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Author(s)	Mouri, Yuka; Takada, Yasuhiro
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Contribution of three different regions of isocitrate dehydrogenases from psychrophilic and psychrotolerant bacteria to their thermal properties

Yuka Mouri[1] • Yasuhiro Takada[2]

[1]Biosystems Science Course, Graduate school of Life Science, Hokkaido University, Kita 10-jo Nishi 8-chome, Kita-ku, Sapporo 060-0810, Japan

[2]Depertment of Biological sciences, Faculty of Science, Hokkaido University, Kita 10-jo Nishi 8-chome, Kita-ku, Sapporo 060-0810, Japan

✉ Yasuhiro Takada: ytaka@sci.hokudai.ac.jp, Fax: +81-11-706-2742

Abstract Monomeric isocitrate dehydrogenases of a psychrophilic bacterium, *Colwellia maris*, and a psychrotolerant bacterium, *Pseudomonas psychrophila*, (*Cm*IDH and *Pp*IDH) are cold-adapted and mesophilic, respectively. On the other hand, previous studies revealed that the monomeric IDH of *Azotobacter vinelandii* (*Av*IDH) is also mesophilic and the regions 2 and 3 among three regions of this enzyme are involved in the thermal properties. Therefore, to examine whether the region(s) responsible for the mesophilic properties are common between *Pp*IDH and *Av*IDH, the genes of chimeric IDHs exchanging three regions of *Pp*IDH and *Cm*IDH in various combinations were constructed and overexpressed as His-tagged recombinant proteins in the *Escherichia coli* cells, and the chimeric and wild-type *Pp*IDH and *Cm*IDH were purified with Ni-chelating affinity column chromatography. The swapping chimeras of the regions 2 or 3 in *Pp*IDH and *Cm*IDH showed lower and higher optimum temperatures for activities and their thermostabilities than the wild-type ones, respectively. On the other hand, the exchange of the respective region 1 hardly influenced these properties of the two IDHs. Therefore, the regions 2 and 3 of the two IDHs were confirmed to be involved in their thermal properties. These results were coincident with those of the previous study on chimeric IDHs between *Av*IDH and *Cm*IDH, indicating that the common regions of *Av*IDH and *Pp*IDH are responsible for their mesophilic properties and the amino acid residues involved in their thermal properties are present in the regions 2 and 3.

Keywords: chimeric enzymes · cold-adapted enzyme · *Colwellia maris* · Isocitrate dehydrogenase · *Pseudomonas psychrophila*

Introduction

$NADP^+$-dependent isocitrate dehydrogenase (IDH; 1.1.1.42) catalyzes oxidative decarboxylation of isocitrate to α-ketoglutarate and CO_2 with a concomitant reduction of $NADP^+$ to NADPH in the TCA cycle present ubiquitously in aerobic organisms. This enzyme controls the metabolic flux between the TCA cycle and the glyoxylate shunt. Bacterial IDHs are classified into two types based on their subunit composition; one has a homodimeric structure consisting of subunits with a molecular mass of 40–45 kDa, the other is monomeric and the molecular mass is 80–100 kDa. Many bacteria, including *Escherichia coli*, possess only one of either type IDH [1, 2, 5, 6, 17]. However, psychrophilic bacteria, *Colwellia maris* strain ABE-1 (JCM 10085) [20, 26] and *Colwellia psychrerythraea* [3, 4], and a psychrotolerant bacterium, *Pseudomonas psychrophila* strain E-3 (JCM 10889) [19, 27], have been found to hold both of the two type IDHs [13–16]. Although these two types of IDH catalyze the same reaction, their amino acid sequences and immunological cross-reactivities are different from each other [6–8, 17].

The monomeric IDH of *C. maris* (*Cm*IDH) is known to be cold-adapted enzyme because it exhibits maximum activity at 20 ºC and is so thermolabile that a half of its activity is lost after incubation for 10 min at 30 ºC [15, 16]. On the other hand, the optimum temperature for activity of the monomeric IDH from *P. psychrophila* (*Pp*IDH) is 60 ºC, and this enzyme retains completely its activity even after incubation for 10 min at 40 ºC, indicating that it is mesophilic [14]. Thus, thermal properties of the two monomeric *Cm*IDH and *Pp*IDH are quite different while their amino acid sequences show a high degree of identity (70%) (Fig. 1).

By crystallographic analysis of monomeric IDH from a nitrogen-fixing bacterium, *Azotobacter vinelandii* (*Av*IDH), this enzyme has been found to be composed of two domains, I and II [23, 24]. The former domain contains the N-terminal region 1 and the C-terminal region 3, while the latter one corresponds to the intermediate region 2. Since the amino acid sequential identities of *Cm*IDH and *Pp*IDH to *Av*IDH are also high (70% [17] and 81%, respectively), the three IDHs are suggested to have similar three-dimensional structure. Furthermore, amino acid residues involved in the binding of isocitrate, metal ion and $NADP^+$ [23, 24] were conserved completely among them (Fig. 1). However, *Av*IDH is also typical mesophilic enzyme and exhibits the maximum activity at 45 ºC, and no activity is lost by incubation for 10 min at 40 ºC [22], showing the same level of

thermostability as *Pp*IDH. Therefore, thermal properties of *Av*IDH are also different from those of *Cm*IDH. A previous study on chimeric enzymes exchanging variously each region between *Av*IDH and *Cm*IDH revealed that their regions 2 and 3 are involved in their thermal properties such as thermostability and optimum temperature for activity whereas their region 1 hardly contributes to them [22]. On the other hand, when five amino acid residues of *Pp*IDH were substituted with the corresponding ones of *Cm*IDH (Fig. 1), only the *Pp*IDH mutant substituted the Glu55 residue in the region 1 by Lys diminished the thermostability for activity, suggesting that this residue is involved in the mesophilic nature of *Pp*IDH [14]. In contrast, the other four substitutional mutations of amino acid residues located in the regions 2 and 3 of *Pp*IDH had no effect on the thermal properties. In addition, the decreased thermostability of *Av*IDH was caused by the substitution of the Pro717 residue located in the region 3 with the corresponding Ala residue of *Cm*IDH, and the Pro residue was reported to contributes to its mesophlic properties [10]. However, the corresponding amino acid residue of *Pp*IDH is Ala, identical to that of *Cm*IDH (Fig. 1). From these results, it was expected that both *Av*IDH and *Pp*IDH are mesophilic but the region(s) contributing to their thermal properties are different from each other. Therefore, to confirm whether common region(s) between *Pp*IDH and *Av*IDH are responsible for the thermal properties, chimeric enzymes exchanging the respective regions in various combinations between *Pp*IDH and *Cm*IDH were constructed, and their optimum temperature and thermostability for activity were examined in this study.

