Running title: NADP$^+$-IDHs of *C. psychrerythraea*

Characterization of NADP$^+$-dependent Isocitrate dehydrogenase isozymes from a psychrophilic bacterium, *Colwellia psychrerythraea* strain 34H

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NADP$^+$-dependent isocitrate dehydrogenase (IDH) isozymes of a psychrophilic bacterium, *Colwellia psychrerythraea* strain 34H, were characterized. The coexistence of monomeric and homodimeric IDHs in this bacterium was confirmed by western blot analysis, the genes encoding two monomeric (IDH-IIa and IDH-IIb) and one dimeric (IDH-I) IDHs were cloned and overexpressed in *Escherichia coli*, and the three IDH proteins were purified. Both of the purified IDH-IIa and IDH-IIb were found to be cold-adapted enzyme while the purified IDH-I showed mesophilic properties. However, the specific activities of IDH-IIa and IDH-IIb were lower even at low temperatures than that of IDH-I. Therefore, IDH-I was suggested to be important for growth of this bacterium. The results of colony formation of *E. coli* transformants carrying the respective IDH genes and IDH activities in their crude extracts indicated that the expression of the IDH-IIa gene is cold-inducible in the *E. coli* cells.

Key words: isocitrate dehydrogenase · isozymes ·cold-adapted enzyme ·*Colwellia psychrerythraea* strain 34H

NADP$^+$-dependent isocitrate dehydrogenase (IDH; EC 1.1.1.42) is a key enzyme in the TCA cycle, which is an important metabolic pathway distributed universally in aerobic organisms, and catalyzes the oxidative decarboxylation of D-isocitrate to α-ketoglutarate and CO_2 concomitantly with the reduction of NADP$^+$. Based on the subunit structure, bacterial IDHs can be categorized into two types. One is homodimer consisting of subunits of 40−45 kDa, and the other is monomer with a molecular mass of 80−100 kDa. Many bacteria possess only one of either type IDH. For example, the IDHs of *Escherichia coli*,[1] *Thermus thermophilus*,[2] *Bacillus stearothermophilus*[3] and *Rhodopseudomonas spheroides*[4] have been found to be dimeric, while those of *Azotobactor vinelandii*,[5,6] *Corynebacterium glutamicum*,[7,8] *Rhodomicrobium vannielii*[9] and *Vibrio parahaemolyticus*[10] are monomeric. On the other hand, psychrophilic bacteria, *Colwellia maris* (formerly, *Vibrio* sp. strain ABE-1) and *Colwellia psychrerythraea* strain NRC1004, and a psychrotrophic bacterium, *Pseudomonas psychrophila*, have been reported to possess both of the two type IDHs.[11-14] In *C. maris* and *C. psychrerythraea* strain NRC1004, the dimeric IDHs are typical mesophilic enzymes, and the optimal temperatures for activity are 40−45 °C. On the other hand, their monomeric IDHs show the maximum activities at 20−25 °C and are markedly thermolabile. In the *C. maris* IDH, about a half of the activity is lost even by incubation for 10 min at 30 °C. These indicate that the monomeric IDHs are typical cold-adapted enzymes. Furthermore, the two IDH isozyme genes of these bacteria are located in tandem with the monomeric IDH genes followed by the dimeric ones on the chromosomal DNAs[13,15] (Fig. 1), but the expressions of the two IDH isozyme genes are independently regulated by different promoters. In the case of *C. maris*, the expressions of the monomeric IDH (IDH-II) gene (*icdII*) and the dimeric IDH (IDH-I) gene (*icdI*) are induced by low temperatures and acetate,

respectively.[16] Furthermore, the IDH-II has higher catalytic activity than the IDH-I. Therefore, the IDH-II has been thought to play an important role in cold adaptation of *C. maris*. On the other hand, in *C. psychrerythraea* NRC1004, the activity of cold-adapted monomeric IDH was much lower than that of mesophilic dimer-type IDH, and the latter maintained higher activity even at low temperatures such as 10 °C than the former.[13] Thus, the mesophilic dimer-type IDH was seemed to be essential for growth of this bacterium. On the other hand, both of the two type IDH isozymes of *P. psychrophila* are mesophilic and show the maximum activity at 60 °C.[14] Furthermore, the two IDH isozyme genes are located tandem in opposite direction from each other on the chromosomal DNA, and the two genes are independently regulated by different promoters similarly to *C. maris* and *C. psychrerythraea* NRC1004. The expressions of the monomeric and dimeric IDH genes of *P. psychrophila* are induced by low temperature and acetate and by low temperature, respectively, but the levels of the cold-inductions are considerably weaker than that of the *C. maris icdII* gene expression.[14]

A psychrophilic bacterium, *Colwellia psychrerythraea* strain 34H, the type species of the genus *Colwellia* belonging to class gammaproteobacteria, has been isolated from Arctic marine sediments,[17] and the complete genomic sequence has been determined.[18] From analysis of the sequence, it is presumed that this bacterium has three genes of $NADP^+$-dependent IDH (two monomeric and one dimeric IDHs are termed IDH-IIa and IDH-IIb and IDH-I, and the genes encoding them are termed *icdIIa, icdIIb* and *icdI,* respectively) (Fig. 1) and one gene of NAD^+-dependent IDH. However, the $NADP^+$-dependent IDHs have not been characterized. In this study, thermal properties of these IDH activities, such as optimum temperature and thermostability, and the expression of the *icd* genes in the *E. coli* cells were examined.

Materials and methods

Bacteria, plasmids and growth conditions. The psychrophilic bacterium, *Colwellia psychrerythraea* strain 34H, was grown at 10 °C with vigorous shaking in Marine Broth medium (Difco). *E. coli* XL1-Blue (Stratagene) was used to propagate plasmids, and a mutant of *E. coli* defective in IDH, DEK2004,[19] which has a glutamate auxotrophic phenotype, was used as a host for overexpression of the *C. psychrerythraea* 34H IDH genes. Morpholinepropanesulfonic acid (MOPS)-based synthetic medium[20] supplemented with 0.5% glucose and 0.5 mM Trp, Luria-Bertani (LB) medium,[21] and Super broth medium[13] are used for growth of these *E. coli* strains. If necessary, ampicillin and tetracycline were added to the culture media at concentrations of 100 and 15 μg/mL, respectively. For the subcloning of the IDH isozyme genes and the overexpression of genes encoding the recombinant IDHs conferred His-tags at the N-terminals, plasmids pBluescript SK(+) (pBS; Stratagene) and pTrcHisB (Invitrogen) were used as vectors, respectively.

