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**Seiya Watanabe · Naoto Yamaoka · Noriyuki Fukunaga**
**Yasuhiro Takada**

# Purification and characterization of a cold-adapted isocitrate lyase and expression analysis of the cold-inducible isocitrate lyase gene from the psychrophilic bacterium *Colwellia psychrerythraea*

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**Abstract** Isocitrate lyase (ICL) from *Colwellia psychrerythraea*, a psychrophilic bacterium, was purified and characterized. The subunit molecular mass was 64 kDa, which is larger than that of other bacterial ICLs. The optimal temperature for its activity was 25°C, the value of $K_m$ for the substrate (DL-isocitrate) was minimum at 15°C, and the catalytic efficiency ($k_{cat}/K_m$) value was maximum at 20°C. Furthermore, the enzyme was remarkably thermolabile and completely inactivated by incubation for 2 min at 30°C. These features indicate that ICL from this bacterium is a typical cold-adapted enzyme. A partial amino acid sequence of the *C. psychrerythraea* ICL was very similar to that of the closely related psychrophile *Colwellia maris*. Expression of the gene encoding the *C. psychrerythraea* ICL was found to be induced by low temperatures and by acetate in the medium. The cold adaptation of the catalytic properties of ICL and the stimulated expression of its gene at low temperatures strongly suggest that this enzyme is important for the growth of this bacterium in a cold environment.

**Key words** Psychrophilic bacterium · *Colwellia psychrerythraea* · Isocitrate lyase · Cold-adapted enzyme · Cold-inducible gene

## Introduction

Extremophiles live under unusual environmental conditions with regard to temperature, pressure, pH, ionic strength, anaerobiosis, and drought. Among these, temperature is critical for their survival because catalytic rates of biochemical reactions are strongly dependent on temperature. Psychrophiles, which inhabit permanently cold environments, have developed some specific strategies to adapt to low temperatures (Gerday et al. 1997). The main strategy is enzymatic adaptation. In particular, enzymatic adaptation of the central metabolism to low temperatures is indispensable for the survival of psychrophiles in a cold environment. However, studies of such enzymes, which include citrate synthase (Russell et al. 1998; Gerike et al. 1997), malate dehydrogenase (Kim et al. 1999), phosphoglycerate kinase (Bentahir et al. 2000), triosephosphate isomerase (Alvarez et al. 1998), and lactate dehydrogenase (Fields and Somero 1998), in psychrophiles are still limited. Biochemical and protein engineering studies have provided information about molecular mechanisms for the achievement of high catalytic activity at low temperatures. Furthermore, crystallographic analyses of the three-dimensional structures of the enzymes have revealed some features of cold-adapted enzymes.

Isocitrate lyase (ICL) (EC 4.1.3.1), a key enzyme of the glyoxylate cycle, catalyzes the cleavage of isocitrate to glyoxylate and succinate and plays important roles in the metabolism of acetate and fatty acids in microorganisms and higher plants (Kornberg 1966; Vanni et al. 1990; Cozzone 1998). It has been reported that the ICL of the psychrophile *Colwellia maris*, which was previously described as *Vibrio* sp. strain ABE-1 (Takada et al. 1979; Yumoto et al. 1998), is very thermolabile and that the expression of the gene encoding the enzyme is induced by low temperature (Watanabe et al. 2001, 2002). These findings suggest the possibility that the metabolic step catalyzed by ICL is important for the cold adaptation and survival of psychrophilic bacteria in cold environments. However, the same enzymes in different psychrophilic bacteria, even in closely related bacteria, do not always display cold-adapted characteristics. In fact, *C. maris* possesses both cold-adapted and mesophilic isocitrate dehydrogenase (IDH) isozymes (Fukunaga et al. 1999), while both IDH isozymes of another psychrophilic *Colwellia* species, *C. psychrerythraea*, which is closely related to *C. maris*, are mesophilic (unpublished data). We report here that *C. psychrerythraea* also possesses

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S. Watanabe · N. Yamaoka · N. Fukunaga · Y. Takada (✉)
Division of Biological Sciences, Graduate School of Science, Hokkaido University, Kita 10-jo Nishi 8-chome, Kita-ku, Sapporo 060-0810, Japan
Tel. +81-11-7062742; Fax +81-11-7064851
e-mail: ytaka@sci.hokudai.ac.jp

a cold-adapted ICL and that the expression of its gene is induced by low temperatures.

## Materials and methods

### Bacterial strains and culture conditions

The psychrophilic bacterium *Colwellia psychrerythraea* (D'Aoust and Kushner 1972; Deming et al. 1988) was cultivated aerobically for 50–75 h at 15°C in a nutrient medium consisting of 1% (w/v) meat extract, 40 mM $MgCl_2$, 3% (w/v) NaCl, and either 1% (w/v) peptone, 1% (w/v) peptone+25 mM sodium acetate, or 25 mM sodium acetate. Cells were harvested, washed twice with chilled 0.5 M NaCl, and stored frozen at –80°C until use. The washed cells were suspended in 50 mM potassium phosphate buffer, pH 6.85, containing 2 mM $MgCl_2$, 0.5 M NaCl, and 1 mM dithiothreitol (DTT), and disrupted by sonication for 4–5 min with brief intervals on ice to obtain a cell-free extract. Protein was measured by the method of Lowry et al. (1951) by using bovine serum albumin as a standard.

### Enzyme assay

ICL was assayed spectrophotometrically by determining the increase of absorbance at 324 nm due to the formation of glyoxyl-phenyl hydrazone, as described previously (Watanabe et al. 2001). Unless otherwise noted, the pH of the reaction mixture was 6.85 and the assay temperature was 20°C. One unit of activity of ICL was defined as the formation of 1 μmol of product per minute.

### Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemmli (1970) by using a 10% (w/v) gel at 25 mV. Proteins on the gel were stained with Coomassie brilliant blue (CBB) R250 and destained with 10% (v/v) acetic acid in 50% aqueous methanol.

### Determination of N-terminal and internal amino acid sequences

The N-terminal amino acid sequence of the *C. psychrerythraea* ICL was determined by the method described previously (Watanabe et al. 2001). For determining the internal amino acid sequences of this enzyme protein, chemical digestion with cyanogen bromide (BrCN) was used (Gross 1967). The purified ICL (710 μg) was dialyzed against deionized water and then lyophilized. The polypeptide was digested chemically at room temperature in 70% (v/v) formic acid containing 1% (w/v) BrCN (100 μl) in the dark and under $N_2$. After 24 h, the solution was diluted with 900 μl of deionized water, frozen with liquid $N_2$, and lyophilized. The sample was dissolved in SDS-PAGE sample buffer (500 mM Tris-HCl, pH 6.8, containing 5% (w/v) SDS, 10% (v/v) glycerol, 0.25% (w/v) bromophenol blue, and 5% (v/v) 2-mercaptoethanol) and separated on a 15% (w/v) acrylamide gel for SDS-PAGE at 25 mA. Peptide fragments on the gel were transferred to an Immobilon polyvinylidene fluoride (PVDF) membrane (Millipore) as described previously (Watanabe et al. 2001). After staining with CBB-R250, the area of the membrane corresponding to the major three peptide fragments of ICL was cut off and analyzed with a model 477A protein sequencer (Applied Biosystems).

### Metal ion requirement

To remove metal ions from the enzyme preparation, the purified ICL was dialyzed at 4°C against a $Mg^{2+}$-free buffer consisting of 66.7 mM Tris-HCl (pH 6.85) and 1 mM DTT. ICL activity was assayed with $MnCl_2$, $CoCl_2$, $CaCl_2$, and $NiCl_2$ at a concentration of 10 mM. Cysteine hydrochloride was omitted from the reaction mixture.

### Purification of ICL

All steps for purification were done below 4°C. The cell-free extract obtained from 14 g wet cells grown on 25 mM acetate as the carbon source was fractionated at between 50% and 70% saturation with $(NH_4)_2SO_4$. The obtained precipitate was dissolved in a small volume of Buffer A (20 mM potassium phosphate, pH 6.85, containing 2 mM $MgCl_2$, 0.5 M NaCl, and 1 mM DTT) and centrifuged to remove insoluble materials. The supernatant was gel-filtered with a column of Sephacryl S-300 (superfine grade, 2.5 × 71 cm) equilibrated with Buffer A. Fractions containing high ICL activity were combined, concentrated with polyethylene glycol #20,000, and dialyzed against Buffer B (Buffer A containing 0.05 mM NaCl instead of 0.5 M NaCl). The dialyzed enzyme solution was applied to a column of DEAE-cellulose (2.6 × 20 cm) that had been equilibrated and washed thoroughly with Buffer B. Proteins were eluted by 200 ml of a linear gradient of 0.05–0.5 M NaCl in Buffer B. The active fractions containing ICL activity were combined, concentrated, and dialyzed against Buffer A. Solid $(NH_4)_2SO_4$ was added to the dialyzed enzyme preparation to give a concentration of 1.0 M. The enzyme preparation was put onto a column of phenyl-Sepharose CL-4B (2.6 × 20 cm) equilibrated with Buffer C [Buffer A containing 1.0 M $(NH_4)_2SO_4$], and the enzyme was eluted by 200 ml of a reversed linear gradient of 1.0–0 M $(NH_4)_2SO_4$ in Buffer A. The fractions with high enzyme activity were pooled, dialyzed against Buffer A containing 10% (w/v) glycerol and 4 mM DL-isocitrate instead of 0.5 M NaCl, and stored at –35°C. To estimate the native molecular mass of the enzyme, the purified enzyme was gel-filtered with a column of Sephadex G-150 (1 × 45 cm) equilibrated with Buffer A. Horse apoferritin (480 kDa), bovine liver catalase (240 kDa), rabbit muscle aldolase (158 kDa), and horse heart cytochrome *c* (12.4 kDa) were used as molecular markers.

### Measurement of kinetic and thermodynamic parameters

The $K_m$ and $V_{max}$ values were determined by a double reciprocal Lineweaver–Burk plot. Steady-state kinetics were examined with variable concentrations of the substrate. The activation energies ($E_a$) of the enzymes were calculated from the slope of an Arrhenius plot: 1/T versus log $k_{cat}$. The thermodynamic activation parameters of the enzyme reactions were calculated by using equations as described previously (Watanabe et al. 2001).

### DNA amplification by PCR and nucleotide sequencing

Genomic PCR analysis was performed to obtain a nucleotide probe for the *icl* gene encoding the ICL of *C. psychrerythraea*. Two upstream primers, 5′-[TC(AGTC)/AG(TC)]AA(TC)TA(TC)CA(AG)[TC(AGTC)/AG(TC)]GC(AGTG)AT(ATC)GA(AG)GC-3′ (26-mer), were designed from SNYQSAIEA, the sequence of the N-terminal amino acids (1–9) of the *C. psychrerythraea* ICL protein. Downstream primers, 5′-CC(AG)TC(CT)TG(AG)TG(ATGC)CC(AG)CA(CT)TG(CT)TT-3′ (23-mer), were designed from KQCGHQDG, the amino acid sequence of the region of the *C. maris* ICL protein corresponding to highly conserved regions of *icl* genes cloned from various organisms (Rehman and McFadden 1996). Chromosomal DNA of *C. psychrerythraea* was purified as described previously (Ishii et al. 1993). The amplification was carried out for 30 cycles in a DNA thermal cycler 4800 (Perkin-Elmer, CT, USA) in a 100-µl reaction mixture containing 1.4 µg of genomic DNA, 200 pmol of each forward and reverse primer, and 2.5 units of Amplitaq DNA polymerase (Roche Molecular Systems, NJ, USA) in a buffer system prepared by the manufacturer. Cycling conditions were as follows: denaturation at 94°C for 1 min, annealing at 47°C for 1 min, and extension at 72°C for 1 min, for each of 30 cycles. As a result, a single PCR product with a predicted length of 660 bp was obtained. The nucleotide sequence of the PCR product was determined by the dideoxy chain termination method by using the primers described above and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech) with an ABI 373 A DNA sequencer (Applied Biosystems). The sequence was analyzed with the Genetyx computer program (Software Development, Tokyo, Japan). The PCR product was used as a probe for Southern and Northern hybridization.

