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# Isocitrate dehydrogenase isozymes from a psychrotrophic bacterium, *Pseudomonas psychrophila*

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**Abstract** The genes encoding monomer- and dimer-type isocitrate dehydrogenase (IDH) isozymes from a psychrotrophic bacterium, *Pseudomonas psychrophila*, were cloned and sequenced. Open reading frames of the genes were 2,226 and 1,257 bp in length and corresponded to polypeptides composed of 741 and 418 amino acids, respectively. The deduced amino acid sequences showed high sequence identity with those of psychrophilic bacteria, *Colwellia maris* and *Colwellia psychrerythraea*, (about 70% identity) and the respective types of the putative IDH genes from other bacteria of genus *Pseudomonas* (more than 80% identity). The two genes were located in opposite direction from each other with a spacer of 463 bases in the order of dimeric and monomeric IDH genes on the chromosomal DNA, but analyses of northern blotting and 5'-terminal regions of the mRNAs revealed that they are transcribed independently. The expression of monomer- and dimer-type IDH genes in *C. maris* are known to be cold- and acetate-inducible, respectively, while only slight inductions by low temperature and/or acetate were observed in the expression of the *P. psychrophila* monomer- and dimer-type IDH genes. Both of these IDH isozymes overproduced in *Escherichia coli* showed mesophilic properties, in contrast with monomer- and dimer-type IDHs of *C. maris* as cold-adapted and mesophilic enzymes, respectively. The substitution of Glu55 residue in the *P. psychrophila* monomeric IDH for Lys, which is the corresponding residue conserved between the cold-adapted monomeric IDHs from *C. maris* and *C. psychrerythraea*, by site-directed mutagenesis resulted in the decreased thermostability and the lowered optimum temperature of activity, suggesting that this residue is involved in the mesophilic properties of the *P. psychrophila* monomeric IDH.

**Keywords** Isocitrate dehydrogenase · Isozymes · Psychrotrophic bacterium · *Pseudomonas psychrophila* · Site-directed mutagenesis

**Introduction**

The $NADP^+$-dependent isocitrate dehydrogenase (IDH; EC 1.1.1.42), which catalyzes the oxidative decarboxylation of D-isocitrate to 2-oxoglutarate and $CO_2$ with a concomitant reduction of $NADP^+$ to NADPH, plays an important role in the central metabolic pathway, TCA cycle. Bacterial IDHs can be classified into two types on the basis of their subunit structure. One is homodimer consisting of subunits of about 40-45 kDa, which exists in many bacterial species including *Escherichia coli* (Burke et al. 1974; Colman 1983; Eguchi et al. 1989; Howard and Becker 1970). The other is monomer with a molecular mass of 80-100 kDa, which has been reported in several bacteria containing *Azotobacter vinelandii* (Chung and Franzen 1969; Sahara et al. 2002), *Vibrio parahaemolyticus* (Fukunaga et al. 1992) and *Corynebacterium glutamicum* (Eikmanns et al. 1995; Chen and Yang 2000). Although bacterial monomeric and dimeric IDHs catalyze the same reaction, homology of amino acid sequence is hardly found between both types of IDHs, and their immunological cross-reactivities are different from each other, in spite of their similarities within each type IDHs (Ishii et al. 1987 and 1993; Fukunaga et al. 1992; Sahara et al. 2002). Generally, bacteria have only one of either type IDH. However, it has reported that psychrophilic bacteria, *Colwellia maris* (Takada et al. 1979; Yumoto et al. 1998) and *Colwellia psychrerythraea* (D'Aout and Kushner 1972; Deming et al. 1988), have both of the two type IDHs (Ochiai et al. 1979, 1984; Maki et al. 2006). Optimum temperature for activity (40~45$^o$C) and thermostability of their dimer-type IDHs indicate that they are mesophilic enzymes. On the other hand, the monomer-type IDHs are extremely labile above 25$^o$C, and their optimum temperature for activity is lower (20$^o$C), revealing that they are cold-adapted. Furthermore, the two IDH isozyme genes of these bacteria are located in tandem with monomer-type IDH genes followed by the dimer-type ones on the chromosomal DNAs (Ishii et al. 1993; Maki et al. 2006). However, the expression of the two genes in these bacteria are independently regulated by different promoters, and the expression of the cold-adapted monomer-type and mesophilic dimer-type IDH genes in *C. maris* are strongly induced by low temperature and acetate, respectively (Suzuki et al. 1995; Maki et al. 2006). In addition, the monomeric

IDH has higher catalytic activity than the dimeric one. Therefore, the cold-adapted monomeric IDH is thought to be important for cold adaptation of *C. maris*. In contrast, physiological role of the dimer-type IDH in this bacterium remains obscure. In fact, a mutant defective in this enzyme isolated by treatment of a mutagen showed normal growth and auxotrophy as well as wild-type strain (Fukunaga et al. 1988). On the other hand, in *C. psychrerythraea*, the cold-adapted monomeric IDH exhibited considerably less catalytic activity than the mesophilic dimer-type IDH, and the latter enzyme maintained higher activity even at low temperatures such as 10°C than the former one (Maki et al. 2006). Therefore, the mesophilic dimer-type IDH seems to be essential for this bacterium.

