis of the anti-inflammatory effects of volatile oils of *Angelica sinensis* on rat model of acute inflammation. Biomed Chromatogr 29:902–910. https://doi.org/10.1002/bmc.3372.
45. Yao W, Zhang L, Hua Y, Peng J, Peng L, Li J, Zhong L, Zhao H, Wei Y. 2015. The investigation of anti-inflammatory activity of volatile oil of *Angelica sinensis* by plasma metabolomics approach. Int Immunopharmacol 29:269–277. https://doi.org/10.1016/j.intimp.2015.11.006.
46. Zhong LJ, Hua YL, Peng J, Yao WL, Zhang WQ, Li J, Wei YM. 2016. Evaluation of the anti-inflammatory effects of volatile oils from processed products of *Angelica sinensis* radix by GC–MS-based metabolomics. J Ethnopharmacol 191:195–205. https://doi.org/10.1016/j.jep.2016.06.027.