### Southern blot analysis

Approximately 14 µg of the *C. psychrerythraea* genomic DNA was digested with *Eco*RV, *Hin*cII, or *Spe*I, separated on a 1% (w/v) agarose gel, and transferred to a nylon membrane (Hybond-N$^+$; Amersham Pharmacia Biotech). Southern blot analysis was performed according to the method of Sambrook et al. (2001). Hybridization was carried out overnight at 60°C with the probe DNA labeled with [α-$^{32}$P]dCTP by using a Random Primer Labeling kit (Takara, Kyoto, Japan). The membrane was washed in SSC (15 mM sodium citrate, pH 7.0, and 0.15 M NaCl) containing 0.1% (w/v) SDS as follows: 2×SSC at room temperature for 20 min and then twice at 60°C for 20 min. Autoradiography was performed by exposing the membrane to a BAS imaging plate (Fuji Film, Tokyo, Japan) for appropriate lengths of time.

### Northern blot analysis

Cultures of *C. psychrerythraea* were grown under different conditions to the mid-exponential phase ($OD_{600}$ = 0.6 – 0.8) and harvested by centrifugation. Total RNA of this bacterium was prepared by using the RNeasy Total RNA kit (QIAGEN, Hilden, Germany), and successively treated with DNaseI. The isolated RNA (4 µg) was separated on 1.2% (w/v) agarose gels containing 0.66 M formaldehyde and transferred onto a nylon membrane (Hybond-N$^+$). The Northern blot analysis (Sambrook et al. 2001) was performed with the same labeled probe as was used for the Southern hybridization. After hybridization, the membrane was washed in SSC containing 0.1% (w/v) SDS as follows: 2×SSC at room temperature for 20 min, and then twice at 42°C for 20 min. Autoradiography was performed by the same method as for the Southern blot analysis. The signal intensities of the bands corresponding to the *icl* mRNA were estimated with Science Lab99 Image Gauge software ver. 3.4 (Fuji Film, Tokyo, Japan).

### Western blot analysis

After SDS-PAGE of the purified ICL and the sonicated extract of the *C. psychrerythraea* cells, the proteins were transferred onto a nitrocellulose membrane (Hybond-C; Amersham Pharmacia Biotech). Western blot analysis was carried out with the ECL Western blotting detection system (Amersham Pharmacia Biotech) and rabbit antibody against the *C. maris* ICL (Watanabe et al. 2002).

### Chemicals

Peptone was obtained from Kyokuto (Tokyo, Japan). Meat extract and formic acid were from Wako (Osaka, Japan). Phenylhydrazine hydrochloride and DL-isocitrate were purchased from Nacarai Chemicals (Kyoto, Japan). DNase I was the product of Amersham Pharmacia Biotech (Uppsala, Sweden). All restriction endonucleases were obtained from Nippon Gene (Toyama, Japan), Toyobo (Osaka, Japan), or New England Biolabs (MA, USA). Marker nucleotides were obtained from Nippon Gene. [α-$^{32}$P]dCTP was purchased from Moravek (CA, USA). All other reagents were of analytical grade.

## Results

### Purification and determination of partial amino acid sequences of the *C. psychrerythraea* ICL

As described in Materials and methods, the ICL of *C. psychrerythraea* was purified by using essentially the same

procedure as was used previously for the *C. maris* ICL (Watanabe et al. 2001). The results of the purification of this enzyme are summarized in Table 1 . The yield of the purified ICL was high (31.7%). The enzyme was purified almost homogeneously, but there was some contaminating protein in the purified enzyme preparation (Fig. 1A). The purity of the enzyme preparation was evaluated as more than 95% by using a densitometric analysis. As judged from the SDS-PAGE results (Fig. 1A), the molecular mass of the ICL subunit proteins was estimated to be 64 kDa. By using gel filtration, the molecular mass of the enzyme was estimated to be 240 kDa, suggesting that the *C. psychrerythraea* ICL is a homotetramer, the same as its counterpart from other organisms, including *Escherichia coli* (Vanni et al. 1990). The N-terminal amino acid sequence of the purified enzyme was very similar to that of *C. maris*, but was obviously different from those of other organisms (Fig. 2). Some internal amino acid sequences of the ICL were determined by using chemical digestion with BrCN, which digests specifically the C-terminals of the methionine residues in the polypeptide. As shown in Fig. 2, the internal amino acid sequences of the purified *C. psychrerythraea* ICL also showed very high homologies with those of the *C. maris* counterparts but not with ICLs from other organisms, including *E. coli*. Furthermore, an antibody against the *C. maris* ICL was able to cross-react with the purified *C. psychrerythraea* ICL, and the immunological cross-reactivity of these two ICLs was almost same (Fig. 1B). These results indicate that the proteins resembled each other very closely. The purified enzyme was stable at –35°C, and more than 80% of the ICL activity remained after more than 1 year.

