Extremophiles (2008) 12:107–117
DOI 10.1007/s00792-007-0115-9

ORIGINAL PAPER

Gene cloning of cold-adapted isocitrate lyase from a psychrophilic bacterium, *Colwellia psychrerythraea*, and analysis of amino acid residues involved in cold adaptation of this enzyme

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Received: 4 July 2007 / Accepted: 20 August 2007 / Published online: 27 October 2007
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Abstract The gene (*icl*) encoding cold-adapted isocitrate lyase (ICL) of a psychrophilic bacterium, *Colwellia psychrerythraea*, was cloned and sequenced. Open reading frame of the gene was 1,587 bp in length and corresponded to a polypeptide composed of 528 amino acids. The deduced amino acid sequence showed high homology with that of cold-adapted ICL from other psychrophilic bacterium, *C. maris* (88% identity), but the sequential homology with that of the *Escherichia coli* ICL was low (28% identity). Primer extension analysis revealed that transcriptional start site for the *C. psychrerythraea icl* gene was guanine, located at 87 bases upstream of translational initiation codon. The expression of this gene in the cells of an *E. coli* mutant defective in ICL was induced by not only low temperature but also acetate. However, *cis*-acting elements for cold-inducible expression known in the several other bacterial genes were absent in the promoter region of the *C. psychrerythraea icl* gene. The substitution of Ala214 for Ser in the *C. psychrerythraea* ICL introduced by point mutation resulted in the increased thermostability and lowering of the specific activity at low temperature, indicating that Ala214 is important for psychrophilic properties of this enzyme.

Keywords Psychrophlic bacterium · *Colwellia psychrerythraea* · Isocitrate lyase · Cold-adapted enzyme · Cold-inducible gene expression

Communicated by K. Horikoshi.

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Introduction

Temperature, especially low temperature, has important effects on growth of living organisms and defines their growth limits because the catalytic activities of enzymes in the cells are strongly dependent on temperature. Nevertheless, microorganisms called as psychrophilic bacteria can survive and live even in permanently cold environment on Earth. The cold-adapted enzymes, which exhibit higher catalytic activity at low temperature and lower thermostability than the counterparts from mesophilic and thermophilic bacteria, are one of critical mechanisms for environmental adaptation of these bacteria because such enzymes can compensate the decrease of catalytic function at low temperature. It has been reported that these common properties of cold-adapted enzymes are attributable to their pronounced structural flexibility, which makes them possible to accomplish easy binding of the substrates to their active sites at low temperature and rapid conformational changes for the catalysis without large energetic loss (Gerday et al. 1997; Fields and Somero 1998; Geolette et al. 2003; Cavicchioli et al. 2002).

Isocitrate lyase (ICL, EC 4.1.3.1) catalyses the cleavage of isocitrate to glyoxylate and succinate, the first step in the glyoxylate cycle designated by Kornberg and Krebs (1957). Then, the second enzyme of this cycle, malate synthase, serves the conversion of glyoxylate and acetyl-CoA to

malate. Thus, the glyoxylate cycle can bypass several reactions for the formation of succinate and malate from isocitrate in the TCA cycle and is known to be important for the metabolism of acetate and fatty acids in plants and microorganisms (Kornberg 1966; Vanni et al. 1990; Cozzone 1998). Furthermore, ICL is known to be highly induced when microorganisms are grown on acetate (Vanni et al. 1990).

It has been reported that ICL (*Cp*ICL) of a psychrophilic bacteria, *Colwellia psychrerythraea* (D'Aoust and Kushner 1972; Deming et al. 1988), exhibits several features as a cold-adapted enzyme, as well as the corresponding enzyme (*Cm*ICL) from *Colwellia maris* (Takada et al. 1979; Yumoto et al. 1998), another psychrophilic bacterial species of the same genus (Watanabe et al. 2001, 2002a, b). Namely, the *Cp*ICL showed maximum activity at 25°C and completely inactivated even by brief incubation for 2 min at 30°C (Watanabe et al. 2002b). Furthermore, the maximum catalytic efficiency of this enzyme was observed at 20°C. Preliminary experiments revealed that the expression of gene encoding this enzyme may be induced by low temperature.

In this study, we cloned the gene encoding the cold-adapted *Cp*ICL and examined effect of sodium acetate and temperature on the gene expression. In addition, to obtain information on the molecular mechanisms for high catalytic function of the cold-adapted ICL at low temperature, several point mutations were introduced into the *icl* gene and several properties of the mutated enzymes were examined.

Materials and methods

Bacteria, plasmids and culture conditions

The psychrophilic *C. psychrerythraea* NRC 1004 (D'Aoust and Kushner 1972; Deming et al. 1988) was grown at 15°C with vigorous shaking in a nutrient medium consisting of 1% meat extract, 40 mM $MgCl_2$, 3% NaCl and 25 mM sodium acetate as a carbon source. *Escherichia coli* XL1-Blue (Stratagene) and TOP10 (Invitrogen) were used for the propagation of plasmids and the production of His-tagged recombinant ICL proteins from expression vectors, respectively. A mutant defective in ICL of *E. coli*, ACA421 (Watanabe et al. 2002a), and its parent strain, KM22 (a Δ*recBCD* mutant; Kenan 1998), were used in the experiments for *icl* gene expression. Unless otherwise stated, these *E. coli* strains were grown at 37°C with vigorous shaking in Luria–Bertani (LB) medium (Sambrook and Russell 2001). Plasmids pBluscript KS(+) (pBS; Stratagene) and pTrcHisB (Invitrogen) were used as vectors for cloning the *icl* gene and conferring N-terminal $(His)_6$-tag on the expressed proteins, respectively.

Cloning of the *C. psychrerythraea icl* gene

Genomic PCR was performed to obtain a nucleotide probe for *icl* gene encoding the *Cp*ICL. Upstream primer, 5′-TCNAAYTAYCARAGYGCNATHGARGC-3′, was designed from SNYQSAIEA, the sequence between the first and ninth amino acid residues from the N-terminal of *Cp*ICL (Watanabe et al. 2002b). Downstream primer, 5′-CCRTCYTGRTGNCCRCAYTGYTT-3′, was designed from KQCGHQDG, the amino acid sequence of the *Cm*ICL corresponding to highly conserved regions of the *icl* genes cloned from various organisms (Watanabe et al. 2002a). Chromosomal DNA of *C. psychrerythraea* was purified as described previously (Ishii et al. 1993). Amplification was carried out for 30 cycles in a DNA thermal cycler 2400 (Perkin–Elmer) in a 50 µl of reaction mixture containing 1.4 µg genomic DNA, 10 pmol each upstream and downstream primer and 1 unit KOD-plus DNA polymerase (Toyobo) in a buffer system prepared by the manufacturer. Cycling conditions were as follows: denaturation at 94°C for 15 s, annealing at 50°C for 30 s and extension at 68°C for 1 min, for each of 30 cycles. PCR products with a predicted length of about 700 bp were purified and ligated to the *Sma*I site of pBS. Nucleotide sequences of the PCR products were determined by using a BigDye terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems) with an ABI PRISM Genetic Analyzer 310. Plasmid carrying the 668 bp PCR product with a nucleotide sequence similar to the *C. maris icl* gene was termed pCP10. This insert DNA was amplified by PCR with pCP10 as template DNA and was used as probe for genomic Southern and colony hybridizations.

