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A role of the transcriptional regulator LldR (NCgl2814) in glutamate metabolism under biotin-limited conditions in *Corynebacterium glutamicum*

Tanyanut Supkulsutra, Tomoya Maeda, Kosuke Kumagai, and Masaaki Wachi*

Department of Bioengineering, Tokyo Institute of Technology, Yokohama 226-8501, Japan

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Corynebacterium glutamicum is a Gram-positive, rod-shaped, aerobic bacterium used for the fermentative production of L-glutamate. LldR (NCgl2814) is known as a repressor for *ldhA* and *lldD* encoding lactate dehydrogenases. LdhA is responsible for production of L-lactate, while LldD is for its assimilation. Since L-lactate production was observed as a by-product of glutamate production under biotin-limited conditions, LldR might play a regulatory role in the glutamate metabolism. Here for the first time, we investigated effects of overproduction or deletion of LldR on the glutamate metabolism under biotin-limited conditions in *C. glutamicum*. It was found that glutamate production under biotin-limited conditions was decreased by overproduction of LldR. In the wild-type cells, L-lactate was produced in the first 24 h and it was re-consumed thereafter. On the other hand, in the overproduced cells, L-lactate was produced like the wild type, but it was not re-consumed. This means that L-lactate assimilation, which is catalyzed by LldD, was suppressed by the overproduction of LldR, but L-lactate production, which is catalyzed by LdhA, was not affected, indicating that LldR mainly controls the expression of *lldD* but not of *ldhA* under biotin-limited conditions. This was confirmed by quantitative real-time RT-PCR. From these results, it is suggested that L-lactate metabolism, which is controlled by LldR, has a buffering function of the pyruvate pool for glutamate production.

Key Words—*Corynebacterium glutamicum*; L-glutamate; L-lactate; lactate dehydrogenase; LdhA; LldD; LldR

Introduction

Corynebacterium glutamicum is a Gram-positive, rod-shaped, aerobic bacterium which was isolated by Japanese researchers in 1956 as an L-glutamate-producing bacterium (Kinoshita et al., 1957; Udaka, 1960). *C. glutamicum* has been used as a producer of L-glutamate, which is used as a flavor enhancer in food industries, for several decades. More than 2.1 million

tons of monosodium glutamate (MSG) is produced annually worldwide by fermentation using *C. glutamicum* or closely related species and its demand is still steadily increasing every year. Several other amino acids, such as lysine and threonine, are also produced by fermentation using this bacterium (Hermann, 2003; Ikeda, 2003; Kinoshita, 2005).

C. glutamicum has a unique mechanism of L-glutamate secretion, in which special treatments or growth conditions are required. *C. glutamicum* is a biotin auxotroph, but the presence of biotin inhibits L-glutamate production (Shiio et al., 1962). As biotin is a co-factor of acetyl-CoA carboxylase, the biotin limitation may affect the fatty acid synthesis. L-Glutamate production is also induced in response to fatty acid ester

* Corresponding author: Dr. Masaaki Wachi, Department of Bioengineering, Tokyo Institute of Technology, 4259 J2-14 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan.

Tel: +81-45-924-5770 Fax: +81-45-924-5820

E-mail: mwachi@bio.titech.ac.jp

surfactants or penicillin treatments (Nara et al., 1964; Takinami et al., 1965). It was recently shown that the NCgl1221 gene encoding a mechanosensitive channel is involved in the mechanism of L-glutamate secretion (Borngen et al., 2010; Hashimoto et al., 2010; Nakamura et al., 2007; Wachi, 2013). Specific mutations of NCgl1221 protein led to a constitutive L-glutamate secretion without any inducing treatments, while disruption of NCgl1221 greatly diminished L-glutamic acid secretion (Nakamura et al., 2007; Nakayama et al., 2012). Very recently, it has been demonstrated that the NCgl1221 channel does indeed serve as a path for glutamate efflux (Hashimoto et al., 2012).

