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**Identification of a methanol-inducible promoter from *Rhodococcus erythropolis*
PR4 and its use as an expression vector**

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Running title : Methanol-inducible promoter from *Rhodococcus*

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erythropolis

25 **Abstract**

26

27 The genus *Rhodococcus* exhibits a broad range of catalytic activity and is tolerant to
28 various kinds of organic solvents. This property makes rhodococci suitable for use as a
29 whole-cell catalyst. Various tools for genetic engineering have been developed to use
30 *Rhodococcus erythropolis* as a host for bioconversion. In this study, we investigated the
31 protein expression responses of *R. erythropolis* strains and found that isocitrate lyase
32 production in *R. erythropolis* PR4 (ICL_{Re}) was induced by methanol. By analyzing the
33 regulation mechanisms of *icl_{Re}* expression, the ~200-bp upstream region from the first
34 nucleotide of the translation initiation codon of *icl_{Re}* was shown to be sufficient for the
35 methanol-inducible expression. Also, the ~100-bp upstream region exhibited strong
36 constitutive promoter activity by an unknown mechanism(s). By investigating proteins
37 that bound to the upstream region of *icl_{Re}* *in vitro*, a RamB homologue of *R.*
38 *erythropolis* PR4 (RamB_{Re}) was identified. Moreover, 2 putative RamB_{Re} binding sites
39 were identified in the upstream region of *icl_{Re}* through pull-down assays. A *ramB_{Re}*
40 knockout experiment suggested that RamB_{Re} negatively controlled the expression of
41 *icl_{Re}* and that RamB_{Re} regulation was dependent on the availability of a carbon source.
42 On the basis of these findings, we were able to create novel methanol-inducible and
43 strong constitutive expression vectors.

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49 **Introduction**

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51 The genus *Rhodococcus* belongs to mycolic-acid containing actinomycetes. Many
52 members of the order *Actinomycetes* are known to be antibiotic producers, while
53 rhodococci are known to exhibit a broad range of catalytic activity that has applications
54 in industrial, pharmaceutical, and environmental biotechnology (1-4). For example,
55 rhodococci have been used as biocatalysts for the industrial production of acrylamide
56 (5). Rhodococci are often isolated from soils contaminated with crude oil and/or
57 xenobiotic compounds, and these organisms can utilize such compounds as carbon
58 sources. Moreover, they exhibit tolerance to various organic solvents (6-9).

59 Their solvent tolerance property makes rhodococci suitable for use as a whole-cell
60 biocatalyst; this is particularly favorable for biocatalytic processes that require cofactor
61 regeneration and/or involve multistep metabolic pathways (10). The 2-phase
62 water-solvent system has been used for whole-cell bioconversion because it permits the
63 efficient conversion of hydrophobic substrates, which are dissolved in the solvent phase,
64 into final products. In most cases, the products are also hydrophobic and accumulate in
65 the solvent phase; this enables continuous reaction and feasible purification (10-13). To
66 date, both gram-positive and gram-negative organic solvent-tolerant bacteria have been
67 used for such reactions (10, 11, 14). Although *Escherichia coli* is not particularly
68 tolerant to solvents, it has been used for whole-cell bioconversion in the presence of
69 toxic solvents because of its ease of handling (15). Nevertheless, rhodococci remain
70 superior candidates for such applications owing to their diverse catabolic abilities and
71 remarkable resistance to various solvents. Solvent-tolerant mechanisms have been
72 investigated in various bacteria, particularly in gram-negative bacteria (16, 17).

Gram-negative bacteria have an outer cell membrane in addition to an inner cell membrane, and these 2 membranes create a periplasmic space between them. Such cell wall structure protects the cell from organic solvents that can damage the cell membrane by attacking the lipid bilayer (18).

To use *Rhodococcus erythropolis* as a host cell for bioproduction, various tools useful for genetic engineering have been developed, such as expression vectors containing an inducible promoter or a constitutive promoter, transposon mutagenesis and/or expression system, and gene expression analysis systems (19-25). To date, such tools have been successfully used in recombinant protein production and bioreactions in aqueous media (26, 27). To fully utilize the abilities of rhodococci, particularly their solvent tolerance property, a solvent-inducible promoter would be useful. If such a promoter is available, organic solvents can be used not only as reservoirs for the substrate and/or the product but also as inducers for proteins that are involved in the catalytic reactions. Therefore, we investigated the responses of various *R. erythropolis* strains to various kinds of organic solvents.

In this report, we identified isocitrate lyase from *R. erythropolis* PR4 (ICL_{Re}) as a methanol-inducible protein, and investigated its expression regulation mechanisms by analyzing the upstream region of icl_{Re} . Also, we identified a *RamB* homologue of *R. erythropolis* PR4 ($RamB_{Re}$) by isolating proteins that were bound to the upstream region of icl_{Re} . Finally, the upstream region of icl_{Re} was used to create methanol-inducible and strong constitutive expression vectors.

Materials and Methods

Bacterial strains and culture conditions

R. erythropolis strains PR4 and NI86/21 were obtained from the National Institute of Technology and Evaluation (NITE, Japan) and the National Collection of Agricultural and Industrial Microorganisms (Hungary), respectively. *R. erythropolis* strains JCM3201 and JCM6824 were obtained from the Japan Collection of Microorganisms. *E. coli* DH5 α and XL1-blue were used as general cloning hosts. *R. erythropolis* and *E. coli* strains were routinely cultured in Luria-Bertani (LB) broth (1% Bacto tryptone, 0.5% Bacto yeast extract, and 1% NaCl) in the presence or absence of antibiotics at 28°C and 37°C, respectively. The antibiotics used to select transformants in the culture media were chloramphenicol (17 μ g/mL), kanamycin (200 μ g/mL), and ampicillin (50 μ g/mL). If necessary, cell growth was monitored by measuring optical density at 600 nm (OD₆₀₀). In the plate culture, the medium was solidified by adding 1.5% agar.

Preparation of total protein

Single colony of *R. erythropolis* was inoculated into 10 mL LB medium and incubated until it reached to a stationary phase. Then, the seed culture was inoculated into 100 mL fresh LB medium and incubated for 20 h at 28°C with shaking at 120 rpm in a 300 mL baffled flask. In order to increase the amount of cell culture, 100 mL

culture that was obtained by the previous step was inoculated into 1 L fresh LB medium and incubated for 20 h at 28°C with shaking at 120 rpm in a 3 L baffled flask. A 20 mL portion that was obtained from the culture in 3 L flask was transferred into a 50 mL screw capped plastic tube (Corning inc., Corning, NY). Fifty microliters of *R. erythropolis* seed culture was inoculated into 15 mL of LB medium and incubated for 2 days. Then, the organic solvent was added into the culture to a proportion of 1% or 5%, and the culture was incubated for an additional 24 h. Cells were grown in a screw-capped 50 mL plastic tube to prevent evaporation of organic solvents. The organic solvents used in this study were ethanol, acetone, dimethyl sulfoxide, propanol, butanol, methanol, acetonitrile, isopropyl acetate, 2-methyl-1-propanol, methyl ethyl ketone, and ethyl acetate. The culture was harvested by centrifugation at $1500 \times g$ for 10 min at 4°C. Then, cells were washed with 50 mM phosphate buffer (pH 7.5) and resuspended in 500 µL of the same buffer. The cells were then disrupted using a Beads shocker (Yasui-kikai, Osaka, Japan). The supernatant was collected by centrifugation at $20,817 \times g$ for 10 min at 4°C and used as the total protein. Protein analysis was performed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Unless otherwise specified, 12.5% acrylamide was used for SDS-PAGE and protein bands were visualized using Coomassie brilliant blue (CBB) staining.

Two-dimensional gel electrophoresis (2-DE)

Three milligrams of total protein was loaded onto a Superose-12 column (GE Healthcare, Buckinghamshire, UK) equilibrated with 25 mM Tris-HCl (pH 7.5)

containing 10% glycerol. Then, a 50 μ L fraction with a high amount of methanol-inducible protein was resuspended in 75 μ L of rehydration buffer containing 8 M urea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 0.5% immobilized pH gradient (IPG) buffer, 0.002% bromophenol blue, 0.28% dithiothreitol, and applied to pH 3–11 IPG strips (GE Healthcare) for overnight rehydration. Isoelectric focusing was performed using Ettan IPGphor II (GE Healthcare) under the following conditions: 500 V for 1 h, 1000 V for 1 h, 8000 V for 3 h. IPG strips were equilibrated with SDS equilibration buffer [6 M urea, 75 mM Tris-HCl (pH 8.8), 29.3% glycerol, 2% SDS, 0.002% bromophenol blue]. The second-dimensional separation was carried out by placing equilibrated IPG strips onto a SDS-PAGE gel that was sealed with 0.5% (w/v) agarose.

Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS)

After 2-DE, the spot was excised and digested as described previously (28, 29). Tryptic peptides were separated by reverse-phase chromatography using a MAGIC C18 column (3 μ m, 200 Å, 0.2 mm i.d. $\times$ 150 mm; Michrom Bioresources, Auburn, CA) connected to a MAGIC 2002 LC system (Michrom Bioresources). Peptides were loaded onto a reverse-phase column as described previously (30) and then eluted during a 40-min linear gradient from 5% to 40% of solvent B (90% acetonitrile, 0.1% formic acid) at a flow rate of 100 μ L/min. The effluent was split by a MAGIC splitter (Michrom Bioresources) to approximately 0.9 μ L/min and introduced directly into an LCQ-DECA XP plus ion trap mass spectrometer (Thermo Fisher Scientific, Waltham,

MA) equipped with a nanoelectrospray ion source (AMR, Tokyo, Japan). All tandem MS spectra were searched against the NCBI non-redundant database and analyzed using the TurboSequest algorithm in the BioWorks 3.2 software package (ThermoFisher Scientific, Waltham, MA) as described previously (30). The identified peptides were further evaluated using the following filters: peptide probability ($\leq 1e-003$), delta correlation score (≥ 0.1), and cross-correlation (X Corr) values ($\geq 1.9, 2.2, 3.75$ for 1+, 2+, 3+ charge-state peptides, respectively).

Cloning of the genes identified with LC-MS/MS

The degenerate primers DGP_S (5'-GAGGCKGCKACKCTKATCACTCKGACGCKGACGA-3') and DGP_AS (5'-CGGATGTGTTGTTGKGTKGGGATKAGKAC-3') were designed corresponding to conserved regions of amino acid sequences of ICL. Degenerate PCR was performed for 25 cycles consisting of 94°C for 30 s, 68°C for 30 s, and 74°C for 2.5 min, with a final extension at 74°C for 7 min by using KOD Dash DNA polymerase (Toyobo, Osaka, Japan) and genome DNA as template. An ~150-bp product was purified and subcloned into a pBS-SK+ vector (Agilent Technologies, Santa Clara, CA) for sequencing. On the basis of the sequence obtained, primers were designed to amplify the full length of *icl_{Re}* by inverse PCR. For the inverse PCR, genomic DNA of *R. erythropolis* PR4 was digested with either *Bam*HI or *Nco*I, purified by a miniprep purification kit (Promega, Madison, WI), and circularized with Ligation kit solution I (Takara, Shiga, Japan). Inverse PCR was performed using KOD FX DNA polymerase

(Toyobo). The sequences of DNA fragments were analyzed by primer walking.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from *R. erythropolis* PR4 grown in the presence of 5% methanol by using RNeasy Midi Kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase I to remove genomic DNA followed by passing through an RNeasy minicolumn (Qiagen). RT-PCR reaction was performed using the Thermo Script RT-PCR System (Invitrogen, Carlsbad, CA) according to the manufacturer's manual. First strand synthesis was carried out using a random primer. PCR was performed using 5 pairs of primers corresponding to the fragments illustrated in Fig. 2B. For fragment a, icl_S (5'-AACCTCTCGGGCCACACGTA-3') and crt_AS (5'-GCTCCTTGAACCTCTTCGTACATGGAGT-3'); b, crt_S (5'-ACCAGCGAAAAAATTCAGCGCG-3') and mhm_AS1 (5'-GCGCGCCTTCCTTGAGTGCTTCGAGAA-3'); c, mhm_S (5'-GGCAAGCGAATCGATCTCTCTCGATCG-3') and adh_AS1 (5'-GCAGAAGTCGAGCATCTCCTGTGTCTG-3'); d, icl_S and mhm_AS2 (5'-AGGACTCGAGGGCGCGCTTGAGCTCA-3'); e, crt_S and adh_AS2 (5'-GTTGATGCCCTCGGGGATCG-3'). RT-PCR products were analyzed on 1.0% agarose gel. For the negative control experiment, water was added instead of RT in the reaction and the product was used as a template for PCR. In the negative control experiment, no band was visible even after 40 cycles of PCR.

212 *ICL activity assay*

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214 ICL activity was assayed spectrophotometrically at 324 nm using the total protein,
215 according to the rate of formation of glyoxylate phenylhydrazone in the presence of
216 isocitrate and phenylhydrazine at room temperature as described previously (31, 32).
217 One unit of enzyme activity corresponds to the production of 1 μ mol of glyoxylate
218 phenylhydrazone per minute under assay conditions.

219

220 *Promoter activity assay*

221

222 Promoter activity was measured by monitoring proline iminopeptidase (PIP) activity
223 as the reporter, as described previously (19). Various lengths of the upstream region of
224 *icl_{Re}* were amplified using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). First,
225 the PCR fragment that was amplified by using the primers icl-303
226 (5'-CGCTGTACAGCGAACTGCCGGAATTTGCAAGTG -3'; *Bsr*GI site is
227 underlined) and icl-1 (5'-CATGCCATGGCCAAGACTCCATCTCTTTGATCTGC -3';
228 *Nco*I site is underlined) was inserted into pHN409 (20) digested with *Bsr*GI and *Nco*I,
229 yielding p303-QC1-PIP. For the deletion series, 8 lengths of the upstream region of *icl_{Re}*
230 were amplified using 8 pairs of primers as follows: for -202 bp, icl-202
231 (5'-AATGTACATACCAGCCGTGTAGGA-3') and pip-AS
232 (5'-GGTCGACTAGTTTATTAATGATGA-3'); -192 bp, icl-192
233 (5'-AATGTACAGTAGGAATCTTCACAG-3') and pip-AS; -182 bp, icl-182
234 (5'-AATGTACATCACAGACTTAGCAAA-3') and pip-AS; -172 bp, icl-172

235 (5'-AATGTACAAAGCAAAGTGTGTTGAA-3') and pip-AS; -152 bp, icl-152
 236 (5'-AATGTACATAGCGAAGATTGGCAT-3') and pip-AS; -102 bp, icl-102
 237 (5'-AATGTACAAATTCTCGGTAAGTACA-3') and pip-AS; -75 bp, icl-75
 238 (5'-AATGTACATGGCAAGTATGTGAAG-3') and pip-AS; and -52 bp, icl-52
 239 (5'-AATGTACACACGTACTGCTCACAA-3') and pip-AS. *Bsr*GI and *Spe*I sites are
 240 underlined and italicized, respectively. Each PCR product was inserted into
 241 p303-QC1-PIP digested with *Bsr*GI and *Spe*I, yielding p202-QC1-PIP, p192-QC1-PIP,
 242 p182-QC1-PIP, p172-QC1-PIP, p152-QC1-PIP, p102-QC1-PIP, p75-QC1-PIP, and
 243 p52-QC1-PIP, respectively. PCR was performed with Pfu turbo DNA polymerase. Cells
 244 harboring these plasmids were cultured according to the method used for the
 245 preparation of total protein, and total protein that was obtained from 20 mL culture was
 246 subjected to PIP activity analysis.

247

248 *Isolation of proteins that were bound to the upstream region of icl_{Re}*

249

250 The upstream region of *icl_{Re}* was amplified using a sense primer, icl-201
 251 (5'-ACCAGCCGTGTAGGAATCTTCACAG-3'), and a biotin-labeled antisense primer,
 252 icl-1+B (5'-CAAGACTCCATCTCTTTGATCTGCT-3'). The biotin-labeled DNA
 253 fragment (50 pmol) was mixed with 200 µL of streptavidin-coated magnet beads
 254 (Invitrogen) containing 30 mg total protein from *R. erythropolis* PR4, which was
 255 prepared in the presence of 5 mM EDTA and concentrated with Amicon Ultra-10,000
 256 NMWL (Millipore, Bedford, MA). The mixture was incubated for 30 min at room
 257 temperature. Then, the magnet beads were washed with TE buffer 4 times and were

boiled in loading buffer containing SDS, followed by SDS-PAGE analysis. Then, the protein band was excised and subjected to LC-MS/MS analysis as previously described.