Genomic Southern hybridization was carried out as described previously (Watanabe et al. 2002b) with some modifications. The chromosomal DNA of *C. psychrerythraea* digested with several restriction enzymes was separated by 1% agarose gel electrophoresis and the DNA fragments on the gel were transferred onto nylon membrane, Hybond-N^+ (Amersham Pharmacia Biotech). Hybridization was carried out overnight at 60°C with the probe labeled with [α-^{32}P]dCTP and a random primer labeling kit (Takara). The blotted membrane was washed successively in 2× SSC (1× SSC is composed of 15 mM sodium citrate pH 7.0 and 0.15 M NaCl) containing 0.1% (w/v) SDS for 10 min at room temperature and then twice with the same solution for 20 min at 60°C. About 3 kbp of *Spe*I fragments hybridized with the probe were cloned into pBS and the resultant plasmids were transformed into *E. coli* XL1-Blue. Colony hybridization was then carried out by using the same probe as Southern hybridization. The blotted membranes were washed as described above. A colony with strongest signal was selected, and the plasmid in this colony, termed pCPB49, was propagated and purified.

The nucleotide sequence of the insert DNA in the pCPB49 was determined in both directions by using appropriate primers according to the method described above and was analyzed with the Genetyx computer program (Software Development Co.). The nucleotide sequence of the *C. psychrerythraea icl* gene has been deposited in the DDBJ database under accession no. AB174852.

Western blot analysis

After SDS-PAGE of the purified ICLs and the cell-free extracts of the *E. coli* cells on 10% polyacrylamide gels (Laemmli 1970), the proteins were transferred onto a nitrocellulose membrane, Hybond-ECL (Amersham Bioscience). Western blot analysis was carried out with the ECL Western blotting detection system (Amersham Bioscience) and rabbit antibody against the purified *Cm*ICL (Watanabe et al. 2002a).

Primer extension analysis

Primer extension analysis was performed as described by Sambrook and Russell (2001) with the following modifications. A synthetic 29-mer oligonucleotide, 5′-TTGAATT TGTTCTGTGCACGCATACGGGC-3′, complementary to the sequence between +91 and +119 from the translation start codon of the *C. psychrerythraea icl* gene, was used as the primer. The 5′-terminal of the primer was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Total RNA (20–50 μg) isolated from *C. psychrerythraea* was incubated overnight at 42°C with the labeled primer in the hybridization buffer. The extension was done with a reverse transcriptase (ReverTra Ace, Toyobo). The reaction products were analyzed on a 6% polyacrylamide sequencing gel with a sequencing ladder of the *C. psychrerythraea icl* gene. Sequencing was performed with the T7 Sequencing Kit (USB) with the same primer as the primer extension reaction.

Construction of expression vector for His-tagged ICL of *C. psychrerythraea* and site-directed mutagenesis

By PCR, restriction sites for *Bam*HI and *Sac*I were introduced at 5′- and 3′-terminals of the ORF of *C. psychrerythraea icl* gene, respectively. For this purpose, the following two primers were synthesized: forward primer 5′-gcgcggatccGTCTAATTATCAGAG-3′ and reverse primer 5′-gcgcgagctcTTAACTGAACTGGTTAG-3′. Small letters indicate additional bases for introducing the underlined cleavage sites of the respective restriction enzymes. The amplification was carried out for 30 cycles in the DNA thermal cycler in a reaction mixture (50 μl) containing 180 ng pCPB49 as template DNA, 10 pmol each forward and reverse primer and 1 unit KOD-plus DNA polymerase in a buffer system prepared by the manufacturer. Cycling conditions were as follows: denaturation at 94°C for 15 s, annealing at 50°C for 30 s, and extension at 68°C for 2 min. After the digestion with *Bam*HI and *Sac*I, the PCR product was ligated to the *Bam*HI–*Sac*I site of pTrcHisB to obtain plasmid pHis-*Cp*WT. Precise insertion of the *C. psychrerythraea icl* gene into the plasmid vector was verified by nucleotide sequencing.

Site-directed mutagenesis was carried out by a standard PCR method with synthetic primers (Table 1). The PCR was performed as described above. Resultant PCR products were ligated into pTrcHisB to obtain the mutated plasmids, pHisQ47K, pHisA214S, pHisA231E, pHisF333L and pHisA341N (pHisQ47K denotes pHis-*Cp*WT introduced the substitutional mutation of Gln47 by Lys in the *Cp*ICL). The introduced mutations were checked by nucleotide sequencing the relevant regions of plasmids as described above.

Table 1 Oligonucleotides used in site-directed mutagenesis

Plasmid	Substitution	Primers	
pHisQ47K	Gln47 → Lys	Forward	5′-GGTCTAGAGATAGCTAAGTACACTGCAG-3′
		Reverse	5′-CTGCAGTGTACTTAGCTATCTCTAGACC-3′
pHisA214S	Ala214 → Ser	Forward	5′-CGAAAACCAAGTATCTGATGAAAAGC-3′
		Reverse	5′-GCTTTTCATCAGATACTTGGTTTTCG-3′
pHisA231E	Ala231 → Glu	Forward	5′-GTAACTGTTCCTCATGAAGATTTCCACTC-3′
		Reverse	5′-GAGTGGAAATCTTCATGAGGAACAGTTAC-3′
pHisF333L	Phe333 → Leu	Forward	5′-GAGCGTTGTGTACTTGACTGTATCGGTGC-3′
		Reverse	5′-GCACCGATACAGTCAAGTACACAACGCTC-3′
pHisA341N	Ala341 → Asn	Forward	5′-CGGTGCACTTAATAATGGCGCTGATTTAC-3′
		Reverse	5′-GTAAATCAGCGCCATTATTAAGTGCACCG-3′

The underlined letters indicate the codons of amino acid residues substituted by site-directed mutagenesis

Overexpression and purification of His-tagged ICLs

E. coli TOP10 transformed with pHis-*Ec*WT (plasmid for His-tagged *E. coli* ICL), pHis-*Cp*WT or the plasmids carrying the mutated *icl* genes was grown at 37°C in Super broth medium [12 g tryptone, 24 g yeast extract, 5 ml glycerol, 3.81 g KH_2PO_4 and 12.5 g K_2HPO_4 per liter (pH 7.0)] containing 50 mg/l ampicillin until OD_{600} of the culture reached 0.6. The cultures were rapidly cooled on ice and were further incubated for 16–24 h at 15°C after the addition of 1 mM isopropyl-β-thiogalactopyranoside (IPTG) to induce the expression of His-tagged ICL proteins. Because of the marked thermolability of *Cp*ICL (Watanabe et al. 2002b), the induction of gene expression by IPTG were carried out at 15°C. As described previously (Watanabe and Takada 2004), cell-free extracts were prepared and His-tagged ICLs were then purified by column chromatography of Ni-NTA agarose (Qiagen). The purified His-tagged ICLs were concentrated with polyethylene glycol #20,000 and dialyzed against 20 mM potassium phosphate (pH 6.85), containing 2 mM $MgCl_2$, 4 mM DL-isocitrate, 10% (v/v) glycerol and 1 mM dithiothreitol (DTT). All His-tagged ICLs were stocked at –35°C until use.

Enzyme assay

The ICL activity was assayed at pH 6.85 by measuring the increase of absorbance at 324 nm due to the formation of glyoxyl-phenylhydrazone as described previously (Watanabe et al. 2001). The reaction mixture contained 66.7 mM potassium phosphate buffer (pH 6.85), 5 mM $MgCl_2$, 3.3 mM phenylhydrazine–HCl, 2 mM cysteine–HCl, 1.67 mM sodium isocitrate and an appropriate amount of enzyme in a final volume of 3 ml. The reaction was started by the addition of enzyme (50 µl) into the mixture previously equilibrated at assay temperatures. One unit of the activity was defined as the formation of 1 µmol of product per min.

Molecular modeling of *C. psychrerythraea* ICL

The three-dimensional structural model of *Cp*ICL was built using ICL from *E. coli* (PDB No. 1IGW) as a homology model with a homology modeling program (http://www.cbs.dtu.dk/services/CPHmodels/index.php).