From analysis of the determined genome sequences, it is expected that several bacteria of genus *Pseudomonas* belonging to class Gammaproteobacteria, in which genus *Colwellia* is also included, possess both of monomeric and dimeric IDH genes. In fact, the two types of IDHs were reported to be existent in a psychrotrophic bacterium *Pseudomonas fluorescens* (Middaugh et al. 2005). However, IDH isozymes from bacteria of genus *Pseudomonas* have been hardly characterized. A Gram-negative bacterium, *Pseudomonas psychrophila*, isolated from the cold room for food preservation, can grow at -1~35°C and shows a maximum growth at 25°C (Saruyama et al. 1978; Yumoto et al. 2001). In this study, we cloned monomeric and dimeric IDH genes of this bacterium and characterized the two type IDH isozymes.

## Materials and methods

### Bacteria, plasmids and culture conditions

The psychrotrophic bacterium, *P. psychrophila*, was grown at 27°C in a nutrient medium consisting of 1% (w/v) meat extract and 1% (w/v) peptone. Two strains of *E. coli*, DH5α (Invitrogen) and XL1-Blue (Stratagene), were used to propagate plasmids. 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 *P. psychrophila* IDH isozyme genes. Unless otherwise noted, *E. coli* strains were cultured at 37°C with vigorous shaking in Luria-Bertani (LB) medium (Sambrook and Russel 2001), Super broth medium (Watanabe et al. 2005) or morpholinepropanesulfonic acid (MOPS)-based synthetic medium (Neidhardt et al. 1974) supplemented with 0.5 mM Trp and 27.8 mM glucose. If necessary, ampicillin and tetracycline were added to the culture media at concentrations of 50 and 15 μg/ml, respectively. Plasmids pBluescript SK(+) (pBS, Stratagene) and pTrcHisB (Invitrogen) were used for the subcloning of the IDH isozyme genes and the overproduction of the genes encoding His-tagged recombinant IDHs, respectively.

Cloning of the IDH isozyme genes and nucleotide sequencing

*P. psychrophila* genomic DNA was isolated and purified as described previously (Ishii et al. 1993). Genomic PCR was performed to obtain a nucleotide probe for the *P. psychrophila* IDH isozyme genes. As the primers for monomeric IDH gene, those for the cloning of monomeric IDH gene of *Colwellia psychrerythraea* NRC1004 reported by Maki et al. (2006) were used. On the other hand, the primers for dimeric IDH gene were designed from a highly conserved region of the dimeric IDH genes deduced from the genome sequences of *Pseudomonas aeruginosa*, *Pseudomonas putida* and *P. fluorescens* (Table 1). The forward primer, Dimer-F, corresponds to the sequences between +979 and +1,005 from the translational start codon of the three putative genes, while the reverse primer, Dimer-R, is complementary to the sequences between +1,162 and +1,190. The two PCR products with predicted length of about 200 bp (probe-M1 and probe-D in Fig. 1) were purified and ligated to the *Eco*RV site of pBS for propagation and were used as probe for genomic Southern and colony hybridizations. Genomic Southern hybridization and colony hybridization (Sambrook and Russel 2001) was carried out with a nylon membrane (Hybond-$N^+$, GE Healthcare) and the probes labeled with AlkPhos Direct Labeling and

Detection system with CDP-*Star* (GE Healthcare), according to the manufacturer's instructions.

The nucleotide sequence was determined in both directions by using appropriate primers and a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) with a sequencer, ABI 3130 Genetic Analyzer. The nucleotide sequences of the monomeric and dimeric IDH genes of *P. psychrophila* have been deposited in the DDBJ database under accession no. AB425997. About 3.2-kbp fragment containing a complete ORF of the monomer IDH gene and its upstream and downstream regions (about 2.2 kbp, 0.8 kbp and 0.2 kbp, respectively) was ligated into the *Eco*RI-*Xho*I site of pBS to obtain plasmid pPpicdM (Fig. 1). Furthermore, about 2.4-kbp fragment containing ORF of the dimer IDH gene and its upstream and downstream regions (about 1.2 kbp, 1.0 kbp and 0.2 kbp, respectively) was ligated into the *Pst*I-*Eco*O109I site of pBS to obtain pPpicdD.