Materials and Methods

Bacterium, plasmids and growth condition

An IDH-defective mutant of *E. coli*, DEK2004 [21], was used as a host for expression of the wild-type and chimeric IDH genes. Unless otherwise noted, this bacterium was cultured at 37 ºC with vigorous shaking in the Luria-Bertani (LB) medium [18] or Super broth medium [22]. If necessary, ampicillin and tetracycline were

added to the culture media at concentrations of 0.1 and 0.015 mg/ml, respectively. For the construction of chimeric IDH genes and the overexpression of genes encoding His-tagged IDH proteins, plasmids pBluescript SK(+) (pBS; Stratagene) and pTrcHisB (Invitrogen) were used, respectively.

Construction of His-tagged wild-type IDH genes

The *Cm*IDH gene possesses a *Nsp*V site between regions 2 and 3, but any digestion site of appropriate restricted enzymes is absent between regions 1 and 2. Thus, a *Bss*HII site had been introduced between regions 1 and 2 of the *Cm*IDH gene without the change of amino acid residues in the translational product by site-directed mutagenesis as described in previous report [22]. Although the pTrcHisB carrying the resultant *Cm*IDH gene had been termed pHis*Cm*-*Bss*HII, it was renamed pHis*Cm*WT in this study. Like the *Cm*IDH gene, the *Pp*IDH gene possesses a *Nsp*V site between region 2 and 3, but not *Bss*HII site between region 1 and 2. On the other hand, a *Bss*HII site and the other *Nsp*V site were present in the coding region of region 1 of this gene. Therefore, to construct chimeric genes exchanging each region between *Pp*IDH and *Cm*IDH, these *Bss*HII and *Nsp*V sites were eliminated, and the novel *Bss*HII site was introduced between region 1 and 2 of the *Pp*IDH gene by site-directed mutagenesis using PCR as described below.

To remove the inherent *Nsp*V site near 5'-terminal of the *Pp*IDH gene with no change of amino acid residues, PCR was performed for 30 cycles in a Verti 96 well Thermal Cycler (Applied Biosystems) in a reaction mixture (50 µl) containing 10 pmol each of forward and reverse primers, PMF0-*Nsp*V and PMR0-*Sac*I, respectively (Table S1), about 40 ng of pHisPPP, harboring the full length of *Pp*IDH gene in the *Bam*HI-*Sac*I site of pTrcHisB, and 1U KOD-plus Neo DNA polymerase (TOYOBO) in a buffer system prepared by the manufacturer. Cycling conditions were as follows: denaturation at 98 ºC for 10 s, annealing at 63 ºC for 30 s, and extension at 68 ºC for 1.3 min. Amplified DNA fragment was purified by a conventional isopropanol precipitation and digested with *Sac*I and *Bam*HI. Then the fragment was purified with Fast Gene Gel/PCR Extraction kit (NIPPON Genetics) and ligated to the *Bam*HI-*Sac*I site of pBS with DNA Ligation Kit Ver. 2.1 (TaKaRa) to obtain plasmid pBS*Pp*WT-Del.*Nsp*V. This plasmid was introduced into *E. coli* DEK2004 by an

electroporation with Gene Pulser II (Bio-Rad). The plasmid amplified in the *E. coli* cells was then extracted and purified with High Pure Plasmid Isolation Kit (Roche Diagnostics). Precise insertion of the gene into pBS was verified by the nucleotide sequencing of the plasmid using M13 primer and Reverse primer as primers and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with a sequencer, 3130 genetic Analyzer (Applied Biosystems).

For the deletion of the inherent *Bss*HII site, Del*Bss*HII-s and Del*Bss*HII-as were used as forward and reverse primers, respectively (Table S1). Amplifications were performed for 30 cycles in a reaction mixture (50 μl) containing 10 pmol each of forward and reverse primer sets, PMF0-*Nsp*V plus Del*Bss*HII-as or Del*Bss*HII-s plus PMR0-*Sac*I, about 40 ng of pBS*Pp*WT-Del.*Nsp*V and 1U KOD-plus Neo DNA polymerase in a buffer system prepared by the manufacturer. Cycling conditions were as follows: for the former primer set, denaturation at 98 ºC for 10 s, annealing at 63 ºC for 30 s, and extension at 68 ºC for 15 s; for the latter primer set, denaturation at 98 ºC for 10 s, annealing at 63 ºC for 30 s, and extension at 68 ºC for 1 min. The two PCR products were used as template DNA for third PCR reaction. Amplification was performed under the same condition as first and second PCRs, except for the use of PMF0-*Nsp*V and PMR0-*Sac*I as primers and 50 ng each of the two PCR products as template DNA, and extension at 68 ºC for 1.3 min. To obtain plasmid pBS*Pp*WT-Del.*Nsp*V,*Bss*HII, the resultant PCR product was digested with *Bam*HI and *Sac*I and ligated to the *Bam*HI-*Sac*I site of pBS as described above. Nucleotide sequence of the insert DNA was determined to confirm the introduced mutation as described above.

To introduce the novel *Bss*HII site between regions 1 and 2 of the *Pp*IDH gene, PCR was performed as described above, except for the use of Add*Bss*HII-s and Add*Bss*HII-as as forward and reverse primers, respectively, (Table S1) and pBS*Pp*WT-Del.*Nsp*V,*Bss*HII as template DNA. The introduced *Bss*HII site was certified by nucleotide sequencing. The obtained plasmid was termed pBS*Pp*WT and used for the construction of chimeric IDH gene. For the overproduction and purification of the His-tagged *Pp*WT protein, pBS*Pp*WT was digested with *Bam*HI and *Sac*I, and DNA fragment of the *Pp*IDH gene ORF was ligated to the *Bam*HI-*Sac*I site of pTrcHisB to obtain pHis*Pp*WT. Since pTrcHisB have additional *Bss*HII and *Nsp*V sites at positions other than the multicloning site, it was difficult to obtain DNA fragments of the respective regions of *Cm*IDH and *Pp*IDH genes by the digestion of IDH genes in pTrcHisB with *Bss*HII and *Nsp*V. Therefore, the *Bam*HI-*Sac*I DNA

fragment obtained by the digestion of pHis*Cm*WT with *Bam*HI and *Sac*I was ligated to the *Bam*HI-*Sac*I site of pBS. This plasmid was termed pBS*Cm*WT and used to construct chimeric IDH genes.