Results

Cloning of the *C. psychrerythraea icl* gene

By genomic PCR described in Materials and methods, the 668 bp DNA fragment was obtained and then sequenced. The deduced amino acid sequence was consistent with the partial amino acid sequence of *C. psychrerythraea* ICL protein reported previously (Watanabe et al. 2002b). Genomic Southern blot analysis using this PCR product as probe revealed that about 3 kbp of single fragment of the *C. psychrerythraea* genomic DNA digested with *Spe*I hybridizes with the probe. Therefore, for cloning of the *icl* gene, the genomic library was constructed with pBS carrying the similar size of *Spe*I-digests of the *C. psychrerythraea* genomic DNA and was screened with the same probe. A clone with strongest signal was selected and the plasmid within the clone (designated as pCPB49) was purified (Fig. 1).

Nucleotide and deduced amino acid sequences of the *C. psychrerythraea icl* gene

Both strands of the insert DNA subcloned in the plasmid pCPB49 were sequenced (Fig. 2). This insert contained an ORF with full length of 1,587 bp, and a putative ribosome-binding site (Shine and Dalgano 1974), GGAG, was found 7–10 bases upstream of the ATG codon. The ORF encodes a polypeptide of 528 amino acids, and the molecular mass was calculated as 58,131 Da. This value was compatible with that of the purified ICL from this bacterium estimated by gel filtration (homotetramer of the 60 kDa subunit) (Watanabe et al. 2002b). Previously, we reported that bacterial ICLs can be classified into two phylogenetic groups, subfamilies 1 and 3, based on their amino acid sequences (Watanabe and Takada 2004). The deduced amino acid sequence showed 24–29% identities with subfamily 1 ICLs from many bacteria, including the *E. coli*

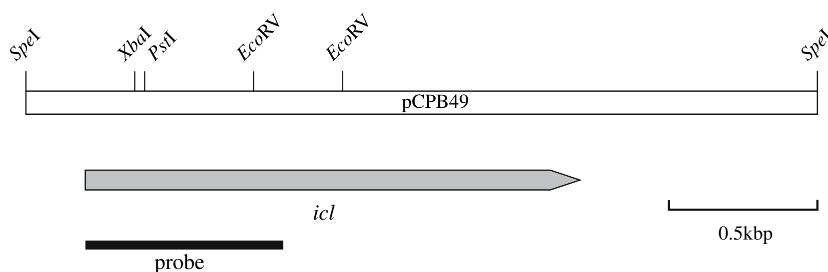

Fig. 1 Physical and restriction map of the genomic DNA fragment containing the *C. psychrerythraea icl* gene. The *black bar* indicates the 668 bp DNA fragment used as the probe for colony hybridization

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   1 CTAGTAAAAATAAGTTTAACTAATTGTTTTTAAATACTTTAAAGTAATATTTATTTGATTTTTGTTTTGGTATTTTCTGTGTGAATGGTG   90

  91 GTAATCATCACTATATATTTCCTAAATACATGGTTAGTAGACTATTATTTTTGCTGTCTAGTGCGAAGACTTAAAAAGTCTGTTACACCA  180
                       -35                     -10

 181 ACCAAGCTAATATAGCTTTTATATACTACAAACAAAGTAAATGGAGATTGATATGTCTAATTATCAGAGTGCAATAGAAGCAGTTCAAGC  270
                                               SD       M  S  N  Y  Q  S  A  I  E  A  V  Q  A

 271 GATCAAAGCAAAAGCCGGTAGCTCTTGGGATGCTATAAACCCAGAATCAATTGCCCGTATGCGTGCACAGAACAAATTCAAAACTGGTCT  360
      I  K  A  K  A  G  S  S  W  D  A  I  N  P  E  S  I  A  R  M  R  A  Q  N  K  F  K  T  G  L

 361 AGAGATAGCTCAGTACACTGCAGACATTATGCGTGCTGATATGGCTGCATTTGATGCAGACAAGACTCAATATACACAATCTCTAGGTTG  450
      E  I  A  Q  Y  T  A  D  I  M  R  A  D  M  A  A  F  D  A  D  K  T  Q  Y  T  Q  S  L  G  C

 451 TTGGCATGGTTTTGTTGGCCAACAAAAAATGATTTCTATCAAAAAACATTTTGATGGTAAAACAGACCGTCGTTATTTATATCTTTCAGG  540
      W  H  G  F  V  G  Q  Q  K  M  I  S  I  K  K  H  F  D  G  K  T  D  R  R  Y  L  Y  L  S  G

 541 TTGGATGGTTGCTGCACTTCGCAGTGAATTTGGTCCTCTTCCTGATCAATCTATGCATGAAAAAACGTCGGTAGCCTCTTTAGTTGCTGA  630
      W  M  V  A  A  L  R  S  E  F  G  P  L  P  D  Q  S  M  H  E  K  T  S  V  A  S  L  V  A  E

 631 GCTTTATACTTTCTTACGTCAAGCTGACGCACGTGAATTAGGCGGTCTTTTCCGTGAACTAGATGCAGCTGCTGAAGGTGATAAAGCTGC  720
      L  Y  T  F  L  R  Q  A  D  A  R  E  L  G  G  L  F  R  E  L  D  A  A  A  E  G  D  K  A  A

 721 TATCCAATCACAGATTGATAACCATGTTACTCACGTTGTGCCAATTGTTGCTGATATCGATGCTGGTTTTGGTAACGCTGAAGCGACTTA  810
      I  Q  S  Q  I  D  N  H  V  T  H  V  V  P  I  V  A  D  I  D  A  G  F  G  N  A  E  A  T  Y

 811 CTTAATGGCTAAGCAAATGATTGAAGCAGGTGCTTGTGCGCTTCAAATCGAAAACCAAGTAGCTGATGAAAAGCAATGTGGTCATCAAGA  900
      L  M  A  K  Q  M  I  E  A  G  A  C  A  L  Q  I  E  N  Q  V  A  D  E  K  Q  C  G  H  Q  D

 901 TGGCAAAGTAACTGTTCCTCATGCTGATTTCCACTCTAAAATTCGTGCATTACGCCATGCATTTTTAGAACTAGGTATAGACAACGGTCT  990
      G  K  V  T  V  P  H  A  D  F  H  S  K  I  R  A  L  R  H  A  F  L  E  L  G  I  D  N  G  L

 991 TATCGTTGCACGTACTGACTCTGAAGGTGCTGGTTTGACTAAAGAAATCGCTGTTGTTAAAGAGCCTGGTGATTCTGGCGATATCTATAA 1080
      I  V  A  R  T  D  S  E  G  A  G  L  T  K  E  I  A  V  V  K  E  P  G  D  S  G  D  I  Y  N

1081 CTCTTATTTAGATGTTGAAGAAATCAGCGCAGCTGATATGGCTGAAGGCGATGTATGCTTTAATCGTGACGGTAAATTAGTTCGTCCTAA 1170
      S  Y  L  D  V  E  E  I  S  A  A  D  M  A  E  G  D  V  C  F  N  R  D  G  K  L  V  R  P  K

1171 GCGTTTACCTTCAGGTTTATACCAATTCCGTCAAGGTACTGGTCATGAGCGTTGTGTATTTGACTGTATCGGTGCACTTAATGCTGGCGC 1260
      R  L  P  S  G  L  Y  Q  F  R  Q  G  T  G  H  E  R  C  V  F  D  C  I  G  A  L  N  A  G  A

1261 TGATTTACTTTGGATTGAAACGGCAGTGCCAACGGTACACGAAATTGCTGGTATGATGGATGACGTTCGTAAAGTTCACCCAGATGCGAA 1350
      D  L  L  W  I  E  T  A  V  P  T  V  H  E  I  A  G  M  M  D  D  V  R  K  V  H  P  D  A  K