L-Lactate is usually produced as a by-product during glutamate production. The excreted L-lactate is re-consumed, which may contribute to glutamate production (Sato et al., 2008; Stansen et al., 2005; Uy et al., 2003). *C. glutamicum* catalyzes the production of L-lactate from pyruvate with a NAD-dependent L-lactate dehydrogenase (EC 1.1.1.27) encoded by *ldhA* (Bott and Niebisch, 2003; Inui et al., 2004), while the quinone-dependent L-lactate dehydrogenase (EC 1.1.2.3) encoded by *lldD* is responsible for oxidation of L-lactate to pyruvate (Bott and Niebisch, 2003; Stansen et al., 2005) (Fig. 1). A recent study reported that overexpression of the *ldhA* gene could restore the growth defect of the Δ *lldD* strain when cells were grown on L-lactate as the sole carbon source. This indicates that LdhA functions in vivo to convert L-lactate to pyruvate (Sharkey et al., 2011). In *C. glutamicum*, *lldD* forms an operon with NCgl2816 encoding a permease for L-lactate utilization. The function of the permease gene NCgl2816 is dispensable, while LldD is essential for growth on L-lactate (Stansen et al., 2005). *lldR* (NCgl2814) encodes a GntR-type transcriptional repressor of the NCgl2816-*lldD* operon. L-Lactate binds to LldR, preventing repression of NCgl2816-*lldD* by LldR (Gao et al., 2008; Georgi et al., 2008). LldR also acts as a repressor of *ldhA* in the absence of L-lactate, but *ldhA* expression is primarily repressed by the DeoR-type transcription regulator, SugR, in the absence of sugar (Engels et al., 2008; Toyoda et al., 2009a, b). Moreover, LldR also represses the *fruR-fruk-ptsF* operon responsible for fructose utilization (Gao et al., 2008). It was reported that overexpression of *lldR* resulted in a decrease in expression of *ldhA* and the NCgl2816-*lldD* operon, while disruption of *lldR* showed a significant increase in expression of the NCgl2816-*lldD* operon (Gao et al., 2008; Georgi et al., 2008). Disruption of

Fig. 1. Schematic of metabolic pathway of L-glutamate in *C. glutamicum*.

L-Lactate is produced as a by-product and re-utilized by lactate dehydrogenases, LdhA and LldD, respectively. Anaplerotic pathways are catalyzed by pyruvate carboxylase (PC) and phosphoenolpyruvate carboxylase (PEPC). Pyruvate kinase (PK) and pyruvate dehydrogenase (PDH) are also indicated.

lldR showed a significant increase in *ldhA* expression in the absence of sugar (Gao et al., 2008) and showed no significant change in *ldhA* expression in the presence of sugar (Georgi et al., 2008).

In this study, we report the role of the transcriptional regulator LldR in the glutamate metabolism under biotin-limited conditions in *C. glutamicum*. By disruption and overexpression of *lldR*, it is suggested that L-lactate metabolism, which is controlled by LldR, has a buffering function of the pyruvate pool for glutamate production.

Materials and Methods

Bacterial strains, media and plasmids. *Corynebacterium glutamicum* ATCC 31831 was used as a wild-type strain (WT) in all experiments. *Escherichia coli* JM109 [*recA1 endA1 gyrA96 thi hsdR17 e14⁻ (mcrA⁻) supE44 relA1 Δ(lac-proAB)/F' (traD36 proAB⁺ lacI^q lacZΔM15)*] and JM110 [*dam dcm supE44 hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr tsx Δ(lac-proAB)/F' (traD36 proAB⁺ lacI^q lacZΔM15)*] were used for plasmid construction. *C. glutamicum* and *E. coli* cells were cultured in

