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ORIGINAL PAPER

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Two isocitrate dehydrogenases from a psychrophilic bacterium, *Colwellia psychrerythraea*

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Abstract Two structurally different monomeric and dimeric types of isocitrate dehydrogenase (IDH; EC 1.1.1.42) isozymes were confirmed to exist in a psychrophilic bacterium, *Colwellia psychrerythraea*, by Western blot analysis and the genes encoding them were cloned and sequenced. Open reading frames of the genes (*icd-M* and *icd-D*) encoding the monomeric and dimeric IDHs of this bacterium, IDH-M and IDH-D, were 2,232 and 1,251 bp in length and corresponded to polypeptides composed of 743 and 416 amino acids, respectively. The deduced amino acid sequences of the IDH-M and IDH-D showed high homology with those of monomeric and dimeric IDHs from other bacteria, respectively. Although the two genes were located in tandem, *icd-M* then *icd-D*, on the chromosomal DNA, a Northern blot analysis and primer extension experiment revealed that they are transcribed independent of each other. The expression of the monomeric and dimeric IDH isozyme genes in *C. maris*, a psychrophilic bacterium of the same genus as *C. psychrerythraea*, is known to be induced by low temperature and acetate, respectively, but no such induction in the expression of the *C. psychrerythraea icd-M* and *icd-D* genes was detected. IDH-M and IDH-D overexpressed in *Escherichia coli* were purified and characterized. In *C. psychrerythraea,* the IDH-M isozyme is cold-active whereas IDH-D is mesophilic, which is similar to *C. maris* that contains both cold-adapted and mesophilic isozymes of IDH. Experiments with chimeric enzymes between the cold-adapted monomeric IDHs of *C. psychrerythraea* and *C. maris* (IDH-M and ICD-II, respectively) suggested that the C-terminal region of the *C. maris* IDH-II is involved in its catalytic activity.

Keywords Psychrophilic bacterium · *Colwellia psychrerythraea* · Isocitrate dehydrogenase · Isozyme · Cold-adapted enzyme

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Introduction

Isocitrate dehydrogenase [IDH; EC 1.1.1.41(42)] is a key enzyme of the TCA cycle and important for controlling the metabolic flux between the TCA cycle and the glyoxylate shunt. This enzyme catalyzes the oxidative decarboxylation of D-isocitrate to 2-oxoglutarate and CO_2 coupled with the reduction of NAD^+ or $NADP^+$. On the basis of subunit structure, bacterial IDHs can be categorized into two types, homodimers consisting of subunits of about 40–45 kDa and monomers with a molecular mass of 80–100 kDa. Many bacteria possess only one type. For instance, the IDHs of *Escherichia coli* (Burke et al. 1974), *Thermus thermophilus* (Eguchi et al. 1989), *Bacillus stearothermophilus* (Howard and Becker 1970) and *Rhodopseudomonas spheroides* (Chung and Braginski 1972) are known to be dimeric, while those of *Azotobacter vinelandii* (Chung and Franzen 1969; Sahara et al. 2002), *Corynebacterium glutamicum* (Eikmanns et al. 1995; Chen and Yang 2000), *Rhodomicrobium vannielii* (Leyland and Kelly 1991) and *Vibrio parahaemolyticus* (Fukunaga et al. 1992) are monomeric. On the other hand, it has been reported that *Colwellia maris*, previously described as *Vibrio* sp. strain ABE-1, and *Ralstonia eutropha* have both dimeric and monomeric IDHs (Ochiai et al. 1979; Wang et al. 2003). In addition, the coexistence of IDH isozyme genes in several bacteria is presumed based on genomic sequences. In the psychrophilic bacterium *C. maris*, the dimeric IDH-I (*Cm*IDH-I) is a typical mesophilic enzyme, while the monomeric IDH-II (*Cm*IDH-II) is cold-adapted and exhibits extreme thermolability above

25°C (Ochiai et al. 1979, 1984). The transcription of the gene encoding IDH-II, *icd-II*, is induced by low temperature while that of *icd-I* encoding IDH-I is acetate inducible (Suzuki et al. 1995). Nevertheless bacterial monomeric and dimeric IDHs catalyze the same reaction, have little homology in amino acid sequence (Ishii et al. 1993) and differ in their immuno-crossreactivity (Ishii et al. 1987; Fukunaga et al. 1992). Recently, the structure of mesophilic IDH of *A. vinelandii* was first resolved as a bacterial monomeric one (Yasutake et al. 2002), and it was revealed that it contains two domains, domain I composed of N-terminal region 1 and C-terminal region 3 and domain II composed of intermediate region 2. Since a mutant of *C. maris* completely lacking IDH-I activity showed the same growth characteristics, with respect to nutrients required and growth temperature, as the wild type, it was concluded that this enzyme is dispensable to this bacterium (Fukunaga et al. 1988). However, the physiological significance of the coexistence of the two IDH isozymes in *C. maris* has not been completely elucidated.

Colwellia psychrerythraea isolated from flounder eggs is classified into the same genus as *C. maris* and is also a psychrophilic bacterium (D'Aoust and Kushner 1972). It is of interest, as well as helpful for understanding the physiological significance of the coexistence of IDH isozymes and cold adaptation of these bacteria, to clarify whether *C. psychrerythraea* possesses IDH isozymes or not. In this study, we present unambiguous evidence of the existence of the two IDH isozymes in this bacterium and report the cloning of the two genes encoding them and their characteristics, including their catalytic properties and gene expression. Furthermore, as a first step toward identifying the structural determinants responsible for the differences in catalytic function between the monomeric IDHs of *C. psychrerythraea* and *C. maris*, chimeric genes in which each region was exchanged between the monomeric IDH of *C. psychrerythraea* and *Cm*IDH-II were constructed based on the three-dimensional structure of *A. vinelandii* IDH, and several characteristics of the chimeric enzymes were examined.

Materials and methods

Bacteria, phage, plasmids and culture conditions

The psychrophilic bacterium *C. psychrerythraea* NRC 1004 (D'Aoust and Kushner 1972) was grown at 15°C with vigorous shaking as described previously (Watanabe et al. 2002b). *E. coli* XL1-Blue (Stratagene) was used to propagate plasmids and cultured at 37°C. A mutant of *E. coli* defective in IDH, DEK2004 (Thorsness and Koshland 1987), which has a glutamate auxotrophic phenotype, was used as a host for expression experiments of the *C. psychrerythraea* IDH genes. For the growth of these *E. coli* strains, Luria-Bertani (LB) medium, Super broth medium (Watanabe et al. 2005) or a morpholinepropanesulfonic acid (MOPS)-based synthetic medium (Neidhardt et al. 1974) supplemented with 0.5 mM Trp and 2% sodium succinate was used. If necessary, ampicillin and tetracycline were added to the culture media at concentrations of 50 and 15 μg/ml, respectively. Phage λEMBL3 (Stratagene) was used as a vector for the construction of a genomic DNA library of *C. psychrerythraea*. Plasmids pBluescript KS(+) (pBS; Stratagene) and pTrcHisB (Invitrogen) were used for further subcloning of the IDH genes and the overexpression of genes encoding His-tagged recombinant IDHs, respectively.

Western blot analysis

Colwellia psychrerythraea was cultured at various temperatures until the OD_{600} of the culture reached 1.0–1.5. The bacterial cells were then harvested and suspended in 50 mM potassium phosphate buffer, pH 7.5, containing 2 mM $MgCl_2$, 0.5 M NaCl and 10 mM 2-mercaptoethanol. The cell-free extract was prepared as described previously (Watanabe et al. 2002b). Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Unless stated otherwise, the Western blot analysis of these crude extracts was performed with rabbit antibodies against *Cm*IDH-I and *Cm*IDH-II as described previously (Suzuki et al. 1995).