1351 ACTTGTTTATAACAACTCTCCATCTTTTAACTGGACACTTAATTTCCGTCAGCAAGCATATGACGCAATGGTTGAAGCGGGTCAAGATGT 1440
      L  V  Y  N  N  S  P  S  F  N  W  T  L  N  F  R  Q  Q  A  Y  D  A  M  V  E  A  G  Q  D  V

1441 ATCTGCATACGTTCGTGCTGACTTAATGAAAGCTGAATATGACGAAACTGAATTGTCTGCAACAGCTGATGAACGCATTCGTACTTTCCA 1530
      S  A  Y  V  R  A  D  L  M  K  A  E  Y  D  E  T  E  L  S  A  T  A  D  E  R  I  R  T  F  Q

1531 AGCTGATACTGCACGTGAAGCGAACGTATTCCATCACTTAATCACTTTACCTACTTACCATACAACTGCGTTATCAGTAGATAACTTAGC 1620
      A  D  T  A  R  E  A  N  V  F  H  H  L  I  T  L  P  T  Y  H  T  T  A  L  S  V  D  N  L  A

1621 TAAAGAGTACTTCGGTGAGCAAGGGATGCTTGGTTATGTTAAAGGTGTACAACGTAAAGAAATCCGTCAAGGTATTGCCTGTGTTAAACA 1710
      K  E  Y  F  G  E  Q  G  M  L  G  Y  V  K  G  V  Q  R  K  E  I  R  Q  G  I  A  C  V  K  H

1711 TCAAAACATGTCTGGTTCAGACATGGGTGATGATCACAAAGAATACTTTGCAGGTGAAAATGCACTTAAAGCGGGTGGCGCAAAAAATAC 1800
      Q  N  M  S  G  S  D  M  G  D  D  H  K  E  Y  F  A  G  E  N  A  L  K  A  G  G  A  K  N  T

1801 GTCTAACCAGTTCAGTTAATCACCAATATTTTAATCTCTTAAGTGTTGCTTAAGGCTGAAATATAAATACGAATCAGATTGTTACTAACG 1890
      S  N  Q  F  S  *

1891 TAGCAATCTGATTTTTTTTGTTAAAAAATAGTATTGTAATTGTAAATTAGTTTACTAGAATCAAGAGAACAAACTAAACAGTTTACACTT 1980

1981 TGAAACAATTGTTGCTATATTGTTTTGATCGTTCGATAAATAAATACATGCCTGATTTTTACTACTTTTGTACGAAGGAAATATGTTGCA 2070

2071 GTCATCTTCATCTAAACAATTGTTGCTACTTAATACCAATGCTGAAAGGCTTACTAAACTTGAAAACTTATTACTTTCTGGCACCCGCTA 2160

2161 TACTATTTTATCCGCAGCAGATACTCGCAGTGCCATAGCGATATTGAAAACAGAAAGCATCGACTTTGTGGTGAGTAATATCAATCTCGA 2250

2251 TAATTTTGATGGCTGGCGTTTGGCCCGAATGGTGCGCTCAGGCGTGTTAAAGTGCTCAGCAGATACACCTTTTGTGGTGGTGGCAA     2336
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Fig. 2 Nucleotide and deduced amino acid sequences of the *C. psychrerythraea icl* gene. The *underlined* sequences are the putative –35 and –10 promoter elements. The *boxed* nucleotides are the putative Shine–Dalgarno sequence. The guanine in *black box* indicates transcriptional start point (Fig. 4). The probable stem-loop structures located downstream of the translational stop codon are shown by two opposite *arrows*

ICL (28% identity). On the other hand, sequence identities with subfamily 3 ICLs shown in Fig. 3 were 70% or above, except for the *Hyphomicrobium methylovorum* ICL (54% identity). In particular, the sequence identity to the ICL of *C. maris*, another psychrophilic bacterium of the same genus *Colwellia*, attained to 88%.

Expression of the *C. psychrerythraea icl* gene

Expression of the *C. psychrerythraea icl* gene in the *icl*-defective mutant of *E. coli*, ACA421, was examined under several conditions by Western blot analysis. As Watanabe et al. (2002b) reported previously, antibody against the purified *Cm*ICL cross-reacted with the ICL protein purified from the *C. psychrerythraea* cells, but not with the *E. coli* ICL. Whereas no protein cross-reactive with this antibody was present in crude extract of the *E. coli* ACA421 cells transformed with a control vector, pBS, a major cross-reactive protein band was detected at the same position as the purified *Cp*ICL in crude extract of the *E. coli* transformant cells carrying pCPB49 grown at 15 and 25°C on LB medium. These results demonstrate that the *C. psychrerythraea icl* gene is cloned in this plasmid. ICL is known to be highly induced in microorganisms grown on acetate (Vanni et al. 1990). When the *E. coli* transformant cells carrying pCPB49 were grown at 15 and 25°C on LB medium supplemented by 50 mM sodium acetate, the gene expression was induced. Furthermore, irrespective of the supplementation of sodium acetate in growth medium, the expression level of this gene in the *E. coli* mutant grown at 15°C was greater than that grown at 25°C, and no expression were detected when it was grown at 37°C. Similar results were obtained by the assay of ICL activity in the crude extract of *E. coli* ACA421 cells transformed with pCPB49 (Table 2). These results were in accord with those of Northern and Western blot analyses carried out using the intact cells of *C. psychrerythraea* (Watanabe et al. 2002b), indicating that the expression of *C. psychrerythraea icl* gene is not only acetate-inducible but also cold-inducible and that the insert DNA subcloned in the plasmid pCPB49 contains ORF of the *icl* gene and its complete promoter region.

Analysis of 5′-terminal region of the *C. psychrerythraea icl* mRNA

The 5′-end of the *C. psychrerythraea icl* mRNA was examined by primer extension analysis (Fig. 4). The result revealed that transcriptional start site of the *C. psychrerythraea icl* gene (TS site in Fig. 4) is guanine, located at 87 bases upstream of translational initiation codon. Furthermore, the putative –35 and –10 promoter elements, TTCCTA and TATTAT, were found 32 and 8 bases upstream of the TS site, respectively (Fig. 2).

Site-directed mutagenesis of *Cp*ICL and properties of mutated ICLs

Cold-adapted enzymes should possess inherent characteristics on protein structure responsible for the common properties, high catalytic function at low temperature and marked thermolability, compared with mesophilic and/or thermophilic counterparts. The introduction of suitable mutation(s) to these enzymes is one of useful approaches for studying this problem. From multiple alignment of amino acid sequences of subfamily 3 ICLs from several mesophilic and psychrophilic bacteria, several substitutions of amino acid residues common to the two cold-adapted ICLs, *Cp*ICL and *Cm*ICL, were found to exist in highly conserved regions of the ICL proteins (Fig. 3). Among them, Gln47 (the 47th Gln residue from N-terminal), Ala214, Ala231, Phe333 and Ala341 of the *Cp*ICL (shown by stars in Fig. 3) were substituted for the conserved amino acid residues of other ICLs by site-directed mutagenesis as described in Materials and methods.