Lennox medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, pH 7.0). For the glutamate production experiment, the preculture medium contained (per liter): 80 g glucose, 30 g $(\text{NH}_4)_2\text{SO}_4$, 1.0 g KH_2PO_4 , 0.01 g $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 200 μg vitamin B₁ HCl, 13.72 ml soybean hydrolysate (total nitrogen, 0.48 g) and 15 μg biotin (pH 8.3). For the main culture, biotin was excluded from the preculture medium and 0.25 g of CaCO_3 was added to the 5 ml culture to maintain pH. The *C. glutamicum* strain cultured in Lennox medium for 24 h was directly inoculated into the preculture medium at 1% volume. After 24 h cultivation, the preculture was directly inoculated into the main culture medium at 5% volume for L-glutamate fermentation. All media were cultivated at 30°C. Plasmids used in this study are all derived from the plasmid pECt (Sato et al., 2008). Kanamycin (20 $\mu\text{g}/\text{ml}$) and 1 mM IPTG was added when needed.

Construction of *lldR*-overexpressing strain. The DNA fragment spanning the *lldR* gene and the Shine-Dalgarno (SD) sequence from the *pyc* gene but not its promoter region was amplified from chromosomal DNA of *C. glutamicum* ATCC 31831 by PCR with the primer pair 5'-CGCCATGGGCGAGCTCGAAAGGAATAATTACTCTAATGAGTGTGAAAGCACATGA-3' (artificially introduced *Nco*I site is underlined) and 5'-TGAGGTCGACAACGGTGTGTTTGG-3' (artificially introduced *Sal*I site is underlined). The DNA fragment was digested and then ligated with the vector plasmid pECt digested with *Nco*I and *Sal*I. The constructed plasmid, pECt-*lldR*, was introduced into ATCC 31831, resulting in the *lldR*-overexpressing strain (WT/pECt-*lldR*) and also introduced into ATCC 31831 *lldR* disruptant (Δ *lldR*) (Gao et al., 2008), resulting in the *lldR*-complemented strain (Δ *lldR*/pECt-*lldR*).

Analysis of protein overexpression. Cells grown in Lennox medium for 24 h were harvested by centrifugation, then suspended in sodium phosphate buffer (50 mM, pH 7.0) and disrupted by sonication. Cell debris was removed by centrifugation. Protein extracts were then separated in 10% polyarylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was stained with Coomassie Brilliant Blue.

Measurement of cell growth, glucose consumption, glutamate production and lactate formation. Cell growth was monitored by measuring the optical density of the culture at 660 nm (OD_{660}) at 0, 6, 12 h and

every 24 h from 24 h to 120 h. For the main culture medium, samples were diluted with 0.1 M HCl to dissolve CaCO_3 prior to measurement. To measure glucose consumption and glutamate or lactate production, the main cultures were sampled every 24 h. The supernatants of cultures were separated from the cell pellets by centrifugation. Glucose, glutamate and lactate concentrations in the culture supernatant were measured using a Biotech-analyzer AS-210 (Sakura Seiki, Tokyo) with a glucose oxidase sensor, glutamate oxidase sensor or lactate oxidase sensor, respectively.

