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Expression of the *alaE* gene is positively regulated by the global regulator Lrp in response to intracellular accumulation of L-alanine in *Escherichia coli*

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The *alaE* gene in *Escherichia coli* encodes an L-alanine exporter that catalyzes the active export of L-alanine using proton electrochemical potential. In our previous study, *alaE* expression was shown to increase in the presence of L-alanyl-L-alanine (Ala-Ala). In this study, the global regulator leucine-responsive regulatory protein (Lrp) was identified as an activator of the *alaE* gene. A promoter less β-galactosidase gene was fused to an *alaE* upstream region (240 nucleotides). Cells that were *lacZ*-deficient and harbored this reporter plasmid showed significant induction of β-galactosidase activity (approximately 17-fold) in the presence of 6 mM L-alanine, L-leucine, and Ala-Ala. However, a reporter plasmid possessing a smaller *alaE* upstream region (180 nucleotides) yielded transformants with strikingly low enzyme activity under the same conditions. In contrast, *lrp*-deficient cells showed almost no β-galactosidase induction, indicating that Lrp positively regulates *alaE* expression. We next performed an electrophoretic mobility shift assay (EMSA) and a DNase I footprinting assay using purified hexahistidine-tagged Lrp (Lrp-His). Consequently, we found that Lrp-His binds to the *alaE* upstream region spanning nucleotide −161 to −83 with a physiologically relevant affinity (apparent K_D, 288.7 ± 83.8 nM). Furthermore, the binding affinity of Lrp-His toward its *cis*-element was increased by L-alanine and L-leucine, but not by Ala-Ala and D-alanine. Based on these results, we concluded that the gene expression of the *alaE* is regulated by Lrp in response to intracellular levels of L-alanine, which eventually leads to intracellular homeostasis of L-alanine concentrations.

[**Key words:** *Escherichia coli*; L-Alanine; Exporter; AlaE; Lrp]

Though it has been known for some time that microorganisms are able to produce extracellular L-glutamic acid (1), the systems that secreted amino acids was not discovered until the identification of the L-lysine exporter, LysE, of *Corynebacterium glutamicum* (2). Since then, a dozen amino acid exporters have been identified in bacteria, which export L-isoleucine, L-glutamic acid, L-threonine, L-cysteine, L-aromatic amino acids, L-leucine, L-arginine, L-valine, and L-homoserine (3–13). The existence of these amino acid exporters in bacteria raises the question of why bacteria possess exporters for various amino acids that are primary metabolites. Their physiological roles are assumed to be (i) a safety valve that prevents abrupt increases in intracellular amino acids to toxic levels and (ii) a route to export intracellular signaling molecules, such as homoserine lactone (14). However, experimental evidence to validate these hypotheses is still lacking.

Recently, we identified the *alaE* (formerly *ygaW*) gene encoding the major L-alanine exporter in *Escherichia coli* (15) and found that an *alaE*-deficient mutant appeared to be hypersusceptible to an L-alanine-containing dipeptide, L-alanyl-L-alanine (Ala-Ala). Moreover, in the presence of 6 mM of Ala-Ala, an *alaE*-deficient mutant, derived from an L-alanine nonmetabolizing strain with deficiencies in three L-alanine synthesizing enzymes and two alanine racemases, accumulated over 160 mM L-alanine, whereas a transformant overexpressing the *alaE* gene accumulated approximately 40 mM L-alanine under the same conditions (15). These results suggested that AlaE exports intracellular L-alanine, which eventually protects the cells from toxic accumulation of L-alanine and its derivatives. Kim et al. (16) found that inverted membrane vesicles prepared from AlaE-overproducing cells accumulated L-alanine in an energy-dependent manner, and this transport activity was inhibited by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), indicating that AlaE exports L-alanine using proton electrochemical potential. Other amino acid exporters such as LeuE (7), ThrE (17), and RhtA (8) in *E. coli* were also reported to export their substrates using proton motive force. In contrast, CydDC in *E. coli* exports its substrate at the expense of ATP (11).