To examine a possibility that His-tagging of the enzyme at the N-terminal alters its original properties, the activity of His-tagged *Cp*ICL (*Cp*-WT) was assayed at various temperatures (Fig. 5). The purified *Cp*-WT showed the maximum activity at 25°C and its k_{cat} value at this temperature was 28.5 s^{-1}. These results were consistent with those of the native *Cp*ICL reported previously (25°C and 27.7 s^{-1}, respectively; Watanabe et al. 2002b). In addition, as well as the native *Cp*ICL and *Cm*ICL, the *Cp*-WT activity could not be measured exactly at 30°C because the enzyme was rapidly inactivated at this temperature even during the enzyme assay. These indicate that the His-

Fig. 3 Alignment of amino acid sequences of subfamily 3 ICLs from various bacteria. Amino acid residues identical and similar to those of *Cp*ICL are shown as *white letters* in *black boxes* and *black letters* in *gray boxes*, respectively. Gaps in the alignments are indicated by *dashes*. *C.m. C. maris* (accession no. for the protein database, BAB62107), *H.m. Hyphomicrobium. methylovorum* GM2 (BAA23678), and *R.e. Ralstonia eutropha* (AAM18124). Putative ICLs, *R.m. Ralstonia metallidurans* (ZP_00022497), *R.p. Rhodopseudomonas palustris* (ZP_00009157), *B.j. Bradyrhizobium japonicum* (NP_709695), *N.a. Novosphingobium aromaticivorans* (ZP_0093836), *P.a. Pseudomonas aeruginosa* PAO1 (NP_251324), *A.v. Azotobacter vinelandii* (ZP_00091825), and *A.c. Acinetobacter calcoaceticus* ADP1 (YP_045792). *Stars* on the sequences indicate the positions of amino acid residues substituted by site-directed mutagenesis (see text) ▶

```
C.p.    1 -------------------------------------------------------------MSNYQSAIEAVQAIKAKAGSSWDAINPES
C.m.    1 -------------------------------------------------------------MSNYQSAIEAVKAIKEKAGNSWDAINPES
R.e.    1 -------------------------------------------------------------MAQYQDDIKAVAGLKENHGSAWNAINPEY
R.m.    1 -------------------------------------------------------------MAQYQDDIKAVAGLKEQHGSAWNAINPEY
A.v.    1 -------------------------------------------------------------MSAYQNEIKALAALKEKNGSAWSAINPEY
P.a.    1 -------------------------------------------------------------MSAYQNEIKAVAALKEKNGSSWSAINPEY
B.j.    1 MLHDITNKKQAIYTKFPFSRLRRDTHFAMHCQECRWPAIAIVSERIRHMNYQPRGISTFQAPASYQSEIDAAKALLETQ-PTWNGVSAEA
R.p.    1 ------------------------------------------------MNFQPRGISAIHAPTSYKSEIEAAEALLKDK-PTWNGVTAEA
N.a.    1 -------------------------------------------------------------MTYHSKIVEAGKAIAPFS-QTWDGIEPES
A.c.    1 --------------------------------------------------------------MTYQSALEHVRAVKNKLGGTWDAIRPED
H.m.    1 -----------------------------------------------------------MAHKKTYSQLRSELLARYPVGLTKGGVSIDD

                         *
C.p.   30 IARMRAQNKFKTGLEIAQYTADIMRADMAAFDADKTQYTQSLGCWHGFVGQQKMISIKKHFDGKTDRRYLYLSGWMVAALRSEFGPLPDQ
C.m.   30 VARMRAQNKFKTGLDIAQYTADIMRADMAAFDADKTKYTQSLGCWHGFIGQQKMISIKKHFDGKTDRRYLYLSGWMVAALRSEFGPLPDQ
R.e.   30 AARMRAQNKFKTGLDIAKYTAKIMRADMAAYDADSSKYTQSLGCWHGFIGQQKMISIKMHFN-STERRYLYLSGWMVAALRSEFGPLPDQ
R.m.   30 AARMRAQNKFKTGLDIAKYTAKIMRADMAAYDANPSKYTQSLGCWHGFIGQQKMISIKKHFK-STERRYLYLSGWMVAALRSEFGPLPDQ
A.v.   30 AARMRIQNRFKTGLDVAKYTAAIMRKDMAEYDADSSVYTQSLGCWHGFIGQQKLISIKKHLK-TTDKKYLYLSGWMVAALRSDFGPLPDQ
P.a.   30 AARMRIQNRFKTGLDIAKYTAAIMRKDMAEYDADSSVYTQSLGCWHGFIGQQKLISIKKHLK-TTNKRYLYLSGWMVAALRSDFGPLPDQ
B.j.   90 VARMRLQNRFRTGLDVARYTAALMRADMAAYDNDPTKYTQSLGCWHGFIAQQKLISVKKHFGGRTDRTYLYLSGWMIAALRSEFGPLPDQ
R.p.   42 VARMRLQNRFKTGLDIARYTAAQMRADMAAYDADPTKYTQSLGCWHGFIAQQKLISIKKHGGGRTDRRYIYLSGWMIAALRSEFGPLPDQ
N.a.   29 VARMRLQNRFHTGLDIARYTAKIMRADMAAYDADPANYTQSLGCWHGFIGQQKMISIKKHFG-TTKGRYLYLSGWMVAALRSEFGPLPDQ
A.c.   29 AARMIVQNRFHTGLDIAKYTAAIMRKDMAEYDADNSSYTQSLGCWHGFIAQQKMIANKKYFG-TTNKRYIYLSGWMVAALRSEFGPLPDQ
H.m.   32 IVQLRLQSPYESHLDVARAMASVMRADMAAYDRDTGKFTQSLGCWSGFHAQQMIKAVKRLRG-TTKGAYVYLSGWMVAGLRNRWGHLPDQ

C.p.  120 SMHEKTSVASLVAELYTFLRQADARELGGLFRELDAAAEG-----DKAAIQSQIDNHVTHVVPIVADIDAGFGNAEATYLMAKQMIEAGA
C.m.  120 SMHEKTSVASLVAELYTFLRQADARELGGLFRDLDAASDG-----DKAAIQDKIDNHVTHVVPIIADIDAGFGNAEATYIMAKQMIEAGA
R.e.  119 SMHEKTSVSALIRELYTFLRQADGRELGGLFRELDDAQGP-----AKAAIQAKIDNHVTHVVPIIADIDAGFGNAEATYLLAKQFIEAGA
R.m.  119 SMHEKTSVSALIRELYTFLRQADARELGGLFRELDAATGA-----AKAAIQEKIDNHVTHVVPIIADIDAGFGNAEATYLLAKQFIEAGA
A.v.  119 SMHEKTAVSSLIEELYTFLRQADARELDLLFSALDDARNAGDK-AKEQEILKQIDGFETHVVPIIADIDAGFGNAEATYLLARKMIEAGA
P.a.  119 SMHEKTAVSGLIEELYTFLRQADARELDLLFTGLDAARAAGDK-AKEAELLAQIDNFETHVVPIIADIDAGFGNAEATYLLAKKMIEAGA
B.j.  180 SMHEKTSVPALIEELYTFLRQADSRELNDIFRSLDKARKEGDK-TREKELIEKIDGFQTHVVPVIADIDAGFGNAEATYLLAKKMIEAGA
R.p.  132 SMHEKTSVPALIEEIYTFLRQADSRELNDLFRQLDKAREAGDK-AKETAIIDQIDNFQTHVVPIIADIDAGFGNAEATYLLAKKMIEAGA
N.a.  118 SMHEKTSVPALIEELYTFLKQADARELGMLFRDLDAAKAAGDA-VNTHRLLHKIDEFETHVVPIIADIDAGFGNAEATYLLAKKFIEAGA