Total RNA isolation and quantitative real-time RT-PCR. Cells grown for 24 h in the main culture medium for glutamate production under biotin-limited conditions were used for total RNA isolation. CaCO_3 was removed by low speed centrifugation before use. Two volumes of RNeasy Protect Bacterial Reagent (Qiagen, Valencia, CA.) were added directly to the culture to stabilize cellular RNAs. Cells were harvested by centrifugation and total cellular RNA was isolated using an RNeasy Mini Kit (Qiagen). To eliminate residual genomic DNA, RNA preparations were treated with RNase-Free DNase I (Qiagen). Quality and concentration of RNA samples were determined by NanoDrop (Thermo Fisher Scientific). Quantitative real-time RT-PCR (qRT-PCR) assays were performed using the QuantiFast SYBR Green RT-PCR kit (Qiagen) and Eco™ Real-Time PCR Systems (Illumina, San Diego, CA). The quality and specificity of the amplification process were verified by melting curve analysis. The target gene transcripts were normalized to the reference gene transcript (16S rRNA) from the same RNA sample. Each gene was analyzed using RNA isolated from three independent samples. The cycle threshold for each sample was generated according to the procedures described in the Eco™ Real-Time PCR Systems user's guide. Primers used were 5'-GATTGGGTCACCGAGGAGCT-3' and 5'-AAATGGTGCCGAGGGCTTCGA-3' for *lldR*, 5'-GGTGAAACGTCAACTGCCCAAC-3' and 5'-CTGGGGTGCGTCGTTTAGCA-3' for *lldD*, 5'-GTCCTCATTGGCGCAGGAGATG-3' and 5'-TCTTCGCAGTCAGCGTAGGTGC-3' for *ldhA*, and 5'-AGAGTTTGATCCTGGCTCA-3', 5'-ACGTGTTACTACCCGTTTG-3', 5'-ACGTTCCCGGGCCTTGACA-3' and 5'-CGGCTACCTTGTTACGAC-3' for 16S rRNA.

Results

Construction of an *LldR* overproducer

To analyze the role of *LldR* in glutamate metabolism in *C. glutamicum*, we constructed an overexpressing strain of *LldR* in *C. glutamicum* as described in MATERIALS AND METHODS. The *lldR* gene was cloned on the *E. coli*-*C. glutamicum* shuttle vector pECt, which carries an IPTG-inducible *trc* promoter and the *lacI* repressor gene. The SD sequence of the *C. glutamicum* *pyc* gene was also introduced just upstream of the *lldR* gene for efficient translation. Overproduction of *LldR* was confirmed by SDS-PAGE. Synthesis of a protein of about 26 kDa, which corresponds to *LldR*, was induced by IPTG induction in wild-type and *lldR*-disruptant cells transformed with pECt-*lldR* (Fig. 2). Overexpression of the *lldR* gene under biotin-limited conditions was also confirmed by qRT-PCR assay as described later (see Fig. 5a).

Effects of overproduction and deletion of *LldR* on growth

Firstly, we examined effects of overexpression and deletion of *lldR* on growth in Lennox broth. Cell growth

Fig. 2. Confirmation of *LldR* overexpression.

Cellular protein expression profiles of WT and $\Delta lldR$ strains carrying the empty vector pECt or the *LldR*-overexpression plasmid pECt-*lldR* were analyzed on 10% SDS-PAGE as described in MATERIALS AND METHODS. A Coomassie Brilliant Blue-stained gel is shown. The signs “–” and “+” represent without and with 1 mM IPTG addition, respectively. Numbers shown on the left of the gel are molecular weights (kDa). An arrow indicates the overproduced *LldR* protein.

was monitored by measuring OD₆₆₀. There were no significant differences in growth rate between *C. glutamicum* strains WT, WT/pECt-*lldR* without and with 1 mM IPTG, and $\Delta lldR$ (Fig. 3). Overproduction and deletion of *lldR* also had no effects on the growth under biotin-limited conditions (Fig. 4a). These results indicate that overexpression and deletion of *lldR* have no effects on the growth.

Effects of overproduction and deletion of *LldR* on glutamate production induced by biotin limitation

To clarify the role of *LldR* in glutamate production under biotin-limited conditions, we examined the effect of overexpression and deletion of *lldR* on glutamate production induced by biotin limitation (Fig. 4, Table 1). After 120 h of cultivation, WT reached its maximal L-glutamate production at about 25 g/L, where glucose was almost depleted (Fig. 4b, c). By disrupting the *lldR* gene, glutamate production was slightly increased; this was observed reproducibly, although it was not significant statistically. Glucose consumption was also slightly increased. *lldR* disruptant reached its maximal L-glutamate production at about 26 g/L after 120 h cultivation, which was 3% increase compared to that of WT (Fig. 4c, Table 1). Overexpression of *lldR* showed a negative effect on L-glutamate production. Slightly decreased glucose consumption was also ob-

Fig. 3. Effects of overproduction and deletion of *LldR* on growth.