Cloning of the IDH genes and nucleotide sequencing

Genomic PCR was performed to obtain a probe for plaque hybridization. Chromosomal DNA was isolated and purified from the *C. psychrerythraea* cells as described previously (Ishii et al. 1993). The primers for *icd-M* and *icd-D* encoding the respective monomeric and dimeric IDHs of this bacterium (*Cp*IDH-M and *Cp*IDH-D, respectively) were designed from a highly conserved region of the *A. vinelandii* monomeric IDH and *Cm*IDH-II and of the *E. coli* dimeric IDH and *Cm*IDH-I, respectively (Table 1). The upstream primer for *icd-M*, *Cp*M-s, corresponds to the sequences between +240 and +263 from the translational start codon of *icd-II*, encoding *Cm*IDH-II, and between +234 and +257 of the *A. vinelandii* IDH gene, while the downstream primer, *Cp*M-as, is complementary to the sequences between +423 and +449 of the *icd-II* gene and between +417 and +443 of the *A. vinelandii* IDH gene. The upstream primer for *icd-D*, *Cp*D-s, corresponds to the sequences between +699 and +722 of *icd-I*, encoding *Cm*IDH-I, and the *E. coli* IDH gene, while the downstream primer, *Cp*D-as, is complementary to the sequences between +1,017 and +1,040 of *icd-I* and the *E. coli* IDH gene. Amplification was carried out for 30 cycles in a DNA thermal cycler 2400 (Perkin-Elmer) in a reaction mixture (50 μl) containing 0.5 μg genomic DNA, 50 pmol each of the forward and reverse primers and 5 U AmpliTaq DNA polymerase

Table 1 Oligonucleotides used in this study

Primer name	Nucleotide sequence (5′ → 3′)
Probe for plaque hybridization	
*Cp*M-s	AAYATCATCAAGYTGCCRAAYATY
*Cp*M-as	TGGCGCRCGRCGRTCRGAGTTRCCTTC
*Cp*D-s	ATGAAGTTYACYGAAGGMGCKTTT
*Cp*D-as	ACCKGCATATTTMGGWGCRGTWCC
Probe for primer extension	
M-primer	GGCTTGGATGATAGGTAATAAAGAATACG
D-primer	GTAATTTTATCACCTATACTGGGAACTG
Construction of IDH isozyme expression vectors	
CPMF0[a]	gataa*ggatcc*gAGCACTGATAACTCAAA
CPMR0[b]	atctc*gagctc*TTATAACAATGCTAACAA
CPDF0[a]	gataa*ggatcc*gACAAGTAAAATCACAGT
CPDR0[b]	atctc*gagctc*TTACATATGTTCAATAAT
Construction of chimeric IDH genes	
CF0[a]	gcgc*ggatcc*gAGCACTGATAACTCAAAAATC
CR0[b]	gcgc*gagctc*TTAAAGTAATGCAGATAAAATGG
*Cm*BssHII-s[c]	GGTAACTCTGATCGTC*GcGCGC*CAGCG
*Cm*BssHII-as[c]	CGCTGG*CGCgCG*ACGATCAGAGTTACC
*Cp*BssHII-s[c]	GTTTAACTGAAGCAG*GcGCgC*GTCTATC
*Cp*BssHII-as[c]	GGTAATTCAGATAGAC*GcGCgC*CTGCTTC
*Cp*NspV-s[d]	GGTGGCGGTTTA*TTcGAA*CTGGTGCAGG
*Cp*NspV-as[d]	CCACCTGCACCAGT*TTCgAA*TAAACCGCC

[a]Small letters indicate additional bases for introducing digestion sites for *Sac*I (italicized letters)
[b]Small letters indicate additional bases for introducing digestion sites for *Bam*HI (italicized letters)
[c]Small letters indicate bases substituted to introduce digestion site for *Bss*HII (italicized letters)
[d]Small letters indicate bases substituted to introduce digestion site for *Nsp*V (italicized letters)

(Roche Molecular Systems) in a buffer system prepared by the manufacturer. Cycling conditions were as follows: denaturation at 94°C for 1 min, annealing at 48°C for 1 min and extension at 55°C for 3 min, for each of 30 cycles. PCR products with predicted lengths of about 200 bp for the *icd-M* gene and 350 bp for the *icd-D* gene were purified and ligated to the *Eco*RV site of pBS for propagation.

Plaque hybridization (Sambrook et al. 2001) was carried out to screen the genomic DNA library of *C. psychrerythraea* constructed with a phage vector λEMBL3 as described previously (Ishii et al. 1993). The PCR products were labeled with [α-^{32}P]dCTP using a random primer labeling kit (Takara) and used as probes. After overnight incubation at 60°C with either of the labeled probes, blotted membranes were washed successively in 2×SSC (1×SSC is 15 mM sodium citrate, pH 7.0, and 0.15 M NaCl) containing 0.1% (w/v) SDS for 20 min at room temperature and then twice with 0.1×SSC containing 0.1% SDS for 20 min at 60°C. Autoradiography was performed by exposing the membrane to X-ray film (RX, Fuji Photo Film) at −80°C. In Southern blot analysis, the *C. psychrerythraea* genomic DNA digested with appropriate restriction enzymes was fractionated by 1% agarose gel electrophoresis, transferred to a nylon membrane and processed as described above.

The nucleotide sequence was determined in both directions using appropriate primers and a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) or using T3 and T7 primers and a Thermo Sequenase pre-mixed cycle sequencing kit (Amersham Biosciences) with a Li-Cor model 4000L sequencer. The sequences were analyzed with the Genetyx computer program (Software Development Co.). The nucleotide sequences of the *icd-M* and *icd-D* genes of *C. psychrerythraea* have been deposited in the DDBJ database under accession no. AB174851.

Northern blot analysis

Total RNA from mid-log phase cells of *C. psychrerythraea* and *E. coli* DEK2004 transformants harboring various plasmids was prepared with an RNeasy Total RNA Kit (Qiagen). Northern hybridization was performed as described previously (Sahara et al. 2002) but with the labeled probes used for plaque hybridization. After hybridization, the membrane was successively washed in 2×SSC containing 0.1% SDS for 20 min at room temperature and then in 2× and 1×SSC containing 0.1% SDS for 20 min at 42°C. Autoradiography was performed by exposing the membrane to a BAS Imaging Plate and using a BAS-2000 Image Reader (Fuji Photo Film).

Primer extension analysis

For the primer extension analysis, two primers, M-primer and D-primer complementary to the internal region between +62 and +90 from the translation start codon of *icd-M* and between +17 and +44 of *icd-D*, respectively, were synthesized (Table 1). The 5′-ends of the primers were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Total RNA (30–50 μg) isolated from *C. psychrerythraea* was incubated for 6 h at 40°C with either of the labeled primers. Extension was performed with a reverse transcriptase (ReverTra Ace, TOYOBO), and the products were analyzed by electrophoresis on a 6%

polyacrylamide sequencing gel with sequencing ladders of the *C. psychrerythraea* IDH genes. Sequencing was performed with the T7 sequence kit (USB) with the same primers as used for the primer extension reaction.

Construction of genes encoding His-tagged IDHs of *C. psychrerythraea* and chimeric IDH genes

The *C. psychrerythraea icd-M* and *icd-D* genes were amplified by PCR to introduce restriction sites for *Bam*HI and *Sac*I at the 5′ and 3′-terminals of the ORFs, respectively. Therefore, the following primers were synthesized: CPMF0 and CPMR0 as forward and reverse primers for *icd-M* and CPDF0 and CPDR0 as forward and reverse primers for *icd-D*, respectively (Table 1). Amplification was carried out for 30 cycles in a DNA thermal cycler 2400 (Perkin-Elmer) in a reaction mixture (50 µl) containing 100 ng pCPM (pBS ligated complete *icd-M* gene to the *Sal*I–*Hin*cII site) or pCPD (pBS ligated complete *icd-D* gene to the *Hin*cII site), 10 pmol each of the forward and reverse primers and 1 U KOD-plus 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 0.5 min and extension at 68°C for 2.5 min. Each PCR product was digested with *Bam*HI and *Sac*I and ligated to the *Bam*HI–*Sac*I site of pTrcHisB (Invitrogen), a plasmid vector for conferring the N-terminal $(His)_6$-tag on the expressed proteins, to obtain plasmids pHis*Cp*IDH-M and pHis*Cp*IDH-D, respectively.

