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...
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 (Borngen et al., 2010; Hashimoto et al., 2010; Nakamura et al., 2007; Wachi, 2013). 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 *∆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 *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 ∆(lac-proAB)F’ (traD36 proAB lacF lacZ.M15)] and JM110 [dam dcm supE44 hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr tsx ∆(lac-proAB)F’ (traD36 proAB lacF lacZ.M15)] were used for plasmid construction. *C. glutamicum* and *E. coli* cells were cultured in...
selected L-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′-CGCCATGGGCGAGCTCGAAAGGAATTACCTAATGAGTGAAGACATGGA-3′ (artificially introduced *Nco*I site is underlined) and 5′-TGAGGTGGAAACGTCAACTGCCCA-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 (ΔlldR) (Gao et al., 2008), resulting in the lldR-complemented strain (ΔlldR/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% polyacrylamide gel by 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 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′-GATTGGA TACCCAGAGAGCT-3′ and 5′-AACTGGTGCCGAGGG CCTCGA-3′ for lldR, 5′-GGTGAACGTCAACTGCCCA AC-3′ and 5′-CTGGGTTGCCTGTTTAGCA-3′ for lldD, 5′-GTTCCTCATTGGCAGAGATTG-3′ and 5′-TCTT GCAGTGCAGCTGTC-3′ for lidhA, and 5′-AGAGTTT GATCCTGAGCTCA-3′, 5′-ACGTGTTACCCCGTTC G-3′, 5′-ACGTTCGCGGGCCTTGAACACA-3′ and 5′-CGGC TACCTGTTACGAC-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 was monitored by measuring OD_{660}. There were no significant differences in growth rate between C. glutamicum strains WT, WT/pECt-lldR without and with 1 mM IPTG, and ΔlldR (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-
served (Fig. 4b, c, Table 1). At 120 h of culture, the LldR overproducer with the addition of IPTG (WT/pECT-

\textit{lldR}+IPTG) reached its maximal \textit{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-\textit{lldR} \textit{−} 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).

\textit{l}-Lactate was simultaneously formed during glutamate production under biotin-limited conditions (Fig. 4d). In the wild-type cells, \textit{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-\textit{lldR}+IPTG), \textit{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. \textit{l}-Lactate reached its maximum at about 7 g/L after 120 h cultivation (Fig. 4d). WT/pECT-\textit{lldR} \textit{−} IPTG showed a delay in re-consumption of \textit{l}-lactate compared to WT (Fig. 4d). These indicate that overproduction of the LldR suppresses the lactate utilization. \textit{lldR} disruptant formed less \textit{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, \textit{lldD} responsible for oxidation of \textit{l}-lactate to pyruvate and \textit{ldhA} responsible for the formation of \textit{l}-lactate from py-

<table>
<thead>
<tr>
<th>Strains</th>
<th>Glucose consumption (g/L)</th>
<th>Lactate production (g/L)</th>
<th>Glutamate production (g/L)</th>
<th>Production yield a (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>71.0 ± 2.5</td>
<td>1.5 ± 1.3</td>
<td>25.3 ± 1.9</td>
<td>43.6 ± 1.9</td>
</tr>
<tr>
<td>\textit{lldR}</td>
<td>74.6 ± 0.4</td>
<td>0.7 ± 0.7</td>
<td>26.0 ± 2.0</td>
<td>42.7 ± 3.5</td>
</tr>
<tr>
<td>WT/pECT-\textit{lldR}−IPTG</td>
<td>71.1 ± 4.9</td>
<td>2.2 ± 1.6</td>
<td>23.2 ± 0.6</td>
<td>40.0 ± 2.4</td>
</tr>
<tr>
<td>WT/pECT-\textit{lldR}+IPTG</td>
<td>66.4 ± 0.4 c</td>
<td>7.5 ± 1.0 b</td>
<td>21.3 ± 1.3 c</td>
<td>39.2 ± 2.6</td>
</tr>
</tbody>
</table>

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

\textsuperscript{a} Production yield was calculated as;

\begin{align*}
\text{Glutamate production (g/L)} / 147 & \times 100. \\
\text{Glucose consumption (g/L)} / 180 &
\end{align*}

\textsuperscript{b, c} Student’s t-test (versus WT): \textsuperscript{b} \( p < 0.01 \) and \textsuperscript{c} \( 0.01 \leq p < 0.05 \).
ruvate. qRT-PCR assay confirmed overexpression and deletion of IlldR: levels of the IlldR mRNA were about 5-fold and 16-fold higher in the WT/pECT-IlldR cells without and with IPTG, respectively, compared to that of WT, while it was negligible in the ∆IlldR cells (Fig. 5a). Overexpression of IlldR with the addition of IPTG caused a significant decrease in IlldD expression, which was about a 2.4-fold decrease compared to WT under biotin-limited conditions (Fig. 5b). Deletion of IlldR caused a significant increase in IlldD expression up to 3.2-fold compared to WT (Fig. 5b). On the other hand, both overexpression and deletion of IlldR had no significant effect on IldhA expression (Fig. 5b). These results suggest that LldR mainly controls the expression of the IlldD gene but not of the IldhA 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 IlldR gene. The overexpression and deletion of IlldR 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 IlldR 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 IlldR disruptant strain, glutamate production was slightly increased reproducibly, although it was not significant statistically. IlldR disruptant produced 3% more glutamate compared to WT (Fig. 4c, Table 1). IlldR 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 IlldD 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. 4d, 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-IlldR—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-IlldR—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 IlldD 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 IlldR with the addition of IPTG decreased IlldD 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 IlldR resulted in an increase in IlldD expression up to 3.2-fold compared to WT (Fig. 5). However, overexpression and deletion of IlldR had no significant effects on IldhA expression compared to WT under biotin-limited conditions (Fig. 5). The previous studies suggest that LldR represses the
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 lower 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 13C-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.

Acknowledgments

This work was supported in part by a grant-in-aid for Scientific Research (B) (20380047 to M. W.) from the Japan Society for Promotion of Science, a grant from the Ministry of Economy, Trade and Industry of Japan entrusted by the New Energy and Industrial Technology Development Organization, and the supporting program of the Skylark Food Science Institute.

References