Pull-down analysis of RamB_{Re} by using the upstream region of icl_{Re}

For the pull-down analysis of RamB_{Re}, 1.5 mg total protein, which was obtained from cells expressing recombinant RamB_{Re}, and 25 pmol biotin-labeled DNA fragments were used. Expression of recombinant RamB_{Re} was performed as follows: first, the PCR fragment was amplified using the primers DBP-Nterm (5'-GGAATTCCATATGTCGAAAACCTTTGTCGGTGTCC-3'; *Nde*I site is underlined) and DBP-Cterm (5'-CGGGATCCTCAGCGAATCACGTAAGGCGAAATC-3'; *Hind*III site is underlined), and was inserted into pTip-QC2 (20) digested with *Nde*I and *Hind*III, yielding pTip-QC2-DBP. Expression of RamB_{Re} was performed using *R. erythropolis* JCM3201 harboring pTip-QC2-DBP in the presence of 0.5 µg/µL thiostrepton as an inducer. Various lengths of the upstream region of *icl_{Re}* were amplified using the following primers corresponding to the fragments illustrated in Fig. 4B. For fragment e, *icl*-201; d, *icl*-151 (5'-AGCGAAGATTGGCATTAGCAACAGG-3'); c, *icl*-101 (5'-TTCTCGGTAAGTACACAAGAAGGGC-3'); b, *icl*-76 (5'-CTGGCAAGTATGTGAAGTAGTACGCA-3'); a, *icl*-61 (5'-AGTAGTACGCACGTACTGCTCACAA-3'). Each primer was used in combination with *icl*-1+B. For fragments f and g, p303(61-76KO)-QC1-PIP, which has a gap between the -76 and -61 bp region of p303-QC1-PIP, was used as template. Then,

281 PCR was performed using the following primers: for f, icl-127
282 (5'-GTAGACGGCACTTTTTTCGATGTGCC-3'); g, icl-142
283 (5'-TGGCATTAGCAACAGGTAGACGGCA-3'). Each primer was used in
284 combination with icl-1+B. p303(61-76KO)-QC1-PIP was constructed by inverse PCR
285 with the primers 61-76KO_S (5'-TAGTACGCACGTACTGCTCACAACAGGCC-3')
286 and 61-76KO_AS (5'-GGCCCTTCTTGTGTACTTACCGAGAATGG-3'). PCR was
287 performed with Pfu turbo DNA polymerase.

288

289 *Construction of a ramB_{Re} knockout mutant*

290

291 To construct an *R. erythropolis* PR4 *ramB_{Re}* knockout mutant, a partial region of
292 *ramB_{Re}*, which was amplified using the primers DBPKO-S (5'-
293 GGGGTACCGAGATCGCCAGACGCCTCGA-3'; *Kpn*I site is underlined) and
294 DBPKO-AS (5'-GCTCTAGACCGGATATGAGAACGTCTCG-3'; *Xba*I site is
295 underlined), was subcloned in pHN267 (20). The vector containing the partial region of
296 *ramB_{Re}* was introduced into wild-type *R. erythropolis* PR4 by electroporation. One
297 hundred transformants were subjected to colony PCR, and 2 of them were confirmed to
298 harbor the gene disruption intended.

299

300 *Construction of expression vectors, and cloning of pip and vdh (vitamin D₃* 301 *hydroxylase)*

302

303 The 303-bp (P_{icl}), 102-bp (P_{epi}, constitutive promoter region of *icl_{Re}*) upstream region

of *icl_{Re}* were amplified using specific primers, as described previously. These DNA fragments were inserted into pNit-QC1, pNit-QC2, pNit-QT1, pNit-QT2, pNit-RC1, pNit-RC2, pNit-RT1, pNit-RT2 (20) digested with *Bsr*GI and *Nco*I, yielding 16 types novel expression vectors, pIcl (or pCpi)-QC1, pIcl (or pCpi)-QC2, pIcl (or pCpi)-QT1, pIcl (or pCpi)-QT2, pIcl (or pCpi)-RC1, pIcl (or pCpi)-RC2, pIcl (or pCpi)-RT1, pIcl (or pCpi)-RT2, respectively (Fig. S1-A).

The *vdh* fragment was isolated by pET29-VDH (26) digested *Nde*I and *Xho*I, and was cloned into *Nde*I/*Xho*I sites of pIcl-QC2 or pCpi-QC2, yielding pIcl-QC2-VDH and pCpi-QC2-VDH, respectively. Those vectors were transformed into *R. erythropolis* JCM 3201 wild-type cells by electroporation as described previously (23). pIcl-QC1-PIP and pCpi-QC1-PIP, which are identical to p303-QC1-PIP and p102-QC1-PIP, respectively, were used for PIP expression.

Results

Protein expression changes in the presence of organic solvents

To select which organic solvent can be a candidate as an inducer of protein expression, we used 11 different organic solvents as described in Materials and Methods. As host cells, we randomly selected 4 strains of *R. erythropolis* from our laboratory collections. *R. erythropolis* NI86/21, JCM6824, JCM3201, and PR4 were grown to a stationary phase and a part of the culture was added to fresh media containing either 1% or 5% of the above-mentioned organic solvents. Although all 4 strains were tolerant to most of these solvents even at 5% concentration, none of the strains grew in the

presence of 5% 1-butanol (data not shown).

Total proteins from cells grown either in the presence or absence of organic solvents were separated using SDS-PAGE. While the protein band patterns were not affected by the organic solvents other than methanol, the amount of the ~46-kDa band was increased by methanol in all strains examined (Fig. 1A and data not shown). The amount of proteins increased was highest in the strain PR4. The induction of ~46-kDa proteins reached almost 80% of its maximum level within 6 h, and it gradually increased until 24 h after the addition of methanol (Fig. 1B). The ~46-kDa band was also induced in the presence of 1% methanol (data not shown).

Identification of 46-kDa proteins induced in the presence of methanol and gene cloning

Since the ~46-kDa band was close to other bands on the acrylamide gel, we performed further separation. Briefly, total proteins that were obtained from the cells grown either in the presence or absence of methanol were subjected to size exclusion column chromatography, and the fractions that contained proteins of around 46 kDa were pooled and separated by 2-DE. By comparing the 2-DE spot patterns between those 2 samples, we found a spot that was observed only in the 2-DE sample obtained from cells grown in the presence of methanol (Fig. 1C). The spot was excised from the acrylamide gel followed by trypsin digestion and was subjected to MS analysis. Three amino acid sequences were obtained (VLIPTQQHIR, TDAEAATLITSDVDER, and LASDVADVPSVIIAR) and were similar to ICL of *Rhodococcus equi*, *Rhodococcus fascians*, and *Corynebacterium efficiens*, and elongation factor Tu (EF-Tu) of

Mycobacterium tuberculosis and *Lactobacillus sakei*, respectively. Since the molecular weight of EF-Tu should be smaller than that estimated by the 2-DE, it seemed to be a contaminated protein.

To identify the gene encoding for ICL_{Re} , we performed inverse PCR by using degenerate primers as described in Materials and Methods. By inverse PCR, we obtained 2 different DNA fragments from genome fragments that were digested with either *NcoI* or *BamHI* (Fig. 2A). These fragments covered an approximately 11-kbp DNA sequence including *icl_{Re}*, corresponding to *R. erythropolis* PR4 chromosome 1710445-1721421, and gene bank accession number is NC_012490. DNA sequencing identified 8 open reading frames (ORFs), and 4 consecutive ORFs were found in the same direction. These 4 ORFs started with *icl_{Re}* followed by 3-hydroxyacyl-CoA dehydrogenase (*hbd*), 5-methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase (*mhm*), and alcohol dehydrogenase (*adh*). Genome information of *R. erythropolis* PR4 is currently available, and the accession numbers for these genes are as follows: *icl_{Re}*, RER_15740; *hbd*, RER_15750; *mhm*, RER_15760, and *adh*, RER_15770. To investigate if these 4 ORFs are polycistronically transcribed, we performed RT-PCR analysis with specific primers that were designed to amplify DNA fragments containing more than 2 consecutive ORFs (Fig. 2B). We detected RT-PCR products when using 5 different primer sets as shown in Fig. 2B, although DNA fragments containing all of the 4 ORFs were not amplified, probably because RT reaction is difficult for long mRNAs.