Construction of chimeric IDH genes between PpIDH and CmIDH

For the construction of chimeric IDH genes, in which the respective region 3 of *Pp*IDH and *Cm*IDH were exchanged, pBS*Pp*WT and pBS*Cm*WT were digested with *Nsp*V and *Sac*I, and the region 3 DNA fragments of the two IDH genes and pBS containing their remaining parts were obtained. The region 3 DNA fragment of the *Pp*IDH gene and pBS carrying the regions 1 and 2 of the *Cm*IDH gene, and the region 3 DNA fragment of the *Cm*IDH gene and pBS containing the regions 1 and 2 of the *Pp*IDH gene were ligated to obtain plasmids harboring chimeric IDH genes, pBSCCP and pBSPPC, respectively. On the other hand, pBS*Pp*WT and pBS*Cm*WT were digested with *Bss*HII and *Sac*I to obtain the regions 2 and 3 DNA fragments of the two IDH genes and pBS carrying their region 1. The DNA fragments containing the regions 2 and 3 of the *Pp*IDH and *Cm*IDH genes was ligated to the region 1 of the *Cm*IDH and *Pp*IDH genes in pBS, and pBSCPP and pBSPCC were obtained, respectively. Similarly, pBSCPC and pBSPCP, consisting of the regions 1 and 3 of the *Cm*IDH gene and the region 2 of the *Pp*IDH gene, and the regions 1 and 3 of the *Pp*IDH gene and the region 2 of the *Cm*IDH gene, respectively, were constructed by the digestion of pBS*Pp*WT and pBS*Cm*WT with *Bss*HII and *Nsp*V and the following exchanges of their region 2 DNA fragments. The precise construction of chimeric IDH genes was confirmed by their nucleotide sequencing as described above. All chimeric IDH genes in pBS were digested with *Sac*I and *Bam*HI, and then ligated to the *Bam*HI-*Sac*I site of pTrcHisB to obtain pHisCCP, pHisPPC, pHisCPP, pHisPCC, pHisCPC, and pHisPCP.

Overproduction and purification of His-tagged IDHs

E. coli DEK2004 transformed with pTrcHisB carrying the *Pp*IDH, *Cm*IDH or chimeric IDH genes were grown at 37 ºC in 1,000 ml of Super broth medium until OD_{600} of the culture reached 0.8. Then, the overproduction of

these IDH proteins and their purification by Ni-NTA agarose (Qiagen) column chromatography were carried out as reported previously [9] except for the following modifications. The *E. coli* transformant cells were disrupted by a sonication (5 times sonication for 2 min at intervals of 5 min on ice). Furthermore, a washing of the column with 50 mM sodium phosphate (pH 8.0), containing 2 mM $MgCl_2$, 0.5 M NaCl, 50 mM imidazole, and 10 mM 2-mercaptoethanol was added before the elution of the IDHs from Ni-NTA column. Then the final elutants containing IDH activity were concentrated with polyethylene glycol #3,000 or #6,000 and dialyzed against 20 mM sodium phosphate (pH 8.0), containing 2 mM $MgCl_2$, 0.5 M NaCl, 0.05 mM sodium citrate, 1 mM dithiothreitol (DTT) and 50% glycerol. All His-tagged recombinant IDHs were stored at –30ºC until use.

Enzyme assay

Unless otherwise noted, the IDH activity was assayed as reported previously [15]. Reaction mixture (2 ml) contained 33 mM Tris–HCl (pH 8.0), 0.67 mM $MnCl_2$, 0.25M NaCl, 0.12 mM $NADP^+$, 2 mM sodium isocitrate and an appropriate amount of enzyme. For *Pp*IDH (*Pp*WT), PPC, PCP, CPC, CPP and CCP, 0.25 M NaCl was omitted from the reaction mixture. In the experiments of thermostability for activity, all purified recombinant IDHs were dialyzed overnight at 4 ºC against 20 mM sodium phosphate (pH 8.0), containing 2 mM $MgCl_2$, 0.5 M NaCl and 1 mM DTT. After incubation for 10 min at various temperatures, the enzyme was immediately cooled on ice for 10 min, and then the residual activity was assayed at the optimum temperature for activity of each enzyme (30 ºC for *Cm*IDH (*Cm*WT), 35 ºC for PCC, 40 ºC for CPC and PPC, 45 ºC for CCP, 50 ºC for PCP and CPP, and 55 ºC for *Pp*WT). One unit of enzyme activity was defined as the amount capable of catalyzing the reduction of 1 μmol of $NADP^+$ per min. Protein concentration was determined by the method of Lowry *et al*. [12] with bovine serum albumin as a standard. All data for activity are the mean values ± SD of duplicate assays from at least two independent experiments.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [11] with 10% gel at 120 V. After electrophoresis, the proteins on the gel were stained by Coomassie Brilliant Blue R250.

Results

Construction and purification of mutated IDHs

As described in "Material and Methods", to construct the genes of six chimeric IDHs exchanging the three regions in various combinations between *Pp*IDH and *Cm*IDH, DNA fragments coding for each region of the two IDH genes were prepared by the digestion of plasmids carrying their intact gene ORFs with *Nsp*V and *Bss*HII. However, the expression vector, pTrcHisB, contains one *Nsp*V site and two *Bss*HII sites outside the multicloning site. Therefore, *Pp*IDH and *Cm*IDH genes were once inserted into pBS, and the resultant chimeric IDH genes were transferred again into pTrcHisB. The pTrcHisB carrying two wild-type (*Pp*WT and *Cm*WT) and six chimeric IDH genes (CPP, PCC, PCP, CPC, PPC or CCP) were introduced into the *E. coli* DEK2004 cells, and their translational products were overproduced and purified. SDS-PAGE of the final elutants of Ni-NTA column chromatography revealed that all wild-type and chimeric enzymes were purified to almost homogeneity, and their molecular masses were estimated to be about 80 kDa (Fig. S1) and were consistent with those of His-tagged *Pp*IDH and *Cm*IDH reported previously [14, 22].