The monomeric IDH of *A. vinelandii* consists of three regions 1–3 (Yasutake et al. 2002). According to a previous report (Watanabe et al. 2005), chimeric genes in which these regions of *Cm*IDH-II and *Cp*IDH-M were exchanged were constructed with the following modifications. Although *Cm*IDH-II has a *Nsp*V restriction site between region 2 and region 3, no restriction site is available between region 1 and region 2. Furthermore, there is no restriction site between region 1 and region 2 or between region 2 and region 3 in *Cp*IDH-M. Therefore, *Bss*HII and/or *Nsp*V sites were introduced into the *Cm*IDH-II and *Cp*IDH-M genes by PCR in a reaction mixture (50 µl) containing 100 ng pHis*Cm*IDH-II harboring the *icd-II* gene in the *Bam*HI and *Sac*I sites of pTrcHisB or pHis*Cp*IDH-M as a template, 10 pmol of each primer and 1 U KOD-plus DNA polymerase in a buffer system prepared by the manufacturer. The forward and reverse primers used to introduce the *Bss*HII site were *Cm*BssHII-s and *Cm*BssHII-as for the *icd-II* gene and *Cp*BssHII-s and *Cp*BssHII-as for the *Cp*IDH-M gene, respectively (Table 1). The primers used to introduce the *Nsp*V site into the *Cp*IDH-M gene were *Cp*NspV-s and *Cp*NspV-as. The PCR products were ligated into pTrcHisB to obtain plasmids pHis*Cm*IDH-II:*Bss*HII and pHis*Cp*IDH-M:*Bss*HII-*Nsp*V. The coding regions of resultant chimeric genes were certified by subsequent sequencing in both directions.

Overexpression and purification of His-tagged IDHs

The IDH-deficient mutant, *E. coli* DEK2004, transformed with pHis*Cp*IDH-M, pHis*Cp*IDH-D or plasmids carrying chimeric IDH genes, was grown, the IDH proteins were overexpressed and cell-free extracts were prepared as described previously (Watanabe et al. 2005) with the following modifications. Cells were suspended in buffer A (50 mM sodium phosphate, pH 8.0, containing 2 mM $MgCl_2$, 0.5 M NaCl, 10 mM 2-mercaptoethanol and 10 mM imidazole) for *Cp*IDH-D and chimeric IDHs or buffer B (50 mM sodium phosphate, pH 8.0, containing 2 mM $MgCl_2$, 0.3 M NaCl, 10 mM 2-mercaptoethanol and 10 mM imidazole) for *Cp*IDH-M. The cell-free extract was loaded onto a Ni-NTA column (25 ml volume; Qiagen). For the purification of *Cp*IDH-D and chimeric IDHs, the column was equilibrated with buffer A. After being thoroughly washed with the same buffer, the column was washed further with 50 ml of buffer C (buffer A containing 10% w/v glycerol and 20 mM imidazole instead of 10 mM imidazole) and next with 50 ml each of buffers D and E (buffer C containing 30 and 50 mM imidazole instead of 20 mM imidazole, respectively). The enzymes were then eluted with 50 ml of buffer F (buffer C containing 250 mM imidazole instead of 20 mM imidazole). For the purification of *Cp*IDH-M, the column was equilibrated with buffer B. After a thorough washing with the same buffer, the column was washed further with buffer G and then H (buffer B containing 20 and 30 mM imidazole instead of 10 mM imidazole, respectively). The *Cp*IDH-M was eluted with buffer E. Each elutant was concentrated and then dialyzed as described previously (Watanabe et al. 2005). All His-tagged recombinant IDHs were stocked at −35°C prior to use.

Enzyme assay

Unless otherwise noted, the IDH activity was assayed by determining spectrophotometrically the increase in absorbance at 340 nm as described previously (Ochiai et al. 1979) with the following modifications. For *Cp*IDH-D, the reaction mixture (2 ml) contained 33 mM Tris–HCl (pH 8.0), 0.67 mM $MnCl_2$, 0.12 mM $NADP^+$, 0.15 M NaCl and 2 mM isocitrate. For *Cp*IDH-M, 33 mM phosphate buffer (pH 7.0) and 0.25 M NaCl were added to the reaction mixture instead of 33 mM Tris–HCl and 0.15 M NaCl. For chimeric IDHs, 0.25 M NaCl was added to the reaction mixture for *Cp*IDH-M instead of 0.15 M NaCl. One unit of enzyme activity was defined as the amount capable of catalyzing the reduction of 1 µmol of $NADP^+$ per min. For heat-inactivation experiments, the purified recombinant IDHs were dialyzed overnight against 20 mM potassium phosphate buffer (pH 8.0) containing 2 mM $MgCl_2$, 100 mM NaCl, 10% (v/v) glycerol and 1 mM DTT. After incubation for 10 min at specific temperatures, the enzyme was immediately cooled on ice

for 10 min. The residual activity was then assayed at each optimal temperature.

Results

Western blot analysis of isocitrate dehydrogenase from *C. psychrerythraea*

A clear difference between the monomeric and dimeric types of bacterial IDHs and similarity within each group have been reported for immunological crossreactivity (Ishii et al. 1987; Leyland and Kelly 1991; Fukunaga et al. 1992; Sahara et al. 2002). To clarify whether *C. psychrerythraea*, another psychrophilic species of the same genus as *C. maris*, has both types of IDH as well, an immunoblot analysis was initially carried out using antibodies against the dimeric *Cm*IDH-I and monomeric IDH from *V. parahaemolyticus* (Fig. 1). Proteins cross-reacting with the respective antibodies were detected in the crude extract of *C. psychrerythraea* and their molecular masses were similar to the same type of IDHs from other bacteria. These results imply the coexistence of the two different types of IDH isozymes in this bacterium. On the other hand, unlike *C. maris* (Ochiai et al. 1979), only one peak of IDH activity in the crude extract was observed at 40°C (data not shown).

Cloning of the *C. psychrerythraea* IDH isozyme genes

Probes used for the cloning of monomeric and dimeric IDH genes of *C. psychrerythraea* were obtained by genomic PCR as described in Materials and methods, and the products were 210 and 342 bp in length, respectively. The deduced amino acid sequences of the two probes were similar to those of the respective IDH isozyme genes from *C. maris*. On the other hand, the genomic library of *C. psychrerythraea* was constructed by ligating 10–22 kbp fragments of the *C. psychrerythraea* chromosomal DNA obtained by partial digestion with *Sau*3AI into a phage vector, λEMBL3, and was

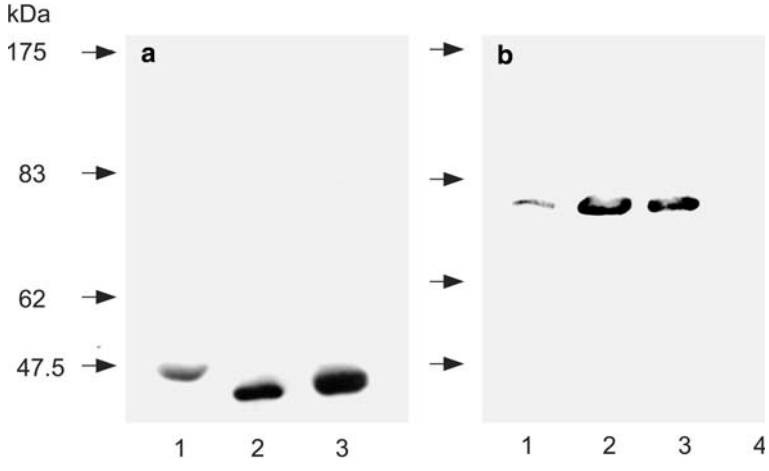

Fig. 1 Western blot analysis of dimeric (**a**) and monomeric (**b**) IDHs of *Colwellia psychrerythraea*. Rabbit antibodies against dimeric *Cm*IDH-I and monomeric IDH from *V. parahaemolyticus* (Fukunaga et al. 1992) were used. In **a**, *lanes 1–3* crude extracts of *C. psychrerythraea*, *C. maris* and *E. coli*, respectively. In **b**, *lanes 1, 3 and 4* crude extracts of *C. psychrerythraea*, *C. maris* and *E. coli*, respectively. *Lane 2* the purified *Cm*IDH-II