## Requirement of divalent cations for the activity of the purified ICL

The effect of divalent cations on the purified ICL activity was examined (Table 2). The *C. psychrerythraea* ICL, like those from other organisms, required the divalent metal cation $Mg^{2+}$ for maximal activity (Vanni et al. 1990), but it was less specific in its requirement since $Mn^{2+}$ and $Co^{2+}$ stimulated the enzyme activity to the same extent as did $Mg^{2+}$.

## Temperature dependence and thermostability of enzyme activity

The activity of the purified ICL was assayed at various temperatures (Fig. 3B). The activity could not be measured at exactly 30°C because the enzyme was markedly thermolabile and was inactivated during the assay. At 35°C, no activity was detected. The apparent optimum temperature for the activity was 25°C, and the $k_{cat}$ value at 20°C (25.2 $s^{-1}$) was higher than that of the *C. maris* ICL (18.1 $s^{-1}$)

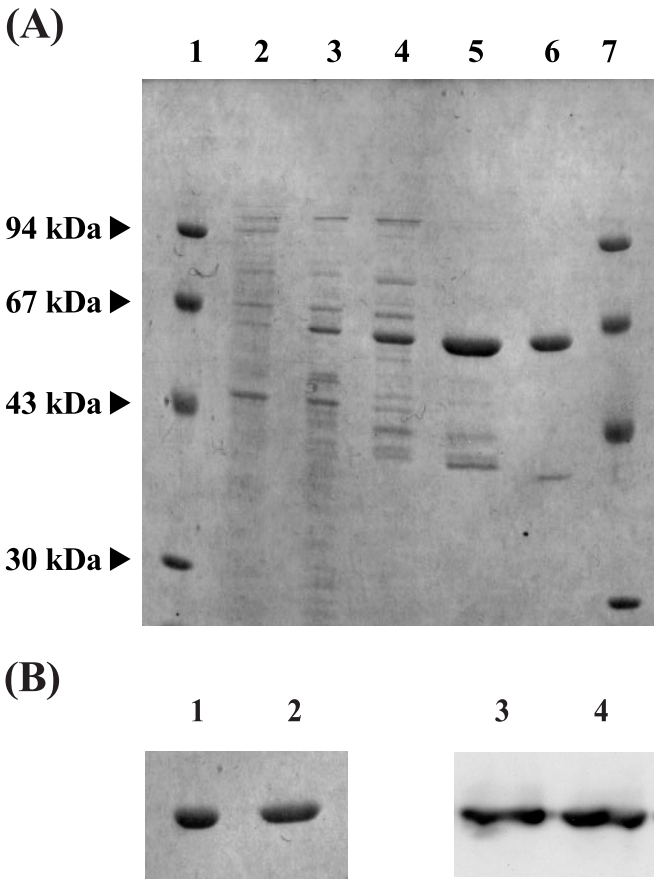


**Fig. 1A,B.** SDS-PAGE (**A**) and immunoblot analysis (**B**) of isocitrate lyase (ICL) from *C. psychrerythraea*. **A** *Lanes 1* and *7*, molecular marker proteins; *lane 2*, cell-free extract (11.1 µg protein); *lane 3*, ammonium sulfate fractionation (10.2 µg); *lane 4*, gel filtration on Sephacryl S-300 (9.6 µg); *lane 5*, anion exchange chromatography on DEAE-cellulose (10.8 µg); *lane 6*, hydrophobic chromatography on phenyl-Sepharose CL-4B (5.6 µg). **B** *Lanes 1* and *3*, the purified *C. maris* ICL (5.5 and 0.2 µg protein, respectively); *lanes 2* and *4*, the purified *C. psychrerythraea* ICL (5.6 and 0.2 µg, respectively). After SDS-PAGE, proteins on the gel were stained with CBB-R250 (*lanes 1* and *2*) or were transferred onto a membrane. Western blotting (*lanes 3* and *4*) was done with antibody against the *C. maris* ICL. The antibody (20 mg/ml) was diluted to 1/2,500

**Table 1.** Purification of isocitrate lyase (ICL) from *C. psychrerythraea*

| Step | Total protein (mg) | Total activity (unit) | Specific activity (unit mg protein$^{-1}$) | Yield (%) | Purification (-fold) |
|---|---|---|---|---|---|
| Cell-free extract | 645 | 56.5 | 0.09 | 100 | 1.0 |
| $(NH_4)_2SO_4$ fractionation | 111 | 42.4 | 0.38 | 75.0 | 4.3 |
| Sephacryl S-300 | 25.9 | 41.5 | 1.60 | 73.5 | 18.2 |
| DEAE-cellulose | 10.2 | 25.1 | 2.47 | 44.4 | 28.0 |
| Phenyl-Sepharose CL-4B | 4.0 | 17.9 | 4.52 | 31.7 | 51.4 |