Growth of WT (open circle), $\Delta lldR$ (closed circle), WT/pECt-*lldR*-IPTG (open triangle) and WT/pECt-*lldR*+IPTG (closed triangle) in Lennox broth are shown. Overnight cultures of each strain were diluted (1 : 50) with fresh Lennox broth and incubated at 30°C with shaking. Growth was monitored by measuring OD₆₆₀. The data shown are representative of three independent experiments that yielded similar results.

served (Fig. 4b, c, Table 1). At 120 h of culture, the LldR overproducer with the addition of IPTG (WT/pECT-

Fig. 4. Effects of overproduction and deletion of LldR on glutamate production induced by biotin limitation.

Cell growth (a), glucose consumption (b), glutamate production (c), and lactate formation (d) are shown. Cell growth from 0 to 24 h is highlighted in (a). WT (open circle), Δ lldR (closed circle), WT/pECT-lldR-IPTG (open triangle) and WT/pECT-lldR+IPTG (closed triangle) were grown under biotin-limited conditions as described in MATERIALS AND METHODS. The mean values of three independent experiments are presented with standard deviations. For growth experiment, the data shown are representative of three independent experiments that yielded similar results.

lldR+IPTG) reached its maximal L-glutamate production at about 21 g/L, which was decreased 16% compared to that of WT (Fig. 4c, Table 1). The yield of glutamate production was also decreased (Table 1). The LldR overproducer without IPTG (WT/pECT-lldR-IPTG) also showed slightly decreased glutamate production at about 23 g/L, which was decreased 8% compared to that of WT, although it was not significant statistically (Fig. 4c, Table 1).

L-Lactate was simultaneously formed during glutamate production under biotin-limited conditions (Fig. 4d). In the wild-type cells, L-lactate was produced in the first 24 h and it was re-consumed thereafter. On the other hand, in the LldR overproducer with the addition of IPTG (WT/pECT-lldR+IPTG), L-lactate was produced like the wild type for the first 24 h and it further increased for the next 24 h. The produced lactate was, however, not re-consumed afterward. L-Lactate reached its maximum at about 7 g/L after 120 h cultivation (Fig. 4d). WT/pECT-lldR-IPTG showed a delay in re-consumption of L-lactate compared to WT (Fig. 4d). These indicate that overproduction of the LldR suppresses the lactate utilization. *lldR* disruptant formed less L-lactate than WT, although it was not significant statistically (Fig 4d, Table 1).

Effects of overproduction and deletion of LldR on gene expression

To confirm that LldR controls the gene expression in lactate metabolism under biotin-limited conditions, we performed the qRT-PCR assays to compare the gene expression involved in lactate metabolism, *lldD* responsible for oxidation of L-lactate to pyruvate and *ldhA* responsible for the formation of L-lactate from py-

Table 1. Effects of overexpression or deletion of *lldR* on lactate and glutamate production under biotin-limited conditions.

Strains	Glucose consumption (g/L)	Lactate production (g/L)	Glutamate production (g/L)	Production yield ^a (%)
WT	71.0 ± 2.5	1.5 ± 1.3	25.3 ± 1.9	43.6 ± 1.9
Δ lldR	74.6 ± 0.4	0.7 ± 0.7	26.0 ± 2.0	42.7 ± 3.5
WT/pECT-lldR-IPTG	71.1 ± 4.9	2.2 ± 1.6	23.2 ± 0.6	40.0 ± 2.4
WT/pECT-lldR+IPTG	66.4 ± 0.4 ^c	7.5 ± 1.0 ^b	21.3 ± 1.3 ^c	39.2 ± 2.6

Glucose consumption, lactate production and glutamate production under biotin-limited conditions at 120 h are shown. Data represent means ± SDs for three independent experiments.