Effect of temperature on activities of wild-type and chimeric IDHs

The *Pp*IDH, *Cm*IDH and chimeric IDH activities were assayed at various temperatures between 10 ºC and 65 ºC to estimate the effect of temperature on their activities (Fig. 2). His-tagging at the N-terminals of *Pp*IDH and *Cm*IDH was reported to have no significant influence on their thermal properties, such as optimum temperature and thermostability for activity [14, 25]. *Pp*WT (PPP) and *Cm*WT (CCC) showed the highest activity (464

unit/mg protein and 84 unit/mg protein) at 55 ºC and 30 ºC, respectively. Thus, as reported previously [14, 22], the two wild-type IDHs showed quite different specific activity and temperature-dependence of activity. As well as *Cm*WT and *Pp*WT, the substitutional chimeras of the region 1, PCC and CPP, showed the maximum activities of 90 unit/mg protein and 417 unit/mg protein at optimum temperatures of 35 ºC and 50 ºC, respectively. On the other hand, the substitutional chimeras of region 2, PCP and CPC, showed the maximum activities at 45 ºC and 40 ºC, and were 10 ºC lower and higher than the optimum temperatures for activities of the *Pp*WT and *Cm*WT, respectively. Specific activity of PCP at 45 ºC was only 36% of that of *Pp*WT, while CPC showed 4.8-fold higher specific activity at 40 ºC than that of *Cm*WT. PPC, in which region 3 of *Pp*IDH was swapped for the corresponding region of *Cm*IDH, showed the maximum activity (252 unit/mg protein) at 40 ºC, and the specific activity was close to that of *Pp*WT rather than *Cm*WT at the same temperature. However, this chimeric IDH activity was markedly decreased over 40 ºC, and no activity was detected at 50 ºC. In contrast, the optimum temperature for activity of CCP (45 ºC) rose by 15 ºC, compared to that of *Cm*WT, and the maximum activity of the former (133 unit/mg protein) was higher than that of the latter.

Thermostability of wild-type and chimeric IDH activities

To examine the thermostability for activities of the *Pp*IDH, *Cm*IDH and their chimeric IDHs, the residual activities after incubation for 10 min at various temperatures were measured at their respective optimum temperatures (Fig. 3). *Pp*WT almost completely maintained its activity (93%) after incubation at 40 ºC, and 13% of the activity was detected even after incubation at 55 ºC. On the other hand, *Cm*WT lost 95% and 100% of its activity by incubation at 35 ºC and 40 ºC, respectively. PCP, CPC, PPC and CCP retained over 90% of their activities after incubation at 30 ºC. However, after incubation at 35 ºC, the residual activities of PCP and PPC were decreased to 59% and 69%, respectively. On the other hand, CPC and CCP completely lost their activity after incubation at 50 ºC whereas PCP and PPC retained 4–5% of their activities after the same incubation. Thus, the substitution of the region 2 or 3 was found to increase and decrease the thermostability for activity in CPC and CCP and in PCP and PPC, respectively. On the other hand, CPP showed almost the same thermostability as

*Pp*WT, and the thermostability of PCC was slightly lower than *Cm*WT. Namely, the remaining activities of *Cm*WT were 89% and 30% after incubation at 25 ºC and 30 ºC whereas those of PCC were 65% and 15% of the activity, respectively.

Discussion

From the experiments for thermal properties of chimeric IDHs, it was found that the regions 2 and 3 of the two IDH proteins are involved in their catalytic activities and thermostability for their activities (Figs. 2 and 3). In particular, the specific activities of PCP and CPC were lower and higher at all temperatures tested than those of their wild-type IDHs, respectively, indicating that the region 2 strongly contributes to exert their catalytic activities. However, the shift of optimum temperature of the PCP and CPC activities was smaller than those of the swapping chimeras of the region 3, PPC and CCP (Fig. 2). On the other hand, the specific activities of PPC at temperatures between 10 ºC and 40 ºC were comparable to those of *Pp*WT, but the former activities above 45 ºC were much lower than the latter ones and were completely lost at 50 ºC. In contrast, the specific activities of CCP up to 30 ºC were similar to those of *Cm*WT, but the former had higher specific activity above 35 ºC than the latter and showed the highest specific activity at 45 ºC where the *Cm*WT activity was completely lost. These results indicate that the region 3 of *Pp*WT is responsible for high specific activity at temperatures above 35 ºC, leading to the shift to higher optimal temperature for activity. Thus, the effects of regions 2 and 3 on the specific activity and optimum temperature for activity are appeared to be different from each other. On the other hand, although thermostability of the CCP activity was slightly lower than that of CPC, the effect of region 2 on thermostability for activity was similar to that of region 3 (Fig. 3).

The swapping chimeras of region 1, CPP and PCC, showed thermal properties similar to the respective wild-type IDHs, except that the specific activity of CPP at the optimum temperature (50 ºC) was 90% of that of *Pp*WT (55 ºC) and the PCC activity was slightly more thermolabile than the *Cm*WT one. These results suggest that the region 1 of the two IDHs is hardly involved in their thermal properties. The substitution of Glu55 residue

located in region 1 of *Pp*IDH by Lys was reported to result in the decreased specific activity and thermostability [14]. However, the effects of this substitutional mutation on the thermal properties was much smaller than those of chimeric enzymes in this study, such as PCP and PPC. Therefore, the regions 2 and 3 of *Pp*IDH are judged to be main determinants for its thermal properties.