Preparation of cell-free extract

*E. coli* DEK2004 transformed with pBS, pPpicdM or pPpicdD was precultured for 16 h at 37°C in LB medium and then cultured at 15, 27 or 37°C to an optical density of 0.7–1.2 at 600 nm in the same medium. Cells were harvested and washed twice with sonication buffer (50 mM potassium phosphate (pH 6.85), containing 2 mM $MgCl_2$, 0.15 M NaCl and 10 mM 2-mercaptoethanol). The washed cells were suspended in the sonication buffer and disrupted by sonication for 2–3 min on ice. The supernatant obtained by centrifugation at 3,360 × *g* for 20 min at 4°C was used as cell-free extract in the following experiments. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Northern blot analysis and determination of transcriptional start site

Total RNA from mid-log phase cells of *P. psychrophila* was prepared with the High pure RNA isolation kit (Roche Diagnostics). The extracted RNA (10 μg) was separated on 1.2% agarose gels containing 0.66 M formaldehyde, and the gels were washed twice with 20× SSC (SSC is composed of 0.15 M NaCl and 15 mM sodium citrate (pH 7.0)) for 15 min at room temperature. The RNAs on the gels were then transferred onto Hybond-$N^+$ membrane according to the manufacturer's instructions. Hybridization and the detection of mRNAs for the two IDH isozyme genes after the cross-linking of RNA to the membrane were carried out as described in colony hybridization, except that the full lengths of these IDH gene ORFs (2.2 kbp and 1.2 kbp for monomeric and dimeric IDH isozyme genes, respectively) labeled with AlkPhos Direct Labeling and Detection system with CDP-*Star* were used as probes.

The 5'-RACE (5'-rapid amplification of cDNA ends) method (Sambrook and Russel 2001) was used to determine transcriptional start sites of the two IDH isozyme genes. Total RNA of *P. psychrophila* (10 μg) was used for synthesis of the template strand of cDNA by using PrimeScript Reverse Transcriptase (Takara) and primers for monomeric and dimeric IDH, H158NR and dts1, respectively (Table 1). The cDNAs were added poly-dC or poly-dG to the 3'-terminal with the terminal deoxynucleotidyl transferase (Takara) and were used as template in PCR. The reaction mixture for PCR (50 μl) contained appropriate amount of the template, 50 pmol each forward and reverse primer (K55ER and poly-dG or poly-dC in the case of monomer-type IDH; dts2 and poly-dC or poly-dG in the case of dimer-type IDH, Table 1) and 1 U KOD-plus DNA polymerase (TOYOBO) in a buffer system prepared by the manufacturer. The 5'-terminals of all primers used for the PCR were phosphorylated with T4 polynucleotide kinase (TOYOBO). The PCR products were purified and ligated into the *Eco*RV site of pBS. Nucleotide sequences of the inserted DNA fragments were determined as described above.

Construction of genes encoding His-tagged IDHs of *P. psychrophila* and site-directed mutagenesis of monomer-type IDH isozyme

The *P. psychrophila* monomeric and dimeric IDH isozyme genes were amplified by PCR to introduce restriction sites for *Bam*HI and *Hin*dIII at the 5' and 3'-terminals of the ORFs, respectively. For this purpose, the following primers were used: PMF0 and PMR0 as forward and reverse primers for monomeric IDH gene and PDF0 and PDR0 as forward and reverse primers for dimeric IDH gene, 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 pPpicdM or pPpicdD, 10 pmol each forward and reverse primer and 1 U KOD-plus DNA polymerase in the 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 *Hin*dIII and ligated to the *Bam*HI-*Hin*dIII site of pTrcHisB, a plasmid vector for conferring the N-terminal $(His)_6$-tag on the expressed proteins, to obtain plasmids pHisPpicdM and pHisPpicdD, respectively.