Preparation of crude extract of C. psychrerythraea 34H. *C. psychrerythraea* 34H cultivated up to the late exponential phase (for about 21 h) at 10 °C were harvested and washed twice with a sonication buffer (20 mM potassium phosphate buffer (pH 7.5), containing 0.5 M NaCl, 2 mM $MgCl_2$ and 10 mM 2-mercaptoethanol). Cells suspended in the sonication buffer were disrupted by twelve times sonication for 0.5 min with intervals of 1 min on ice. The cell lysate was centrifuged at 16,000 × *g* for 20 min at 4 °C, and the supernatant was then stored at –30 °C and used as crude extract in the following

experiments. Protein concentration was measured by the method of Lowry et al.[22] using bovine serum albumin as a standard.

Western blot analysis. SDS-PAGE[23] of crude extract of the *C. psychrerythraea* 34H cells was carried out on 10% polyacrylamide gel at 120 V. The proteins on the gel were then transferred onto a polyvinylidene fluoride membrane (Immobilon-P, Millipore) at 4 °C, according to the manufacturer's instructions. Western blot analysis was carried out as reported previously[24] except for using rabbit antibodies against the purified IDH-I and IDH-II of *C. maris*[25] diluted to 1:30,000 and 1:50,000, respectively.

Cloning of the IDH isozyme genes. Genomic DNA of *C. psychrerythraea* 34H was isolated and purified with FastPure DNA Kit (TaKaRa). For the cloning of IDH isozyme genes, genomic PCR was carried out as described below. The forward primers, IIa-around-f, IIb-around-f and I-around-f, correspond to the sequences between –995 and –969 from the translational start codon of *icdIIa*, between –995 and –971 from that of *icdIIb* and between –994 and –968 from that of *icdI*, respectively. The reverse primers, IIa-around-r, IIb-around-r and I-around-r, are complementary to the sequences between +2382 and +2407 from the translational start codon of *icdIIa*, between +2393 and +2417 from that of *icdIIb* and between +1423 and +1450 from that of *icdI*, respectively (Table 1). Amplification was performed for 30 cycles in a Verti 96 well Thermal Cycler (Applied Biosystems) in a reaction mixture (50 μL) containing 0.22 μg genomic DNA as the template, 15 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: for *icdIIa*, denaturation at 98 °C for 10 s, annealing at 45 °C for 0.5 min and

extension at 68 °C for 3.2 min; for *icdIIb*, denaturation at 98 °C for 10 s, annealing at 43 °C for 0.5 min and extension at 68 °C for 3.4 min; for *icdI*, denaturation at 98 °C for 10 s, annealing at 48 °C for 0.5 min and extension at 68 °C for 2.4 min, for each of 30 cycles. For the cloning of *icdIIa* and *icdIIb*, about 3.4-kbp PCR product, of which the size corresponds to that of a complete ORF of *icdIIa* or *icdIIb* containing its upstream and downstream regions (about 2.2 kbp, 1.0 kbp and 0.2 kbp, respectively), was purified by the extraction of DNA bands from the agarose gel after electrophoresis, phosphorylated with T4 polynucleotide kinase (TOYOBO) and then ligated into the *Sma*I site of pBS with DNA Ligation Kit Ver. 2.1 (TaKaRa) to obtain plasmid pIDH-IIa-ar or pIDH-IIb-ar. Plasmid pIDH-I-ar was also obtained by similar experimental procedures except that the size of PCR product containing a complete ORF of *icdI* and its upstream and downstream regions (about 1.2 kbp, 1.0 kbp and 0.2 kbp, respectively) was about 2.4 kbp. Then, these plasmids were purified with High Pure Plasmid Isolation Kit (Roche Diagnostics) and introduced into *E. coli* DEK2004 by an electroporation with Gene Pulser II (Bio-Rad). Precise insertion of these genes into pBS was certified by the nucleotide sequencing of these plasmids by using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with a sequencer (3130 genetic Analyzer, Applied Biosystems).

Construction of genes encoding His-tagged IDHs of C. psychrerythraea 34H. The *icdIIa*, *icdIIb* and *icdI* genes of *C. psychrerythraea* 34H were amplified by PCR to introduce restriction sites for *Bam*HI and *Sac*I at the 5’ and 3’-terminals of their ORFs, respectively. Therefore, the following primers were used: IDH-IIa-f and IDH-IIa-r as forward and reverse primers for *icdIIa*, IDH-IIb-f and IDH-IIb-r as forward and reverse primers for *icdIIb*, IDH-I-f and IDH-I-r as forward and reverse primers for *icdI*, respectively (Table 1).

Amplification was performed for 30 cycles in the DNA thermal cycler in a reaction mixture (50 μL) containing 0.22 μg genomic DNA as the template, 15 pmol each of the forward and reverse primers and 1 U KOD-plus DNA polymerase in a buffer system prepared by the manufacturer. Cycling conditions were as follows: for *icdIIa*, denaturation at 98 °C for 10 s, annealing at 51 °C for 0.5 min and extension at 68 °C for 2.2 min; for *icdIIb*, denaturation at 98 °C for 10 s, annealing at 48 °C for 0.5 min and extension at 68 °C for 2.2 min; for *icdI*, denaturation at 98 °C for 10 s, annealing at 49 °C for 0.5 min and extension at 68 °C for 1.2 min. Each PCR products was digested with *Bam*HI and *Sac*I and ligated to the *Bam*HI-*Sac*I site of pTrcHisB, which is a plasmid vector for conferring the N-terminal $(His)_6$-tag on the expressed proteins, to obtain plasmids pHisCp34IDH-IIa, pHisCp34IDH-IIb and pHisCp34H-I, respectively. Precise insertion of these genes into the vector was certified by the nucleotide sequencing as described above.

Overexpression and purification of His-tagged IDHs. *E. coli* DEK2004 transformed with pHisCp34IDH-IIa, pHisCp34IDH-IIb or pHisCp34H-I was grown at 37 °C in Super broth medium until OD_{600} of the culture reached 0.8. For the overexpression of IDH proteins, the culture was then immediately cooled on ice and further incubated for 18−21 h at 15 °C for IDH-IIb and IDH-I and at 10 °C for IDH-IIa after the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After cells were harvested at 4 °C, the His-tagged IDH proteins were purified by Ni-NTA column chromatography (Quiagen) according to the procedure for the *C. maris* IDH-II reported by Kobayashi et al.,[26] except that the column was further washed with 50 mL of 50 mM sodium phosphate (pH 8.0), containing 2 mM $MgCl_2$, 0.5 M NaCl, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol and 50 mM imidazole. The eluted

solution was concentrated and then dialyzed as described previously.[27] All His-tagged recombinant IDHs were stored at –30 °C.

