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The cold-inducible *icl* gene encoding thermolabile isocitrate lyase of a psychrophilic bacterium, *Colwellia maris*

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The gene encoding isocitrate lyase (ICL; EC 4.1.3.1) of a psychrophilic bacterium, *Colwellia maris*, was cloned and sequenced. The ORF of the gene (*icl*) was 1584 bp long, and the predicted gene product consisted of 528 aa (molecular mass 58 150 Da) and showed low homology with the corresponding enzymes from other organisms. The analyses of amino acid content and primary structure of the *C. maris* ICL suggested that it possessed many features of a cold-adapted enzyme. Primer extension and Northern blot analyses revealed that two species of the *icl* mRNAs with differential lengths of 5'-untranslated regions (TS1 and TS2) were present, of which the 5' end (TS1 and TS2 sites) were G and A, located at 130 and 39 bases upstream of the translation start codon, respectively. The levels of TS1 and TS2 mRNAs were increased by both acetate and low temperature. The induction of *icl* expression by low temperature took place in the *C. maris* cells grown on succinate as the carbon source but not acetate. Furthermore, a similar manner of inductions was also found in the levels of the translation and the enzyme activity in cell-free extract. These results suggest that the *icl* gene, encoding thermolabile isocitrate lyase, of *C. maris* is important for acetate utilization and cold adaptation.

Keywords: cold-inducible gene, cold-adapted enzyme, isocitrate lyase, psychrophilic bacterium

INTRODUCTION

Temperature is an important environmental factor for organisms on Earth. Although many parts of Earth's surface are cold environments, organisms called psychrophiles successfully live in such environments (Low *et al.*, 1973). One area of the study of psychrophiles is to clarify the molecular mechanism of cold-adapted enzymes, which exhibit high catalytic activity at low temperatures, because of the biotechnological and industrial benefits. Although only a limited number of these enzymes have been thoroughly investigated, it has been revealed that these enzymes display a pronounced thermolability, probably due to more structural flexibility than their mesophilic and thermophilic counter-

parts (Gerday *et al.*, 1997; Hochachka & Somero, 1984). The higher flexibility of the enzymes is expected to be able to accommodate their substrates and to convert the enzyme into the activated state with minimum energetic loss.

Several cold-shock proteins that function as RNA chaperones and DNA helicases, and that associate with ribosomes are overexpressed in mesophilic organisms, including *Escherichia coli*, after a shift to low temperature (Thieringer *et al.*, 1998). On the other hand, psychrophilic organisms prefer to adopt other mechanisms such as cold-adapted enzymes for survival under permanently cold environments. In addition to the enzymology studies for cold-adapted enzymes, information on the expression of genes encoding these enzymes at low temperatures is also limited. In this paper, we focused on a gene encoding a psychrophilic enzyme and its expression at low temperatures. A psychrophilic bacterium, *Colwellia maris* (Takada *et al.*, 1979; Yumoto *et al.*, 1998), possesses a cold-adapted

Abbreviations: ICL, isocitrate lyase; IDH, isocitrate dehydrogenase; MS, malate synthase.

The GenBank accession number for the sequence reported in this paper is AB066287.

isocitrate lyase (ICL; EC 4.1.3.1) characterized by a lower optimum temperature for activity and higher thermolability than the counterparts from mesophilic and thermophilic bacteria (Watanabe *et al.*, 2001).

When micro-organisms grow on C₂ compounds or fatty acids, the glyoxylate cycle is required for biosynthesis of cellular components (Cozzzone, 1998; Kornberg, 1966). ICL, a key enzyme of this cycle, catalyses the cleavage of isocitrate to glyoxylate and succinate, and competes with isocitrate dehydrogenase (IDH; EC 1.1.1.42) of the TCA cycle for their common substrate, isocitrate. By the activity of malate synthase (MS; EC 4.1.3.2), the other essential enzyme of the glyoxylate cycle, glyoxylate is condensed with acetyl CoA to produce malate. In *E. coli* cells, the flux of isocitrate between these two metabolic cycles is controlled by different affinities of ICL and IDH for isocitrate and by a reversible phosphorylation to modulate IDH activity (Cozzzone, 1998). The gene *aceK*, encoding IDH-specific kinase/phosphatase that catalyses this phosphorylation, forms an operon together with *aceB* and *aceA* encoding MS and ICL, respectively (Cozzzone, 1998). When *E. coli* cells grow on acetate as the sole carbon source, the *aceBAK* operon is upregulated by the release of a specific repressor, IclR, from the promoter. On the other hand, *C. maris* has two IDH isozymes; a dimeric IDH-I with mesophilic and a monomeric IDH-II with psychrophilic characteristics (Ochiai *et al.*, 1979). The expression of *icdI* encoding IDH-I was induced by acetate. On the other hand, the expression of *icdII* encoding IDH-II was induced by low temperature, but not acetate, in the cells of *C. maris* and an *E. coli* mutant defective in IDH (Suzuki *et al.*, 1995). We report here that the *icl* gene of *C. maris* is also induced by low temperature. Cold adaptation at the levels of catalytic activity and gene expression of IDH and ICL, catalysing the first reaction in the TCA and glyoxylate cycles, respectively, suggests that the flux of isocitrate at pivotal branch between the two metabolic cycles is very important for the survival of this bacterium at low temperature.

METHODS

Bacteria, plasmids and culture conditions. The psychrophilic bacterium *Colwellia maris* was grown at 15 °C with vigorous shaking in nutrient medium (Ochiai *et al.*, 1979) or synthetic medium containing succinate and/or acetate as the carbon source (Ishii *et al.*, 1987). The strains of *E. coli* and the plasmids used are shown in Table 1. The strains of *E. coli* were grown in Luria-Bertani (LB) medium or M9 medium (Sambrook & Russell, 2001) supplemented with appropriate carbon source(s), amino acids and antibiotics. Cells were harvested, washed twice with a chilled sonication buffer (50 mM potassium phosphate, pH 6.85, containing 2 mM MgCl₂, 0.5 M NaCl and 1 mM DTT) and disrupted by sonication. The supernatant obtained by centrifugation at 11000 g for 30 min at 4 °C was used as cell-free extract.

Enzyme assay. The activity of ICL was assayed at 20 °C by the method described previously (Watanabe *et al.*, 2001). Protein was measured by the Lowry method using BSA as the standard. One unit of activity of ICL was defined as the

amount of enzyme that forms 1 μmol glyoxylic phenyl-hydrazone min⁻¹.