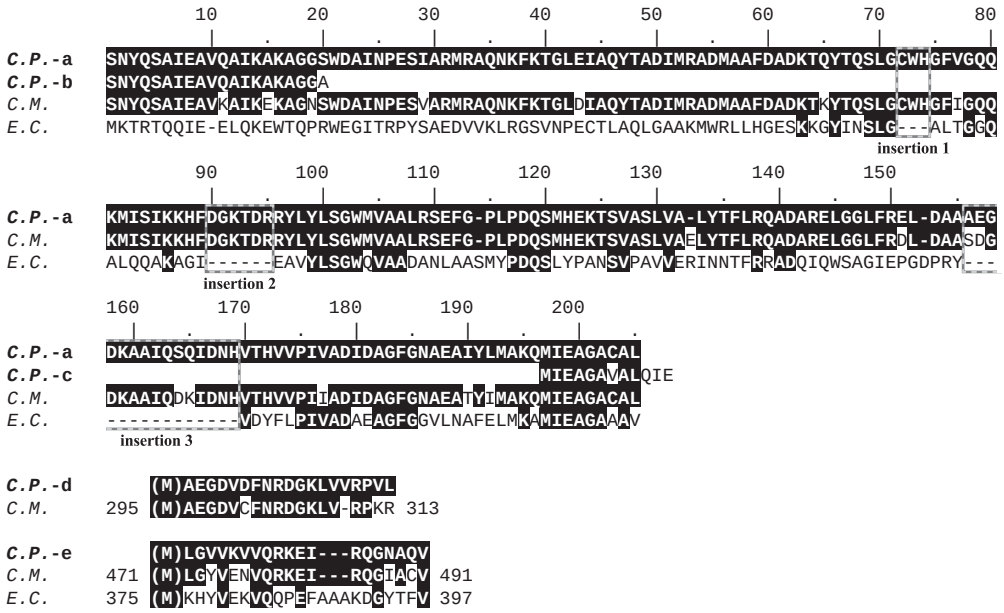


**Fig. 2.** Alignment of partial amino acid sequences of ICLs from *C. psychrerythraea* and other organisms. Amino acid sequences of the *C. psychrerythraea* ICL and residues identical with them are shown by *white letters on black*; *C.P.*, *C. psychrerythraea*; *C.M.*, *C. maris* [DNA Data Bank of Japan (DDBJ) accession number, AB066287]; *E.C.*, *E. coli* (P05313); *C.P.-a*, amino acid sequence deduced from the DNA product amplified by genomic PCR as described in Materials and methods; *C.P.-b*, N-terminal amino acid sequence determined from the purified protein; *C.P.-c–e*, internal amino acid sequences determined by chemical digestion of the purified protein. The *numbers* show the positions relative to the N-terminal of amino acid residues of the *C. maris* ICL

**Table 2.** Requirement of divalent metal cations for ICL activity

| Metal ion | Relative activity (%) |
|---|---|
| None | 0 |
| $MgCl_2$ | 100 |
| $MnCl_2$ | 82.4 |
| $CoCl_2$ | 100 |
| $NiCl_2$ | 23.5 |
| $CaCl_2$ | 0 |

The activity of ICL was measured in the presence of 10 mM of the divalent cations indicated. Enzyme activities were expressed as values relative to the maximum activity (100%) measured in the presence of $Mg^{2+}$

at 20°C, which is the optimum temperature for the *C maris* enzyme (Watanabe et al. 2001).

In addition, we examined the thermostability of the purified ICL by measuring its activity at 20°C after incubation for various lengths of time at 30°C (Fig. 3A). As might be expected, this enzyme was very rapidly inactivated at 30°C, and its half-life for inactivation at this temperature was estimated to be no more than 10 s. The *C. maris* ICL was reported to maintain 26% of its activity after incubation for 2 min at 30°C (Watanabe et al. 2001), whereas no activity of the *C. psychrerythraea* ICL was detected under the same conditions, indicating that the ICL of *C. psychrerythraea* was more thermolabile than that of *C. maris*. NaCl significantly increased the thermostability of the ICL of *C. psychrerythraea* as well as that of other halobacterial enzymes (Ochiai et al. 1979); after incubation for 10 min at 30°C, 73% of the remaining activity was detected in the presence of 0.5 M NaCl. However, even in the presence of NaCl, the thermostability of the *C. psychrerythraea* ICL was lower than that of its *C. maris* counterpart. On the other hand, the ICL activity was inhibited with increasing concentrations of NaCl (data not shown), suggesting that NaCl inversely affects ICL activity and thermostability.

## Kinetic and thermodynamic parameters

The Michaelis-Menten constant ($K_m$) and catalytic efficiency ($k_{cat}/K_m$) of the purified ICL were determined at various temperatures (Fig. 3C). The $K_m$ values for the DL-isocitrate of the ICL were minimum at 15°C (633 μM) and increased with elevating temperature. The maximum $k_{cat}/K_m$ value (29.0 $s^{-1}$ $mM^{-1}$) was observed at 20°C, and 60% of the maximum value was retained at 15°C, which has been reported to be the optimum temperature for growth of *C. psychrerythraea* (Hagen et al. 1964). The thermodynamic parameters of ICL are shown in Table 3. The value for the free energy of activation ($E_a$; 45.4 kJ $mol^{-1}$) of the purified ICL at 20°C, the optimum temperature for the activity, was at the same level as that of the *C. maris* ICL (64.6 kJ $mol^{-1}$) (Watanabe et al. 2001).

## Partial nucleotide sequence of the gene encoding the *C. psychrerythraea* ICL

As a first step toward cloning and sequencing the *icl* gene encoding the *C. psychrerythraea* ICL, genomic PCR was performed with the primers designed as follows. Down-