Enzyme assay. The IDH activity was assayed as reported previously.[11] The reaction mixture (2 mL) contained 33 mM Tris-HCl (pH 7.5), 0.67 mM $MnCl_2$, 0.15 M NaCl, 0.12 mM $NADP^+$, 2 mM sodium isocitrate and an appropriate amount of enzyme. Thermostability of the IDH activity was examined as described previously,[27] except that all purified IDHs were dialyzed overnight at 4 °C against 20 mM potassium phosphate buffer (pH 8.0) containing 2 mM $MgCl_2$, 0.3 M NaCl and 1 mM dithiothreitol. One unit of enzyme activity was defined as the amount capable of catalyzing the reduction of 1 μmol of $NADP^+$ per min.

Results

Thermal properties of the IDH activity in crude extract of C. psychrerythraea *34H and Western blot analysis*

IDH activity in crude extract of *C. psychrerythraea* 34H grown at 10 °C was assayed at various temperatures (Fig. 2A). IDH in the crude extract showed the maximum activity at 50 °C (0.26 U/mg protein) and decreased at above 60 °C. In addition, the curve of temperature-dependence showed a slight swelling between 20 and 40 °C, implying that IDH(s) with an optimum temperature for activity of about 30 °C is also present in the crude extract. Furthermore, about 80% of the activity is maintained after incubation of the crude extract for 10 min at 40 °C (Fig. 2B). After incubation under the same condition, the cold-adapted IDH-II of *C. maris* showed no activity while the mesophilic monomer-type IDH

of *A. vinelandii* kept the activity completely.[11,27] These results indicate a possible coexistence of cold-adapted and mesophilic IDHs in the crude extract of this bacterium. Immunological cross-reactivity of monomeric and dimeric bacterial IDHs have been reported to be different from each other.[10,25] To confirm the existence of the two type IDH isozymes in *C. psychrerythraea* 34H, western blot analysis was carried out with rabbit antibodies against the *C. maris* dimeric IDH-I and monomeric IDH-II (Fig. 3). Proteins crossreacted with the respective antibodies were detected in the crude extract of *C. psychrerythraea* 34H. The molecular masses of these proteins were estimated to be 47.5 and 80 kDa and were compatible with those of IDH-I and IDH-II from *C. maris*, respectively. These results indicate that at least both of the two type IDH isozymes are present in the crude extract of this bacterium.

Expression of the C. psychrerythraea 34H IDH isozyme genes

Two monomeric IDH gene ORFs (*icdIIa* and *icdIIb*) and one dimeric IDH gene ORF (*icdI*) containing their upstream and downstream regions, which were presumed from the determined complete genome sequence of this bacterium, were cloned into pBS as described in MATERIALS AND METHODS. The expression of the three *C. psychrerythraea* 34H IDH isozyme genes in an IDH-defective mutant of *E. coli*, DEK2004, was examined at 15, 27 and 37 °C by colony formation on the MOPS-based synthetic agar medium. Although the transformant harboring pIDH-IIb-ar was not able to grow at all temperatures tested, colonies were formed within 6 days only at 15 °C in the transformant with pIDH-IIa-ar and within 4 days at 27°C and within 5 days at 37 °C in the transformant with pIDH-I-ar after their inoculations, respectively. On the other hand, the *E. coli* DEK2004 cells transformed with pBS were not able to grow under these conditions because of a lack of glutamate in the growth medium.

These results indicate that the expression of these genes complements the auxotrophy of glutamate in the *E. coli* mutant defective in IDH. Furthermore, similar results were obtained by the comparison of the IDH activities in the crude extracts of these *E. coli* transformants grown at 15, 25 and 37 °C. No IDH activity was detected in the crude extract of transformant with pIDH-IIb-ar grown at all temperatures tested. On the other hand, the transformant with pIDH-IIa-ar showed the IDH activity (0.64×10^{-3} U/mg protein) when only grown at 15 °C. The activity of the transformant with pIDH-I-ar grown at 25 °C (2×10^{-3} U/mg protein) were larger than those grown at the other temperatures (0.44 and 0.16×10^{-3} U/mg protein at 15 and 37 °C). From these results, it is implied that the *icdIIb* gene could not be expressed in *E. coli* under the conditions tested in this study while the expressions of the *icdIIa* and *icdI* genes are induced by low and moderate temperatures, respectively. Moreover, IDHs in the crude extracts of transformants with pIDH-IIa-ar and pIDH-I-ar showed the maximal activities at 30 °C and 50 °C, respectively (data not shown).

Properties of His-tagged IDH isozymes

The *icdIIa*, *icdIIb* and *icdI* genes were introduced into the expression vector, pTrcHisB. The IDH proteins conferred the N-terminal His-tags, which consist of 32 amino acid residues containing six His residues, were overexpressed in the *E. coli* DEK2004 cells and then purified by Ni-affinity column chromatography. SDS-PAGE of the purified His-tagged IDHs revealed that each major protein has a slightly larger molecular mass than that calculated from the nucleotide sequence of the gene because of the additional amino acid residues by the His-tagging at N-terminal (Fig. 4). When the IDH-IIa was overexpressed at 15 °C by IPTG, several protein bands with lower molecular masses were detected in SDS-PAGE of the purified enzyme protein, implying

the degradation of the enzyme protein during its overexpression and/or purification. Therefore, the overexpression of IDH-IIa was carried out at lower temperature, 10 °C. As a result, it led to the decrease of the degraded protein and the improved yield of the enzyme protein. Activities of the purified IDHs were assayed at various temperatures (Fig. 5A). The His-tagged IDH-I showed the highest activity (85.05 U/mg protein) at 50 °C and this result was similar to the optimal temperature for IDH activity in the crude extract of *C. psychrerythraea* 34H. Furthermore, about 90% of IDH-I activity was maintained after incubation for 10 min at 40 °C, indicating that it is a mesophilic enzyme (Fig. 5B). On the other hand, the two monomeric IDHs showed thermal properties different from IDH-I. Maximum activities of IDH-IIa and IDH-IIb (8.4 and 8.2 U/mg protein, respectively) were observed at 30 °C and 25 °C, respectively. Furthermore, about 60% of the IDH-IIa activity and about 70% of the IDH-IIb activity were lost after incubation for 10 min at 20 °C and 30 °C, respectively. These results indicate that both of monomeric IDHs are typical cold-adapted enzymes and IDH-IIa is more thermolabile than IDH-IIb. On the other hand, IDH-I was considerably higher specific activity than IDH-IIa and IDH-IIb (Fig. 5A). Optimum temperatures for the His-tagged IDH-IIa and IDH-I activities were consistent with those of the native IDHs overproduced in the *E. coli* DEK2004 cells transformed with pIDH-IIa-ar and pIDH-I-ar described above, indicating that His-tagging at the N-terminals did not significantly affect their thermal properties.