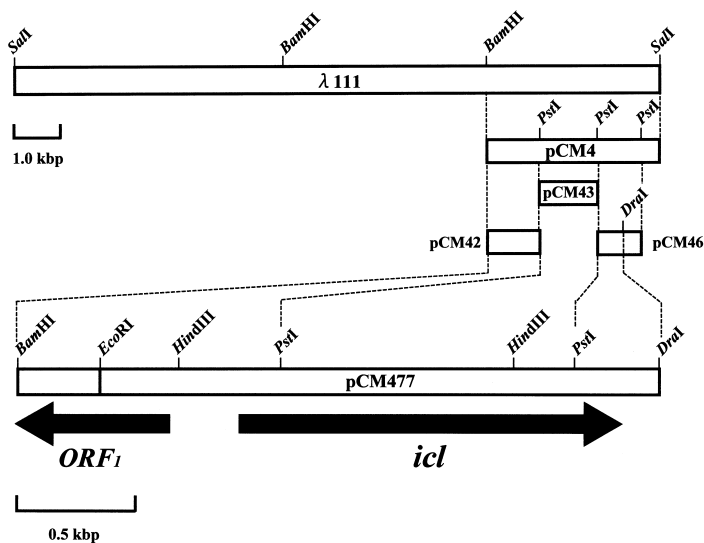
PCR. Genomic PCR was performed to obtain a nucleotide probe for the *C. maris icl* gene encoding ICL. Four upstream primers, 5'-(TCN or AGY)AAYTAYCAR(TCN or AGY)GCNATHGARGC-3' (26-mer) were designed from SNYQSAIEA, the sequence of amino acids 1–9 of the *C. maris* ICL protein (Watanabe *et al.*, 2001). Two downstream primers were designed from the sequences of highly conserved regions of ICL genes cloned from various organisms (Rehman & McFadden, 1996); 5'-SCCATRTGNCRCAYTTYTT-3' (20-mer) corresponding to KKCGHMA and 5'-CNARRTGNCCRCAYTTYTT-3' (19-mer) corresponding to KKCGHLG. *C. maris* genomic DNA was isolated as described by Ishii *et al.* (1993). Amplification was carried out for 30 cycles in a DNA thermal cycler 4800 (Perkin-Elmer) in 100 μl reaction mixture containing 1.3 μg genomic DNA, 200 pmol each forward and reverse primer and 2.5 U KOD DNA polymerase (Toyobo) in a buffer system prepared by the manufacturer. Cycling conditions were as follows: denaturation at 94 °C for 2 min, annealing at 50 °C for 2 min and extension at 72 °C for 2 min, for 30 cycles. PCR products with a predicted length of 600–700 bp were purified and ligated to the *Sma*I site of pBluescript SK(+) (Stratagene). DNA sequencing identified a plasmid harbouring an insert with a sequence that resembled ICLs of various organisms (pCM19). A 665 bp PCR product amplified with primers described above using pCM19 as template DNA was purified and utilized as a probe for plaque and Southern hybridization.

Cloning of the *C. maris icl* gene. The oligonucleotide probe was labelled with [α -³²P]dCTP by using a random primer labelling kit (Takara). Plaque hybridization (Sambrook & Russell, 2001) was carried out to screen the *C. maris* genomic DNA library constructed with a phage vector λ EMBL3 (Stratagene) as described previously (Ishii *et al.*, 1993). After overnight incubation at 42 °C with a labelled probe, blotted membranes were washed successively in 2 × SSC, 0.1% (w/v) SDS (1 × SSC is 15 mM sodium citrate, pH 7.0, and 0.15 M NaCl) at room temperature for 20 min and then twice with 0.1 × SSC/0.1% (w/v) SDS at 60 °C for 20 min. Autoradiography was performed by exposing the membrane to X-ray film (RX, Fuji Photo Film) at –80 °C. In Southern blot analyses, *C. maris* genomic DNA digested with appropriate restriction enzymes was fractionated by 1% agarose gel electrophoresis, transferred to a nylon membrane and processed as described above.

Isolation of the *E. coli aceA* mutant. For the directed disruption of *aceA* to obtain an *E. coli* mutant defective in ICL, *E. coli* strain KM22 (Δ *recBCD* mutant) (Kenan, 1998), plasmids pICL1 (Matsuoka & McFadden, 1988) (harbouring the *E. coli aceA* gene) and pICL1::Tet (tetracycline resistance gene inserted in *aceA* of pICL1) were used. For amplification of the *aceA::tet* insertion in pICL1::Tet, two primers were designed as follows; *aceA*-sense primer (5'-TTCCTGACCC-TGCCAGGCTACCG-3'; 23-mer), sequence of the upstream region between –43 and –65 from the translation start codon of *E. coli aceA* gene, and *aceA*-antisense primer (5'-AGGCCACGCGGCATTTAGCGC-3'; 21-mer), complementary to the downstream region between +175 and +196 from translation termination codon. The PCR was carried out for 30 cycles in 50 μl reaction mixture containing 25 ng pICL1::Tet digested with *Pst*I, 10 pmol each primer and 2.5 U KOD-plus polymerase in the manufacturer's buffer system. Cycling conditions were as follows: denaturation at 94 °C for 2 min, annealing at 50 °C for 30 s and extension at 68 °C for 2 min, for 30 cycles. The 2.4 kbp fragment amplified by PCR

Table 1. Strains, λ phages and plasmids used in this study

Strain/plasmid	Relevant characteristic(s)	Reference/source
Strains		
<i>Colwellia maris</i>	Psychrophilic bacterium	Takada <i>et al.</i> (1979); Yumoto <i>et al.</i> (1998)
<i>Escherichia coli</i>		
XL1-Blue	<i>recA1 lac endA1 gryA96 thi-1 hsdR17 supE44 relA1</i> (F' <i>proAB lacI^qZΔM15</i> Tn10)	Stratagene
KM22	<i>argE3 his-4 leuB6 proA2 thr-1 ara-14 galK2 lacY1 met-1 xyl-5 thi-1 rpsL31 tsx-33 supE44 Δ(recC ptr recB recD)::Plac-bet exo kan</i>	Kenan (1998)
ACA421	KM22 with <i>aceA::tet</i>	This study
Plasmids or λ phages		
λ EMBL3-based genomic library	λ EMBL3 containing 16 kbp chromosomal <i>C. maris</i> <i>Sau3AI</i> inserts	Ishii <i>et al.</i> (1993)
λ 111	λ EMBL3 containing <i>C. maris icl</i>	This study
pBluescript SK(+)	<i>ori</i> ColE1; ampicillin resistant	Stratagene
pCM4	pBluescript SK(+) containing 3.5 kbp <i>Bam</i> HI– <i>Sal</i> I insert from λ 111	This study
pCM477	2.3 kbp <i>Eco</i> RI– <i>Dra</i> I fragment derived from pCM4 ligated into the <i>Eco</i> RI/ <i>Sma</i> I sites of pBluescript SK(+)	This study
pICL1	pDR540 containing <i>E. coli aceA</i>	Matsuoka & McFadden (1988)