Promoter activity assay with the 5' upstream region of icl_{Re}

The promoter activity of the upstream region of *icl_{Re}* was analyzed using *pip* as a reporter gene. Different lengths of the upstream region of *icl_{Re}* were connected to *pip*, and the protein amount and enzymatic activity of PIP were monitored before and after the addition of methanol (Fig. 3A). When the 303-bp upstream region of *icl_{Re}* was used, the protein amount and enzymatic activity were both induced by methanol in a time-dependent manner. The induction profile of PIP was similar to that of *ICL_{Re}*.

To find the minimal promoter region, we constructed different sizes of the upstream region of *icl_{Re}* connected to *pip* (Fig. 3B). A similar response was observed for the 303- and 202-bp upstream regions of *icl_{Re}*, and PIP activity was induced in a time-dependent manner. Shortening the length of the upstream region decreased the inducibility and expression level, except for the 2 constructs containing the -152-bp and -102-bp upstream regions. In these cases, the expression levels were high even in the absence of methanol. The expression level of the -102-bp upstream region was 1.8 times higher than that observed for the methanol-inducible promoter.

Isolation of proteins that were bound to the putative promoter region of icl_{Re}

To identify regulatory factors for *icl_{Re}* expression, pull-down analysis with the biotin-labeled upstream region of *icl_{Re}* was performed as described in Materials and Methods. Proteins that were bound to the biotin-labeled DNA fragments were collected using streptavidin-coated magnet beads and were analyzed by SDS-PAGE (data not shown), which detected 2 major bands (~53 and ~65 kDa). Since the ~65-kDa band was also detected in the negative control experiment in which the total protein was incubated with only streptavidin beads, we focused on only the ~53-kDa protein for the further

analysis.

To identify the ~53-kDa protein, LC-MS/MS analysis was performed. A database search matched peptide sequences that were deduced from the MS analysis to some different protein sequences, including the RamB homologue from *Rhodococcus jostii* RHA1 and *Mycobacterium smegmatis*, and acyl-coenzyme A carboxylase from *Corynebacterium diphtheriae*. We chose only RamB_{Re} for the further analysis, because the other proteins were not putative transcriptional regulators. RamB from *Corynebacterium glutamicum* (RamB_{Cg}) is a transcriptional regulator and negatively controls the expression of some genes, including *icl* (33).

In the case of *C. glutamicum*, the 13-bp binding motif for RamB_{Cg} (5'-AA/GAACTTTGCAAA-3') is identified in the upstream region of *icl*_{Cg}. However, homologous sequence was not found in the upstream region of *icl*_{Re}. Therefore, we confirmed the binding ability of RamB_{Re} to the upstream region of *icl*_{Re} by pull-down experiments; the biotin-labeled upstream region of *icl*_{Re} was mixed with the total proteins obtained from *R. erythropolis* PR4 cells expressing recombinant RamB_{Re} (Fig. 4C). By the experiments, at least 2 possible binding sites were found: one is located between -61 and -76 bp (5'-TGGCAAGTATGTGAAG-3') and the other is located between -127 and -142 bp (5'-TGGCATTAGCAACAGG-3') (Fig. 4A).

Effect of ramB_{Re} disruption on R. erythropolis PR4

The above pull-down experiments suggested that *ramB*_{Re} was involved in the regulation of *icl*_{Re} expression. To investigate the role of RamB_{Re} in *icl*_{Re} expression, we analyzed the ICL_{Re} activity of wild-type and *ramB*_{Re} knockout mutant in the presence of

various carbon sources (Table 1). Gene knockout was done by a single crossover homologous recombination. Since random recombination of exogenous DNA fragments often occurs in *R. erythropolis*, we checked the candidate clones in the gene arrangement around *ramB_{Re}* by using PCR, and obtained a knockout mutant (data not shown).

In *C. glutamicum* and *M. tuberculosis*, it has been reported that the expression of *icl* is negatively regulated by RamB and is dependent on the carbon source availability in the culture media (33-37). In wild-type *R. erythropolis* PR4, ICL_{Re} activity was induced in the presence of methanol and acetate, while these inductions were suppressed by the addition of glucose. In contrast, in the knockout mutant, we did not observe a significant difference among them.

Expression vector construction by using the promoter from the 5' upstream region of icl_{Re}

Since the activity of the 303-bp upstream region of *icl_{Re}* was methanol inducible and that of the 102-bp upstream region was constitutively very high, we believe they could be used as a methanol-inducible and strong constitutive promoter for recombinant protein expression, respectively. We have recently cloned antibiotic resistance genes, 2 different types of replication origins, and constitutive or inducible promoters from the genus *Rhodococcus* (19, 20). By using these elements, we have constructed a wide variety of expression vectors. Here, we combined these elements with the promoter region of *icl_{Re}*, and constructed constitutive or methanol-inducible expression vectors (representative constructs are shown in Fig. 5A). One of the promoters was P_{icl}, which

includes the 303-bp upstream region of *icl_{Re}*, and another one was P_{*cpi*} (constitutive promoter region of *icl_{Re}*), which includes the 102-bp upstream region of *icl_{Re}*. By combining 2 types of selection markers, replication origins, and multiple-cloning sites as described previously (20), we constructed 16 types of vectors (Fig. S1). In order to test the expression ability of these expression vectors, PIP and VDH were expressed as model cases. When P_{*icl*} was used for the expression, protein expression was inducible depending on the presence of methanol, and when P_{*cpi*} was used, constitutive high expression was observed in both cases of PIP and VDH (Fig. 5B). As shown in Figure 5C, the expression level of P_{*cpi*} was quite high and its activity exhibited approximately 3- and 2-fold higher than P_{*icl*} and P_{*tip*}, respectively.

Discussion

All of the *R. erythropolis* strains used in this study exhibited tolerance to various kinds of organic solvents at certain concentrations, and all strains tested grew in the presence of 5% methanol. By analyzing cell responses to organic solvents through SDS-PAGE, we found a protein that was induced in the presence of methanol. The protein was identified as ICL_{*Re*} by LC-MS/MS analysis, and its methanol-inducible regulation was revealed to depend on the at least 200-bp upstream region of *icl_{Re}*. We also found *ramB_{Re}* as one of the regulatory factors of *icl_{Re}* expression.

RT-PCR data suggested that *icl_{Re}* would be cotranscribed with 3 other neighboring genes. Thus, it would be reasonable to think that the other 3 proteins would also be induced in the presence of methanol. However, in this study, only ICL_{*Re*} was found to be induced in the presence of methanol by using acrylamide gels and 2-DE. This may be

due to the differences in protein turnover rate among these proteins or the differences in translation rate of mRNA even in a single cistron.

ICL is a key enzyme in the glyoxylate cycle and is essential for the shunt pathway of the TCA cycle. ICL is induced in the presence of acetate in *Corynebacterium* and *Mycobacterium* (35, 38, 39). In *R. erythropolis* PR4, ICL_{Re} was induced by the addition of sodium acetate, suggesting a similar regulation of *icl*_{Re} expression as that of *Corynebacterium* or *Mycobacterium*. Also, *ramB*_{Re} knockout experiment supports the idea that RamB_{Re} functions as a suppressor of *icl*_{Re}.

In the analysis of the minimal promoter of *icl*_{Re}, reporter expression was gradually decreased in both of the methanol-inducibility and the amount of protein expression as the upstream region used was shortened, except for constructs containing the -152 and -102 bp upstream regions. This would suggest that there are at least 2 different regulation mechanisms: one involves positive regulation, the function of which depends on the length of the upstream region, and the other involves negative regulation, the function of which depends on the region between -152 and -102 bp. The expression would be regulated not only by RamB_{Re}, as described below, but also by another factor that would regulate the expression by interacting with the upstream region of *icl*_{Re} between -152 and -102 bp. The details of the regulation mechanism(s) of *icl*_{Re} by RamB_{Re} in *R. erythropolis* remain to be elucidated.