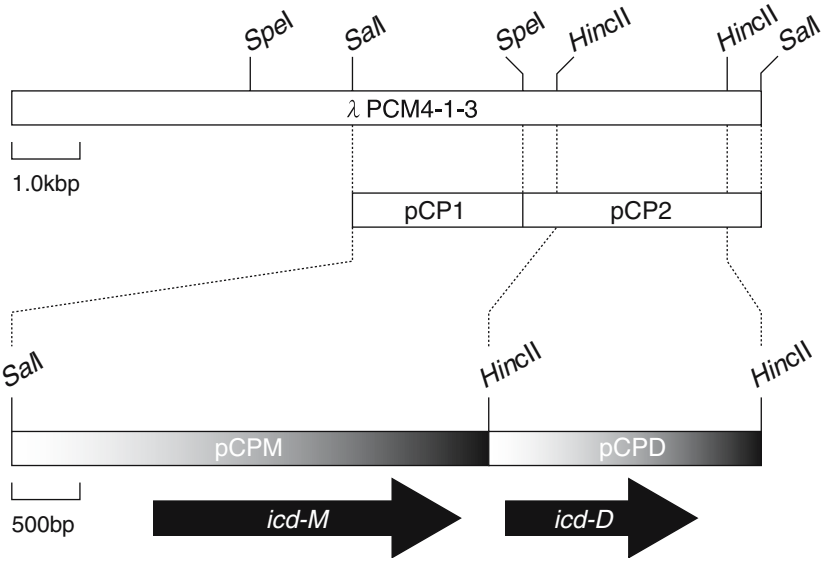

Fig. 2 Physical and restriction map of the genomic DNA fragment containing the *icd-M* and *icd-D* genes. *Arrows* represent the ORFs of *icd-M* and *icd-D*

screened with the respective probes. Consequently, many positive signals were obtained in both cases. Among them, the respective phage plaques with the strongest signal were selected for further characterization. From analyses of restriction enzyme maps of the recombinant DNAs, it was found that a phage clone, λCPM4-1-3, contains both IDH isozyme genes (Fig. 2). A 2.6 kbp *Sal*I–*Spe*I fragment and a 3.5 kbp *Spe*I–*Sal*I fragment of this phage clone were subcloned into a plasmid vector, pBS, to yield pCP1 and pCP2.

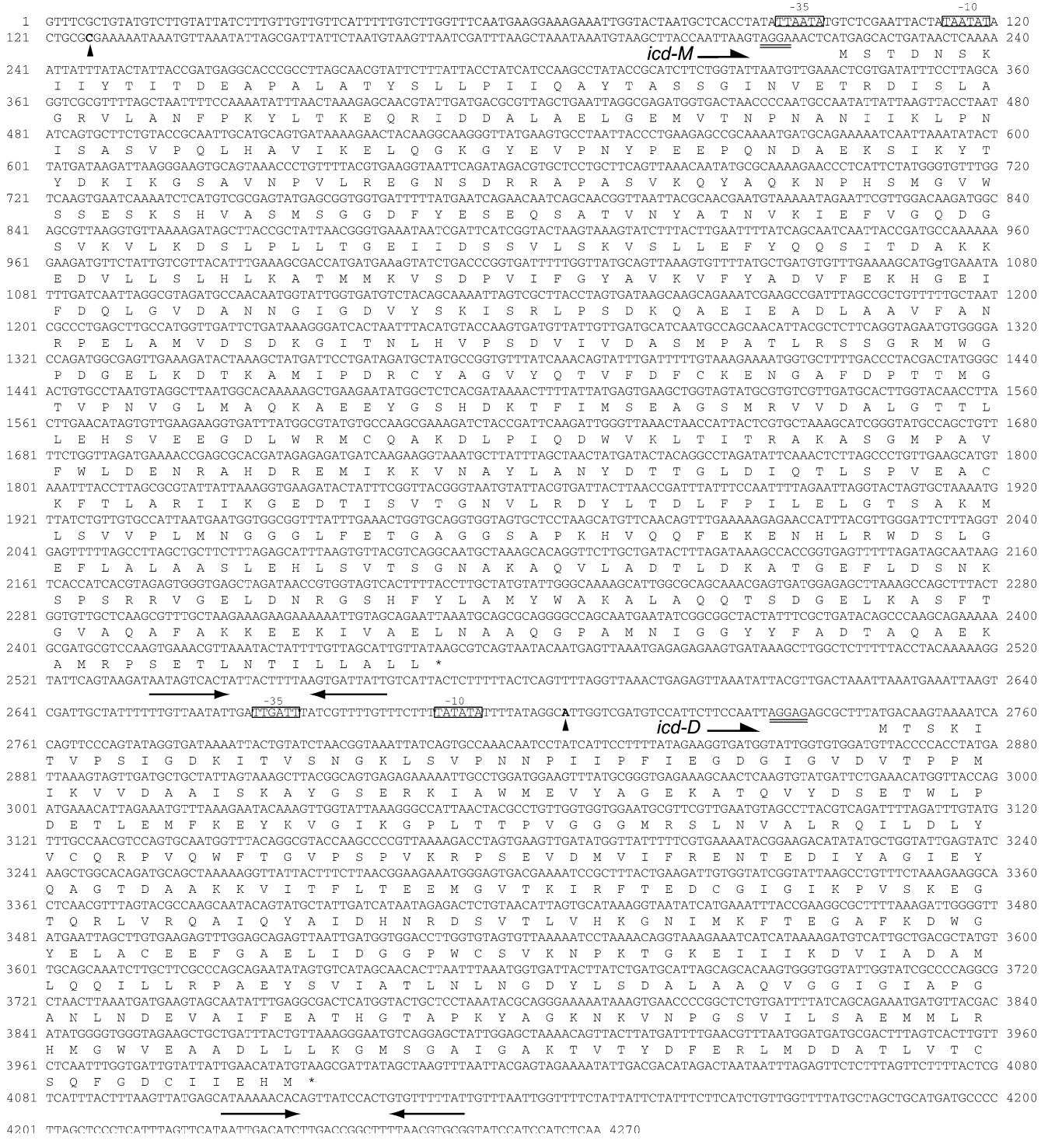

Fig. 3 Nucleotide and deduced amino acid sequences of the *icd-M* and *icd-D* genes from *C. psychrerythraea*. *Arrowheads* indicate the transcriptional start points of the respective genes. The *double underlined sequences* are the putative ribosome-binding sites. The *boxed nucleotides* show putative −35 and −10 sequences of the respective promoters. The probable stem-loop structures located downstream of the stop codons for translation are shown by *two opposite arrows*

Nucleotide and deduced amino acid sequences of the IDH isozyme genes

The DNA fragments subcloned in pCP1 and pCP2 were sequenced (Fig. 3). Like *icd-I* and *icd-II* of *C. maris* (Ishii et al. 1993), the genes encoding monomeric and dimeric IDHs of *C. psychrerythraea* are located in tandem with *icd-M* followed by *icd-D*, on the chromosomal DNA. *icd-M* was 2,232 bp in length, and a putative ribosome-binding site, AGGA, was found 6–9 bases upstream of the ATG codon. The ORF encodes a polypeptide of 743 amino acids with a calculated molecular mass of 81,033 Da. On the other hand, the ORF of *icd-D* contains 1,251 bases, and a putative ribosome-binding site, AGGAG, was also present 9–13 bases upstream of the ATG codon. *icd-D* encodes a polypeptide composed of 416 amino acids, whose molecular mass is calculated as 45,322 Da. The sequence of a terminator-like stem-loop structure was found downstream of each ORF. The former is composed of a 10 bp stem with a 10-nucleotide loop, while the latter is composed of a 10 bp stem with a 11-nucleotide loop. The deduced amino acid sequences of *Cp*IDH-M and *Cp*IDH-D showed high degrees of homology to monomeric and dimeric IDHs of other bacteria, respectively; 77, 68, 60 and 73% identity with *Cm*IDH-II and monomeric IDHs of *A. vinelandii*, *C. glutamicum* and *V. cholerae*, respectively, and 86, 79, 76 and 64% identity with *Cm*IDH-I and dimeric IDHs of *Pseudomonas aeruginosa*, *E. coli* and *Bacillus subtilis*, respectively (Figs. 4, 5). The amino acid residues involved in the binding of substrate, metal ion and coenzyme were completely conserved between the two IDH isozymes of *C. psychrerythraea* and the same type of IDHs of other bacteria, respectively. On the other hand, no sequential similarity was found between *Cp*IDH-M and *Cp*IDH-D as reported previously in other bacterial IDHs (Ishii et al. 1993; Eikmanns et al. 1995; Sahara et al. 2002).