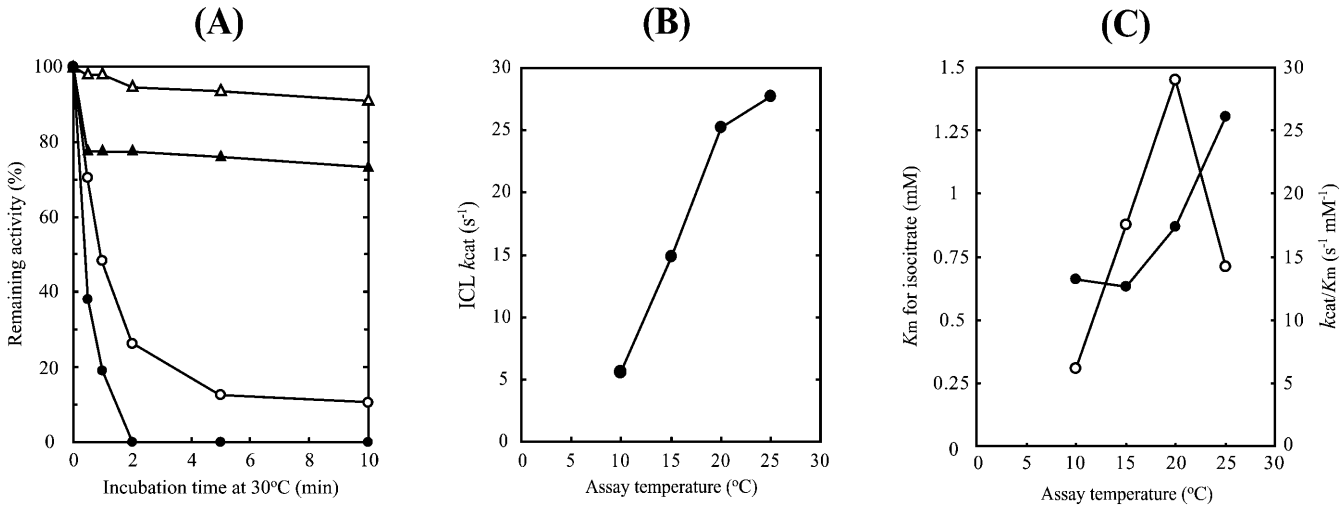


**Fig. 3A–C.** Thermostability (**A**) and effects of temperature on the activity (**B**) and $K_m$ and $k_{cat}/K_m$ (**C**) of the purified ICL. **A** The purified enzymes (*C. psychrerythraea* ICL, *solid symbols*; *C. maris* ICL, *open symbols*) were dialyzed against 20 mM potassium phosphate (pH 6.85) containing 2 mM $MgCl_2$ and 1 mM DTT (*circles*) or the same buffer supplemented with 0.5 M NaCl (*triangles*) for 2 h at 4°C. After incubation for the indicated times at 30°C, the dialyzed enzymes were withdrawn and immediately cooled for 10 min on ice. The remaining enzyme activity was then measured at 20°C. Dialyzed enzyme samples without incubation were used as controls. ICL activities were expressed as values relative to the controls as follows; *C. psychrerythraea* ICL, 5.3 and 6.7 units mg protein$^{-1}$ in the absence and presence of NaCl, respectively; *C. maris* ICL, 4.2 and 5.0 units mg protein$^{-1}$ in the absence and presence of NaCl, respectively. **B** ICL activity ($k_{cat}$) was measured at the temperatures indicated. Other enzyme assay conditions were the same as described in Materials and methods. For the calculation of the $k_{cat}$ value, a molecular mass of 240 kDa was used. The maximum $k_{cat}$ value at 25°C was 27.7 s$^{-1}$. **C** *Solid circles*, $K_m$; *open circles*, $k_{cat}/K_m$. To prevent heat inactivation of the enzymes, the reaction mixture without enzyme was preincubated for appropriate times for temperature equilibration. The reaction was started by the addition of the purified ICL (4.5 µg protein) and isocitrate. All values were the averages of at least two independent experiments

**Table 3.** Thermodynamic parameters of ICL

| | 15°C | 20°C |
|---|---|---|
| *E*a (kJ mol$^{-1}$) | 45.4[a] | |
| $\Delta H^{\ddagger}$ (kJ mol$^{-1}$) | 43.0 | 43.0 |
| $\Delta S^{\ddagger}$ (kJ K$^{-1}$ mol$^{-1}$) | –78.6 | –77.0 |
| $\Delta G^{\ddagger}$ (kJ mol$^{-1}$) | 65.6 | 65.5 |

[a]The activation energy (*E*a) was calculated from the approximate straight line of 1/T versus log $k_{cat}$ between 10° and 20°C. The $k_{cat}$ values at 15° and 20°C were 14.8 and 25.2 s$^{-1}$, respectively. The gas constant (R) was 8.31 J K$^{-1}$ mol$^{-1}$

$\Delta H^{\ddagger}$, activation enthalpy; $\Delta S^{\ddagger}$, activation entropy; $\Delta G^{\ddagger}$, activation free energy

stream primers were designed from the amino acid sequence of the region of the *C. maris* ICL protein (KQCGHQDG) corresponding to the regions highly conserved among ICLs from other organisms (KKCGHMA) because the determined N-terminal and internal amino acid sequences of the purified *C. psychrerythraea* ICL showed higher similarities to those of the *C. maris* ICL. On the other hand, upstream primers were designed from the N-terminal amino acid sequence of the purified *C. psychrerythraea* ICL protein determined as described in Materials and methods. About 660 bp of the resultant PCR product was sequenced. The determined nucleotide sequence (*C.P.*-a in Fig. 2) showed good agreement (92%) with that of the corresponding region of the *C. maris* ICL and contained one of the internal amino acid sequences determined by the digestion of the purified ICL with BrCN (*C.P.*-c in Fig. 2). These results indicate that the PCR product is a part of the *C. psychrerythraea icl* gene. Genomic Southern hybridization with this fragment as a probe showed that each single fragment of 2.9, 5.5, and 3.1 kbp, which resulted from digestions of the *C. psychrerythraea* genomic DNA with *Eco*RV, *Hin*cII, and *Spe*I, respectively, hybridized with this probe (data not shown).