Discussions

The three IDH isozyme genes of *C. psychrerythraea* 34H, *icdIIa* and

icdIIb encoding the two monomeric IDHs and *icdI* encoding the dimeric IDH, were cloned in this study. From the determined genome sequence of this bacterium,[18] it was found that the two genes, *icdIIa* and *icdI*, are located in tandem with the former followed by the latter, similarly to *C. maris* and *C. psychrerythraea* NRC1004, while the *icdIIb* is located far apart in opposite direction on the chromosomal DNA (Fig. 1). Therefore, the *icdIIa* gene of *C. psychrerythraea* 34H is appeared to correspond with the monomeric IDH genes of *C. maris* and *C. psychrerythraea* NRC1004. On the other hand, the *icdIIb* gene might be added by horizontal transfer of IDH gene from other organism or gene duplication. As data for supporting this possibility, the amino acid sequence of IDH-IIa showed higher degrees of homology to the monomeric IDHs from other bacteria than that of IDH-IIb, particularly 92% of the sequential identity to the *C. maris* IDH-II (Table 2). Furthermore, the amino acid residues involved in the binding of substrate, metal ion and coenzyme were completely conserved in the three IDH isozymes of *C. psychrerythraea* 34H.

Similarly to *C. maris* and *C. psychrerythraea* NRC1004, the one dimeric and both of two monomeric IDHs of *C. psychrerythraea* 34H were mesophilic and cold-adapted enzymes, respectively (Fig. 4). However, specific activities of the two monomeric IDHs were considerably lower at all temperatures tested than that of the dimeric IDH. Similar results have been reported in the IDH isozymes from *C. psychrerythraea* NRC1004.[13] In contrast, the cold-adapted monomeric IDH-II of *C. maris* showed much higher activity than the mesophilic dimer-type IDH-I.[11,12] Therefore, the mesophilic IDH-I is suggested to contribute to the growth of *C. psychrerythraea* 34H.

From colony formation of the *E. coli* DEK2004 transformant carrying pIDH-IIa-ar on MOPS-based synthetic agar medium and the IDH activity in the crude extract of this transformant grown at various temperatures, the expression

of the *icdIIa* gene in the *E. coli* cells was shown to be cold-inducible. In addition, it is noteworthy that only the *E. coli* DEK2004 transformant carrying pIDH-IIa-ar, but not pIDH-I-ar, was able to grow at 15 °C on MOPS-based synthetic agar medium. A “CCAAT” sequence, which has been reported to be a *cis*-element involved in the cold-induction of the *C. maris icdII* gene expression,[28] is present 737-733 bases upstream of the translational initiation codon of *icdIIa* gene. 5’-RACE analysis of the *icdI* mRNA revealed that the expression of this gene was regulated independently to the *icdIIa* gene in spite of the tandem location of the two genes on the chromosomal DNA (data not shown). Further experiments are required to clarify the expression of the *icdIIa* gene in the *C. psychrerythraea* 34H and the function of the “CCAAT” sequence in this gene expression.

Since the molecular mass of IDH-IIb calculated from the nucleotide sequence (82,103 Da) was almost the same as that of IDH-IIa (80,885 Da), the content of the two IDHs in the crude extract of *C. psychrerythraea* 34H grown on Marine Broth medium was not able to be estimated by western blot analysis with antibody against the purified IDH-II of *C. maris* (Fig. 3B). On the other hand, no expression of the *icdIIb* gene in the *E. coli* DEK2004 transformant carrying pIDH-IIb-ar was detected at all temperatures tested in its colony formation on MOPS-based synthetic agar medium and the IDH activity in the crude extract. However, the purified His-tagged IDH-IIb protein was found to have the catalytic activity and show cold-adapted properties (Fig. 5). Therefore, isolation of a mutant of *C. psychrerythraea* 34H defective in IDH-IIb is required to understand the physiological role of this enzyme in growth of this bacterium under various conditions.

Interestingly, nevertheless IDH-IIa showed very high level of amino acid sequential identity to the IDH-II of *C. maris* as described above, the IDH

specific activity of the former is much lower than that of the latter (about 10% in comparison of the maximum activity). Therefore, the experiments on chimeric enzymes between the *C. psychrerythraea* 34H IDH-IIa and the *C. maris* IDH-II should be very important to clarify their psychrophilic properties, particularly catalytic activity at low temperature, and are in progress.

Author contributions

Y.T. designed the research plan; K.S. performed the experiments; K.S. and Y.T. analyzed the data; and K.S and Y.T. wrote the manuscript.

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Legends to figures

Fig. 1. Location of IDH isozyme genes on the chromosome DNA in *C. maris*. *C. psychrerythraea* NRC1004 and *C. psychrerythraea* 34H. Relative configurations of IDH isozyme genes of these bacteria on their chromosomes are shown. Gene structures for *C. maris* and *C. psychrerythraea* NRC1004 have been reported previously,[13,15] but *icd-D* and *icd-M* of the latter bacterium are renamed as *icdI* and *icdII*, respectively. Information for IDH gene in *C. psychrerythraea* 34H was obtained from Genome Information Broker (GIB) in DNA Data Bank of Japan (DDBJ).

Fig. 2. Temperature dependence (A) and thermostability (B) of the IDH activity in crude extract of *C. psychrerythrea* 34H.

In (A), IDH activity in the crude extract of *C. psychrerythraea* 34H, which was cultured at 10°C, assayed at various temperatures. In (B), residual activity of IDH in the crude extract of *C. psychrerythrea* 34H after incubation for 10 min at various temperatures was assayed at 30°C. Values are represented as percentages of the activity of IDH without incubation.

Fig. 3. Western blot analysis of dimeric (A) and monomeric (B) IDHs of *C. psychrerythraea* 34H.

Rabbit antibodies against the *C. maris* IDH-I and IDH-II were used in (A) and (B), respectively. Lanes 1, 2 and 3; crude extract of *C. psychrerythraea* 34H (24 μg protein), the purified *C. maris* IDH-I and IDH-II, respectively.