The substrates of exporters like AlaE are primary metabolites for protein biosynthesis. In regards to L-alanine, its metabolite D-alanine is an essential component of peptidoglycan. Therefore, from a biological viewpoint, the unintended export of amino acids out of the cells using metabolic energy could be futile. To avoid energy loss, the expression of amino acids exporters needs to be tightly regulated. Indeed, expression of several amino acid exporters, such as *leuE* (7), *argO* (18), *rhtB*, *rhtC* (19), and *ygaZH* (10) in *E. coli* is known to be regulated by the global regulator leucine-responsive regulatory protein (Lrp). In addition, *lysE* in *C. glutamicum* and

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argO in *E. coli* were reported to be regulated by their specific regulators *lysG* (20) and *argP* (18), respectively.

In our earlier study, Hori et al. (15) demonstrated that the expression of the *alaE* gene was induced under conditions where the intracellular L-alanine level increased, i.e., in the presence of Ala-Ala. In this study, we addressed how L-alanine regulates the gene expression of *alaE*.

MATERIALS AND METHODS

Bacterial strains and plasmid The *E. coli* strains and plasmids used in this study are listed in Supplementary Table S1 and the primers used in Supplementary Table S2. Cells were grown in Luria–Bertani (LB) broth containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl (pH 7.2), or minimal medium containing 22 mM glucose, 7.5 mM $(NH_4)_2SO_4$, 1.7 mM $MgSO_4$, 7 mM K_2SO_4, 22 mM NaCl, and 100 mM sodium phosphate (pH 7.2). When necessary, ampicillin (AP, 100 μg/ml), kanamycin (KM, 12.5 μg/ml), and chloramphenicol (CP, 25 μg/ml) were added to the media.

Construction of the gene deletion mutants To construct the *lrp*-deletion *E. coli* mutant MG1655Δ*lrp*, we first amplified the *lrp* gene using primers, lrp-F and lrp-R, with MG1655 genomic DNA as a template and PrimeSTAR HS DNA polymerase (Takara, Japan). The amplified DNA fragment (1104 bp) was digested with BamHI and cloned into pSTV29 pretreated with BamHI and bacterial alkaline phosphatase (Toyobo, Japan). To remove an internal region of approximately 200 bp within the *lrp* gene of the resulting recombinant plasmid, we performed an inverse PCR reaction using primers (Δlrp-F and Δlrp-R) and PrimeSTAR HS DNA polymerase, digestion of the amplified DNA fragment with EcoRV, and relegation to yield pΔLrp. Subsequently, the BamHI fragment isolated from pΔLrp was cloned into the BamHI site of pTH18cs1 (21) to generate pTH18ΔLrp. The pTH18ΔLrp plasmid was then transformed into MG1655. The *lrp*-deficient mutant MG1655Δ*lrp* was selected as described by Hori et al. (15). The *lacZ*-deletion mutants, MG1655Δ*lacZ* and MG1655Δ*lrp*Δ*lacZ*, were constructed by the one-step inactivation method (22) using primers, lacZ-del-F and lacZ-del-R, and pKD13 as a template. The resulting DNA fragment was then transformed into MG1655 and MG1655Δ*lrp* harboring pKD46 followed by selection on LB-agar containing 100 μg/ml AP at 30°C. We then screened for KM-resistant and CP-sensitive clones at 37°C and verified deletion of the *lacZ* gene by PCR analysis.

Construction of the reporter plasmids for the β-galactosidase assay We amplified upstream regions of the *alaE* gene with various lengths using individual alaE-up primers (alaEup300-F, alaEup240-F, alaEup180-F, alaEup120-F, and alaEup60-F) and the reverse primer alaE-R with the MG1655 chromosome as a template. The resulting DNA fragments were digested with HindIII/XbaI and then subcloned into a promoter probe vector pCB192 (23), which had been treated with HindIII/XbaI, to generate reporter plasmids (Supplementary Table S1).