Mutated monomeric IDH genes were constructed by a standard PCR method as previously reported (Watanabe and Takada 2004). At first, 5'-terminal fragment of the monomeric IDH isozyme gene containing the mutated region was amplified in 50 μl of reaction mixture containing 50 ng pHisPpicdM as a template, 10 pmol forward primer (primer A in Table 1), 10 pmol reverse primer to introduce each substitution of amino acid residue (primers B) and 1 U KOD-plus DNA polymerase in the buffer system prepared by manufacturer. Next, 3'-terminal fragment of the IDH gene containing the mutated region was amplified in the same reaction mixture as the first PCR, except that 10 pmol forward primer to introduce each substitution (primers C, complementary to primers B) and 10 pmol reverse primer (primer D) were used. Cycling condition of first and second PCR was as follows: denaturation at 94°C for 15 s, annealing at 50°C for 30 s and extension at 68°C for 2.5 min. Then, the full lengths of mutated IDH genes were amplified in the same reaction mixture as the first PCR, except for using both products of the first and second PCR as templates and primers A and D. Cycling conditions of this PCR were the same as first and second PCR except for annealing at 64°C for 30 s. All PCR was carried out for 30 cycles in the DNA thermal cycler. The final PCR products were digested with *Bam*HI and *Hin*dIII and ligated into the *Bam*HI-*Hin*dIII site of pTrcHisB. The introduced mutations were verified by nucleotide

sequencing the relevant regions of plasmids as described earlier.

Overproduction and purification of His-tagged IDHs

According to the method of Watanabe et al. (2005), *E. coli* DEK 2004 transformed with pHisPpicdM, pHisPpicdD or pTrcHisB carrying the mutated monomeric IDH genes was grown, and the IDH proteins were overproduced and then purified by Ni-NTA agarose column chromatography (Quiagen). However, cell-free extract was prepared with the following modifications. The cell suspension added hen-egg lysozyme (final concentration of 2 mg/ml) was gently shaken for 2 h at 4°C. The cells were then disrupted by a sonication for about 10 min. After centrifugation of the cell lysate at 16,000 × *g* for 20 min at 4°C to remove cell debris, the supernatant was further centrifuged at 64,500 × g for 1 h at 4°C. All His-tagged recombinant IDHs were stocked at –30°C until use.

Enzyme assay

Unless otherwise noted, the IDH activity was assayed at 40°C by the method of Ochiai et al. (1979). Reaction mixture (2 ml) contained 33 mM Tris-HCl (pH 8.0), 0.67 mM $MnCl_2$, 0.12 mM $NADP^+$, 2 mM sodium isocitrate and enzyme. To examine thermostability of the IDH activity, all purified wild-type and mutated IDHs were diluted to a protein concentration of about 0.01 mg/ml with 20 mM potassium phosphate buffer (pH 8.0), containing 2 mM $MgCl_2$, 0.1 M NaCl, 10% (v/v) glycerol and 1 mM dithiothreitol, on ice. After incubation for 10 min at various temperatures, the enzyme was immediately cooled on ice for 10 min, and then the residual activities were assayed at 40°C. One unit of enzyme activity was defined as the amount capable of catalyzing the reduction of 1 μmol of $NADP^+$ per min.

**Results**

Cloning of the *P. psychrophila* IDH isozyme genes

As probes used for the cloning of monomeric and dimeric IDH genes of *P. psychrophila*, about 200 bp of each DNA fragment (probe-M1 and probe-D in Fig. 1) was obtained by genomic PCR described in "Materials and methods" and then sequenced. The deduced amino acid sequences were similar to those of the respective type IDH genes from other bacteria. Genomic Southern blot analysis revealed that about 2.5 kbp and 2.7 kbp of single fragment of the *P. psychrophila* genomic DNA digested with *Eco*RI and *Pst*I hybridize with the probe-M1 and probe-D, respectively. Because the 2.5-kbp *Eco*RI fragment was found to contain only about 80% of ORF of the monomeric IDH gene by subsequent sequencing (Fig. 1), genomic Southern hybridization was further performed by using fragment obtained by the digestion of the *Eco*RI fragment with *Sma*I (probe-M2) as a probe. A 3.2-kbp *Eco*RI-*Xho*I and 2.4-kbp *Pst*I-*Eco*O109I fragments were subcloned into pBS to yield pPpicdM and pPpicdD, respectively (Fig. 1).