Thermal properties of the His-tagged *Cm*IDH and *Pp*IDH shown in this study and the His-tagged *Av*IDH reported previously [22] are summarized in Table1. By constructing chimeric IDHs between *Cm*IDH and mesophilic *Av*IDH instead of *Pp*IDH, Watanabe *et al*. [22] elucidated that the regions 2 and 3 of *Av*IDH are critical for its high specific activity and thermostability for activity. These results correspond with those of this study. Therefore, the common regions of the *Av*IDH and *Pp*IDH proteins are concluded to contribute to their mosophilic properties. Coincidentally, the amino acid sequences of regions 2 and 3 between *Pp*IDH and *Av*IDH (84% and 79% of identity, respectively) are more analogous than those of region 1 (73% of identity). In addition, the active site of the monomeric *Av*IDH is be located at the interface of the two domains [23], and the amino acid residues involved in the binding of isocitrate, metal ion and $NADP^+$, essential for the catalytic function [23, 24], are present in all three regions of the IDH proteins (Fig. 1). They are localized in a small and highly conserved area of region 1, which is adjacent to region 2, while are scattered within regions 2 and 3, in particular region 2. Thus, this might also participate in the different contribution of the three regions in *Pp*IDH and *Av*IDH to their thermal properties. On the other hand, the results of this study suggest that the protein regions involved in thermal properties are identical among the bacterial mesophilic monomer-type IDHs. To verify this possibility, studies on chimeras of the other combinations between the cold-adapted and the mesophilic monomer-type IDHs are necessary.

On the other hand, from the analysis of mutated enzymes, in which the two Pro residues in the region 3 of *Av*IDH (Pro708 and Pro717) were substituted by Ala residues at the corresponding positions of *Cm*IDH, these amino acid residues were reported to contribute to their thermal properties and catalytic activity [10]. The 708th amino acid residue of *Pp*IDH is also Pro (Fig. 1), but the substitutional mutation of this Pro residue in *Pp*IDH by Ala resulted in no significant change in its thermal properties and catalytic activity [14]. Furthermore, the amino acid residue of *Pp*IDH corresponding to the other Pro residue of *Av*IDH, Pro717, is Ala, identical to that of

*Cm*IDH (Fig. 1). These indicate that at least a part of the amino acid residues responsible for the mesophilic properties present in the common regions (regions 2 and 3) of *Av*IDH and *Pp*IDH are different from each other. In previous study, no significant change of thermal properties in *Pp*IDH was found to be brought about by the substitutions of His158 and Pro490 residues in the region 2 and Glu595 residue in the region 3 by the corresponding ones of *Cm*IDH [14]. Therefore, to identify the amino acid residues involved in the mesophilic properties, further study on the substitutional mutants of amino acid residues in the regions 2 and 3 of *Pp*IDH and *Cm*IDH is in progress. Recently, it was observed that optimum temperature for activity lowerss by 5 ºC, compared to the wild-type enzyme, in the *Pp*IDH mutant substituted the Leu594 residue by the corresponding Glu of *Cm*IDH (Nagai and Takada, unpublished data).

Conflict of interest The authors declare no conflict of interest.

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Legends to figures

Fig. 1 Alignment of deduced amino acid sequences of *Pp*IDH, *Cm*IDH, and *Av*IDH. The deduced amino acid sequence of *Pp*IDH (Database accession No. AB425997) is aligned with those of *Cm*IDH (No. BAA03134) and *Av*IDH (No. D73443). The amino acid residues involved in the binding of isocitrate and metal ion are indicated with asterisks. The amino acid residues involved in the binding of $NADP^+$ are indicated with closed triangles. Letters in boxes indicate the amino acid residues substituted in previous studies [10, 14]. Each region (1, 2 and 3) of the IDH proteins is enclosed with lines.

Fig. 2 Effect of temperature on activities of *Pp*WT, *Cm*WT and chimeric IDHs.

Fig. 3 Thermostability of *Pp*WT, *Cm*WT and chimeric IDH activities. Residual activities are assayed at the optimal temperature of each IDH after incubation for 10 min at the indicated temperatures and are represented as percentages of those without the incubation.

Table 1 Thermal properties of *Cm*IDH, *Pp*IDH and *Av*IDH

	*Cm*IDH	*Pp*IDH	*Av*IDH [22]
Optimum temperature for activity	30 ºC	55 ºC	45 ºC
Remaining activity after incubation for 10 min at 40 ºC	0 %	93 %	98 %