By using various lengths of the biotin-labeled upstream region of *icl*_{Re}, we identified 2 possible binding sites of RamB_{Re}. However, we could not find any sequence similarity between these 2 sites, and neither of these sequences contained the core motif of *C. glutamicum* RamB binding sites, 5'-TTTGCAAA-3' (36). As shown in Fig. 4, the amount of RamB_{Re} binding to the upstream region of *icl*_{Re} was apparently different

among the 101-, 151-, and 201-bp upstream regions. The amount of binding protein seems higher for the longer regions, suggesting there would be at least 3 binding sites, which would be located between -61 and -101 bp, -101 and -151 bp, and -151 and -201 bp, respectively. Although we did not obtain experimental data, we found a sequence similar to that between -142 and -127 bp in the region between -101 and -151 bp (Fig. 4D). This sequence was located between -179 and -164 bp (5'-CAGACTTAGCAAAGTG-3'). Also, these 2 sites contain 5'-TTAGCAA-3', which is similar to the core-binding motif of RamB_{Cg}, suggesting a possibility of RamB_{Re} binding to this region. RamB_{Re} has 2 functional domains, the helix turn helix (HTH) motif and transcriptional regulation domains (Fig. S2). Since the HTH motif, which is important for recognizing the target binding sequence, was not highly conserved among related genera, the relation between the HTH motif and RamB binding sequence remains to be elucidated. Also, the genome structure around *icl* is highly diverse among related genera (Fig. S3). This diversity would be related to the regulation difference of *icl* among related genera.

The response to solvent stress has not been well understood, although *R. erythropolis* is often said to be tolerant to organic solvents. The general mechanism of solvent tolerance has been reported to include rigidification of the cell membrane, change in the membrane's protein content or composition, active export of solvent, adaptation of energetic status, changes in cell wall and outer membrane composition, modification of cell surface properties, morphological changes, and metabolism or transformation of the solvent (11). In the case of *R. erythropolis* PR4 methanol tolerance, assimilation of methanol would be related to the mechanism because the shunt pathway of the TCA cycle, which is also needed for C1 carbon assimilation,

would be activated in the presence of methanol. To investigate this possibility, we performed 2 preliminary experiments: one was HPLC measurement of the amount of methanol before and after the incubation of *R. erythropolis* PR4, and the other was observation of growth of *R. erythropolis* PR4 in minimal media containing methanol as a sole carbon and energy source. However, neither of these experiments supported the possibility (data not shown). These data suggest that *icl_{Re}* regulation by methanol would be different from what is done by acetate through glyoxylate cycle. This would be supported by the difference of *icl* response to acetate or methanol in the presence of glucose (Table1).

Methanol-inducible promoters have been identified from methanol-assimilating bacteria such as *Methylobacterium extorquens* and *Candida boidinii* (40-42). However, such promoters were never applied to create expression vectors. Although functional analysis of *icl_{Re}* induction has not been completed, the novel vectors described here would be useful for bioreactions.

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Figure legends

Fig. 1. SDS-PAGE and 2-DE analyses of the total protein of *Rhodococcus erythropolis* PR4 incubated either in the presence or absence of methanol. (A) Total proteins were extracted from PR4 cells grown for 24 h either in the presence or absence of 5% methanol, and 15 µg protein was separated on an acrylamide gel. The arrowhead indicates proteins induced by the addition of 5% methanol. Lanes – and +, total protein obtained from cells grown in the absence and presence of 5% methanol, respectively. (B) A time course analysis of the ~46-kDa protein was performed using 15% acrylamide gel. Fifteen micrograms of total protein was subjected to SDS-PAGE analysis. The arrowhead indicates proteins that were induced by the addition of 5% methanol. Lane numbers indicate the incubation time after the addition of 5% methanol. (C) 2-DE analysis of the total proteins. The arrow indicates proteins that were induced by 5% methanol.

Fig. 2. A 11-kbp *R. erythropolis* PR4 DNA fragment including a probable *icl_{Re}* operon. (A) Schematic drawing made using the sequence analysis data obtained by inverse PCR. The recognition sites of restriction enzymes used for the inverse PCR are indicated above the drawing. The direction of the open arrow indicates the direction of each ORF. *ramB*, transcriptional regulator of acetate metabolism; *aad*, acetamidase; *prp*, putative regulatory protein; *icl*, isocitrate lyase; *hbd*, 3-hydroxyacyl-CoA dehydrogenase; *mhm*, 5-methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase; *adh*, alcohol dehydrogenase. The accession numbers for these genes are as follows: *ramB*, RER_15700; *aad*, RER_15710; *prp*, RER_15720. The white arrow indicates

706 hypothetical protein; the gray arrows indicate 4 ORFs of probable polycistron. (B)

707 RT-PCR was performed using 1 µg of total RNA as a starting material, and the PCR

708 product was subjected to electrophoresis using 1% agarose gel (left). Lanes “a” to “e”

709 correspond to the gene fragment that is indicated on the right side of the panel.

710

711 Fig. 3. Promoter activity analysis by using deletion series of the upstream region of *icl_{Re}*

712 and time course analysis. (A) Promoter activity analysis of the 303-bp upstream region

713 of *icl_{Re}*. SDS-PAGE was performed using 15 µg of total protein from PR4 cells

714 expressing PIP. The arrowhead indicates PIP. The specific activity of PIP was

715 colorimetrically measured using proline-7-amino-4-methylcoumarin as a substrate. (B)

716 PIP activity in *R. erythropolis* PR4 cells carrying plasmids with various deletions of the

717 upstream region of *icl_{Re}* were measured. The cells were incubated in the presence of 5%

718 methanol for 0, 2, and 8 h, and subjected to PIP activity assay.

719

720 Fig. 4. *Cis*-element analysis of the *icl_{Re}* promoter region. (A) Schematic drawing of the

721 *icl* promoter region containing putative *cis* elements. The *cis* elements that were

722 identified by the binding assays are indicated by 2 black boxes, while a probable *cis*

723 element that was deduced from the sequence similarity is indicated by an open box.

724 Nucleotide positions were calculated from the first base of translation initiation codon

725 (ATG, +1). (B) DNA fragments used for the pull-down analysis are schematically

726 illustrated. (C) The protein that was bound to the DNA fragment was analyzed on an

727 acrylamide gel. The lane names correspond to the construct names. The arrowhead

728 indicates RamB_{Re}. (D) Alignment of the sequence of RamB_{Re} binding sites in the

729 upstream of *icl_{Re}* -61 to -76 bp, -127 to -142 bp, and -164 to -179 bp. Boxed sequences

(black) represent the identities of at least 2 members of the group.

Fig. 5. Expression vector construction and application by using the promoter from the upstream region of *icl_{Re}*. (A) Schematic map of pIcl-QC1 and pCpi-QC1. *cm^r*, chloramphenicol-resistant gene; *amp^r*, ampicillin-resistant gene; *ColE1*, replication origin for *E. coli*; *T_{thcA}*, *thcA* transcriptional terminator; MCS, multiple-cloning site; *P_{icl}*, 303-bp upstream region of *icl_{Re}* (methanol-inducible promoter); *P_{cpi}*, 102-bp upstream region of *icl_{Re}* (constitutive strong promoter); *repAB*, essential region for stable maintenance of the plasmid in *R. erythropolis*. (B) (Left) Expression profiles of PIP or VDH in *R. erythropolis* JCM3201 containing the pIcl-QC1-PIP or pIcl-QC2-VDH, respectively. Total proteins (15 µg) were analyzed by SDS-PAGE. Black and gray arrowheads indicate PIP and VDH, respectively. Lane – and +, total protein obtained from cells grown in the absence and presence of 5% methanol, respectively. (Right) Expression profiles of PIP or VDH in *R. erythropolis* JCM3201 containing the pCpi-QC1-PIP or pCpi-QC2-VDH, respectively. Total proteins (15 µg) were analyzed by SDS-PAGE. Black and gray arrowheads indicate PIP and VDH, respectively. NC, negative control. (C) Activity of PIP in *R. erythropolis* JCM3201 containing the pIcl-QC1-PIP, pHN380 (20), or pCpi-QC1-PIP. The transformant was incubated in the presence of 5% methanol for 24 h or 0.5 µg/µL thiostrepton for 20 h, and subjected to PIP activity assay. NC, negative control, in which the total protein from *R. erythropolis* JCM3201 containing an empty vector was used.