Nucleotide and deduced amino acid sequences the *P. psychrophila* IDH isozyme genes

Nucleotide and deduced amino acid sequences of the DNA fragments subcloned in the pPpicdM and pPpicdD are shown in Fig. 2. The genes encoding monomeric and dimeric IDH isozymes are located in opposite direction from each other with a spacer of 463 bases in the order of dimeric and monomeric IDH genes on the chromosomal DNA of this bacterium. The DNA fragment in pPpicdM contained an ORF with full length of 2,226 bp, and a putative ribosome binding site, AGAG, was found 11-14 bases upstream of the ATG codon. The ORF was found to encode a polypeptide of 741 amino acids, and its calculated molecular mass was 80,630 Da. On the other hand, ORF in pPpicdD was 1,257 bp in length,

and a putative ribosome binding site, GGAG, was present 7-10 bases upstream of the ATG codon. The ORF encodes a polypeptide composed of 418 amino acids, and the molecular mass was calculated as 45,384 Da. The sequences of a terminator-like stem-loop structure were found downstream of each ORF. The former is composed of a 13-bp stem with a 7 nucleotide loop, and the latter is composed of an 18-bp stem with a 8 nucleotide loop. The deduced amino acid sequences of monomer- and dimer-type IDHs of *P. psychrophila* showed high degrees of homology to the respective type IDHs from other bacteria, particularly those of genus *Pseudomonas* (Figs. 3 and 4): 87, 82 and 84 % identity with putative monomeric IDHs of *P. fluorescens* Pf-05, *P. putida* KT2440 and *P. syringae*, respectively, and 94, 91 and 88 % identity with putative dimeric IDHs of *P. fluorescens* Pf-05, *P. putida* KT2440 and *P. aeruginosa* PAO1, respectively. Furthermore, their identities with monomeric and dimeric IDHs of *C. maris* and *C. psychrerythraea* were about 70 % in each case. The amino acid residues involved in the binding of substrate, metal ion and coenzyme were conserved between the two IDH isozymes of *P. psychrophila* and the same type of IDHs from other bacteria.

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

Each transcriptional start point of the two *P. psychrophila* IDH isozyme genes was determined by the 5'-RACE method. By sequencing of the PCR products, it was confirmed that the initiation sites for monomer- and dimer-type IDH genes were G-584 and G-933, located 220 and 33 bases upstream of translational initiation codon, respectively (triangles in Fig. 2). The putative promoter motifs at -10 and -35 for monomer- and dimer-type IDH genes were TATACT (nucleotides between 572 and 577) and TCGCAG (nucleotides between 548 and 553), and TACGCT (nucleotides between 920 and 925) and TAAGTT (nucleotides between 893 and 898), respectively (boxed nucleotides in Fig. 2). These results indicate that the two genes are independently expressed each other.

Expression of the IDH isozyme genes

Expression of the two *P. psychrophila* IDH isozyme genes in an IDH-defective mutant of *E. coli*, DEK2004, which has the phenotype of a glutamate auxotroph attributable to the loss of IDH (Thorsness and Koshland 1987), was examined by colony formation on the MOPS-based synthetic agar medium and the assay of the IDH activity. The *E. coli* DEK2004 cells transformed with pPpicdM or pPpicdD, harboring the full lengths of the *P. psychrophila* monomer- and dimer-type IDH genes, respectively, with their 5'-flanking regions into pBS, were able to grow at 37$^{o}$C on the MOPS-based synthetic agar medium and formed colonies within about 1 day and 2 days, respectively. Growth of these transformants at 15$^{o}$C was slower, and the colonies appeared at 8 days and 12 days after their inoculations, respectively. On the other hand, the *E. coli* DEK2004 cells transformed with pBS as a control were not able to grow under the same conditions because glutamate was not contained in the growth medium. These results indicate that the auxotrophy of the *E. coli* mutant was complemented by the expression of *P. psychrophila* IDH isozyme genes. Furthermore, the IDH activity was detected in the cell-free extract of the *E. coli* DEK2004 transformants carrying pPpicdM and pPpicdD, demonstrating that the cloned genes in this study encode IDHs. The two IDHs in the cell-free extract showed their maximum activities at 50$^{o}$C, and these activities were completely kept after incubation for 10 min at 40$^{o}$C (data not shown). From these results, it was concluded that the monomer- and dimer-type IDHs of this bacterium are mesophilic enzymes.

Northern blot analysis was carried out with total RNA isolated from the *P. psychrophila* cells grown under different conditions (Fig. 5). Two single bands hybridized to the probes for the *P. psychrophila* monomeric and dimeric IDH genes, and sizes of the transcripts estimated from the mobility of rRNA on the gel were compatible with those of the respective ORFs with their franking regions. These results support that the two genes are transcribed independently. Transcription of the monomeric and dimeric IDH genes was induced by low temperature (1.5-folds by densitometric comparisons of lanes 1 and 4 to