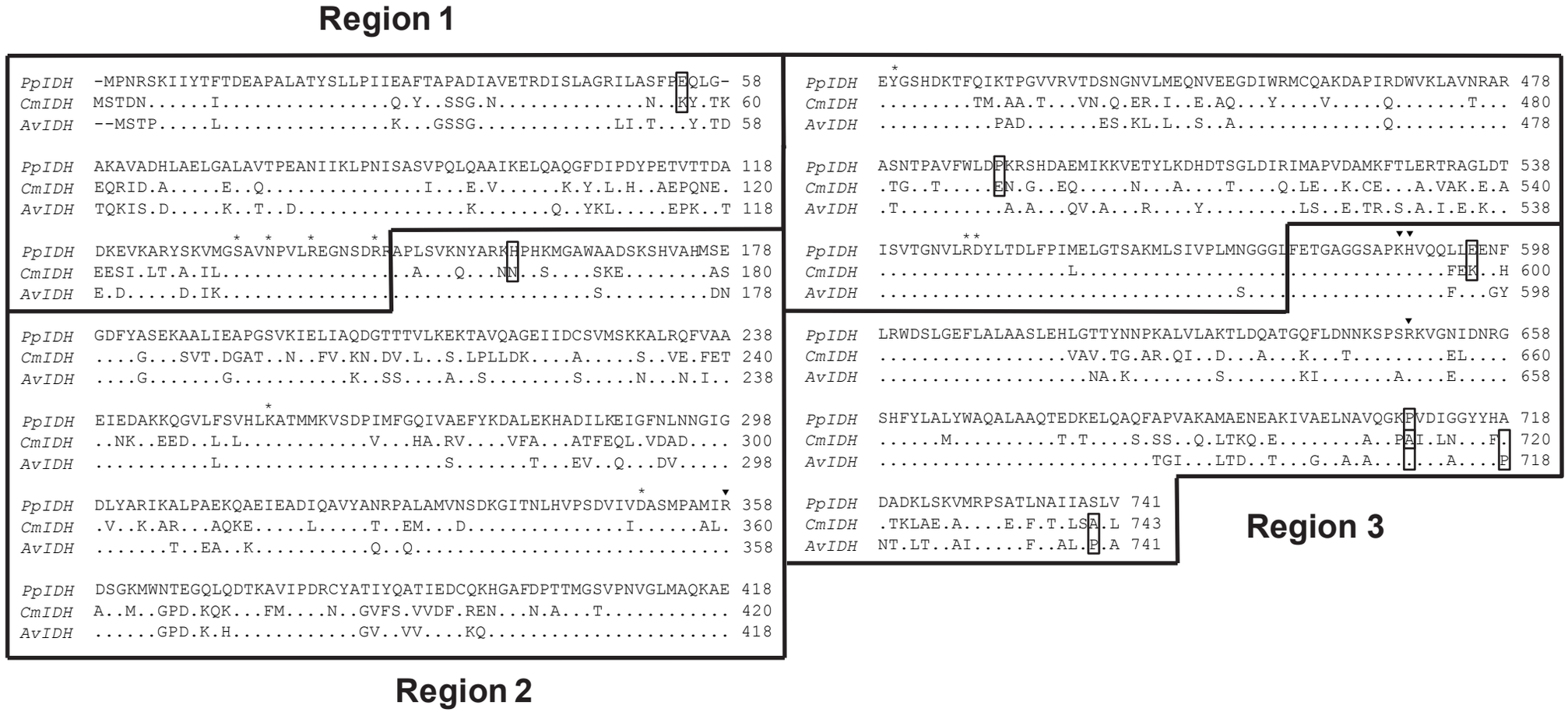
Region 1
PpIDH -MPNRSKIIYTFTDEAPALATYSLLPIIEAFTAPADIAVETRDISLAGRILASFPEQLG- 58
CmIDH MSTDN.....I.............Q.Y..SSG.N.............N..KY.TK 60
AvIDH --MSTP.....L.............K...GSSG.............LI.T...Y.TD 58
PpIDH AKAVADHLAELGALAVTPEANIIKLPNISASVPQLQAAIKELQAQGFDIPDYPETVTTDA 118
CmIDH EQRID.A.....E..Q.............I...E.V......K.Y.L.H..AEPQNE. 120
AvIDH TQKIS.D.....K..T..D.............K.......Q..YKL.....EPK..T 118
PpIDH DKEVKARYSKVMGSAVNPVLREGNSDRPAPLSVKNYARKHPHKMGAWAADSKSHVAHMSE 178
CmIDH EESI.LT.A.IL.................A...Q...NN..S....SKE........AS 180
AvIDH E.D.....D.IK.....................................S..........DN 178
PpIDH GDFYASEKAALIEAPGSVKIELIAQDGTTTVLKEKTAVQAGEIIDCSVMSKKALRQFVAA 238
CmIDH ....G...SVT.DGAT..N..FV.KN.DV.L..S.LPLLDK....A.....S..VE.FET 240
AvIDH ....G.......G...........K..SS....A..S........S.....N...N.I.. 238
PpIDH EIEDAKKQGVLFSVHLKATMMKVSDPIMFGQIVAEFYKDALEKHADILKEIGFNLNNGIG 298
CmIDH ..NK..EED..L.L............V...HA.RV....VFA...ATFEQL.VDAD.... 300
AvIDH ..........L.....................S.......T...EV..Q...DV..... 298
PpIDH DLYARIKALPAEKQAEIEADIQAVYANRPALAMVNSDKGITNLHVPSDVIVDASMPAMIR 358
CmIDH .V..K.AR...AQKE.....L.....T..EM...D..............I......AL. 360
AvIDH .......T..EA..K...........Q..Q.............................. 358
PpIDH DSGKMWNTEGQLQDTKAVIPDRCYATIYQATIEDCQKHGAFDPTTMGSVPNVGLMAQKAE 418
CmIDH A..M..GPD.KQK...FM....N..GVFS.VVDF.REN...N.A...T............ 420
AvIDH ......GPD.K.H............GV..VV....KQ...................... 418
Region 2
PpIDH EYGSHDKTFQIKTPGVVRVTDSNGNVLMEQNVEEGDIWRMCQAKDAPIRDWVKLAVNRAR 478
CmIDH .........TM.AA.T...VN.Q.ER.I..E.AQ...Y....V.....Q.......T... 480
AvIDH ...........PAD.......ES.KL.L..S..A.............Q........... 478
PpIDH ASNTPAVFWLDPKRSHDAEMIKKVETYLKDHDTSGLDIRIMAPVDAMKFTLERTRAGLDT 538
CmIDH .TG..T.....EN.G..EQ.....N...A....T....Q.LE..K.CE...A.VAK.E.A 540
AvIDH .T..........A.A...QV.A...R....Y.........LS..E.TR.S.A.I.E.K.. 538
PpIDH ISVTGNVLRDYLTDLFPIMELGTSAKMLSIVPLMNGGGLFETGAGGSAPKHVQQLIEENF 598
CmIDH ..................L.....................................FEK..H 600
AvIDH .....................................S.....................F...GY 598
PpIDH LRWDSLGEFLALAASLEHLGTTYNNPKALVLAKTLDQATGQFLDNNKSPSRKVGNIDNRG 658
CmIDH ..................VAV.TG.AR.QI..D...A...K...T.........EL.... 660
AvIDH ....................NA.K........S.......KI.......A....E..... 658
PpIDH SHFYLALYWAQALAAQTEDKELQAQFAPVAKAMAENEAKIVAELNAVQGKPVDIGGYYHA 718
CmIDH ......M..........T.T....S.SS..Q.LTKQ.E........A..PAI.LN...F. 720
AvIDH ..........................TGI...LTD..T...G..A.A.....A....P 718
PpIDH DADKLSKVMRPSATLNAIIASLV 741
CmIDH .TKLAE.A....E.F.T.LSA.L 743
AvIDH NT.LT..AI.....F..AL.P.A 741
Region 3