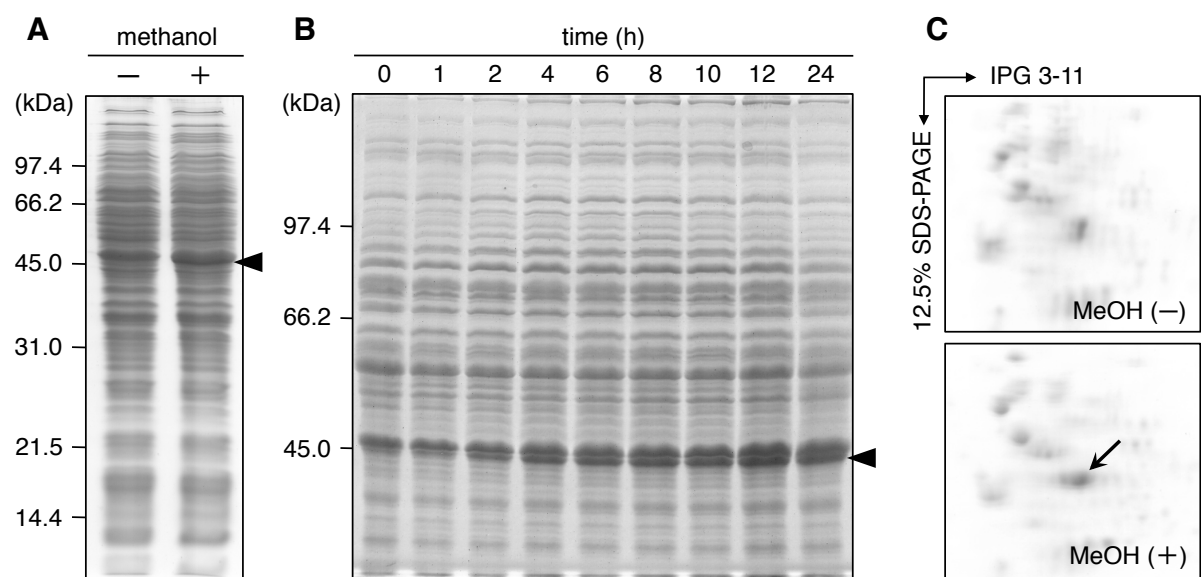


Fig. 1

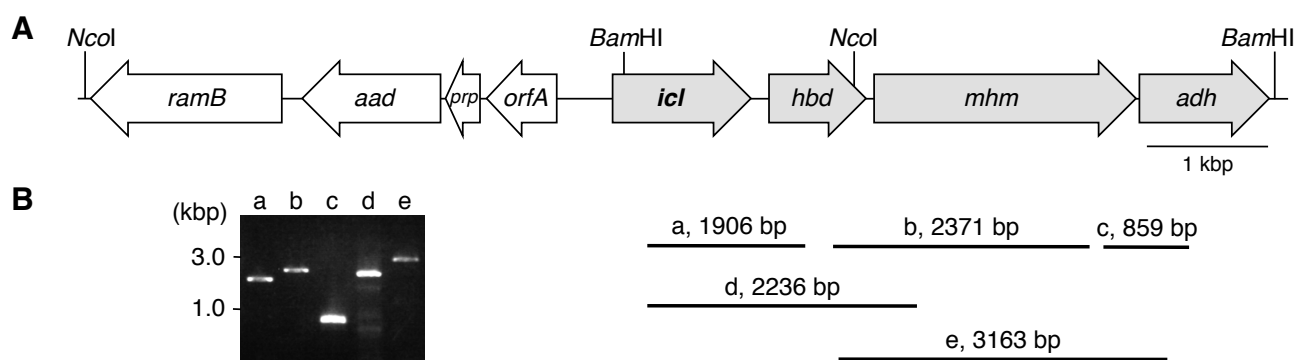


Fig. 2

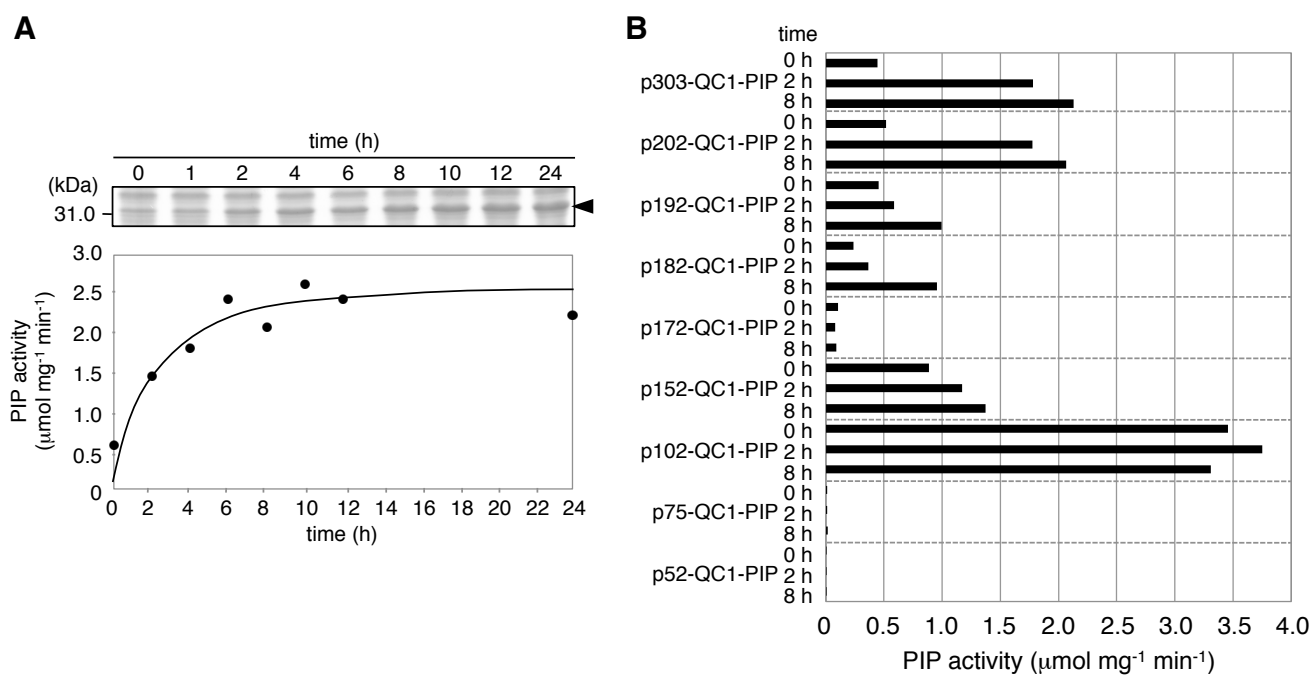


Fig. 3

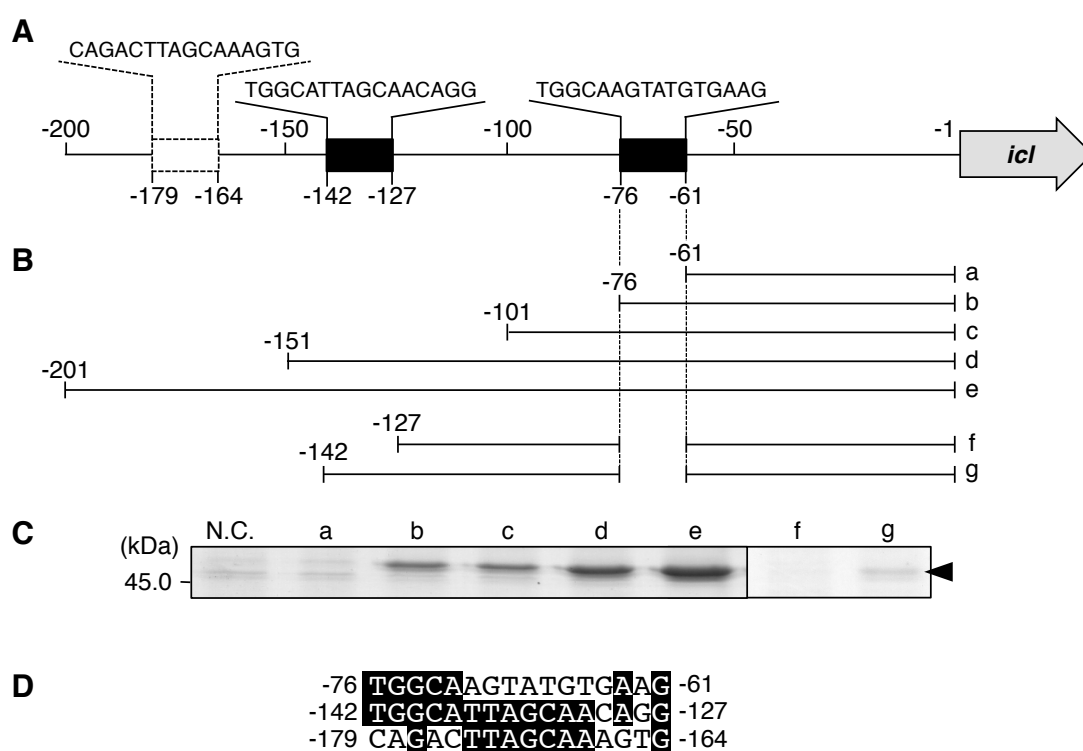


Fig. 4

Fig. 5

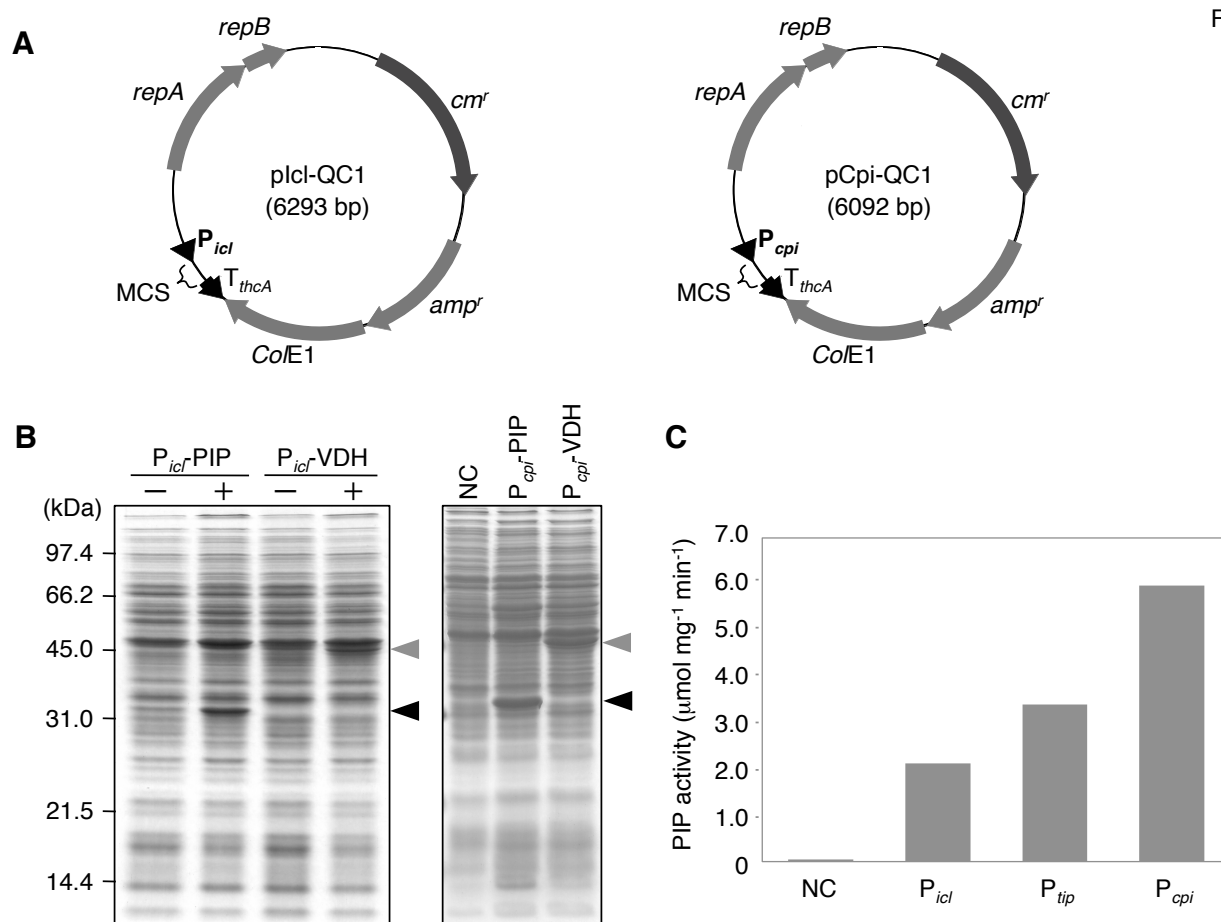


Table 1. Isocitrate lyase activities in total proteins of *R. erythropolis* PR4 wild-type and *ramB_{Re}* knockout mutant grown with supplements and glucose

Growth supplements	<i>R. erythropolis</i> PR4	
	WT	<i>ramB_{Re}</i> KO
No supplement	8.4 ± 0.7	24.5 ± 2.6
1% Methanol	21.4 ± 3.8	21.4 ± 4.6
1% Sodium acetate	22.4 ± 2.0	23.4 ± 1.7
1% Glycerol	6.5 ± 0.5	ND
1% Glucose	5.4 ± 0.8	26.0 ± 3.1
1% Methanol and 1% glucose	6.4 ± 0.8	24.8 ± 5.6
1% Sodium acetate and 1% glucose	14.2 ± 2.4	23.6 ± 2.0
1% Glycerol and 1% glucose	5.7 ± 0.8	ND

Activities are indicated in U/mg protein: mean ± standard deviation. ND, not determined (n = 3).

Fig. S1. Expression vector construction and sequence of P_{icl} , P_{cpi} , MCS type1 and type2, and T_{thcA} . (A) Schematic map of expression vectors. Sixteen expression vectors were constructed, pIcl-QC1, pIcl-QC2, pIcl-QT1, pIcl-QT2, pIcl-RC1, pIcl-RC2, pIcl-RT1, pIcl-RT2, pCpi-QC1, pCpi-QC2, pCpi-QT1, pCpi-QT2, pCpi-RC1, pCpi-RC2, pCpi-RT1, and pCpi-RT2, which indicates Q, *repA* and *B*; R, *rep*; C, chloramphenicol-resistant; T, tetracycline-resistant; 1, MCS