**Fig. 1.** Physical map of the chromosomal region containing the *icl* gene and its flanking region. Arrows represent the ORFs of *icl* and *ORF₁* (see text). The upper portion in this figure shows the restriction map of the isolated clones (pCM4, pCM42, pCM43, pCM46 and pCM477).

was electroporated at a voltage of 1.8 kV and capacitance 25 μ F with an electroporation system (Gene Pulser II; Bio-Rad) into electrocompetent *E. coli* strain KM22 cells prepared following the Gene Pulser manufacturer's instructions. Transformants resistant to both kanamycin and tetracycline were selected. Successful disruption of *aceA* was verified by genomic PCR and Southern blot analysis. One of the resultant strains was termed ACA421 and used for the expression of the *C. maris icl* gene.

Northern blot analysis. Bacterial cells were cultured to mid-exponential phase (OD₆₀₀ 0.6–0.8) and harvested by centrifugation. Total RNAs from *C. maris* and *E. coli* were prepared with RNeasy Total RNA Kit (Qiagen), and treated with DNase I. Northern hybridization was done as described by Sambrook & Russell (2001). The 1215 bp *Pst*I fragment of pCM4 (Fig. 1) was used as a specific probe for the *icl* gene.

Autoradiography was performed by exposing the membrane to a BAS Imaging Plate and analysed with a BAS-2000 Image Reader (Fuji Photo Film). The signal intensities of the bands corresponding to the *icl* mRNA were estimated with Science Lab99 Image Gauge v. 3.4 (Fuji Photo Film).

Primer-extension analysis. This was performed as described by Sambrook & Russell (2001) with the following modifications. A synthetic 30-mer oligonucleotide (5'-AAACCAGT-TTTGAATTTATTTTGTGCTCGC-3'), complementary to an internal region between +98 and +128 from the translation start codon, was used as the primer. Total RNA (10–100 μ g) isolated from *C. maris* cells was incubated for 6 h at 40 °C with the primer labelled by ³²P in the hybridization buffer. The primer-extension reaction was done with Rous-associated virus 2 reverse transcriptase (Takara). The products were analysed on a 6% (w/v) polyacrylamide sequencing gel

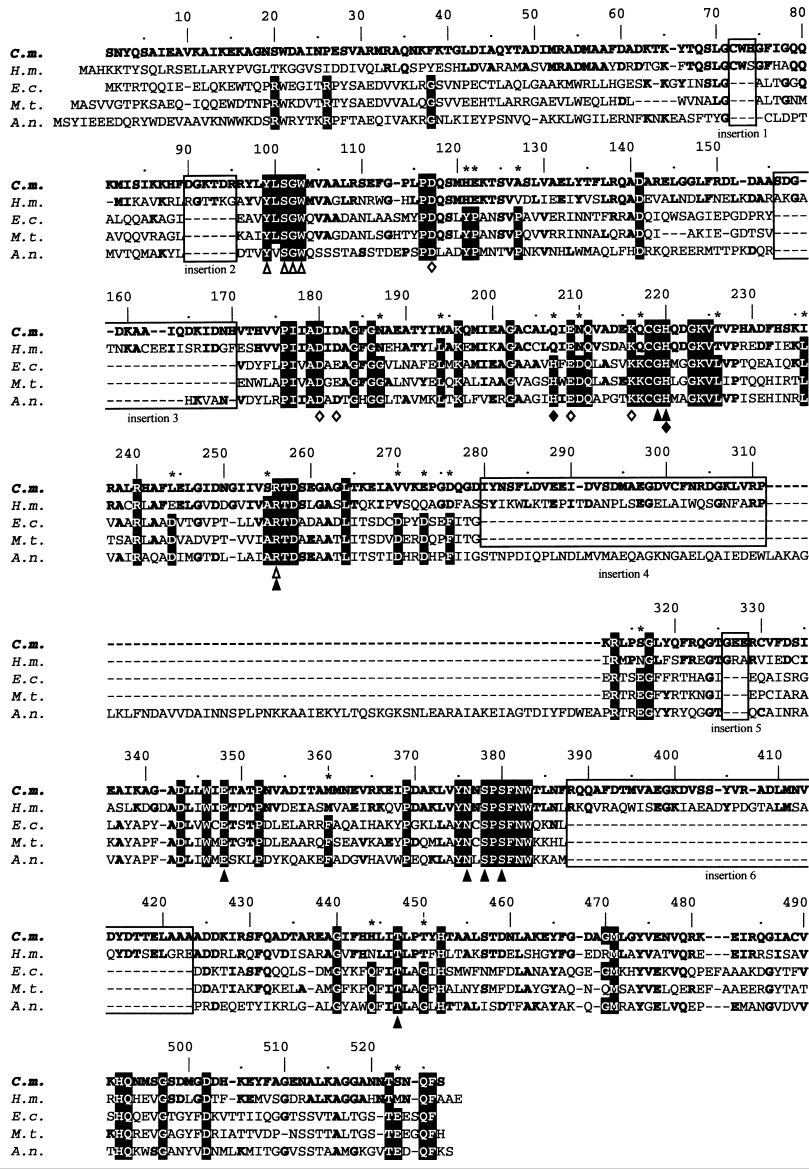


Fig. 2. Alignment of amino acid sequences of ICLs from different organisms. The protein sequences are from *C. maris* (*C.m.*; database accession no. AB066287), *H. methylovorum* GM2 (*H.m.*; BAA23678), *E. coli* (*E.c.*; P05313), *M. tuberculosis* (*M.t.*; O53752) and *A. nidulans* (*A.n.*; CAA44572). Identical amino acids with ICL of *C. maris* are in bold. Complete conserved amino acid sequences from 22 different ICLs (source organisms are in Britton *et al.*, 2000) are shown as white letters in black boxes. In the *C. maris* ICL, amino acids substituted for these conserved amino acids are shown by asterisks. Amino acids forming hydrogen bonds with substrates [glyoxylate (Δ) or succinate (▲)] and with metal ions or with H₂O coordinating with metal ion (◇), or contributing to tetrameric assembly (◆) are indicated below the sequences.

with a sequencing ladder of the *C. maris icl* gene. Sequencing was performed with the Sequenase Version 2.0 Labelled dCTP Kit (USB/Amersham Pharmacia Biotech) with the same primer as the primer-extension reaction.