Fig. 1

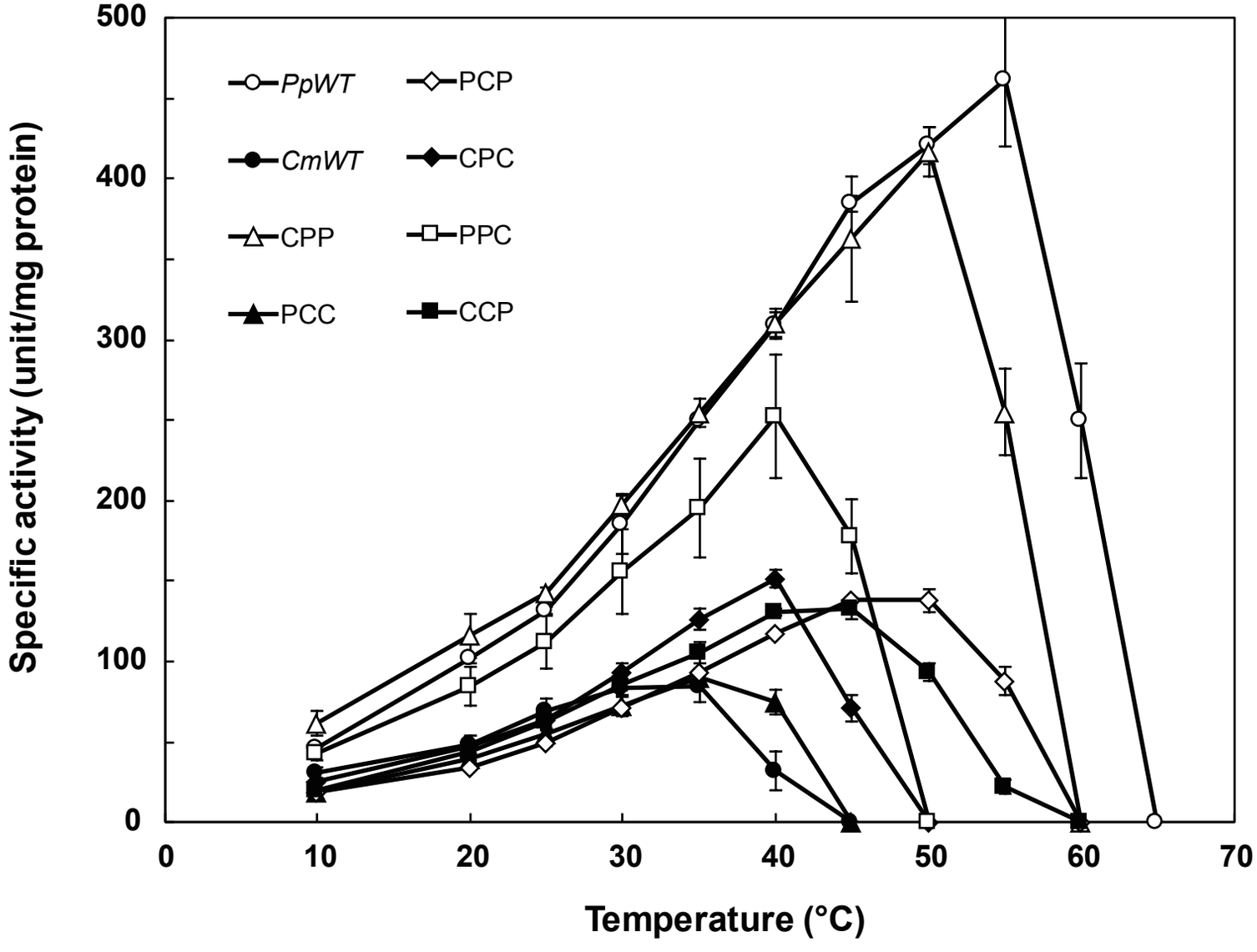
Specific activity (unit/mg protein)
500
400
300
200
100
0
0
10
20
30
40
50
60
70
Temperature (°C)
PpWT
CmWT
CPP
PCC
PCP
CPC
PPC
CCP

Fig. 2

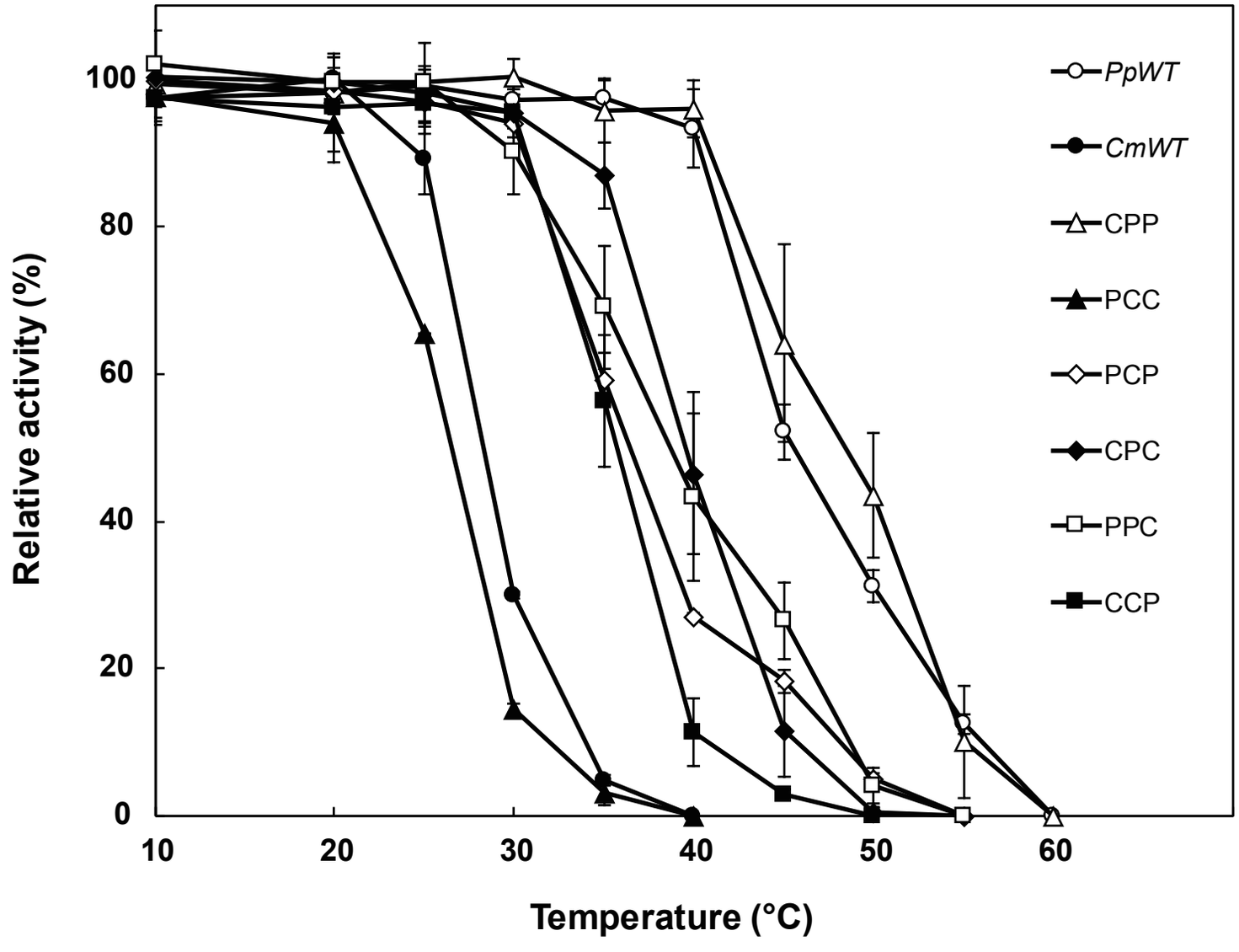
Relative activity (%)
100
80
60
40
20
0
10
20
30
40
50
60
Temperature (°C)
PpWT
CmWT
CPP
PCC
PCP
CPC
PPC
CCP