type1; 2, MCS type2, respectively. *cm^r*, chloramphenicol-resistant gene; *tet^r*, tetracycline-resistant gene (each pIcl and pCpi vector has either *cm^r* or *tet^r*); *amp^r*, ampicillin-resistant gene; *ColE1*, replication origin for *E. coli*; T_{thcA} , *thcA* transcriptional terminator; MCS, multiple-cloning site (each pIcl and pCpi vector has either MCS type1 or MCS type2); P_{icl} , 303-bp upstream region of *icl_{Re}*; P_{cpi} , 102-bp upstream region of *icl_{Re}*; *repAB* and *rep*, essential region for stable maintenance of the plasmid in *R. erythropolis*. (B) Sequences of P_{icl} , P_{cpi} , MCS, and T_{thcA} . All of the restriction enzyme sites indicated in the MCS, except for *SnaBI* in the R type vectors are unique. Gray arrows indicate the positions of the perfect IR sequences in the *thcA* terminator. Note that neither conserved Shine-Dalgarno sequence nor -35 and -10 promoter elements were found in the P_{icl} and P_{cpi} .

Fig. S2. Schematic drawing of the primary structure of RamB_{Re}, and alignment of its HTH domains. Black and gray boxes indicate the HTH and transcriptional regulation domains, respectively. Identical residues are shaded black, and similar residues are shaded gray. Abbreviations: aa, amino acids; RE, *R. erythropolis* PR4; MT, *M. tuberculosis*; CG, *C. glutamicum*. The HTH domain region is underlined.