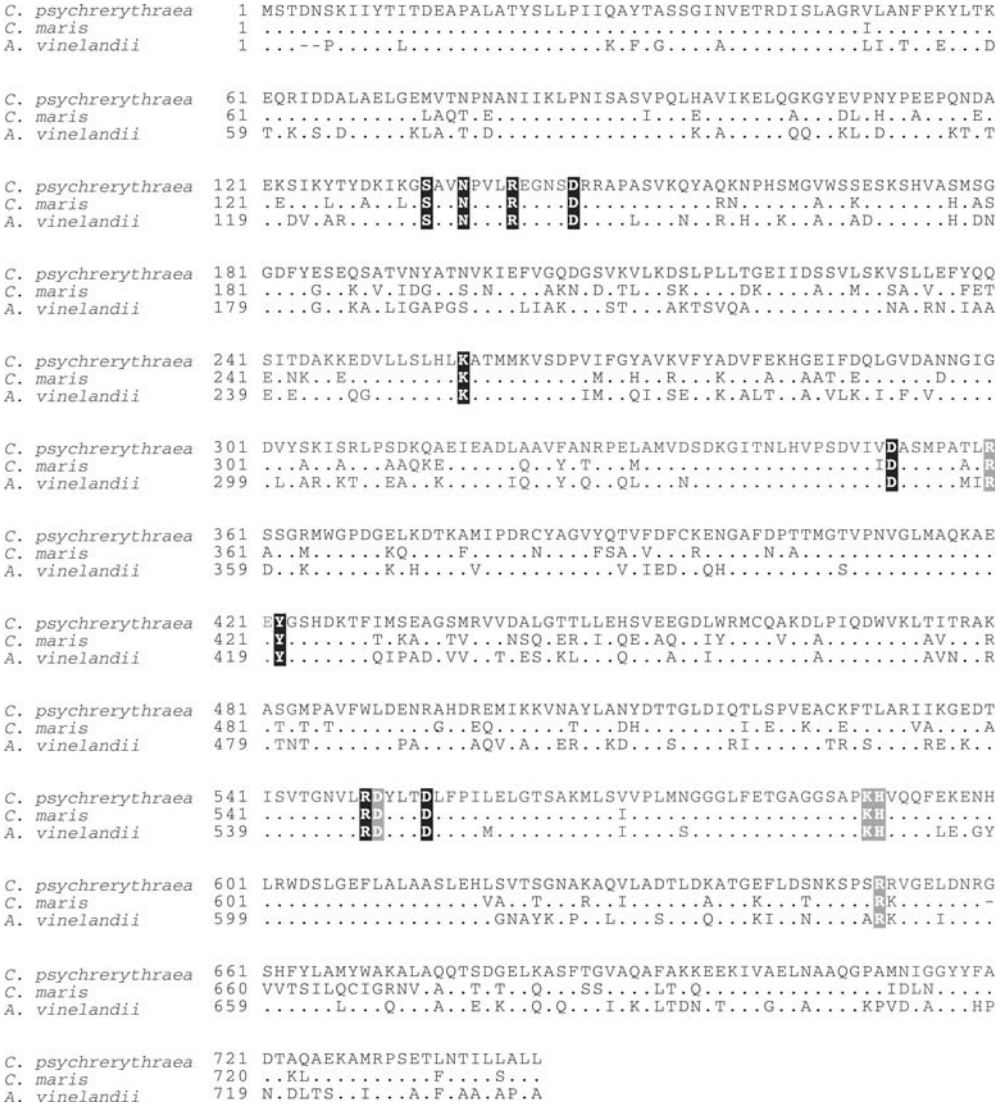

Fig. 4 Alignment of amino acid sequences of monomeric IDHs from different organisms. The deduced amino acid sequence of *Cp*IDH-M is aligned with that of *Cm*IDH-II (database accession no. D14047) and the *A. vinelandii* IDH (D73443). The amino acids involved in the binding of isocitrate and metal ion are indicated in *block boxes*. *Letters in gray boxes* are amino acids of the binding sites for $NADP^+$

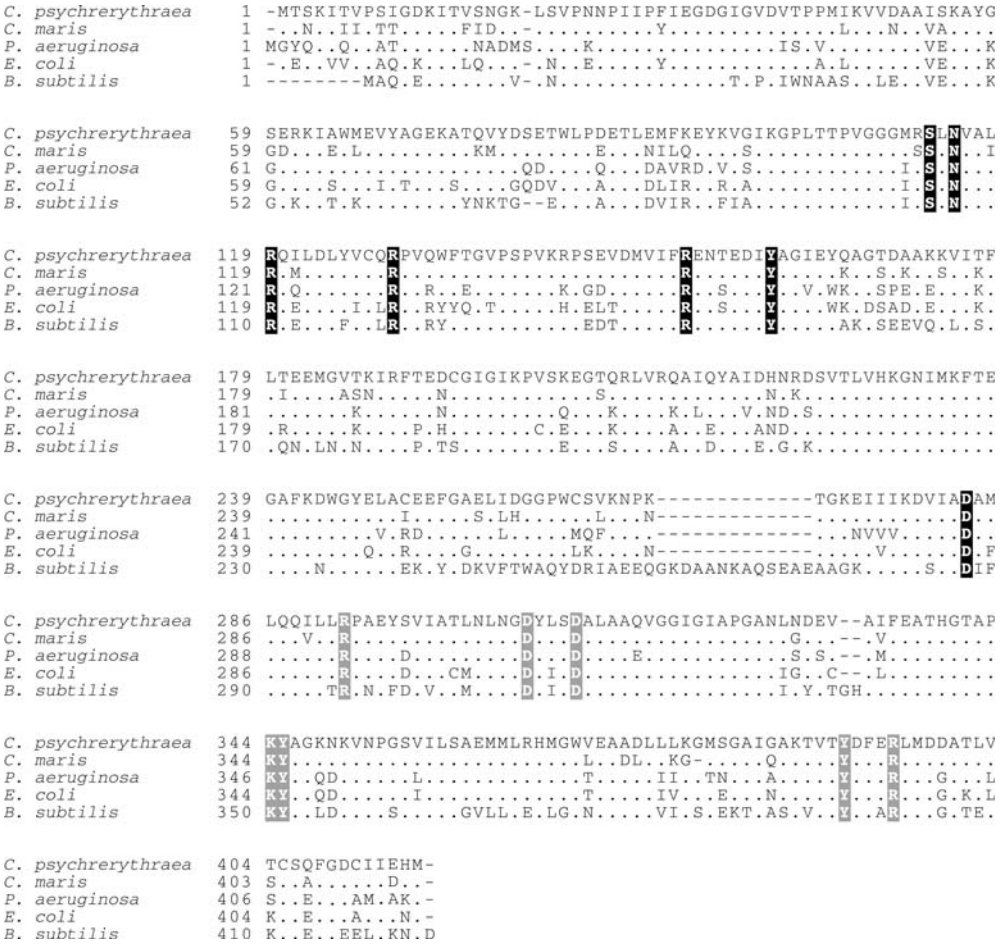

Fig. 5 Alignment of amino acid sequences of dimeric IDHs from different organisms. The deduced amino acid sequence of *Cp*IDH-D is aligned with that of *Cm*IDH-I (database accession no. D14047) and IDHs of *E. coli* (J02799), *P. aeruginosa* (AE004691) and *B. subtilis* (U05257). The *letters in boxes* are the same as in Fig. 4

Expression of the IDH isozyme genes

To examine the expression of *icd-M* and *icd-D*, the *Sal*I–*Hin*cII fragment and *Hin*cII fragment of λCPM4-1-3 containing *icd-M* and *icd-D*, respectively, were subcloned into pBS to obtain pCPM and pCPD (Fig. 2). Each plasmid was introduced into an IDH-defective mutant of *E. coli*, DEK2004, which has the phenotype of a glutamate auxotroph (Thorsness and Koshland 1987). The transformants harboring either pCPM or pCPD were able to grow at 25°C on a MOPS succinate medium and formed colonies within about 9 and 3 days, respectively. However, no growth under the same conditions was observed for DEK2004 transformed with the control vector, pBS, because of a lack of glutamate in the growth medium. These results indicate that the expression of these genes complements the auxotrophy of the *E. coli* mutant. The IDH activity in cell-free extracts of transformants carrying pCPM or pCPD grown under such conditions was 0.002 and 0.08 units/mg protein, respectively, and the latter showed higher activity than the former.