Immunological studies. ICL of *C. maris* was purified as described previously (Watanabe *et al.*, 2001). The purified ICL (1.2 mg) was emulsified with Freund's complete adjuvant and injected subcutaneously into a young (approx. 10 weeks old) New Zealand white rabbit. After 2 and 3 weeks, the rabbit was boosted twice with half the amount of the enzyme first injected, which was emulsified with incomplete adjuvant. From 20 days after the last injection, blood was intermittently collected from an ear vein. The IgG fraction was purified as described previously (Ishii *et al.*, 1987). Double immunodiffusion in 1.2% agarose was performed by the method of Ouchterlony (1968).

Western blot analysis. After SDS-PAGE of the purified ICL and the cell extracts of the *C. maris* and *E. coli* cells on 10% (w/v) acrylamide gels, the proteins were transferred to 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 *C. maris* ICL.

RESULTS

Cloning of the *C. maris icl* gene

A 655 bp PCR product was obtained by the genomic PCR as described in Methods and its deduced amino acid sequence was similar to those of ICLs of various organisms. Genomic Southern blot analysis using this PCR product as an *icl*-specific probe indicated that each single 2.7, 0.8 and 1.0 kbp fragment of the *C. maris* genomic DNA digested with *EcoRV*, *HincII* and *HindIII*, respectively, hybridized with the probe (data not shown). To isolate the *icl* gene, the genomic library constructed with phage carrying fragments of the *C.*

maris chromosomal DNA was screened with the same probe. A phage clone with strongest positive signal was selected (termed λ 111) and analysis of restriction map was carried out (Fig. 1). A 3.5 kbp *Bam*HI–*Sal*I fragment of λ 111 was subcloned into pBluescript SK(+) to yield a plasmid pCM4. As shown in Fig. 1, 1.2 and 1.0 kbp *Pst*I fragments and a 1.1 kbp *Bam*HI–*Pst*I fragment of pCM4 were further subcloned into pBS SK(+) (termed pCM43, pCM46 and pCM42, respectively).

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

Both strands of the three subcloned fragments were sequenced. The *icl* gene was 1584 bp long and a putative ribosome-binding site, GGAG (Shine & Dalgarno, 1974), was found 7–10 bases upstream of the ATG codon. The ORF encodes a polypeptide of 528 aa with a calculated molecular mass of 58 150 Da. This value is slightly smaller than that of the purified ICL determined by SDS-PAGE (64 kDa) but is close to that by the gel filtration (240 kDa; tetramer of the 60 kDa subunit). The N-terminal amino acid sequence agreed completely with that determined from the purified ICL (SNYQS-AIEAVKAIKAIKEKAGNS) (Watanabe *et al.*, 2001), except for a deletion of amino acids at positions 11–13 (KAI) in the sequence of the purified ICL, probably due to misvaluation of the N-terminal amino acid sequence by Edman degradation.

The molecular masses of bacterial and eukaryotic ICL subunits are about 46–48 kDa and 62–67 kDa, respectively (Vanni *et al.*, 1990). A comparison of amino acid sequences of these two types of ICLs revealed the existence of a long insertion of about 100 aa residues in the middle region of eukaryotic ICLs (Fig. 2). In castor beans, this insertion has been reported to take part in the import of ICL into the glyoxysome (Matsuoka & McFadden, 1988; Vanni *et al.*, 1990). In contrast, alignment of the deduced amino acid sequence of the *C. maris* ICL revealed that, instead of this additional insertion, several short insertions of 3–36 aa residues were scattered in the enzyme (Fig. 2, boxed regions), suggesting that they play a role different from the longer insertion of the eukaryotic ICLs. Such insertions of amino acid residues, which are not found in mesophilic counterparts, also exist in some psychrophilic enzymes (Davail *et al.*, 1994; Russell *et al.*, 1998). Although no ICL gene has been cloned from thermophilic bacteria, putative ICLs in their determined genome sequences do not possess such insertions. The amino acid sequence homology between the *C. maris* ICL and any other bacterial or eukaryotic ICL was low (24–29% identity). Indeed, no cross-reactivity of antibody against ICL from *C. maris* with ICL in cell-free extract of *E. coli* was detected (Fig. 3). These results indicate that the *C. maris* ICL is not closely related to other bacterial ICLs. However, ICL of a methylotrophic bacterium, *Hyphomicrobium methylovorum* GM2, showed a relatively high homology of amino acid sequence (54% identity) with that of *C. maris* despite the large phylogenetic

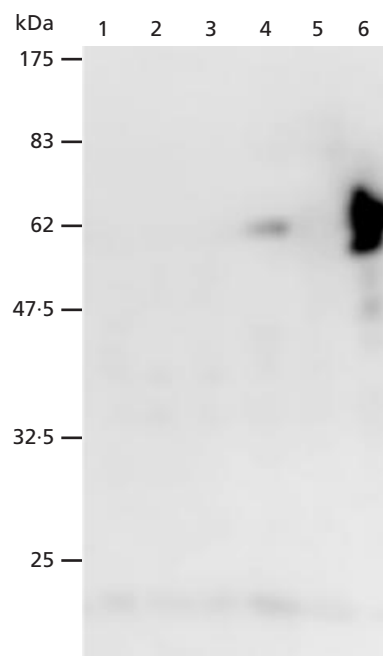


Fig. 3. Western blot analysis of ICL in the sonic extract of *E. coli* cells transformed by a plasmid harbouring *icl*. Lane 1, *E. coli* KM22 (ICL-positive control; 20 μ g protein); lane 2, *E. coli* ACA421 (ICL-negative control; 20 μ g protein); lane 3, pBS SK(+)-transformed *E. coli* ACA421 cells (20 μ g protein); lanes 4 and 5, pCM477-transformed *E. coli* ACA421 cells grown at 15 °C and 37 °C, respectively (20 μ g protein); lane 6, purified *C. maris* ICL (0.02 μ g protein).

distance between these two bacteria (Tanaka *et al.*, 1997).

A partial ORF transcribed divergently from *icl* was present in the flanking region of *icl* (Fig. 1). The predicted amino acid sequence of this partial ORF exhibited 30–40% identity with the LysR family of bacterial transcriptional activators (Henikoff *et al.*, 1988).