Fig. 4. SDS-PAGE of purified His-tagged IDHs.

Ten μg of protein was applied to each lane. Lanes 1, 2 and 3; the purified

IDH-IIb, IDH-IIa and IDH-I, respectively. Lane M; marker proteins.

Fig. 5. Temperature dependence (A) and thermostability (B) of the His-tagged IDH activities.

In (A), Activities of the purified IDH isozymes were assayed at various temperatures. In (B), residual activities of His-tagged IDHs after incubation for 10 min at various temperatures were assayed at 20°C for IDH-IIa and IDH-IIb, and at 30°C for IDH-I. Values are represented as percentages of IDH activity without incubation. □, IDH-IIa; Δ, IDH-IIb; ○, IDH-I

Table 1 Oligonucleotides used in this study

Primer name	Nucleotide sequence (5'→3')
Used for the cloning of IDH isozyme genes	
IIa-around-f	CGCTTTATGGCTGCAGTAAGACTTTC
IIa-around-r	GGCACTAATCACTATTTAGTGATACC
IIb-around-f	CCTACCGAGTGAAAATTTCCAATAGC
IIb-around-r	CCGAACGTAAGATATCTGAAAATAG
I-around-f	CGTGGCCATGATGCAGAAATGATCAAG
I-around-r	GGATATCGAACATTAAAAGCAGGACAGG
Used for the construction of pHisIDH-IIa, pHisIDH-IIb and pHisIDH-I	
IDH-IIa-f	gcgc*ggatccg*AGCACTGATAACTC
IDH-IIa-r	gcgc*gagctc*TTAGAGCAATGCAG
IDH-IIb-f	gcgc*ggatccg*AGTTCAAAAATAATTTAC
IDH-IIb-r	gcgc*gagctc*TTATTTAAATGCTGC
IDH-I-f	gcgc*ggatccg*ACTAATCAAATCATCATCC
IDH-I-r	gcgc*gagctc*TTACATATGATCAATAATAC

Small letters indicate additional bases for introducing the digestion sites for *Bam*HI and *Sac*I (italicized letters)

Table 2 Homology of amino acid sequence between *C. psychrerythraea* 34H IDHs and other bacterial IDHs

	Colwellia psychrerythraea 34H		
	IDH-IIa	IDH-IIb	IDH-I
Colwellia maris	92%	62%	93%
Colwellia psychrerythraea NRC1004	78%	61%	86%
Pseudomonas psychrophila	65%	63%	73%
Azotobacter vinelandii	66%	62%	—
Escherichia coli	—	—	71%

Values indicate percentages of the identical amino acid residues between the amino acid sequences of two type IDHs.

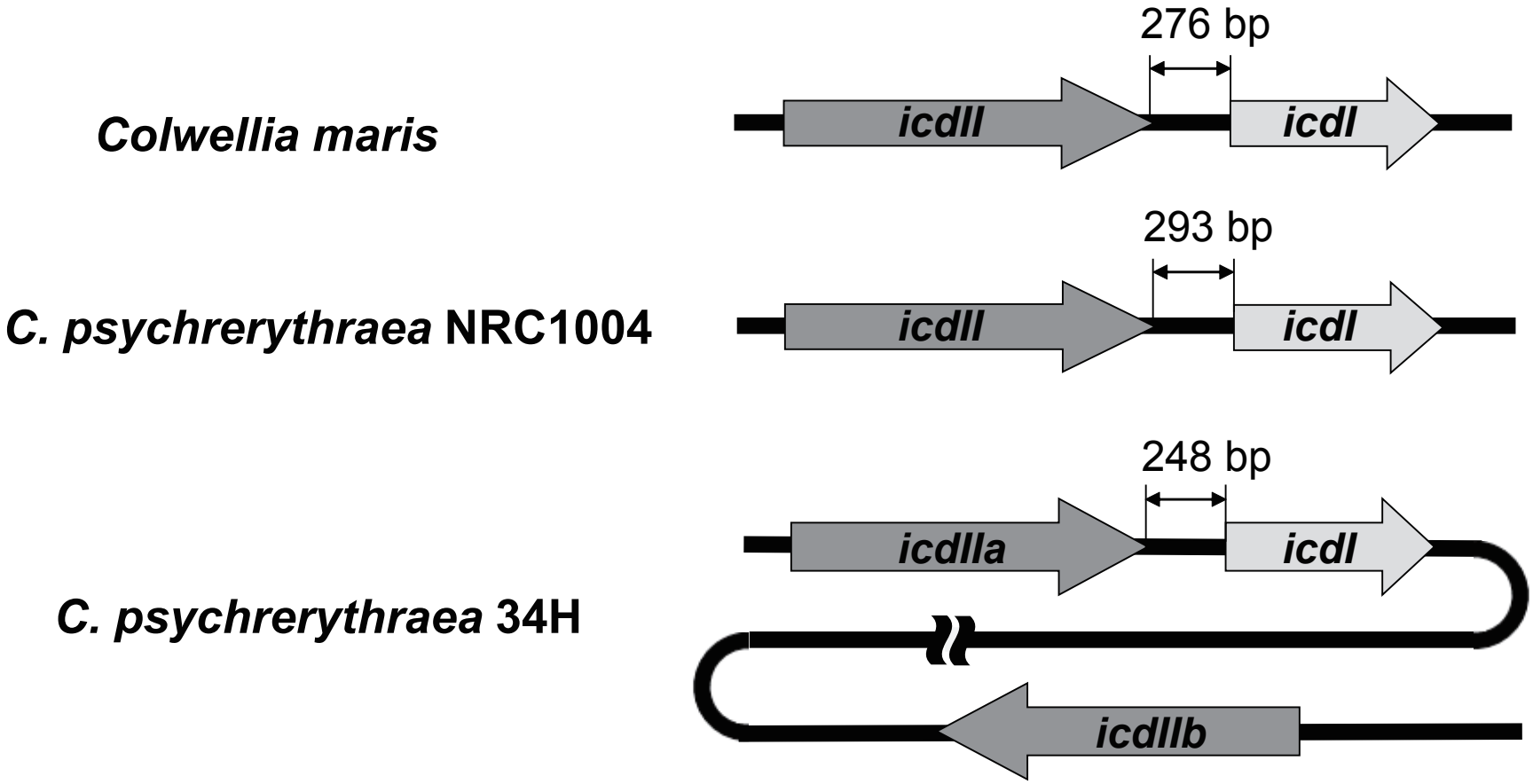
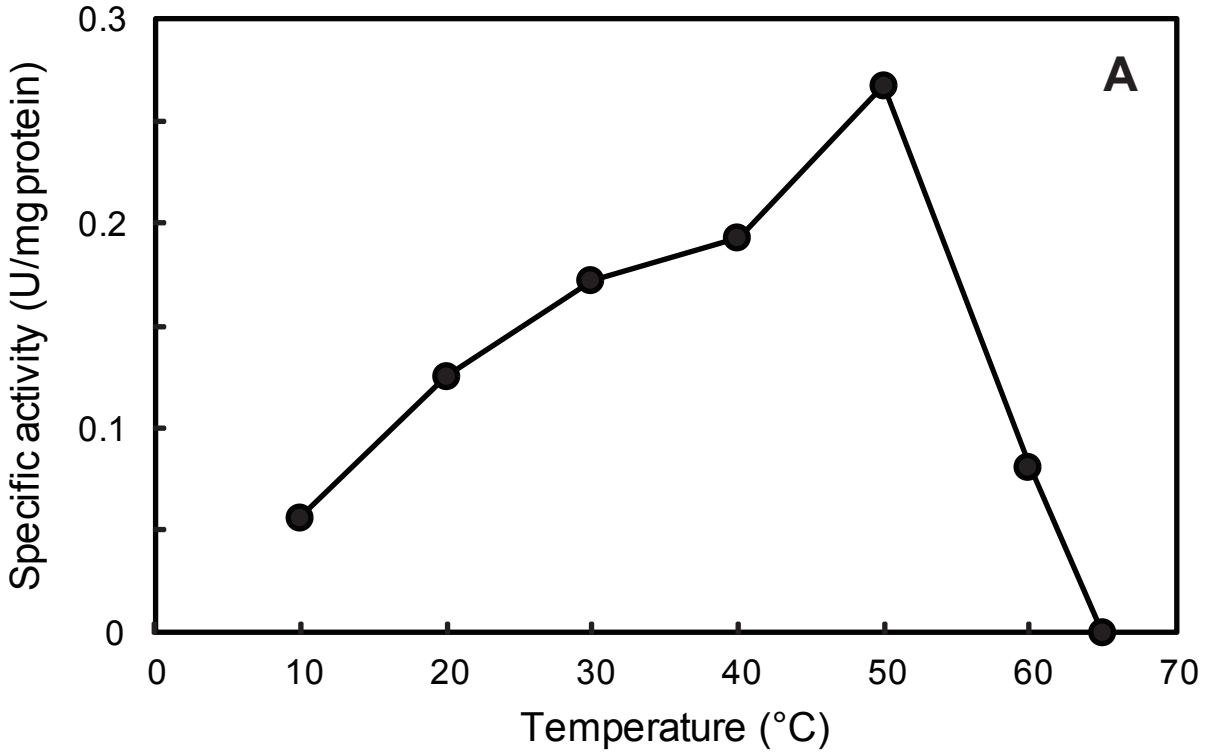