Transcription of the adjacent IDH genes was examined by Northern blot analysis (Fig. 6). Two single bands with lengths of approximately 2.4 and 1.4 kb hybridized to the probes for *icd-M* and *icd-D* prepared by PCR, respectively. No transcript larger than 2.4 kb was detected. The estimated sizes of the transcripts were well consistent with those of the respective ORFs plus their flanking regions. These results reveal that the two IDH genes are transcribed separately. In addition to the RNA from the *C. psychrerythraea* cells, the probes for *icd-M* and *icd-D* hybridized to the RNA isolated from the DEK2004 cells harboring pCPM and pCPD, respectively, but not to that carrying the control vector, indicating that both IDH genes were expressed precisely in the transformants carrying pCPM and pCPD.

Analysis of 5′-terminal regions of the IDH isozyme mRNAs

Each transcriptional start point of the two *icd* genes was examined by primer extension analysis (Fig. 7). The initiation sites for the *icd-M* and *icd-D* genes were found to be C-126 and A-2706, respectively (Fig. 3). The deduced promoter motifs at −10 and −35 for *icd-M* and *icd-D* were TTAATA (nucleotides 93–98) and TAATAT (114–119), and TTGATT (2,671–2,676) and TATATA

(2,694–2,699), respectively. From these results, the independent expression of *icd-M* and *icd-D* described above was confirmed.

Expression of the IDH isozyme genes under various conditions

To elucidate the effects of temperature and the carbon source for growth on the expression of the *icd* genes, Northern and Western blot analyses were performed using *C. psychrerythraea* cells grown at various temperatures between 0 and 15°C (Fig. 8). The transcriptional and translational levels of the *icd-M* gene were almost the same at all temperatures tested, whereas a slight induction of *icd-D* transcription was observed upon lowering the growth temperature from 15 to 0°C. However, no difference in translational level was found between the cells grown at these temperatures. The addition of sodium acetate into the growth medium faintly increased the transcriptional levels of *icd-M* and *icd-D* (about 1.2 and 1.5-folds, respectively), but not their translational levels. These results suggest that the expression of the two genes is little affected by low temperature or acetate.

Properties of His-tagged IDH isozymes

The *icd-M* and *icd-D* genes were introduced into the expression vector, pTrcHisB, conferring a $(His)_6$-tag on the N-terminal of the translated protein, and His-tagged *Cp*IDH-D and *Cp*IDH-M were overexpressed in the *E. coli* DEK2004 cells and then purified by Ni-affinity column chromatography. Activities of the purified enzymes were assayed at various temperatures (Fig. 9a). The His-tagged *Cp*IDH-D showed the highest activity at 45°C (121.8 units/mg protein) and this result was similar to the optimal temperature for IDH activity in the crude extract of *C. psychrerythraea* cells. Furthermore, 73% of the maximum activity was retained even at 55°C. These results indicate that *Cp*IDH-D is a typical mesophilic enzyme, like the dimeric *Cm*IDH-I. NaCl stimulated its activity which in the presence of 0.15 M NaCl was increased 1.9-fold. On the other hand, *Cp*IDH-M showed different properties from *Cp*IDH-D. Its maximal activity (8.8 units/mg protein) was observed at 25°C, and the activity of this enzyme could not be precisely measured at 30°C because of marked thermolability (Fig. 9b), indicating that, in contrast to *Cp*IDH-D, *Cp*IDH-M is a cold-adapted enzyme. However, whereas the cold-adapted monomeric *Cm*IDH-II has higher activity than the mesophilic *Cm*IDH-I (Ochiai et al. 1979), *Cp*IDH-M was much less active at all temperatures than *Cp*IDH-D. The activity of *Cp*IDH-M was also, but only slightly, stimulated by NaCl (1.2-fold higher in the presence of 1 M NaCl than in the absence of NaCl). IDH activity in the cell-free extract of *E. coli* DEK2004 cells harboring either pCPM or pCPD overexpressing the IDH proteins by isopropyl-*β*-D-thiogalactopyranoside was most active at 20 or 45–50°C, respectively. These results were analogous to the optimal temperature for activity of the respective His-tagged IDHs, indicating that the attachment of His-tag to the IDH proteins does not have significant effect on their thermal properties.

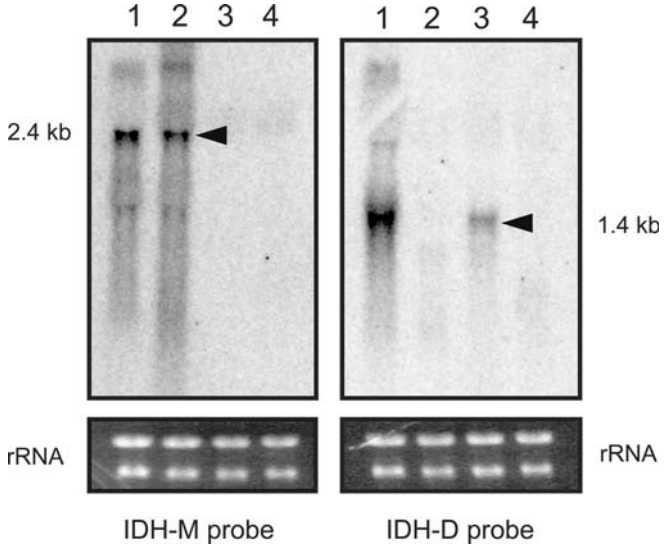

Fig. 6 Northern blot analysis of *icd-M* and *icd-D*. Total RNA (6 μg per lane) was isolated from cells of *C. psychrerythraea* and *E. coli* DEK2004 transformed with a plasmid bearing either *icd-M* or *icd-D*. *Lane 1*, *C. psychrerythraea*; *lane 2*, *E. coli* DEK2004 transformed with pCPM; *lane 3*, *E. coli* DEK2004 transformed with pCPD; *lane 4*, *E. coli* DEK2004 transformed with pBS

Properties of chimeric enzymes

Although *Cp*IDH-M and *Cm*IDH-II are cold-adapted enzymes and showed a high degree of homology in amino acid sequence, their catalytic activities differed as described above. As a first step toward clarifying the structural characteristics behind such a difference, His-tagged chimeric enzymes were constructed and characterized (Fig. 10). Each chimeric enzyme was termed *Cp*IDH-M (P) or *Cm*IDH-II (M) based on regions 1–3. For example, the chimera consisting of regions 1 and 3 of *Cp*IDH-M and region 2 of *Cm*IDH-II is named PMP. The purification of the chimeric IDHs with region 3 of *Cp*IDH-M (MMP and MPP) was unsuccessful. This probably results from poor expressions of the chimeric genes and/or the remarkable lability of the expressed proteins, but further study is required to certify these possibilities. The levels of activity of *Cm*IDH-II (MMM) and all chimeric IDHs were almost the same until 20°C. However, at higher temperatures, PMM and PPM were more active than MMM and MPM, respectively. The optimal temperature for the activity (T_{opt}) of PMM and PPM rose by 10°C, compared to that of MMM and MPM, respectively. Furthermore, the former two chimeras were more stable