^a Production yield was calculated as;

$$\frac{\text{Glutamate production (g/L)} / 147}{\text{Glucose consumption (g/L)} / 180} \times 100.$$

^{b, c} Student's *t*-test (versus WT): ^b $p < 0.01$ and ^c $0.01 \leq p < 0.05$.

ruvate. qRT-PCR assay confirmed overexpression and deletion of *lldR*: levels of the *lldR* mRNA were about 5-fold and 16-fold higher in the WT/pECt-*lldR* cells without and with IPTG, respectively, compared to that of WT, while it was negligible in the Δ *lldR* cells (Fig. 5a). Overexpression of *lldR* with the addition of IPTG caused a significant decrease in *lldD* expression, which was about a 2.4-fold decrease compared to WT under biotin-limited conditions (Fig. 5b). Deletion of *lldR* caused a significant increase in *lldD* expression up to 3.2-fold compared to WT (Fig. 5b). On the other hand, both overexpression and deletion of *lldR* had no significant effect on *ldhA* expression (Fig. 5b). These results suggest that LldR mainly controls the expres-

sion of the *lldD* gene but not of the *ldhA* gene, at least, under biotin-limited conditions.

Discussion

In this study, we investigated the role of LldR in the glutamate production under biotin-limited conditions by overexpression and disruption of the *lldR* gene. The overexpression and deletion of *lldR* had no effect on the growth in Lennox medium (Fig. 3) nor under biotin-limited conditions (Fig. 4a). It was also reported that overexpression and deletion of *lldR* have no significant effects on growth rate or biomass formation compared to WT in minimal medium containing glucose as a sole carbon source (Georgi et al., 2008). In the *lldR* disruptant strain, glutamate production was slightly increased reproducibly, although it was not significant statistically. *lldR* disruptant produced 3% more glutamate compared to WT (Fig. 4c, Table 1). *lldR* disruptant formed less L-lactate than WT (Fig. 4d, Table 1), probably because L-lactate re-utilization is more efficient without the repression of the *lldD* gene by LldR. On the other hand, the LldR overproducer without or with IPTG produced less L-glutamate, an 8% and 16% decrease respectively, compared to WT (Fig. 4c, Table 1). WT produced L-lactate in the first 24 h, and it was re-consumed thereafter. On the other hand, in the LldR overproducer with IPTG (WT/pECt-*lldR*+IPTG), L-lactate production increased for 48 h but it was not re-consumed (Fig. 4d). The increase of L-lactate production was roughly comparable to the decrease of glutamate production (Fig. 4c, d, Table 1). The LldR overproducer without IPTG (WT/pECt-*lldR*-IPTG) showed a delay in re-consumption of L-lactate compared to WT (Fig. 4d). These results suggest that LldR controls L-lactate utilization by regulating *lldD* expression under biotin-limited conditions and that L-lactate formed during glutamate production is re-consumed for glutamate production.

qRT-PCR revealed that overexpression of *lldR* with the addition of IPTG decreased *lldD* expression significantly, which was about a 2.4-fold decrease under biotin-limited conditions compared to WT (Fig. 5). On the other hand, deletion of *lldR* resulted in an increase in *lldD* expression up to 3.2-fold compared to WT (Fig. 5). However, overexpression and deletion of *lldR* had no significant effects on *ldhA* expression compared to WT under biotin-limited conditions (Fig. 5). The previous studies suggest that LldR represses the

Fig. 5. Effects of overproduction and deletion of LldR on gene expression.