Expression of the *C. maris icl* gene in the *E. coli aceA* mutant

No ICL activity was detected in cell extract of the ICL-defective *E. coli* ACA421 cells grown at 37 °C on LB medium and even on this medium containing 50 mM acetate to induce the ICL gene expression in *E. coli*. The specific activity of ICL in the cell extract of the ACA421 cells transformed by a plasmid (pCM477) harbouring the *C. maris icl* gene was 0.028 ± 0.002 U (mg protein)⁻¹ (three independent experiments) following growth at 15 °C on M9 medium containing 50 mM acetate and 0.1% (w/v) Casamino acid as carbon sources. This was comparable to that of the *C. maris* cells grown at 15 °C on nutrient medium supplemented with 25 mM acetate [0.03 U (mg protein)⁻¹] (Watanabe *et al.*, 2001). The transformant was not able to grow at 37 °C in M9 medium containing acetate as the sole carbon source. Northern and Western blot analyses showed that mRNA

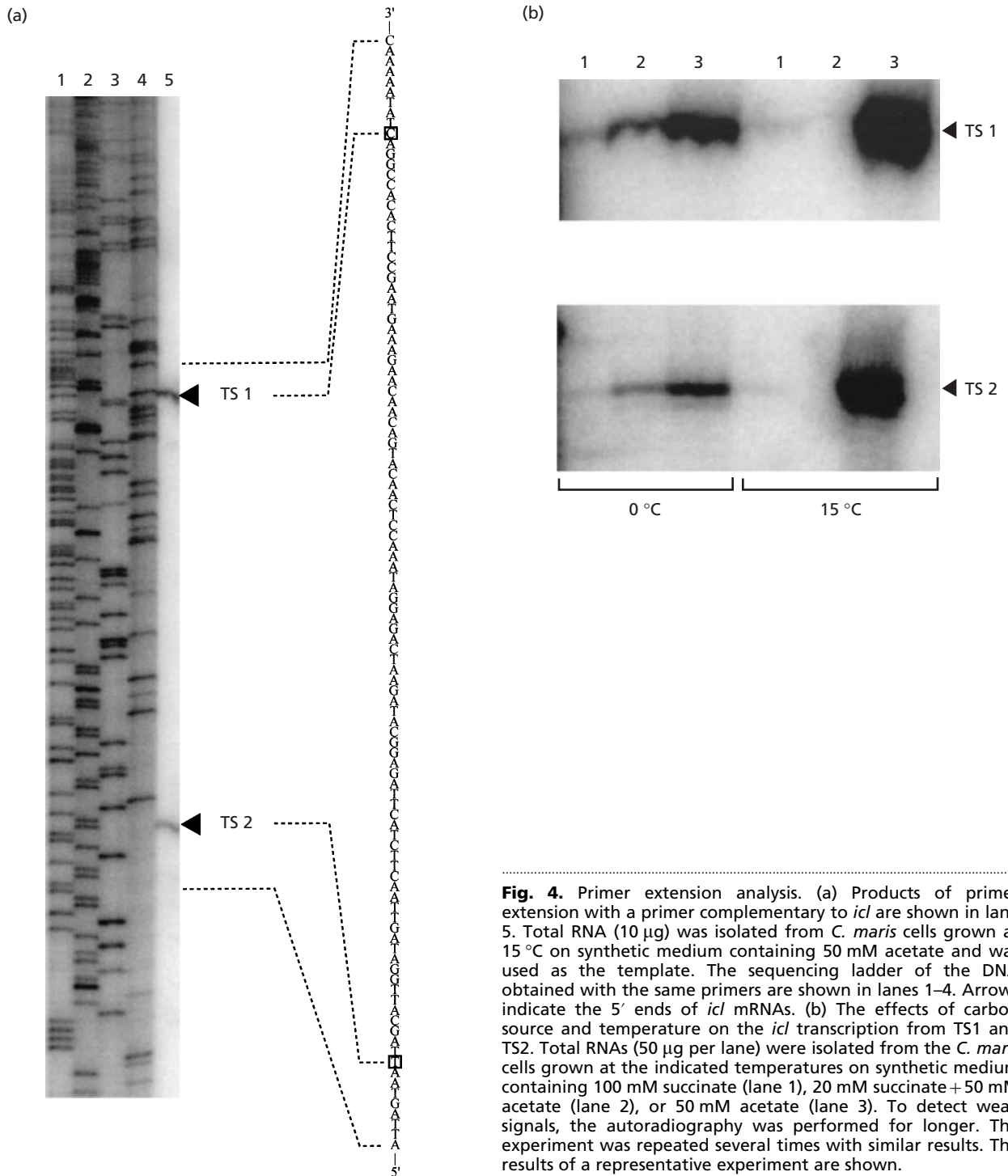


Fig. 4. Primer extension analysis. (a) Products of primer extension with a primer complementary to *icl* are shown in lane 5. Total RNA (10 µg) was isolated from *C. maris* cells grown at 15 °C on synthetic medium containing 50 mM acetate and was used as the template. The sequencing ladder of the DNA obtained with the same primers are shown in lanes 1–4. Arrows indicate the 5' ends of *icl* mRNAs. (b) The effects of carbon source and temperature on the *icl* transcription from TS1 and TS2. Total RNAs (50 µg per lane) were isolated from the *C. maris* cells grown at the indicated temperatures on synthetic medium containing 100 mM succinate (lane 1), 20 mM succinate + 50 mM acetate (lane 2), or 50 mM acetate (lane 3). To detect weak signals, the autoradiography was performed for longer. The experiment was repeated several times with similar results. The results of a representative experiment are shown.

of the *icl* gene, the ICL protein and the ICL activity were detected in the cells grown at 15 °C, but not at 37 °C, on M9 medium containing 50 mM acetate and 0.1% (w/v) Casamino acid (data not shown). These results suggested that no transcription of the *icl* gene was responsible for the inability of the transformant to grow at 37 °C. Western blot analysis revealed that a single protein cross-reacting with antibodies against the *C. maris* ICL was detected in the transformant grown at

15 °C at the same position as the purified ICL on the gel of SDS-PAGE (Fig. 3).