## Expression of the *C. psychrerythraea icl* gene under various conditions

To evaluate the expression of the *C. psychrerythraea icl* gene, a Northern blot analysis was carried out with the PCR product as a probe and RNA isolated from the *C. psychrerythraea* cells grown under different conditions (Fig. 4A). Expression of the *icl* gene was induced when the cells were grown in the presence of acetate, particularly when the medium was supplemented with acetate only, at all temperatures tested, indicating that this gene was acetate inducible. Induction of gene expression was also observed by reducing the growth temperature from 15° to 10°C regardless of carbon source(s) in the growth media. In the cultures grown on peptone, ICL expression at 5°C was higher than that at 10°C, but not when there was acetate in the growth medium. The amount of the *C. psychrerythraea* ICL in sonic extracts of cells grown under various conditions was examined by Western blot analysis with the antibody against the *C. maris* ICL (Fig. 4B). Induction of gene

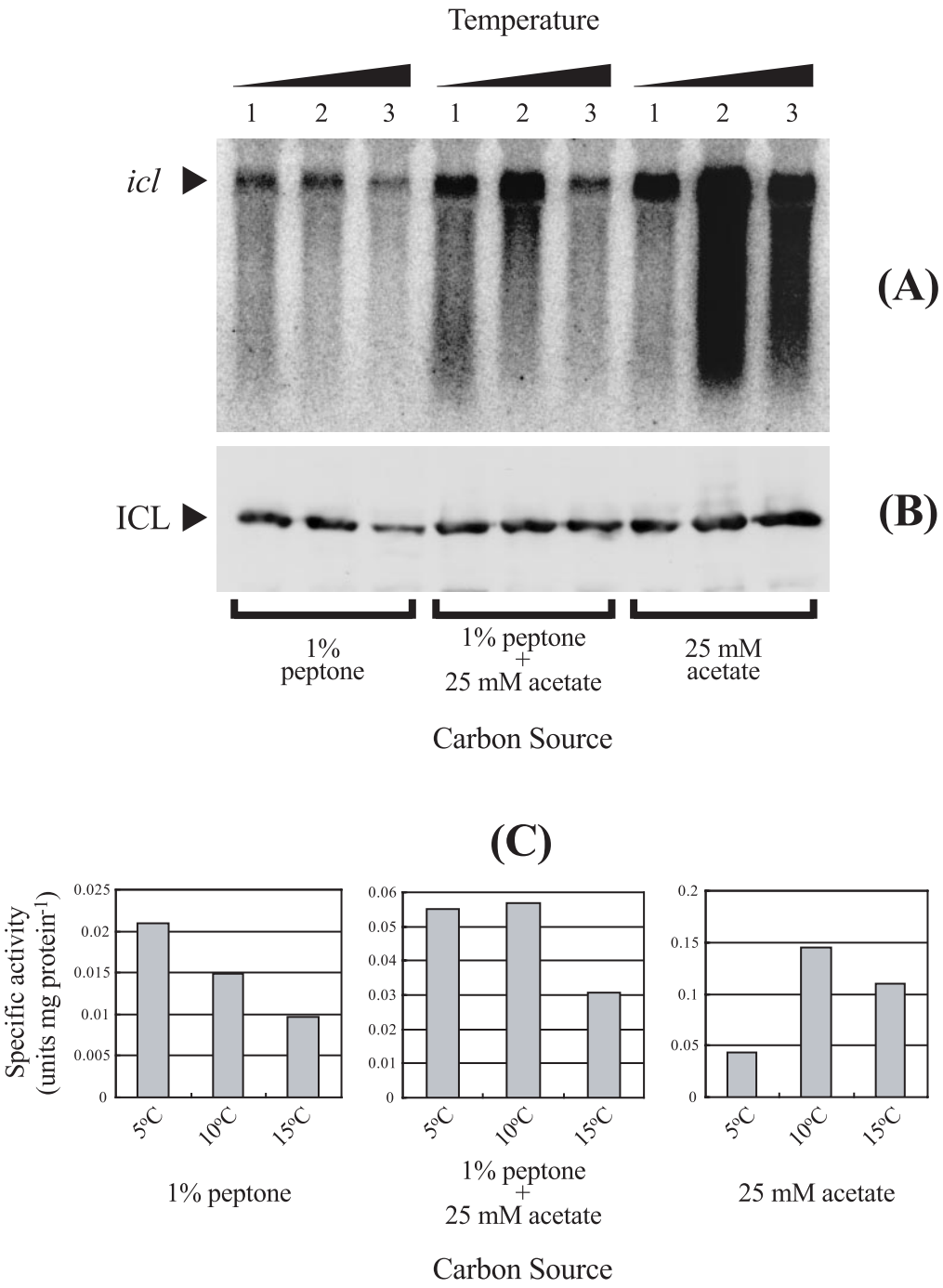


**Fig. 4A–C.** Expression analyses of the *icl* gene at transcriptional, translational, and enzyme activity levels. **A** Northern hybridization. After electrophoresis of total RNA (4 µg per lane) of *C. psychrerythraea* cells grown at 5° (*lane 1*), 10° (*lane 2*), and 15°C (*lane 3*) in nutrient medium containing the indicated carbon source(s), the RNA was blotted onto a nylon membrane and hybridized with the radiolabeled probe as described in Materials and methods. **B** Effects of growth temperature and carbon source on the translation of the *icl* gene. Cell-free extract of cells grown on the carbon source(s) indicated (10 µg protein) was applied to each lane. A Western blot analysis was performed as described in Materials and methods and in the legend to Fig. 1B. **C** The ICL activity in the cell-free extract

expression by acetate and low temperatures was also observed at the translational level, but the intensity of the induction was lower than that at the transcriptional level. Furthermore, similar results were also obtained by measuring ICL activities in the sonic extracts (Fig. 4C).

## Discussion

We previously reported that the psychrophilic bacterium *C. maris* possesses a cold-adapted ICL and that expression of the *icl* gene encoding this enzyme was induced by low temperatures (Watanabe et al. 2001, 2002). In this paper, we showed that ICL from the psychrophilic *C. psychrerythraea*, which is closely related to *C. maris*, was also thermolabile and that the *icl* gene was cold-inducible.