A.c.  118 SMHEKTAVPKLIAEIYTFLRQADAKELNDLFRALQKAEAAGDS-AKVKDIESQIDNFETHVVPIIADIDAGFGNEEATYLLTKQMIEAGA
H.m.  121 SMHEKTSVVDLIEEIYVSLRQADEVALNDLFNELKDARAKGATNKACEEIISRIDGFESHVVPIIADIDAGFGNEHATYLLAKEMIKAGA

                    *           *
C.p.  205 CALQIENQVADEKQCGHQDGKVTVPHADFHSKIRALRHAFLELGIDNGLIVARTDSEGAGLTKEIAVVKEPGDSGDIYNSYLDVEEISAA
C.m.  205 CALQIENQVADEKQCGHQDGKVTVPHADFHSKIRALRHAFLELGIDNGIIVSRTDSEGAGLTKEIAVVKEPGDQGDIYNSFLDVEEIDVS
R.e.  204 CCIQIENQVSDEKQCGHQDGKVTVPHEDFLAKIRAIRYAFLELGVDDGIIVARTDSLGAGLTKQIAVTNTPGDLGDQSNYFLACEELSAD
R.m.  204 CCIQIENQVSDEKQCGHQDGKVTVPHEDFLAKIRAIRYAFLELGVDDGVIVARTDSLGAGLTKQIAVTATPGDLGDQYNSFLDCEELSAD
A.v.  208 CCIQIENQVSDEKQCGHQDGKVTVPHEDFLAKINAVRYAFLELGVDDGVIVARTDSLGAGLTKQIAVTRQEGDLGDKYNAFLDCEEISPA
P.a.  208 CCIQIENQVSDEKQCGHQDGKVTVPHIDFLAKINAVRYAFLELGVDDGVIVARTDSLGAGLTKQIAVTNEPGDLGDLYNSFLDCEEISES
B.j.  269 CALQIENQVSDEKQCGHQDGKVTVPHDVFLAKIRACRHAFLELGVEDGVVVTRTDSLGAGLTQQIAVSHKPGDIGDQYNSFLDCEEITAE
R.p.  221 CALQIENQVSDEKQCGHQDGKVTVPHDVFIAKVRAIRHAFLEMGVEDGIIVTRTDSLGAGLTQQIAVSHQPGDIGDQYNSFLDCEEVDAS
N.a.  207 CCIQIENQVSDEKQCGHQDGKVTVPHEDFLAKIRAVRYAFMELGVDDGVIVARTDSLGAGLTKQIAFVKEPGDIGDQYNSFLDCEEVDAA
A.c.  207 CAIQIENQVSDAKQCGHQAGKVTVPHEDFLAKINAVRYAFLELGVDEGVIVARTDSEGADLTQKIPVSKEKGDLASQYISYLDTQEIDIA
H.m.  211 CCLQIENQVSDAKQCGHQDGKVTVPREDFIEKLRACRLAFEELGVDDGVIVARTDSLGASLTQKIPVSQQAGDFASSYIKWLKTEPITDA

                                          *       *
C.p.  295 D-MAEGDVCFNRDGKLVRPKRLPSGLYQFRQGTGHERCVFDCIGALNAG-ADLLWIETAVPTVHEIAGMMDDVRKVHPDAKLVYNNSPSF
C.m.  295 D-MAEGDVCFNRDGKLVRPKRLPSGLYQFRQGTGEERCVFDSIEAIKAG-ADLLWIETATPNVADITAMMNEVRKEIPDAKLVYNNSPSF
R.e.  294 Q-LGNGAVIITRDGKLLRPKRLPSNLFQFRAGTGEARCVLDCVTALQNG-ADLLWIETEKPHIAQIGGMVSEIRKVIPNAKLVYNNSPSF
R.m.  294 Q-LGNGDVIIKRDGKLLRPKRLPSNLFQFRAGTGEARCVLDCVTALQNG-ADLLWIETEKPHIAQIGGMVSEIRKVIPNAKLVYNNSPSF
A.v.  298 E-MKNGDVVLNRNGKLLRPKRLASGLFQFRKGTGEDRCVLDCITSLQNG-ADLLWIETEKPHIGQIKGMVDRIREAIPNAKLVYNNSPSF
P.a.  298 E-LGNGDVVIKREGKLLRPKRLASNLFQFRKGTGEDRCVLDCITSLQNG-ADLLWIETEKPHVGQIKAMVDRIREVIPNAKLVYNNSPSF
B.j.  359 N-ARNGDVIINRNGKMMRPKRLPSNLYQFRPGTGEDRCVLDCITSLQNG-ADLLWIETEKPHIEQIAKMVDRIREVVPNAKLAYNNSPSF
R.p.  311 K-IGNGDVIISRNGKLLRPKRLPSNLYQFRPGTGADRCVLDCITSLQNG-ADLLWIETEKPHIEQIASMVDRIREVVPNAKLAYNNSPSF
N.a.  297 N-LGHGDVLISRDGKLMRPRRLPSNLYQFRAGTGEDRCVLDCITSLQNG-ADLLWIETEKPHIGQIGGMVDRIREVIPNAKLVYNNSPSF
A.c.  297 D-AQEDEILIKRDGKLHRPTRLASGLYQFREGTQHDRVVLDCVTSLQNG-ADMIWIETPTPDVAGIAGFVNDIKKQVPNAKLVYNNSPSF
H.m.  301 NPLSEGELAIWQSGNFARPIRMPNGLFSFREGTGRARVIEDCIASLKDGDADLIWIETDTPNVDEIASMVAEIRKQVPDAKLVYNNSPSF

C.p.  383 NWTLNFRQQAYDAMVEAGQDVSAYVRA--DLMKAEYDETELSATADERIRTFQADTAREANVFHHLITLPTYHTTALSVDNLAKEYFGEQ
C.m.  383 NWTLNFRQQAFDTMVAEGKDVSSYVRA--DLMNVDYDTTELAAAADDKIRSFQADTAREAGIFHHLITLPTYHTAALSTDNLAKEYFGDA
R.e.  382 NWTLNFRQQAYDAMKAAGKDVSAYDRA--QLMSVEYDQTELAKLADGKIRTFQGDASREEGIFHHLITVATYHNAALSTDNLAKEYFGDQ
R.m.  382 NWTLNFRQQAYDAMKAAGKDVSAYERT--QLMSVEYDDSELAKIADEKIRTFQADASREAGIFHHLITLPTYHTAALSTDNLAKEYFGDQ
A.v.  386 NWTLNFRQQVFDTWAAEGKDVAKYDRA--KLMSVEYDETELALEADARIRTFQRDAAAQAGIFHHLITLPTYHTAALSTDNLAKGYFAEE
P.a.  386 NWTLNFRQQVFDAFVAEGKDVSAYDRN--KLMSVEYDDTELAKVADEKIRTFQRDGSAHAGIFHHLITLPTYHTAALSTDNLAKGYFADE
B.j.  447 NWTLNFRWQVYDAMKEAGKDVSKYNRA--ELMKAEYDDTPLAKEADERIRTFQADSAKRAGIFHHLITLPTYHTAALSTDNLAREYFGEQ
R.p.  399 NWTLNFRWQVYDAWKEEGKDVSKYNRA--ELMKAEYDETPLAIEADARIRTFQADSAKRAGIFHHLITLPTYHTAALSTDNLAREYFGDQ
N.a.  385 NWTLNFRQQVFDAWQAEGRDTSAYDRA--KLMSVDYDGTELGVEADERIRTFQRDAAAQAGIFHHLITLPTYHTAALSTDNLAKRYFGEE
A.c.  385 NWTLNFRQQAYDRWAAEGKDVSAYDRA--KLMSAEYDNTELAADADEKIRTFQADAAREAGVFHHLITLPTYHTAALSTHELAKGYFGEE
H.m.  391 NWTLNLRKQVRAQWISEGKIAEADYPDGTALMSAQYDTSELGREADDRLRQFQVDISARAGVFHNLITLPTFHLTAKSTDELSHGYFGED

C.p.  471 GMLGYVKGVQRKEIRQGIACVKHQNMSGSDMGDDHKEYFAGENALKAGGA-KNTSNQFS-
C.m.  471 GMLGYVEGVQRKEIRQGIACVKHQNMSGSDMGDDHKEYFAGENALKAGGA-NNTSNQFS-
R.e.  470 GMLGYVAGVQRKDIRQGIACVKHQNMSGSDIGDDHKEYFSGEAALKLAGK-DNTMNQF--
R.m.  470 GMLGYVAGVQRKEIRQGIACVKHQNMSGSDIGDDHKEYFSGEAALKAAGK-DNTMNQF--
A.v.  474 GMLAYVKGVQRQEIRQSIACVKHQNMAGSDIGDNHKEYFAGEAALKAGGK-DNTMNQFH-
P.a.  474 GMLAYVKGVQRQELRQGIACVKHQNMAGSDIGDNHKEYFAGEAALKASGK-DNTMNQFH-
B.j.  535 GMLGYVKNVQRAEIRQGIACVKHQNMAGSDIGDDHKEYFAGEAALKAGGA-HNTMNQFG-
R.p.  487 GMLGYVKNVQRQEIRQGIACVKHQNMAGSDIGDDHKEYFAGDAALKAGGE-HNTMNQFS-
N.a.  473 GMLGYVKQVQREEIRQGIACVKHQNMAGSDIGDDHKEYFAGEAALKAGGA-HNTMNQFAA
A.c.  473 GMLAYVAGVQRKEIRGGIACVKHQAMAGSDIGDDHKEIFAGEQALKAGDASKNTMNQFAH
H.m.  481 RMLAYVATVQREEIRRSISAVRHQHEVGSDLGDTFKEMVSGDRALKAGGA-HNTMNQFAA
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Table 2 ICL activity of *E. coli icl*-defective mutant transformed with the *C. psychrerythraea icl* gene

Growth medium	Growth temperature		
	37°C	25°C	15°C
LB	1.40×10^{-3}	2.08×10^{-3}	6.07×10^{-3}
LB + acetate	3.72×10^{-3}	7.29×10^{-3}	1.70×10^{-2}