Analysis of 5'-terminal region of the *C. maris icl* mRNA

The 5' end of the *C. maris icl* mRNA was examined by primer-extension analysis (Fig. 4a). The results showed that two species of the *icl* mRNA with different lengths

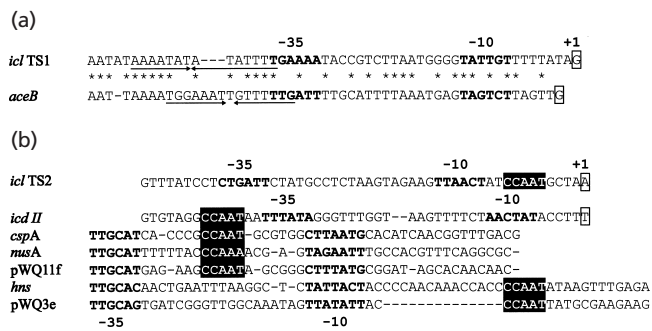


Fig. 5. Alignment of the sequences of the promoter upstream of *icl* TS1 and TS2 sites. (a) Alignment of the sequences of the promoter regions upstream of the *icl* TS1 site and the *E. coli* *ace* operon (*aceB*). Identical nucleotides are shown by asterisks. Inverted arrows indicate palindromic repeats. In *aceB*, this sequence is the IclR repressor binding site (Gui *et al.*, 1996). The putative -35 and -10 promoter elements and the putative transcriptional start points (+1) are indicated by bold and boxed letters, respectively. (b) Alignment of the sequences of the promoter regions upstream of the *C. maris icl* TS2 site and *iclII* and the *E. coli* cold-inducible genes. The CCAAT sequences are shown as white letters in black boxes.

of 5'-untranslated regions (TS1 and TS2) were present in the *C. maris* cells grown at 15 °C on acetate and the 5' ends of these RNAs (TS1 and TS2 sites) were G and A, located at 130 and 39 bases upstream of the translational start codon of the *icl* gene, respectively. The level of TS1 was 2.2 times that of TS2. The putative promoter motifs upstream of the TS1 and TS2 sites are shown in Fig. 5. We also examined effects of growth temperature and

carbon source(s) on the levels of both mRNAs (Fig. 4b). At temperatures of both 0 °C and 15 °C, acetate more strongly induced the expression of both the mRNAs than succinate as well as acetate-inducible ICL genes generally known in other organisms; the expression of TS1 and TS2 in the cells grown at 15 °C on acetate were 27- and 39-fold higher than those on succinate, respectively. However, when the *C. maris* cells were grown on succinate, the expression levels of both mRNAs at 0 °C were more than those at 15 °C, regardless of the presence of acetate. The relative ratio of TS1 to TS2 was constant at both temperatures (1.6 and 1.7 at 0 °C and 15 °C, respectively, in the presence of succinate). These results indicate that the expressions of both mRNAs are also induced by low temperature.

Expression of the *C. maris icl* gene under different growth conditions

To evaluate the expression of the *icl* gene in more detail, Northern blot analysis was carried out with RNAs isolated from *C. maris* cells grown under different conditions (Fig. 6a). At all temperatures tested, maximum gene expression was observed in cells grown on 50 mM acetate as the sole carbon source, and the expression level of these cells at 15 °C was about 67 times that of the cells grown on succinate. This induction of the expression by acetate was hardly affected by temperature (the expression level at 15 °C was 1.1 times that at 0 °C). The induction was also observed in the cells grown on succinate plus acetate, but the level of the induction (16 times that of the cells grown on succinate) was smaller than that in the cells grown on acetate

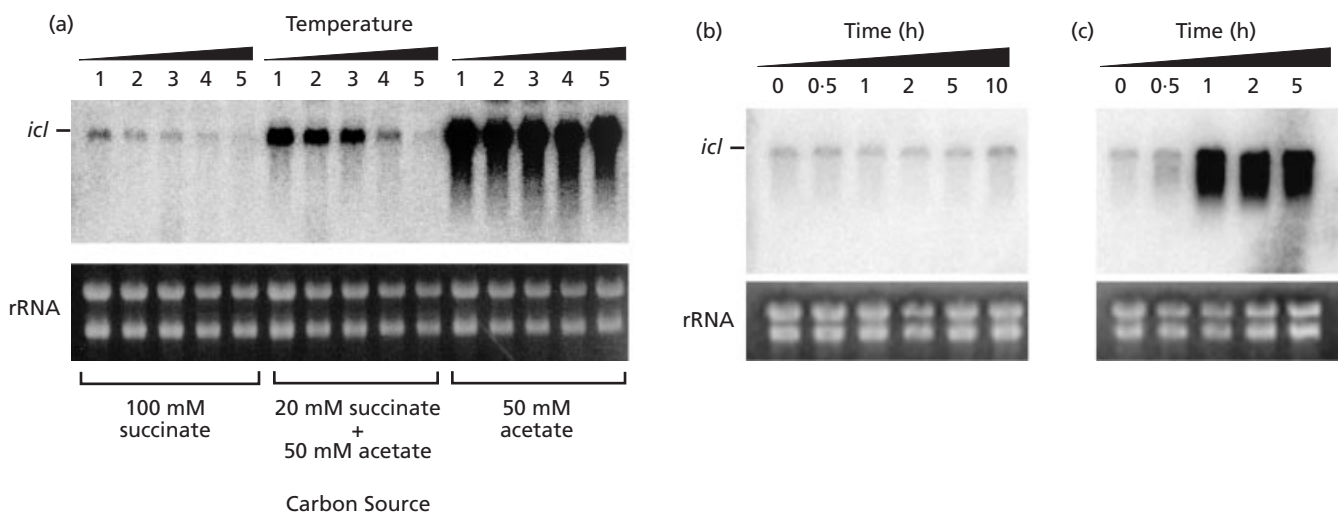


Fig. 6. Northern hybridization of *C. maris icl*. (a) Total RNAs (4 µg per lane) were isolated from *C. maris* cells grown at 0 °C (lane 1), 5 °C (lane 2), 10 °C (lane 3), 15 °C (lane 4) and 20 °C (lane 5) on synthetic medium containing indicated carbon source(s). (b) Temperature shift from 15 °C to 0 °C. *C. maris* was grown at 15 °C on succinate until mid-exponential phase, transferred to 0 °C and growth continued. RNA was collected at 0, 0.5, 1, 2, 5 and 10 h after the temperature shift, and 6 µg RNA per lane was electrophoresed. (c) Shift of carbon source from succinate to acetate. *C. maris* cells were grown at 15 °C on 100 mM succinate until mid-exponential phase and collected by centrifugation. The cells were suspended in fresh medium containing 50 mM acetate, and growth continued at 15 °C. RNA was collected at 0, 0.5, 1, 2 and 5 h after the shift, and 4 µg RNA per lane was electrophoresed. The experiment was repeated several times with similar results. The results of a representative experiment are shown.