Total RNA isolation and reverse transcription-PCR analysis Total RNA was isolated as described by Hori et al. (15). Briefly, MG1655 was grown in minimal medium supplemented with 1% tryptone at 37°C until the optical density at 660 nm (OD_{660}) reached 0.7. Cells were harvested by centrifugation (13,700 ×*g*, 5 min, 4°C), washed twice with ice-cold minimal medium, and resuspended in minimal medium to give an OD_{660} of 3.0. After preincubation at 37°C for 10 min, amino acids or Ala-Ala were added to a final concentration of 6 mM and the cells were incubated at 37°C for an additional 5 min. Cells were harvested by centrifugation (13,700 ×*g*, 5 min, 4°C) and total RNA was isolated using NucleoSpin RNA II (MACHEREY-NAGEL, Germany). The RNA concentration was quantified spectrophotometrically by the absorbance at 260 nm (A_{260}). A reverse transcription-PCR (RT-PCR) reaction was performed using a PrimeScript RT reagent kit (Takara) with 1 μg of total RNA as a template in a 20 μl reaction mixture.

β-Galactosidase assay To measure promoter activity, we performed β-galactosidase assays with MG1655Δ*lacZ* and MG1655Δ*lrp*Δ*lacZ* harboring each promoter probe vector. Cells were grown in minimal medium supplemented with 1% tryptone, 100 μg/ml AP, and 12.5 μg/ml KM at 37°C overnight. The fully-grown cells were harvested by centrifugation (13,700 ×*g*, 5 min, RT), washed twice with 0.85% (w/v) NaCl and resuspended in the original volume of the 0.85% (w/v) NaCl. The cells were then inoculated into a minimal medium (2% v/v) and incubated at 37°C for 1 h. L-Alanine, L-leucine, D-alanine or Ala-Ala were then added to a final concentration of 6 mM and incubation was continued for further 3 h at 37°C. Cells were harvested by centrifugation (13,700 ×*g*, 5 min, 4°C) and β-galactosidase activity was determined as described by Miller (24).

Purification of histidine-tagged Lrp Purification of Lrp-His was performed as described by Matthews et al. (25) with some modifications. To generate hexahistidine-tagged Lrp (Lrp-His), we amplified the *lrp* gene using primers, pQELrp-F and pQELrp-R, and the MG1655 genomic DNA as a template with PrimeSTAR HS DNA polymerase. The resulting DNA fragment was digested with BamHI and HindIII and cloned into pQE30 (Qiagen) pretreated with BamHI and HindIII to yield pQE-Lrp. pQE-Lrp was then transformed into *E. coli* JM109 cells. *E. coli* JM109/pQE-Lrp was grown in LB broth at 37°C containing 100 μg/ml AP until the OD_{660} reached 0.6. Isopropyl-β-D-galactopyranoside (IPTG) was added to the cells to a final concentration of 0.5 mM. After a 4 h incubation at 37°C, cells were harvested by centrifugation (6800 ×*g*, 10 min, 4°C) and resuspended in a solution containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. The resulting cell suspension was sonicated with a Bioruptor UCD-250 (Cosmo Bio Co., Japan) and centrifuged at 15,000 ×*g*, for 20 min at 4°C to remove intact cells and debris. The clear supernatant was mixed with Ni-NTA agarose (Qiagen, Japan) and incubated at 4°C for 16 h. The resin was centrifuged (1000 ×*g*, 1 min, 4°C) and washed three times with a buffer containing 50 mM sodium phosphate (pH 6.0), 300 mM NaCl, 100 mM imidazole, 10% (v/v) glycerol. Lrp-His was then eluted with a solution containing 50 mM sodium phosphate (pH 6.0), 300 mM NaCl, 10% (v/v) glycerol and imidazole (150, 200, 250, 300, 350, 400 or 450 mM) in a step-wise manner. Fractions containing Lrp-His were combined and dialyzed at 4°C against an excess volume of solution containing 10 mM Tris–HCl (pH 8.0), 50% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol and 100 mM NaCl. Purified Lrp-His was subjected to SDS-PAGE, and the protein concentration was analyzed by ImageJ software by comparing the signal intensity of Lrp-His with those of bovine serum albumin standards. Purified Lrp-His was stored at −20°C until use.