lanes 3 and 6 in Fig. 5, respectively) and acetate (1.8~1.9-folds by the comparisons of lanes 1, 2 and 3 to lanes 4, 5 and 6, respectively) and by low temperature (2- and 2.1-folds by the comparisons of lanes 7 and 10 to lanes 9 and 12, respectively), respectively. However, in contrast to strong induction in the expression of the cold-adapted monomer-type and mesophilic dimer-type IDH genes from *C. maris* by low temperature and acetate (12-folds and 12-folds, respectively) (Suzuki et al. 1995), the observed inductions were considerably weak. These results indicate that the expression of the *P. psychrophila* IDH isozyme genes is hardly influenced by low temperature and acetate. Similar results were obtained by the following gene expression experiments in the *E. coli* DEK2004 transformants carrying pPpicdM and pPpicdD. These transformants were grown at 15, 27 and 37$^{o}$C on LB medium, and the IDH activities in the cell-free extracts were assayed at 40$^{o}$C (Table 2). The monomeric and dimeric IDH activities of the transformants grown at 15$^{o}$C were slightly larger and smaller than those at 25 and 37$^{o}$C, respectively, indicating that the expression of the two IDH genes in the *E. coli* mutant cells was not strongly dependent on temperature. Furthermore, Western blot analysis by using antibody against the purified monomeric IDH protein of *C. maris* also revealed that the intensity of the cross-reactive band was almost constant when the transformant carrying pPpicdM was grown at 15, 25 and 37$^{o}$C (a ratio of 1.00:1.15:1.03, respectively, by densitometric analysis) (data not shown).

Properties of His-tagged IDH isozymes and monomeric IDH mutants

The monomeric and dimeric IDH genes of *P. psychrophila* were introduced into the expression vector, pTrcHisB, to confer a $(His)_6$-tag on the N-terminal of the translated protein. The His-tagged IDHs were overproduced in the *E. coli* DEK2004 cells and then purified as described in “Materials and methods”. SDS-PAGE of the purified His-tagged IDHs revealed that the enzymes were homogenously purified, and their molecular mass were slightly larger than those calculated from the nucleotide sequences of the respective genes due to the additional amino acid residues by the His-tagging at N-terminals (data not

shown). Temperature-dependence and thermostability of the purified His-tagged monomer- and dimer-type IDH activities are shown in Fig. 6. The His-tagged monomer- and dimer-type IDHs showed the highest activity at 60°C. Furthermore, the two IDHs kept about 90 % of the respective maximum activities even after incubation for 10 min at 45°C. These results are compatible with those of the native IDHs overeproduced in the *E. coli* mutant cells transformed with pPpicdM and pPpicdD described above, indicating that His-tagging at the N-terminals does not significantly affect their thermal properties.

Monomeric IDHs of *C. maris* and *C. psychrerythraea* have been reported as cold-adapted enzymes (Ochiai et al. 1979, Maki et al. 2006). When amino acid sequences of these enzymes were compared with that of mesophilic monomer-type IDH of *P. psychrophila* (PpIDH-M), several substitutions of amino acid residues specific for the later IDH were found to be present (Fig. 3), implying that such substitutions may be responsible for difference of thermal properties between the former two IDHs and the latter one. Among them, Glu55 (the 55th Glu residue from N-terminal), His158, Pro490, Glu595 and Pro709 residues of the PpIDH-M have different properties (such as charge, hydrophobicity and so on) from the corresponding amino acids residues of the two cold-adapted IDHs. Therefore, these residues of PpIDH-M were substituted for amino acids residues conserved in the cold-adapted IDHs by site-directed mutagenesis, and the mutated enzymes were overproduced and purified as described previously. Although only P709A (an IDH mutant substituted Pro709 by Ala) was partially degraded during the purification step, it was protected by the addition of 1 mM phenylmethylsulfonyl fluoride into buffers used for the purification. This implies that the Pro709 residue is involved in the structural stability of the PpIDH-M. Except for E55K, temperature-dependence and thermostability of the mutated IDH activities were similar to those of the wild-type IDH (Fig. 7). On the other hand, optimum temperature for the E55K activity decreased by about 5°C, compared to wild-type enzyme. Furthermore, this mutant was more thermolabile than wild-type and the other mutated IDHs, that is, about a half of the E55K activity was lost by incubation for 10 min at 45°C. These results suggest that the Glu55 residue is involved in the mesophilic properties of the PpIDH-M.