Colwellia maris
276 bp
icdII
icdI
C. psychrerythraea NRC1004
293 bp
icdII
icdI
C. psychrerythraea 34H
248 bp
icdIIa
icdI
icdIIb

Fig. 1


A
Specific activity (U/mg protein)
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
Temperature (°C)

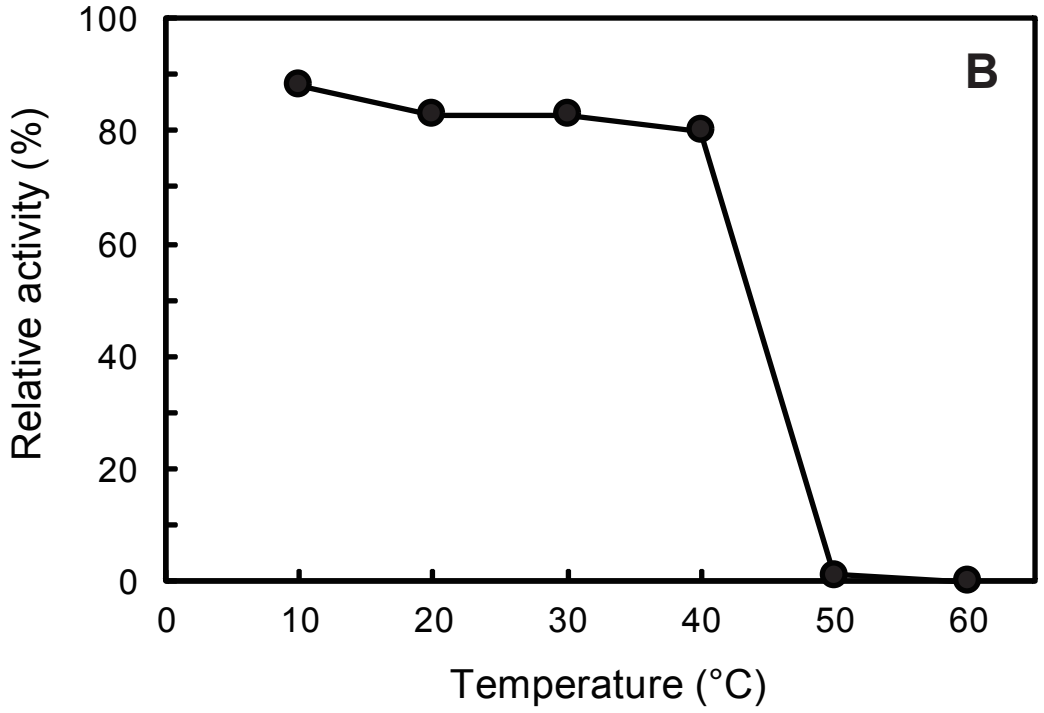

B
Relative activity (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Temperature (°C)