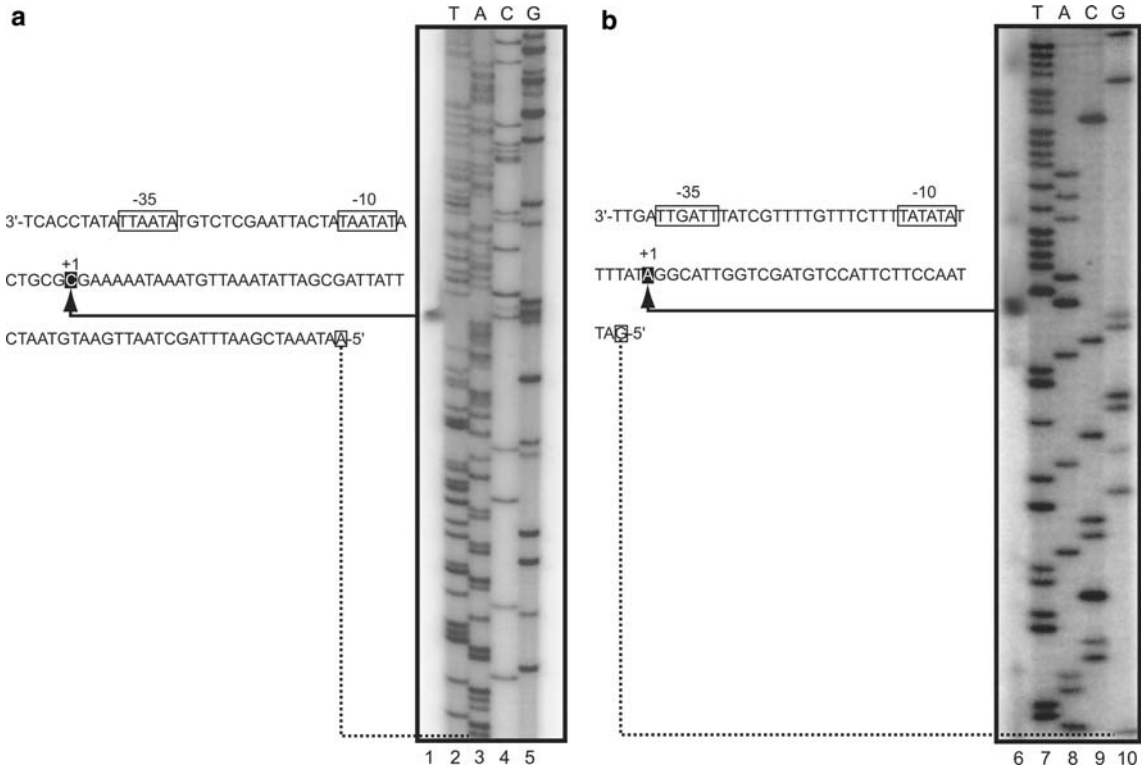

Fig. 7 Primer extension analysis of *icd-M* (**a**) and *icd-D* (**b**). Products of the extension using primers complementary to *icd-M* and *icd-D* are shown in *lanes 1 and 6*, respectively. The total RNA (30 μg) used in these experiments was obtained from *C. psychrerythraea* cells grown in the nutrient medium. The sequencing ladders are shown in *lanes 2–5 and 7–10*. *Arrows* indicate the 5′-ends of respective *icd* mRNAs. The *boxed nucleotides* show putative −35 and −10 sequences of the respective promoters. The *broken lines* show the ends of sequence ladders in each IDH isozyme gene

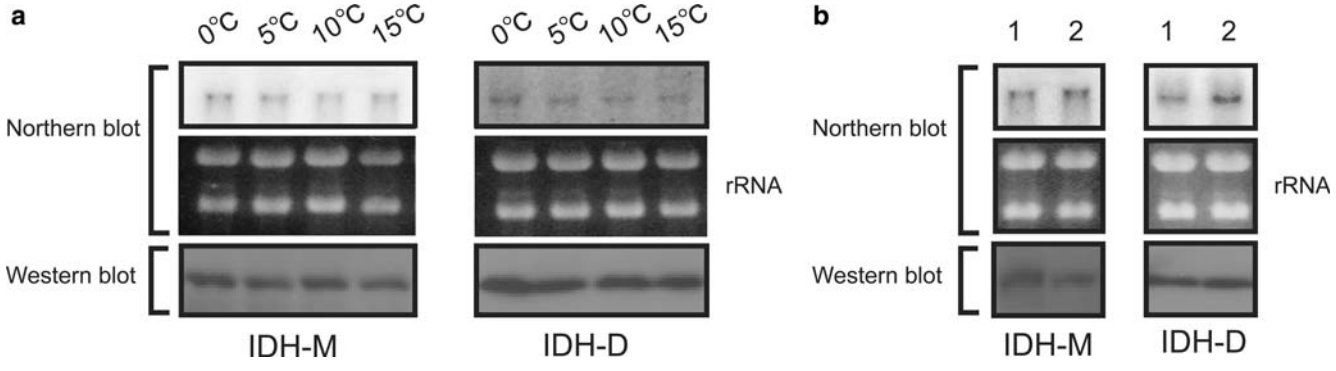

Fig. 8 Effects of temperature and carbon source for growth on transcriptional and translational levels of the IDH isozymes. Northern and Western blot analyses were carried out with RNAs and cell-free extracts prepared from the *C. psychrerythraea* cells grown at the indicated temperatures (**a**) and at 15°C on the nutrient medium (**b**; *lane 1*) or that supplemented with 25 mM sodium acetate (**b**; *lane 2*)

than the latter ones. These results imply that the change to region 1 of *Cp*IDH-M increased the thermostability and T_{opt}. On the other hand, above 25°C, the activities of PPM and MPM were lower than those of PMM and MMM, respectively. Although the T_{opt} values of PMM and MMM (35 and 25°C, respectively) were higher than those of PPM and MPM (30 and 20°C, respectively), the former IDHs showed almost the same thermostability as the latter two, respectively. Therefore, the change to region 2 of *Cm*IDH-II was found to result in an improvement in activity above 25°C. Furthermore, PPM, with region 3 of *Cm*IDH-II, had much higher activity at all temperatures than the wild type of *Cp*IDH-M (PPP).

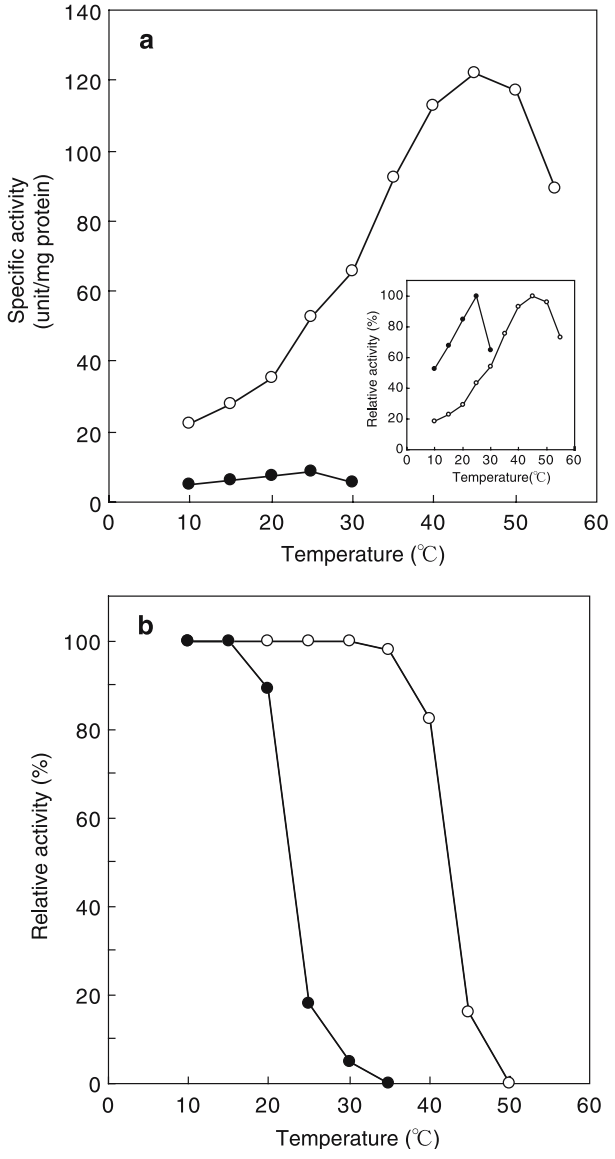

Fig. 9 Effect of temperature on activity (**a**) and thermostability (**b**) of the His-tagged IDH isozymes. *Cp*IDH-M and *Cp*IDH-D are indicated by *closed and open symbols*, respectively. In **b**, residual activity after the incubation is represented as a percentage of that without the incubation