The values indicate ICL activity (unit/mg protein) in crude extract of *E. coli* ACA421 transformed with pCPB49. The enzyme activity was assayed at 25°C. The *E. coli* transformant was grown on LB medium supplemented with none (LB) or 50 mM sodium acetate (LB + acetate). No ICL activity was detected in the *E. coli* ACA421 cells grown at 37°C on LB or LB + acetate medium.

tagging at the N-terminal does not affect the activity and characteristics of *Cp*ICL.

The activities of recombinant *Cp*ICLs and His-tagged *E. coli* ICL (*Ec*-WT) at various temperatures were shown in Fig. 5. The ICL activity of F333L, which is *Cp*ICL substituted Phe333 by Leu, was not able to be exactly measured because of its very low activity, suggesting that Phe333 of the *Cp*ICL is important for the catalytic function. Temperature dependence of ICL activities of Q47K and A341N was similar to that of *Cp*-WT. Furthermore, as well as *Cp*-WT, optimum temperature for the two enzyme activities was 25°C, and their exact activities could not be measured at 30°C because they were rapidly inactivated even during the enzyme assay at this temperature. At the optimum temperature, the activity of Q47K (k_{cat} value, 26.6 s^{-1}) was comparable to that of *Cp*-WT, but A341N (13.3 s^{-1}) exhibited lower activity than *Cp*-WT. The ICL activities of A231E below 20°C were similar to those of A341N, but the former enzyme showed the maximum activity at 20°C. Further lowering of ICL activity at low temperatures was observed in A214S, but optimum temperature for this enzyme activity increased by about 5°C, compared to that of *Cp*-WT. However, above 30°C, this enzyme was also rapidly inactivated. These results imply that Ala214 of the *Cp*ICL is involved in its high activity at low temperatures.

To estimate whether the mutations of *Cp*ICL affect the thermal stability, the residual activities of wild and mutated enzymes after the incubation for various times at 25°C were examined (Fig. 6). A214S and A231E were more thermostable and more thermolabile than *Cp*-WT, respectively.

Discussion

In this study, the *icl* gene encoding the cold-adapted ICL of *C. psychrerythraea* was cloned and sequenced. From expression analysis, DNA fragment cloned in the plasmid pCPB49 was confirmed to contain the ORF of this gene and

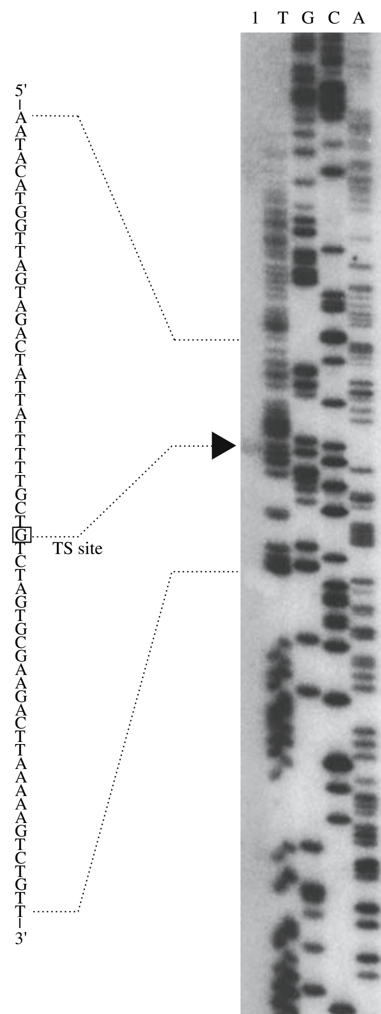

Fig. 4 Primer extension analysis. Product of primer extension with a primer complementary to *icl* is shown in *lane 1*. Total RNA (35 μg) was isolated from the *C. psychrerythraea* cells grown at 15°C on nutrient medium containing 50 mM acetate and was used as the template. The sequence ladders are shown in lanes *T*, *G*, *C* and A

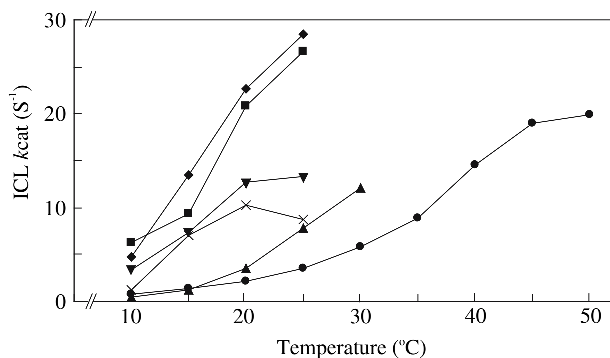

Fig. 5 Effect of temperature on activities of wild and mutated ICLs. Symbols; *Cp*-WT (*filled diamond*), Q47K (*filled square*), A214S (*filled triangle*), A231E (×), A341N (*filled inter triangle*) and *Ec*-WT (*filled circle*). The k_{cat} values of ICLs were measured under standard assay condition.

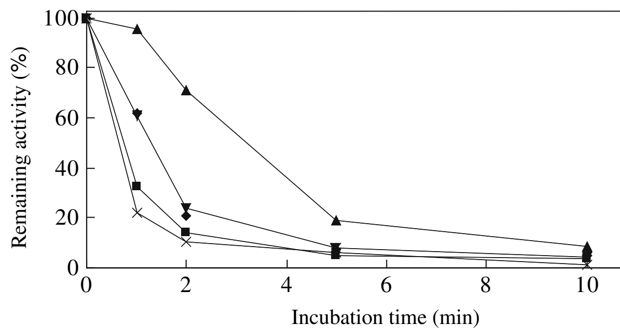

Fig. 6 Thermostability of wild and mutated ICL activities. *Symbols* used in this figure are the same as Fig. 6. The purified enzymes were dialyzed at 4°C against 20 mM potassium phosphate (pH 6.85) containing 2 mM $MgCl_2$ and 1 mM DTT. After the incubation for the indicated time periods at 25°C, the dialyzed enzymes were withdrawn and immediately cooled for 10 min on ice. The ICL activities were then measured at optimum temperatures of the respective enzymes

full length of the promoter region, because the expression of this gene in the *E. coli* cells was induced by acetate and low temperature and this result was consistent with that in the *C. psychrerythraea* cells reported previously (Watanabe et al. 2002b). The CCAAT sequence is present in the upstream region of the *C. maris icl* gene whose expression is induced by acetate and low temperature as well as the *C. psychrerythraea icl* gene (Watanabe et al. 2002a). This sequence has been reported to be *cis*-acting element for cold-inducible expression of several genes including the *icd-II* encoding the cold-adapted isocitrate dehydrogenase isozyme II of *C. maris* (Qoronfleh et al. 1992; Sahara et al. 1999). Furthermore, several *cis*-acting elements for cold-inducible gene expression was found by promoter analysis of the genomic DNA of a Gram-negative psychrophilic bacterium, *Pseudomonas haloplanktis* strain TAC125, which are about 20 bp of upstream AT-rich sequences adjacent to the –35 consensus element of promoters (Duilio et al. 2004). However, such sequences are absent in the upstream region of the *C. psychrerythraea icl* gene despite its cold-inducible expression (Fig. 2). Therefore, it is suggested that other novel *cis*-element is involved in the cold-inducible expression of this gene or the stabilization of its mRNA at low temperature influences the gene expression as reported for *cspA* and *otsA/B* mRNA of *E. coli* (Tanabe et al. 1992; Kandror et al. 2002).