**Discussion**

In this study, we cloned and sequenced monomer- and dimer-type IDH genes of *P. psychrophila*. These genes are located tandem in opposite direction from each other on the chromosomal DNA of this bacterium, and the dimer-type IDH gene is followed by the monomer-type IDH gene (Fig. 1). This arrangement of the *P. psychrophila* IDH isozyme genes is different from those of *C. maris* and *C. psychrerythraea* (Ishii et al. 1993; Maki et al. 2006), but putative two type IDH isozyme genes from other bacteria of genus *Pseudomonas*, such as *P. aeruginosa*, *P. fluorescens* and *P. putida*, are located with the same arrangement as those of *P. psychrophila* (Fig. 8). Furthermore, similar to the case of *C. maris* and *C. psychrerythraea*, the putative IDH isozyme genes of *Burkholderia cenocepacia*, belonging to class Betaproteobacteria, are located tandem in the same direction, but the order of the isozyme genes are different from those of these two bacteria. In bacteria possessing both type IDH isozymes, relationship between their phylogenetic classification and the arrangement of IDH isozyme genes on the chromosomal DNA are very interesting. However, further studies for other bacteria sharing the two type IDHs are required to clarify it. On the other hand, as previously reported (Ishii et al. 1987; Sahara et al. 2002), the monomeric and dimeric IDHs of *P. psychrophila* showed high homology with the same types of IDHs from other bacteria, in particular the putative IDHs from bacteria of genus *Pseudomonas* (Figs. 3 and 4).

The expression of the *P. psychrophila* two type IDH isozyme genes was found to be controlled independently by different promoters (Fig. 2). Furthermore, the results of northern blot analysis revealed that the expression of monomer- and dimer-type IDH genes is slightly induced by low temperature and acetate and by low temperature, respectively (Fig. 5). However, since these inductions were considerably smaller than cold- and acetate-inducible expression of the *C. maris* monomeric and dimeric IDH genes, respectively (Suzuki et al. 1995), they are judged to have substantially no physiological significance for *P. psychrophila*. From analysis of promoters in a psychrotrophic bacterium, *Pseudoalteromonas haloplanktis*, a consensus sequence of cold-inducible -35 and -10

promoter has been proposed (Duilio et al. 2004). Since putative promoter sequences of monomeric and dimeric IDH genes of *P. psychrophila* showed about 58 and 68% identity with this consensus sequence, the slight cold-induction might be derived from these promoters. Furthermore, the CCAAT sequence, which has been reported to be a *cis*-element involved in the strong cold-induction of the *C. maris* monomer-type IDH gene (Sahara et al. 1999), was found upstream of the promoter of the monomeric IDH gene (Fig. 2). On the other hand, the expression of isocitrate lyase gene from *E. coli* is known to be acetate inducible. This gene expression is regulated by complicated mechanisms, and many *cis*-elements and *trans*-factors are involved in this regulation (Cozzone 1998). Among them, the nucleotide sequence involved in the binding of IclR repressor protein has been determined (Gui et al. 1996; Pan et al. 1996; Yamamoto and Ishihama 2003). However, such a sequence is not present in the 5'-flanking region of monomeric IDH gene of *P. psychrophila*.

The monomer- and dimer-type IDHs of *C. maris* and *C. psychrerythraea* are cold-adapted and mesophilic, respectively (Ochiai et al. 1979, 1984; Maki et al. 2006). On the other hand, both the IDH isozymes of *P. psychrophila* were mesophilic nevertheless this bacterium is psychrotrophic (Fig. 6). The catalytic activities of the His-tagged monomeric and dimeric IDHs from *P. psychrophila* at optimum temperature (681 and 185 U/mg protein; Fig. 6) were considerably higher than those of the cold-adapted monomeric IDHs from *C. maris* (about 70 U/mg protein; Watanabe et al. 2005). Furthermore, these IDH isozymes in the cell-free extract of the *E. coli* transformants carrying pPpicdM and pPpicdD showed the maximum activity at 50°C but retained about 20% of their activity even at 10°C (data not shown). The specific activity of IDH at 10°C in the cell-free extract of the *C. maris* and *P. psychrophila* grown on the nutrient medium containing 1% peptone and 1% meat extract was about 0.2 (Ochiai et al. 1979) and 0.4 U/mg protein, respectively. On the other hand, the growth rate of *C. maris* at 10°C on the same medium was similar to that of *P. psychrophila* (Takada et al. 1979; Saruyama et al. 1978). Therefore, similar to the case of *C. psychrerythraea* (Maki et al. 2006), the catalytic abilities of mesophilic IDH isozymes from *P. psychrophila* seem sufficient to sustain the growth of this bacterium at low temperatures. Since the monomer-type IDH has significantly higher catalytic activity than the dimer-type one (Fig. 6),

the former IDH may contribute to such growth.