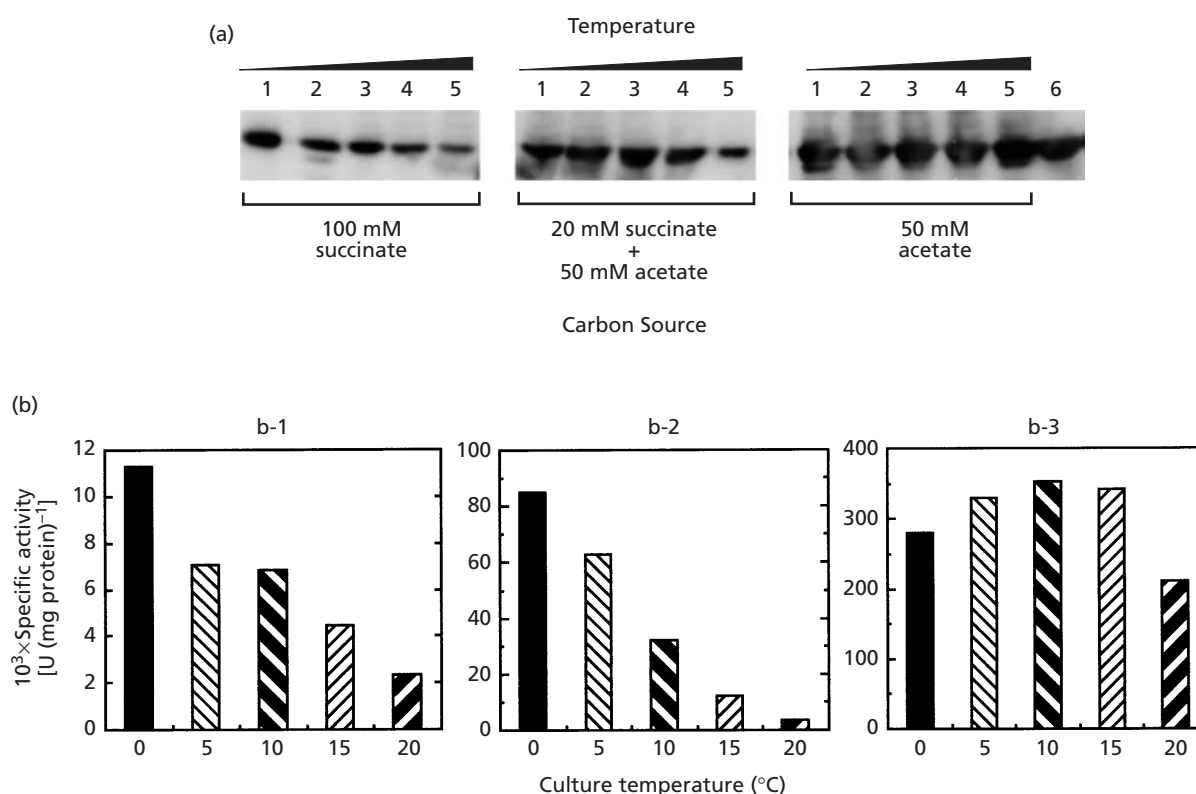


Fig. 7. Effects of temperature and carbon source on translational level of ICL. (a) Western blotting analysis of ICL in cell-free extract of *C. maris*. Lanes 1–5; 20 μg cell-free extract, lane 6; purified ICL (2 μg protein). The cells were grown at 0 °C (lanes 1), 5 °C (lanes 2), 10 °C (lanes 3), 15 °C (lanes 4) and 20 °C (lanes 5) on the indicated carbon source(s). (b) Specific activity of ICL in the cell-free extract. Cells were grown at the indicated temperatures on 100 mM succinate (b-1), 20 mM succinate + 50 mM acetate (b-2), and 50 mM acetate (b-3) as carbon source(s). The mean values of three independent experiments are shown.

alone. The level of *icl* mRNA was also increased by the exchange of succinate for acetate as the carbon source and was maximized (75-fold) at 5 h after the exchange (Fig. 6c).

When the cells were grown on succinate regardless of the presence of acetate, the transcriptional level was increased with decreasing temperature. Ratios of the expression level at 0 °C to that at 15 °C were about 4.5 and 7.7 in the cells grown on succinate and succinate plus acetate, respectively. These results were consistent with those of the primer-extension analysis described above. Furthermore, a slight induction of the expression was also observed by decreasing the cultivation temperature from 15 °C to 0 °C (1.3-fold at 10 h after the shift) (Fig. 6b).

The amount of ICL protein in cell extracts of *C. maris* cells was examined by Western blot analysis (Fig. 7a). The results were consistent with those of the mRNA levels shown in Fig. 6. However, the extents of the induction at protein levels by acetate and low temperature were lower than those of transcription (the ratio of acetate to succinate at 15 °C was 13; the ratio of 0 °C to 15 °C in the presence of succinate was 8). In contrast, changes of the ICL activity in the cell-free

extract dependent on carbon source and growth temperatures (Fig. 7b) were analogous to those observed at the level of transcription (the induction by acetate at 15 °C was 77-fold higher than that by succinate; the relative ratio of the ICL activity at 0 °C to that at 15 °C was 7 when the *C. maris* cells were grown on succinate and acetate) (Figs 4b and 6).

DISCUSSION

This paper reports the cloning and sequencing of the *icl* gene encoding the cold-adapted ICL protein of *C. maris* and demonstrated that the expression of this gene was induced by low temperature and acetate. This is the first report of an ICL gene from a psychrophilic bacterium. In *E. coli* the *aceA* gene (encoding ICL) forms an operon together with the *aceB* and *aceK* genes encoding MS and IDH-specific kinase/phosphatase, respectively, in the order *aceB*–*aceA*–*aceK* (Cozzzone, 1998). A similar organization of genes has also been found in *Salmonella typhimurium* (Wilson & Maloy, 1987) and *Yersinia pestis* (database accession no. NC_003143). On the other hand, in *Corynebacterium glutamicum*, *aceA* is clustered with *aceB*, which is transcribed in the opposite direction (Reinscheid *et al.*, 1994a, b). In addition, in the

genomes of some bacteria, *aceA* and *aceB* cluster in the order *aceB-aceA* [*Vibrio cholerae* (NC_002505) and *Sulfolobus solfataricus* (NC_002754)] or *aceA-aceB* [*Haloferax volcanii* (Serrano & Bonete, 2001) and *Caulobacter crescentus* (AE005673)]. However, *aceA* and *aceB* of many bacteria are separated from each other. Among them, several bacteria such as *Bacillus halodurans* (NC_002570) and *Deinococcus radiodurans* (NC_001263), possess no *aceK*. *aceB*- and *aceK*-like genes were not present in the flanking regions of the *C. maris icl* gene (Fig. 1), and it has been reported that *C. maris* does not contain an IDH kinase (Suzuki *et al.*, 1995).