Fig. 2

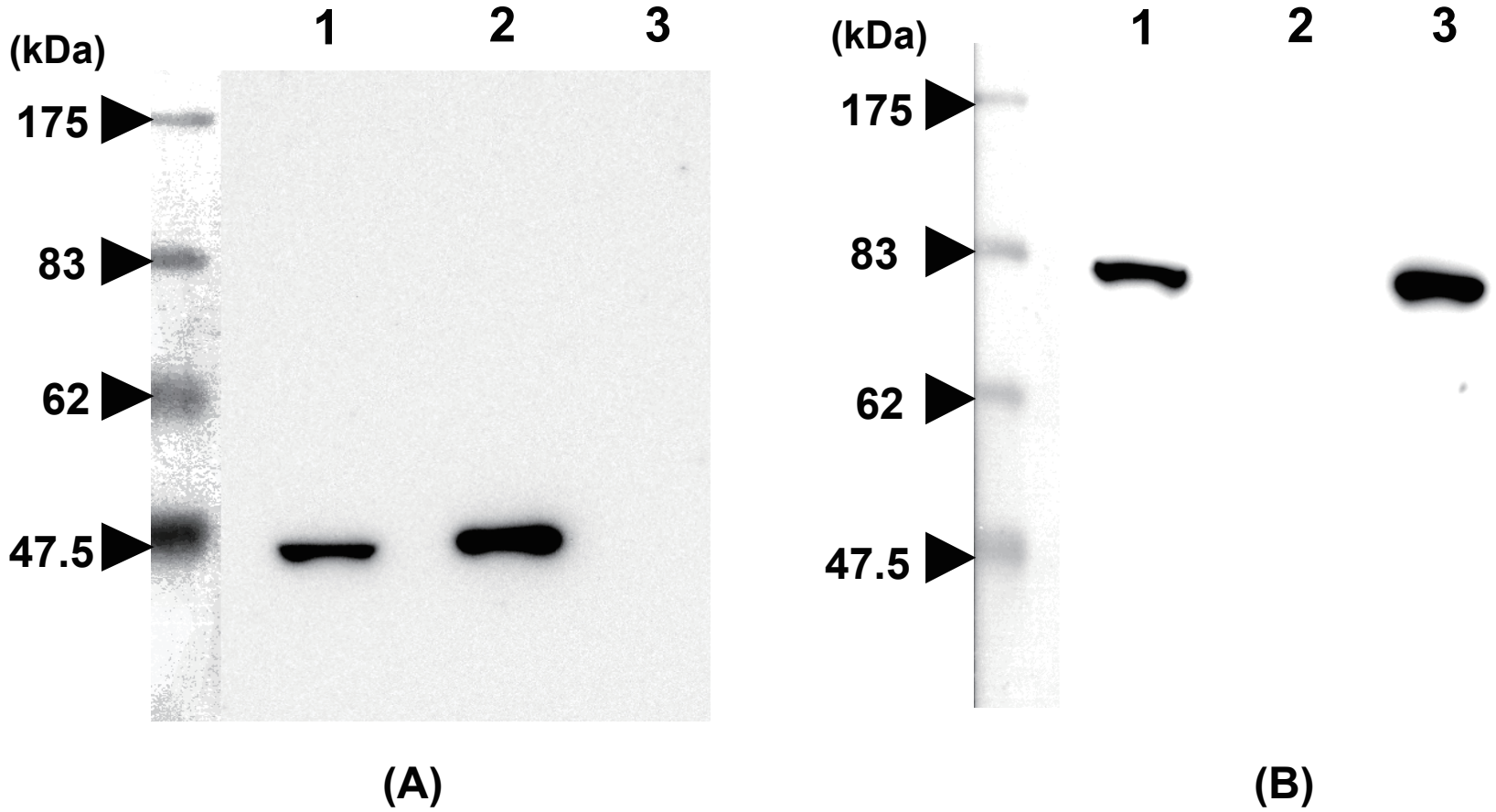


Fig. 3

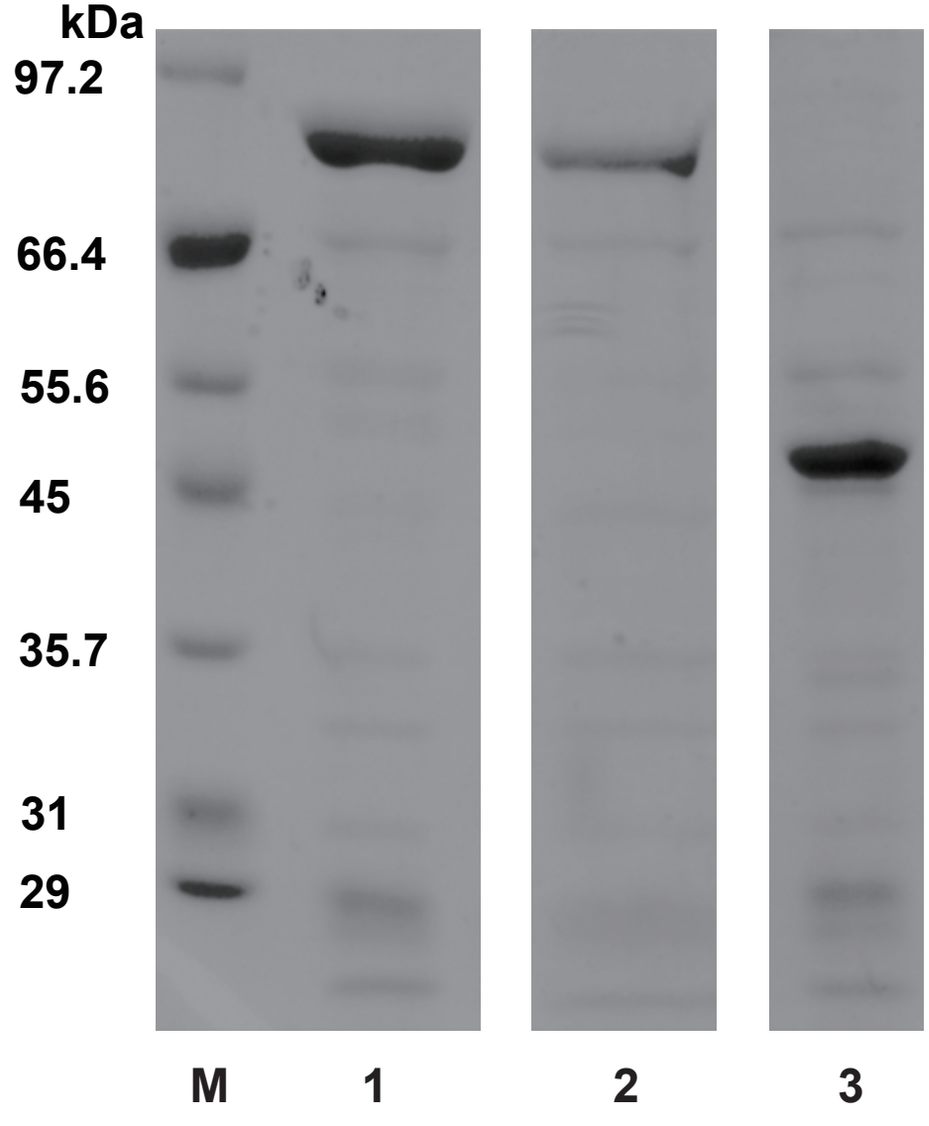
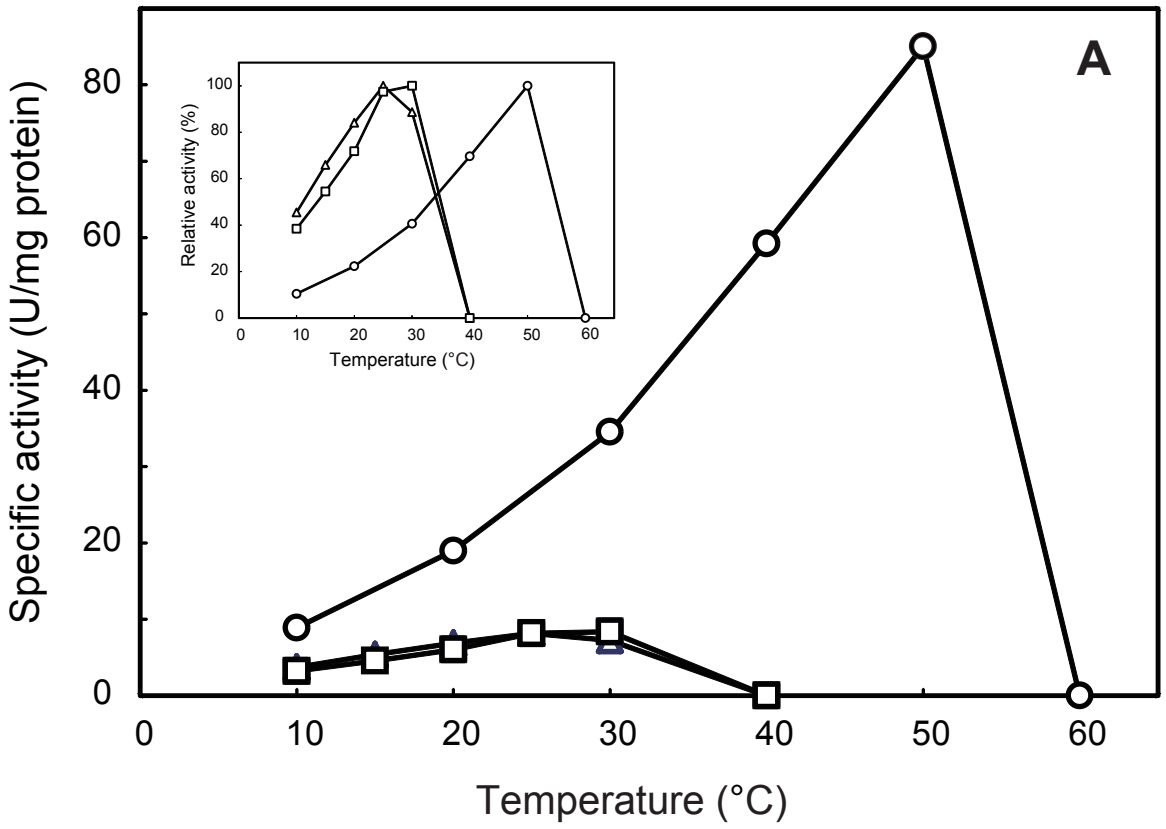