The subunit molecular mass of the purified ICL (64 kDa) was larger than those of typical bacterial ICLs (approximately 46–48 kDa), but was same as that of the *C. maris* ICL (Fig. 1B). The *C. maris* ICL has six unique insertions of 3–36 amino acid residues that are lacking in other bacterial ICLs (Fig. 2), and their possible contribution to the marked thermolability of this ICL as a result of the enhanced interac-

tions of the enzyme with the solvent has been reported (Watanabe et al. 2002). Partial amino acid sequences of the *C. psychrerythraea* ICL in the N-terminal and internal regions showed very high homology to those of the *C. maris* ICL, and the predicted amino acid sequence of the DNA fragment of *C. psychrerythraea* amplified by genomic PCR included these unique insertions at the same positions as in the *C. maris* ICL (Fig. 2). Furthermore, one of the internal amino acid sequences determined from the purified *C. psychrerythraea* ICL (*C.P.*-d in Fig. 2B) did not align with those of bacterial ICLs other than that of *C. maris*, because it corresponded to a part of unique insertion 4 of the *C. maris* ICL. These results suggest that the amino acid sequence of the *C. psychrerythraea* ICL has a high homology over the whole protein with that of the *C. maris* ICL and that those unique insertions of amino acid residues that are present in the *C. maris* ICL are also present in the *C. psychrerythraea* ICL. Thus, these unique insertions might also be responsible for the marked thermolability of the *C. psychrerythraea* ICL.

Catalytic rate constant ($k_{cat}$) values of the enzymes from cold-adapted organisms show a high degree of temperature compensation compared with those of orthologs from their warm-adapted counterparts; at any assay temperature, the $k_{cat}$ values of cold-adapted enzymes tend to be higher than those of warm-adapted ones (Fields and Somero 1998). We recently purified the ICL of *E. coli* overexpressed in cells and examined the effect of temperature on the *E. coli* ICL activity (unpublished data). The $k_{cat}$ value of the *C. psychrerythraea* ICL (25.2 $s^{-1}$) was 1.7-fold higher at 20°C than that of the *E. coli* ICL (15.2 $s^{-1}$), and the relative activity of *C. psychrerythraea* ICL at 20°C (91.0% of the maximum activity) was 3.6-fold higher than that of *E. coli* ICL (25.5% of the maximum activity at 45°C) at the same temperature, suggesting that the former ICL adapts to low temperature better than its mesophilic counterpart. On the other hand, the Michaelis-Menten constant ($K_m$) of the *C. psychrerythraea* ICL (633 µM at 15°C) was significantly higher than those of ICLs from *E. coli* (130 µM at 37°C), *Corynebacterium glutamicum* (280 µM at 40°C), and *Mycobacterium tuberculosis* (145 µM at 37°C) (Diehl and McFadden 1994; Reinscheid et al. 1994; Höner zu Bentrup et al. 1999), but almost same as that of the *C. maris* ICL (510 µM at 20°C, Watanabe et al. 2001). It has been reported that $K_m$ values of cold-adapted enzymes tend to be higher than those of their mesophilic and thermophilic counterparts because high flexibilities are necessary for the accommodation of the enzymes with their substrates at low temperatures but result in poor binding to the ligand (Fields and Somero 1998). However, the $K_m$ value of the *C. psychrerythraea* ICL was lowest at 15°C, the optimum temperature for growth of this bacterium (Fig. 3C); this result is consistent with results of an extensive study that showed that the $K_m$ values of pyruvate kinases of fish and crabs were minimum at their living temperatures (Hochachka and Somero 1984). These findings suggest that this bacterial ICL also adapts to low temperatures with respect to its affinity for the substrate as well as its catalytic activity. The adaptation of intracellular enzymes to low temperatures can be achieved by the following three strategies: an increase in the $k_{cat}$ value, a decrease in the $K_m$ value, or a combination of these two changes (Feller and Gerday 1997). The *C. psychrerythraea* ICL may thus reflect the first strategy.

It is noteworthy that the expression of the gene encoding the cold-adapted ICL of this bacterium is induced by low temperatures, but its physiological significance is probably different from that of the genes for cold-shock proteins such as the Csp family proteins, CsdA, RbfA, and NusA (Thieringer et al. 1998). These cold-shock proteins are known to function as RNA chaperones and to be involved in the formation of cold-acclimated ribosomes. The induction of expression by low temperature of genes encoding metabolic enzymes such as ICL has been little studied in psychrophiles, and we suggest that not only the molecular mechanism for cold adaptation of enzyme proteins but also the cold-inducible expression of genes for cold-adapted proteins may be essential for survival in a permanently cold environment.

The ICLs of both psychrophilic bacteria, *C. psychrerythraea* and *C. maris,* are cold-adapted enzymes, and the expression of both *icl* genes for these ICLs are induced by low temperature. These findings indicate that the reaction step catalyzed by ICL may be generally essential for acetate metabolism in a cold environment. The shift of growth temperature from 10° to 5°C decreased the expression of the *icl* gene in the presence of acetate, while expression was slightly increased in the absence of acetate (Fig. 4). Coincidentally, when *C. psychrerythraea* was grown on acetate, the growth rate at 5°C was markedly less than those at 10° and 15°C (data not shown). Furthermore, the exhaustion of succinate in the growth medium was found to be necessary to induce the expression of the *C. maris icl* gene by low temperature, but the cause remains unclear (Watanabe et al. 2002). These findings suggest that the regulation of the isocitrate flux between the tricarboxylic acid (TCA) and glyoxylate cycles is complex and dependent on both the carbon source and temperature in psychrophilic *Colwellia* species. A promoter analysis of the *C. psychrerythraea icl* gene should give more detailed insight into the mechanism of induction by low temperature. Therefore, we are currently attempting to clone and sequence the complete *icl* gene from *C. psychrerythraea*.

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