It was reported that there are four highly conserved regions in the aligned amino acid sequences of eukaryotic and prokaryotic ICLs (Rehman & McFadden, 1996). From several investigations of chemical modifications and site-directed mutagenesis within these regions of the *E. coli* ICL, amino acid residues at the active site of the enzyme were tentatively identified (Diehl & McFadden, 1993, 1994; Ko & McFadden, 1990; Ko *et al.*, 1991, 1992; Rehman & McFadden, 1996, 1997a, b, c). Furthermore, the three-dimensional structures of eukaryotic and prokaryotic ICLs were recently determined by X-ray crystallography (Britton *et al.*, 2000, 2001; Sharma *et al.*, 2000). Many amino acid residues essential for catalytic activity, including K216, C218, H220, E348, S378, P379, S380 and H452, and for binding with substrates (Fig. 2, \triangle and \blacktriangle) and divalent metal ion (Fig. 2, \diamond) were conserved in the *C. maris* ICL despite the low overall homology with ICLs from other organisms. These facts indicate that the tetrameric structure and catalytic mechanism of the *C. maris* ICL may be fundamentally similar to those from other organisms. However, several substitutions of strictly conserved amino acid residues were detected in the *C. maris* ICL (Fig. 2, asterisks). Several of these may be involved in forming the active site and maintaining the three dimensional structure of the protein. For example, the substitution of His for Gln at position 184 of the *E. coli* ICL, corresponding to Q207 in the *C. maris* ICL, leads to a dramatic decrease in enzyme activity and prevents the formation of a tetrameric structure (Diehl & McFadden, 1994). Therefore, the Gln residue at this position in the *C. maris* ICL may be responsible for its marked thermolability. Also, Lys at position 194 in the *E. coli* ICL (Rehman & McFadden, 1997b), suggested to be an important amino acid residue for catalytic function, was replaced by Gln at position 217 in the *C. maris* enzyme (Fig. 2). In addition, a KKCGH motif containing this residue is conserved completely in ICLs from other organisms and was reported to form a flexible 'active site loop', moving reversibly by the binding of substrate, in three dimensional structure (Sharma *et al.*, 2000; Britton *et al.*, 2001). For high catalytic activity at low temperatures, enzymes are known to increase their flexibilities and consequently their accessibility for substrates (Gerday *et al.*, 1997). Our preliminary studies that Q207H and Q217K mutants of the *C. maris* ICL exhibited considerably lower activities at relatively low

temperature ranges (between 10 and 25 °C), and were more thermostable than wild-type enzyme may support this possibility. Furthermore, the *C. maris* ICL contains insertions consisting of 3–36 aa residues previously seen in some cold-adapted enzymes (Davail *et al.*, 1994; Russell *et al.*, 1998). These differences in amino acid sequence may endow the *C. maris* ICL with high activity at low temperatures and the high structural flexibility. In fact, the ICL of *H. methylovorum* with the highest amino acid sequence homology to the *C. maris* ICL was found to share these substitutions and the insertions of amino acid residues (Fig. 2) and showed a relatively low thermostability in spite of being a mesophilic enzyme (Tanaka *et al.*, 1997).

Analyses of RNA and protein levels, and enzyme activity showed that the *icl* gene and ICL protein of *C. maris* were induced transcriptionally and translationally by acetate as was also observed for ICL genes of other organisms (Figs 4, 6 and 7). These results suggest that this enzyme is important for acetate metabolism. Primer-extension analysis of the *C. maris* total RNA revealed that two different mRNAs (TS1 and TS2) could be produced from the *icl* gene. This result give rise to two hypotheses; TS1 and TS2 mRNAs may be transcribed independently of each other from different start points (TS1 and TS2 sites, respectively), or TS2 might be derived from a post-transcriptional processing of TS1. Further studies are required to distinguish between these possibilities. However, the promoter region upstream of the TS1 site was similar to that of the *E. coli ace* operon, and contained a similar palindrome structure to that of the *ace* operon, to which its specific repressor protein, IclR, binds (Fig. 5a) (Gui *et al.*, 1996; Negre *et al.*, 1992). The promoter region of *icdI* encoding acetate-inducible IDH-I isozyme of *C. maris* was also reported to resemble that of the *E. coli ace* operon (Ishii *et al.*, 1993; Suzuki *et al.*, 1995). The CCAAT sequence is present 5–9 bases upstream of the TS2 site (Fig. 5b). This sequence is reported to be a common motif in the promoter regions of the *E. coli* cold shock genes (Qoronfleh *et al.*, 1992) and is also present in the upstream region of *icdIII* (Ishii *et al.*, 1993). Recently, we found direct evidence that the CCAAT sequence was essential for the induction of the *icdIII* gene expression by low temperature and the deletion of the sequence at upstream region of the *icdIII* gene resulted in a complete loss of the ability of the gene expression responsive to low temperature in *icdIII* transformant of the *E. coli* mutant strain defective in *icd* (Sahara *et al.*, 1999).

It is interesting that a partial ORF exhibiting 30–40% identity with the LysR family of bacterial transcriptional factors (referred to as *ORF₁*) is adjacent to the *icl* gene (Fig. 1) because the ICL protein may be regulated by this ORF product. We determined the 5' sequence of the *ORF₁* mRNA and examined the effects of temperature and carbon source on the expression of *ORF₁* by primer-extension analysis (data not shown). A main putative transcriptional starting point of *ORF₁* was located 130 bp upstream of the *icl* TS1 site, and the expression was induced by succinate, but not acetate and low

temperature. Further studies are required to clarify the function of *ORF₁*.

In this study, it was found that the expression of *icl* was induced by not only acetate but also low temperature (Figs 4, 6 and 7). Furthermore, the cold-induction of *icl* expression took place in the presence of succinate but not acetate, suggesting that it is closely linked with carbon metabolism. It was reported that IDH-II isozyme of this bacterium is also a cold-adapted enzyme and the expression of the IDH-II gene is induced by low temperature (Ochiai *et al.*, 1979; Suzuki *et al.*, 1995). Considering the metabolic importance for the flows of carbon compounds between the TCA and glyoxylate cycles, it may be reasonable that both IDH and ICL, key enzymes of the respective metabolic cycles, are adapted to cold environment in respect of gene expression of the enzymes as well as their catalytic functions. Furthermore, we found recently that ICL of another psychrophile, *Colwellia psychrerythraea*, was also very thermolabile and the gene encoding the protein was induced by low temperature (Watanabe *et al.*, 2002). Psychrophilic characters of the ICL genes and proteins from *C. maris* and *C. psychrerythraea* indicate that the metabolic step catalysed by ICL may be important for the abilities of cold adaptation and survival under cold environments of psychrophilic bacteria.

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