Fig. 4


A
Specific activity (U/mg protein)
Temperature (°C)
Relative activity (%)
Temperature (°C)

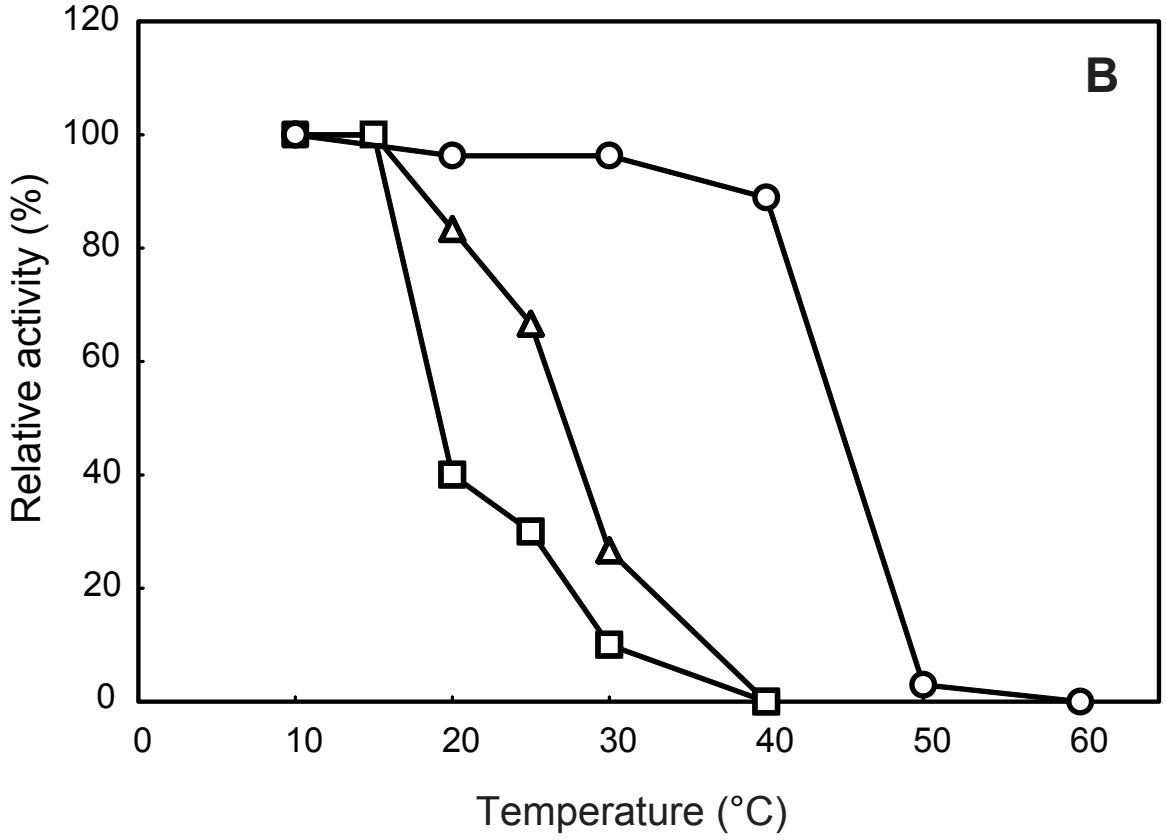

B
Relative activity (%)
Temperature (°C)

Fig. 5