From the comparison of amino acid sequence with various bacterial ICLs, it was found that the *Cp*ICL belongs to subfamily 3, as well as the *Cm*ICL (Fig. 3). As reported previously (Watanabe et al. 2002a), six additional short insertions of 3–36 amino acids are scattered in the *Cm*ICL, compared to subfamily 1 ICLs from *E. coli* and *Mycobacterium tuberculosis*. The same insertions were also present in the *Cp*ICL. In spite of such a sequential difference between the *Cm*ICL and *E. coli* ICL, many amino acid residues essential for catalytic function and for binding with substrates and divalent metal ion, which were identified in the *E. coli* ICL, are conserved in the *Cm*ICL (Watanabe et al. 2002a). In this study, these amino acid residues of the *Cm*ICL were found to be completely conserved in the *Cp*ICL.

Multiple alignments of amino acid sequences of subfamily 3 ICLs and the putative ones from several bacteria, including the cold-adapted *Cp*ICL and *Cm*ICL, revealed that several substitutions of amino acid residues peculiar to these two enzymes are present in the conserved regions of the ICL proteins. From the following reasons, five amino acid residues of Gln47, Ala214, Ala231, Phe333 and Ala341 were selected and were substituted by point mutations; Gln47, the corresponding amino acid residues of other ICLs are basic Lys or Arg; Ala214, in a three-dimensional model of the *Cp*ICL protein constructed with the homology modeling program, this residue is located closely to TIM barrel structure involved in the binding of substrate (Britton et al. 2001), and the corresponding residue is more hydrophilic Ser in other ICLs; Ala231 and Phe333, the corresponding residues are acidic Glu or Asp and less hydrophobic Leu in most of other ones, respectively; Ala341, the corresponding residues of other ones are Asn. Although the A214S mutation of *Cp*ICL led to lowering of the enzyme activity, the thermostability and optimum temperature for ICL activity of the mutated enzyme were elevated, compared to those of wild-type enzyme. These results indicate that Ala214 is involved in thermolability of the *Cp*ICL and its high activity at low temperatures. Possible hydrogen bonds of this residue predicted by a program SWISSPDB VIEWER were shown in Fig. 7. The mutated ICL, A214S, can form two hydrogen bonds between main chain of the substituted Ser214 and side chain of Gln119, and side chain of Ser214 and main chain of Gln119, whereas only one hydrogen bond is allowed between main chain of native Ala214 and side chain of Gln119 in the *Cp*-WT. Therefore, this may be responsible for the decrease of structural flexibility of the A214S and resultant increase of its thermostability. On the other hand, the decreased thermostability and lowering of catalytic activity were observed in the A231E, and Q47K and A341N, respectively. These three substitutions of amino acid residues have negative effects to thermostability and specific activity of *Cp*ICL, suggesting that these amino acid residues are also involved in the thermostability and catalytic activity of *Cp*ICL. In particular, the F333L mutation of *Cp*ICL resulted in almost complete loss of its catalytic function, implying that Phe333 might play an important role for catalytic function of this enzyme. Furthermore, various combinations of these amino acid substitutions may be able to bring some different effects on the

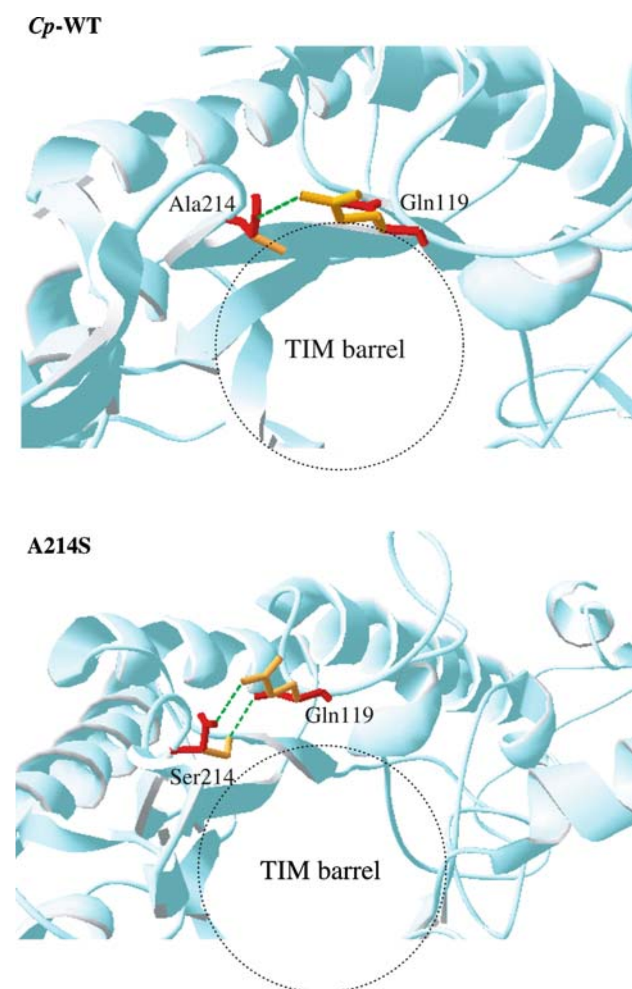

Fig. 7 Molecular models of *Cp*-WT and A214S. The models were built by the homology modeling program. The main chains and side chains of Gln119 and Ala214 or Ser214 are indicated by *red* and *orange*, respectively. Hydrogen bonds predicted by a program SWISSPDB VIEWER is indicated by *green* dashes

psychrophilic properties of *Cp*ICL. On the other hand, it was reported that the surface of cold-adapted enzyme tends to have more nonpolar amino acid residue, in particular Ala, compared with the corresponding mesophilic and thermophilic ones (Siddiqui and Cavicchioli 2006; Sælensminde et al. 2007). Besides A214, A231 and A341, several substitutions by Ala are found to be still present in the two cold-adapted *Cp*ICL and *Cm*ICL (Fig. 3). Some of such Ala residues might be responsible for the psychrophilic nature of these enzymes. Thus, further studies are planned to clarify the role of these residues of cold-adaptation and thermolability of the *Cp*ICL.

Acknowledgments We sincerely thank Dr. Isao Yumoto of the National Institute of Advanced Industrial Science and Technology for the donation of *C. psychrerythraea*.

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