25 Fig. S3. Comparison of *icl_{re}* organizations in *R. erythropolis* PR4 and other bacteria.
26 The gene names are as follows: *ramB*, transcriptional regulator involved in acetate
27 metabolism; *aad*, acetamidase; *prp*, putative regulatory protein; *icl*, isocitrate lyase; *hbd*,
28 3-hydroxyacyl-CoA dehydrogenase; *mhm*,
29 5-methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase; *adh*, alcohol
30 dehydrogenase; *abc*, branched-chain amino acid ABC transporter substrate-binding
31 protein; *egc*, endoglycosylceramidase; *ndt*, Na⁺-dependent transporter; *aceB*, malate
32 synthase; *thiX*, protein potentially involved into thiamin biosynthesis; *lcoP*, ectoine
33 betaine transporter; *umaA*, mycolic acid synthase; *dbp*, DNA binding protein. The ORF
34 of white arrows indicate hypothetical protein; the ORF of black arrows indicate *icl* and
35 *icl* homolog; and the ORF of gray arrows indicate *ramB* and *ramB* homolog.

36

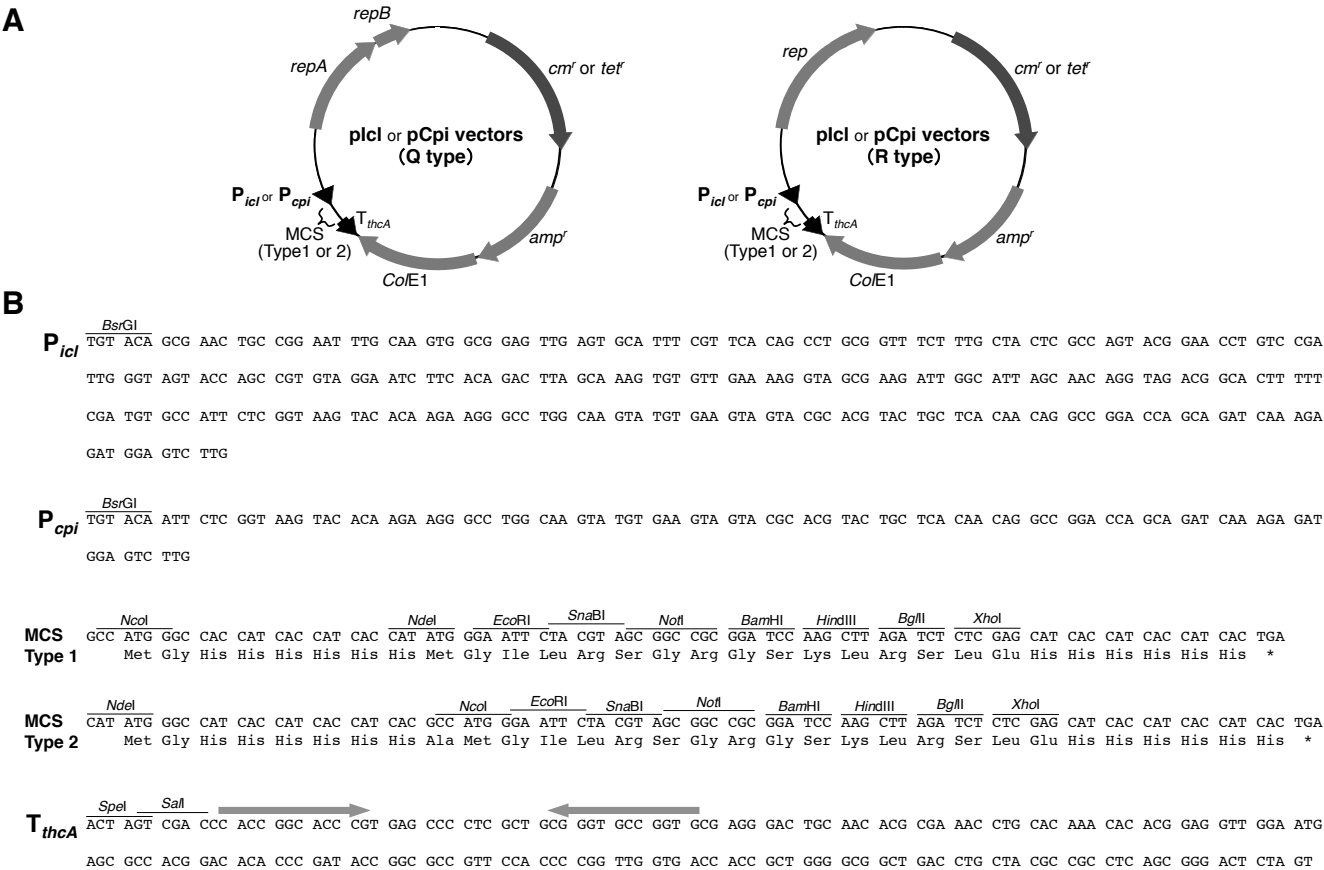


Fig. S1

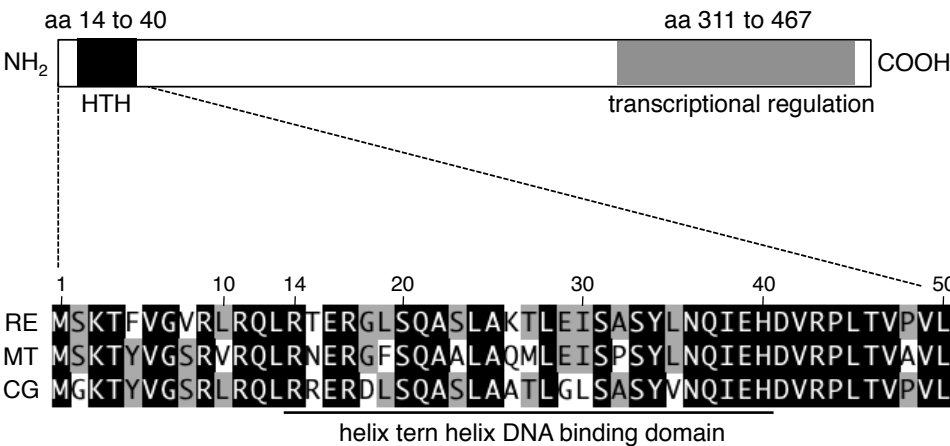


Fig. S2

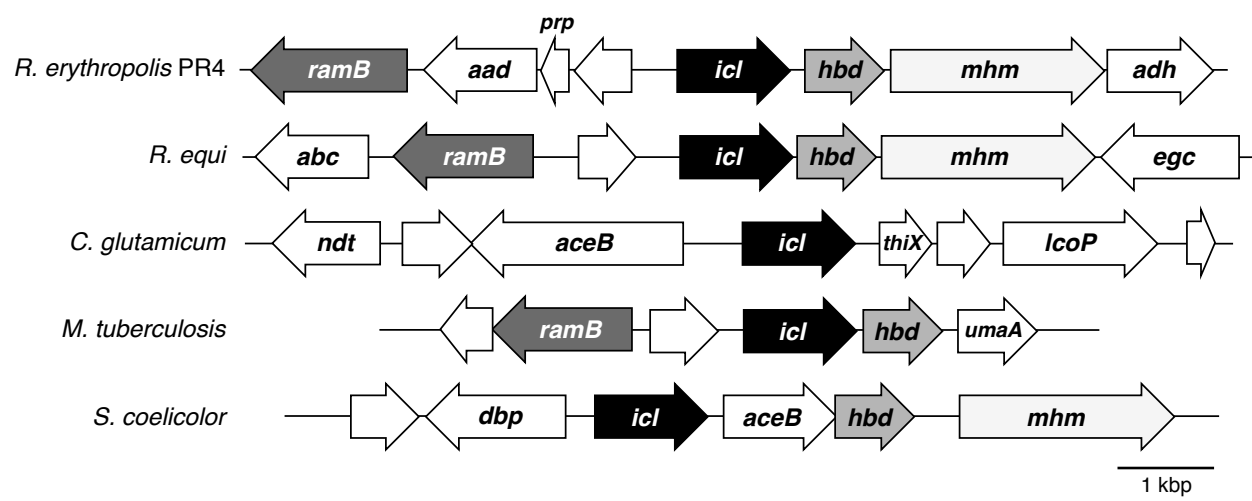


Fig. S3