(a) Relative expression levels of *lldR*, and (b) relative expression levels of *lldD* (closed bar) and *ldhA* (open bar) of WT, WT/pECt-*lldR*-IPTG, WT/pECt-*lldR*+IPTG and Δ *lldR* under biotin-limited conditions are shown. Total RNAs were prepared and relative expression levels of *lldR*, *lldD* (L-lactate dehydrogenase responsible for oxidation of L-lactate to pyruvate) and *ldhA* (L-lactate dehydrogenase catalyzing the formation of L-lactate from pyruvate) were determined by qRT-PCR as described in MATERIALS AND METHODS. The mean values of three independent experiments are presented with standard deviations.

ldhA gene depending on the culture conditions, i.e., sugar and L-lactate availability. Gao et al. (2008) used a culture medium containing a trace amount of glucose. In this case, SugR is a major repressor of *ldhA* in the absence of sugar. Moreover, the absence of sugar results in low lactate; therefore LldR also represses *ldhA*. Georgi et al. (2008) and Toyoda et al. (2009a, b) used culture media containing higher amounts of glucose. In this case, lactate is formed in higher levels and therefore repression by LldR is relieved. Under these conditions, deletion of *lldR* did not cause a change in expression levels of *ldhA*. Our result is consistent with those of Georgi et al. (2008) and Toyoda et al. (2009a, b). It was reported that repression by LldR is relieved in the presence of L-lactate at the extent of 40 mM (3.6 g/L) (Georgi et al., 2008), which is comparable to the levels in our experimental conditions (Fig. 4d). However, LldR was extensively overproduced in our experiments, i.e. about 16-fold higher compared to WT (Fig. 5a), and therefore it seems that higher lactate levels were required for complete derepression of *lldD* by overproduced LldR. Then, why was not the expression of *ldhA* affected by the overproduction of LldR? In the presence of glucose, repression by SugR is relieved only partially, because *ldhA* promoter activity becomes higher when grown on sucrose or fructose, which generates fructose-1-phosphate, a negative effector of SugR (Toyoda et al., 2009b). It is possible that partial repression by SugR affects the binding of LldR to the *ldhA* promoter. Cooperative binding of these two regulators was suggested although they were capable of binding to the *ldhA* promoter region simultaneously (Toyoda et al., 2009a). It is also possible that affinity of the LldR repressor to the *ldhA* promoter is lower than that for the *lldD* promoter. This point should be investigated further.

It is well known that pyruvate carboxylase (PC) is a biotin-containing enzyme which converts pyruvate to oxaloacetate (Fig. 1). Therefore, biotin limitation causes the decreased intracellular level of biotin-bound PC and results in the intracellular accumulation of pyruvate. Under these conditions, glutamate production solely depends on the anaplerotic reaction catalyzed by phosphoenolpyruvate carboxylase (PEPC), which converts phosphoenolpyruvate to oxaloacetate (Fig. 1) (Sato et al., 2008). Enzymatic activities of pyruvate kinase (PK) and pyruvate dehydrogenase (PDH) decrease during glutamate production under biotin-limited conditions. Moreover, the enzymatic activity of

PEPC and carbon flux from phosphoenolpyruvate to oxaloacetate increase under biotin-limited conditions, while the enzymatic activity of PC significantly decreases (Hasegawa et al., 2008). Disruption of *pyc*, which encodes PC, showed increased L-lactate production in addition to glutamate production under biotin-limited conditions. The increased glutamate production suggested that the produced lactate was reused by the cells to produce glutamate (Sato et al., 2008).

Taken altogether, we concluded that LldR mainly controls the expression of *lldD* but not of *ldhA*, at least, under biotin-limited conditions. The produced lactate is reused by the cells to produce glutamate. L-Lactate metabolism, which is controlled by LldR, may have a buffering function of the pyruvate pool for glutamate production under biotin-limited conditions. Glutamate production assay with overproducer/deletion strains of *ldhA* and *lldD* as well as flux analysis with ^{13}C -labeled substrates will be necessary to confirm this conclusion. Disruption of *lldR* with the manipulation of genes involved in the flux toward glutamate production might give a new approach to improve glutamate production under biotin-limited conditions.

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