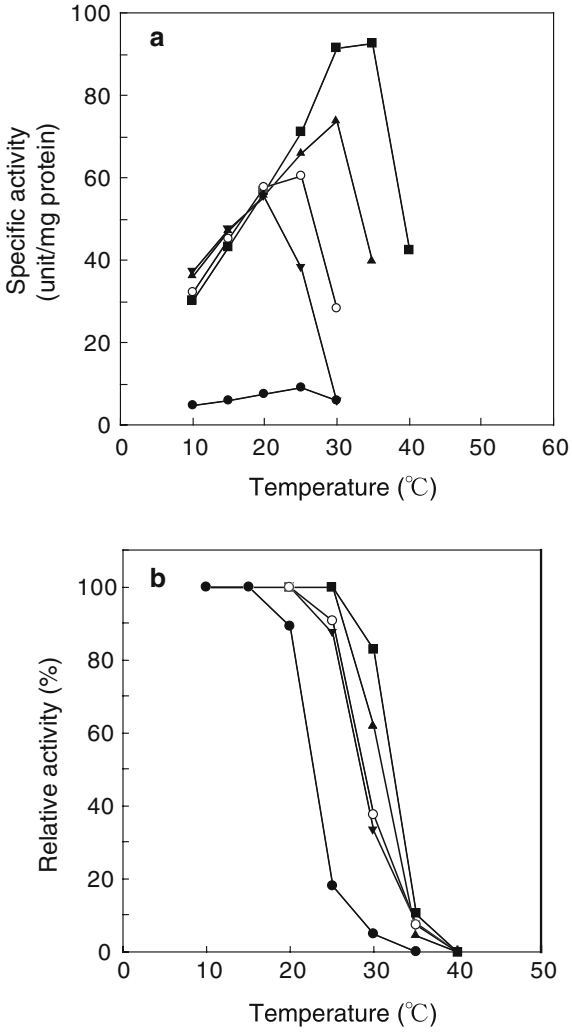

Fig. 10 Temperature dependence of the activity (**a**) and thermostability (**b**) of the chimeric IDHs. *Cp*IDH-M (PPP), *Cm*IDH-II (MMM), MPM, PPM and PMM are indicated by *closed and open circles*, *reversed triangles*, *triangles* and *squares*, respectively

Discussion

In this report, it was elucidated that the psychrophilic bacterium, *C. psychrerythraea*, possesses both monomeric and dimeric IDH isozymes as does *C. maris*. Furthermore, the two genes, *icd-M* and *icd-D*, encoding the monomeric and dimeric IDHs (*Cp*IDH-M and *Cp*IDH-D, respectively) were cloned and sequenced. Like the two IDH isozyme genes of *C. maris*, *icd-I* and *icd-II*, the *icd-M* and *icd-D* genes of *C. psychrerythraea* were located adjacent to but expressed independent of each other (Figs. 3, 6, 7). Furthermore, multiple alignments of the deduced amino acid sequences revealed that *Cp*IDH-D and *Cp*IDH-M have a high degree of homology with the corresponding types of IDH from *C. maris* (86 and 77% identity, respectively). The amino acid residues involved in the binding of substrate, metal ion and coenzyme were conserved between the two IDH isozymes of *C. psychrerythraea* and the same types of IDH from other bacteria (Figs. 4, 5). On the other hand, the expression of the *C. maris icd-I* and *icd-II* is induced by acetate and low temperature, respectively (Suzuki et al. 1995), and the "CCAAT" sequence located upstream of *icd-II* has been confirmed to be responsible for the cold-inducible expression (Sahara et al. 1999). In *C. psychrerythraea*, a "CCAAT" sequence was also present upstream of both the *icd* genes, but the expression levels of the two genes were almost constant at all temperatures tested (Fig. 8), indicating that the CCAAT sequences of these genes do not function as a *cis*-element for the cold-inducible expression. Furthermore, the expression of the two

genes was not induced by acetate. Therefore, it is concluded that the expression of IDH isozyme genes is regulated differently in the two bacteria.

The low optimal temperature for activity (25°C) and marked thermolability of *Cp*IDH-M reveals that it is a typical cold-adapted enzyme similar to *Cm*IDH-II (Fig. 9). However, the former had much less catalytic ability than the latter (only about 15% in comparison to the maximum activity at the respective T_{opt}) (Fig. 10). In contrast, *Cp*IDH-D showed mesophilic characteristics similar to *Cm*IDH-I. Nevertheless, *Cp*IDH-D retained a much higher level of activity even at low temperatures than did the cold-adapted *Cp*IDH-M and its activity at 10°C was comparable to about 70% of the *Cm*IDH-II activity at the same temperature (Figs. 9, 10). In fact, only one peak of IDH activity in the crude extract of *C. psychrerythraea* cells was observed at 40°C when its temperature dependence was examined (data not shown). Furthermore, Western blot analysis revealed that at least as much *Cp*IDH-D as *Cp*IDH-M is contained in the *C. psychrerythraea* cells (Fig. 8). These results suggest that the mesophilic *Cp*IDH-D, but not the cold-adapted *Cp*IDH-M, contributes to the growth and survival of *C. psychrerythraea* at low temperatures. Since the growth rates of this bacterium on a nutrient broth medium supplemented with 2.75% NaCl and 0.4% $MgCl_2$ and on Bacto marine broth 2216 medium (Difco) were about 40 and 70% those of *C. maris*, respectively, at 15°C, which is the optimal temperature for their growth, the catalytic ability of *Cp*IDH-D seems sufficient to sustain the growth of *C. psychrerythraea* under cold conditions.

The chimeric IDH, PPM, in which region 3 of *Cp*IDH-M was replaced with the corresponding region of *Cm*IDH-II, was more active above 20°C and was more thermostable than the wild type *Cp*IDH-M, and the optimal temperature for its activity was elevated (Fig. 10). The further exchange of region 2 in this chimeric IDH (yielding PMM) enhanced these characteristics. These results indicate that regions 2 and 3, in particular region 3, of *Cm*IDH-II may responsible for its higher catalytic ability than *Cp*IDH-M. The study of chimeric IDHs in which each region was exchanged between *Cm*IDH-II and the mesophilic monomeric IDH of *A. vinelandii* indicated that region 3 of *Cm*IDH-II plays an important role in the thermolability (Watanabe et al. 2005). The Arg residue is known to stabilize a protein structure since one molecule of this amino acid residue can form five hydrogen bonds, and some cold-active enzymes have been reported to have a low Arg content (Gerday et al. 1997). On the other hand, Gly residues make a protein flexible because of a lack of side chain. However, little difference in the content of these amino acid residues was detected between the two regions of *Cp*IDH-M and *Cm*IDH-II, and amino acid residues involved in the binding of isocitrate, metal ion and $NADP^+$, essential for the catalytic function, are completely conserved between the two IDHs (Fig. 4). The amino acid sequences of regions 2 and 3 of *Cp*IDH-M have a high degree of homology to those of *Cm*IDH-II (85 and 86%, respectively), but substituted amino acid residues are also present in these regions. Several experiments have demonstrated that the substitution of small numbers of amino acid residues can cause drastic changes in catalytic activity and thermostability (Mavromatis et al. 2002; Miyazaki et al. 2000). This may also be the case for *Cp*IDH-M. Experiments to test this possibility are underway in our laboratory.

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