Electrophoretic mobility shift assay A DNA fragment containing the upstream region of the *alaE* gene, that encompassing from −300 to +50 bp from the initiation codon, was amplified using primers, alaE-300 6-FAM and alaE+50 6-FAM, which had 6-carboxyfluorescein (6-FAM) labels at their 5′ ends. Binding reaction between the labeled DNA fragment (final concentration of 4.6 nM) and various amounts of purified Lrp-His (final concentrations of 11.9 nM to 3.034 μM) was performed in a solution containing 20 mM Tris–HCl (pH 8.0), 12.5% (v/v) glycerol, 0.3 mM EDTA, 0.1 mM dithiothreitol, 50 mM NaCl, 5 mM $MgCl_2$, and 50 μg/ml salmon sperm DNA. After incubation at 37°C for 30 min, DNA fragments were resolved by electrophoresis on a polyacrylamide gel (5% w/v). Gels were then scanned with a Molecular Imager Fx (Bio Rad, USA), and the amounts of free and Lrp-bound 6-FAM-labeled DNA were quantified by Gel Analyzer 2010a software (http://www.gelanalyzer.com/).

DNase I footprinting assay A DNase I footprinting assay was performed as described by Michael Zianni et al. (26). Briefly, 6-FAM labeled-DNA (37.5 ng) and Lrp-His (515 or 257.5 ng) were mixed in a 200 μl of reaction solution containing 20 mM Tris–HCl (pH 8.0), 12.5% (v/v) glycerol, 0.3 mM EDTA, 0.1 mM dithiothreitol, 50 mM NaCl, and 5 mM $MgCl_2$, and preincubated at 37°C for 30 min. As a control, the labeled DNA was incubated with bovine serum albumin (final concentration of 0.1 mg/ml). After incubation at 37°C for 30 min, 2 μl of DNase I (0.2 mg/ml, 0.08–0.04 Kunitz unit/μl; Sigma, Japan) was added, and the resulting mixture was incubated at 25°C for 3 min. The reaction was stopped by mixing with 200 μl of phenol-chloroform-isoamyl alcohol (25:24:1), and the DNA fragments were recovered in the aqueous phase by centrifugation (15,000 ×*g*, 30 min, 4°C). After precipitation of the DNA with ethanol, the resulting pellets were dissolved in 9.9 μl of Hi-Di Formamide (Life Technologies, Japan) and mixed with 0.1 μl of GeneScan 600LIZ dye Size Standard (Life Technologies). DNA samples were then analyzed with a 3500xL Genetic Analyzer (Applied Biosystems, Japan). DNA patterns were analyzed with GeneMapper software version 4.0 (Life Technologies).

Primer extension assay Two micrograms of total RNA isolated as described above was used for RT-PCR reaction by using PrimeScript II 1st strand cDNA Synthesis Kit (Takara) and alaE+60 6-FAM primer. The products were purified by ethanol precipitation and the resulting pellets were dissolved in 9.9 μl of Hi-Di Formamide. After mixing with 0.1 μl of GeneScan 600LIZ dye Size Standard, samples were analyzed with the 3500xL Genetic Analyzer. DNA patterns were analyzed with GeneMapper software version 4.0.

RESULTS

L-Alanine induces the *alaE* gene expression We previously reported that Ala-Ala enhanced *alaE* expression and that a level of intracellular L-alanine was increased to approximately 120 mM in the presence of 1 or 6 mM Ala-Ala in the L-alanine non-metabolizing mutant (15,27), suggesting that induction of the *alaE* gene could be responsive to intracellular accumulation of L-alanine derived from the dipeptide (15). We thus determined the *alaE* expression in *E. coli* MG1655 in the presence of 6 mM L-alanine by the RT-PCR analysis. The *alaE* gene expression was significantly induced in the presence of 6 mM L-alanine (Fig. 1, lane 3). Ala-Ala also induced *alaE* expression as reported previously (15). This result clearly indicates that an intracellular accumulation of L-alanine induces *alaE* gene expression.

***alaE* expression depends on its upstream region** To elucidate the mechanism by which the *alaE* gene expression is regulated, we employed β-galactosidase assays using reporter plasmids

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