Fig. 3

Supplementary Material (Figure and Table)

Contribution of three different regions of isocitrate dehydrogenases from psychrophilic and psychrotolerant bacteria to their thermal properties

Current Microbiology

Yuka Mouri[1] • Yasuhiro Takada[2]

[1]Biosystems Science Course, Graduate school of Life Science, Hokkaido University, Kita 10-jo Nishi 8-chome, Kita-ku, Sapporo 060-0810, Japan

[2]Depertment of Biological sciences, Faculty of Science, Hokkaido University, Kita 10-jo Nishi 8-chome, Kita-ku, Sapporo 060-0810, Japan

✉ Yasuhiro Takada: ytaka@sci.hokudai.ac.jp, Fax: +81-11-706-2742

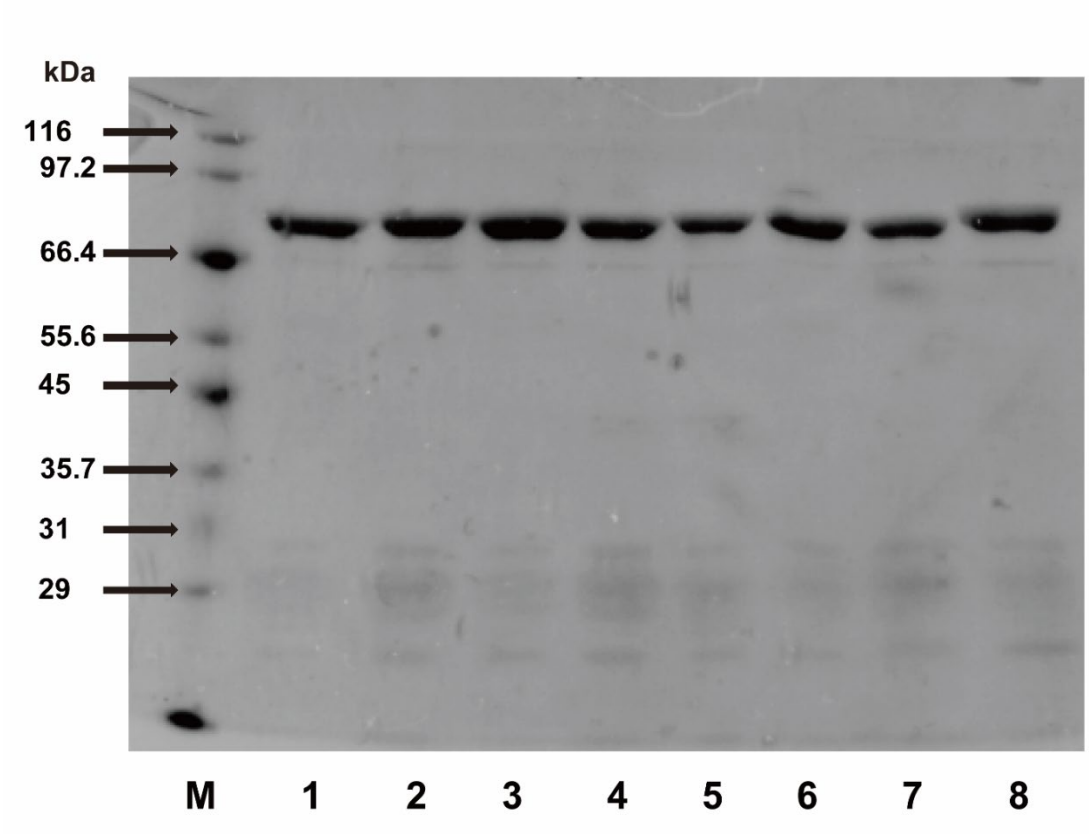

Fig. S1 SDS-PAGE of wild-type and chimeric IDHs. Five μg of proteins were applied into each lane. Lane M, marker proteins; lane 1, *Pp*WT; lane 2, *Cm*WT; lane 3, CPP; lane 4, PCC; lane 5, PCP; lane 6, CPC; lane 7, PPC; lane 8, CCP.

Table S1 Oligonucleotides used in this study

Primer name	Nucleotide sequence (5' to 3')
PMF0-*Nsp*V	5'-gcgcggatccgCCCAACCGTTC**C**AAGATC-3'
PMR0-*Sac*I	5'-gcgcgagctcTTACACCAGTGAGGCG-3'
Del*Bss*HII-s	5'-GTCAAAGC**A**CGCTACAGCAAGGTTATGGG-3'
Del*Bss*HII-as	5'-CCCATAACCTTGCTGTAGCG**T**GCTTTGAC-3'
Add*Bss*HII-s	5'-CAACTCTGACCGCCGCGC**G**CCGCTGCTGG-3'
Add*Bss*HII-as	5'-CCGACAGCGG**C**GCGCGGCGGTCAGAGTTG-3'

Small letters indicate the additional bases for introducing digestion sites for *Bam*HI and *Sac*I (underlined and double-underlined letters), respectively. Letters in gray boxes indicate the removed *Nsp*V and *Bss*HII sites, respectively, and letters in boxes indicate the introduced *Bss*HII site (bold letters are the substituted bases). Bold letters are the substituted bases.