Several conserved amino acid residues in the cold-adapted monomeric IDHs from *C. maris* and *C. psychrerythraea* were found to be substituted by other ones in the mesophilic monomer-type IDH of *P. psychrophila* (PpIDH-M) from the comparison with the amino acid sequences of the monomeric IDHs (Fig. 3). Among them, five amino acid residues, Glu55, His158, Pro490, Glu585 and Pro709, were selected to be exchanged by site-directed mutagenesis for the following reasons; acidic Glu55 and Glu585, the corresponding amino acid residues of the cold-adapted IDHs are basic Lys; basic His158, the corresponding residues are Asn; Pro490 and Pro709, this amino acid is known to have effect on the structure of protein and the corresponding residues are acidic Glu and Ala, respectively. Among them, only the E55K mutation resulted in the shift-down of optimum temperature for activity and the decreased thermostability (Fig. 7), suggesting that the Glu55 residue is involved in the mesophilic properties of the PpIDH-M. From the predicted structures of wild-type and mutated IDHs built by a program SWISSPDB VIEWER, the Glu55 residue is expected to be located on the surface loop structure far from the catalytic site. This residue of wild-type IDH can form a salt bridge and also one hydrogen bond between Arg125 in α-helix structure, but not in the substituted Lys55 residue of the E55K mutant. These bonds may cause the increased structural lability of the enzyme and the resultant decrease of its thermostability. On the other hand, study of chimeric enzymes between mesophilic and cold-adapted monomeric IDHs from *Azotobacter vinelandii* (*Av*IDH) and *C. maris*, respectively, which were constructed based on the three-dimensional structure of *Av*IDH, revealed that region 2 and particularly region 3 (the central and C-terminal parts of the IDH protein, respectively) are involved in thermal properties of the two enzymes (Watanabe et al. 2005). Since the Glu55 residue of the PpIDH-M is located in region 1 (the N-terminal part of the protein), different amino acid residues and/or regions of PpIDH-M and *Av*IDH are suggested to be involved in their mesophilic properties. Therefore, experiments of chimeric enzymes between PpIDH-M and cold-adapted monomeric IDH from *C. maris* are planned to confirm this possibility.

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**Table 1** Oligonucleotides used in this study

| Name | | Nucleotide sequence (5' → 3') |
|---|---|---|
| Cloning of IDH genes | | |
| Monomer-F | | AAYATCATCAAGYTGCCRAAYATY |
| Monomer-R | | TGGCGCRCGRCGRTCRGAGTTRCCTTC |
| Dimer-F | | GGCGCCAACCTGTCCGAYWCSGTGGCC |
| Dimer-R | | CGYTCGAARTCRTAGGTCACGGTCTTGGC |
| Determination of transcriptional start site | | |
| dts1 | | GTTGTCAGCGGACCTTTG |
| dts2 | | CATAGACTTCCATCCAGGAAATC |
| poly-dG | | GGGGGGGGGGGGGGG |
| poly-dC | | CCCCCCCCCCCCCCC |
| Construction of pHisPpicdM and pHisPpicdD[a] | | |
| PMF0 | | gcgc*ggatcc*gCCCAACCGTTCGAAG |
| PMR0 | | gcgc*aagctt*cCACCAGTGAGGCGATGATC |
| PDF0 | | gcgc*ggatcc*gGGATACAACAAGATC |
| PDR0 | | gcgc*aagctt*cCATATGTGCGATCAGGG |
| Site-directed mutagenesis of monomer-type IDH gene[b] | | |
| primerA | | |
| pTrcHisB-F | | CCATGGGGGGTTCTCATCATCATCATC |
| primerB | | |
| E55KR | Glu55 → Lys | CCCAATTG<u>CTT</u>GGGGAAG |
| H158NR | His158 → Asn | GTGCGG<u>GTT</u>CTTGCG |
| P490ER | Pro490 → Glu | GCGCTT<u>TTC</u>ATCAAGCCAC |
| E595KR | Glu595 → Lys | GAAGTTCTC<u>TTT</u>CAGCAGCTG |
| P709AR | Pro709 → Ala | GTCGAC<u>AGC</u>CTTGCC |
| primerC | | |
| E55KF | Glu55 → Lys | CTTCCCC<u>AAG</u>CAATTGGG |
| H158NF | His158 → Asn | CGCAAG<u>AAC</u>CCGCAC |
| P490EF | Pro490 → Glu | CTGGCTTGAT<u>GAA</u>AAGCGC |
| E595KF | Glu595 → Lys | CAGCTGCTG<u>AAA</u>GAGAACTTC |
| P709AF | Pro709 → Ala | GGCAAG<u>GCT</u>GTCGAC |
| primerD | | |
| pTrcHisB-R | | GGCAAATTCTGTTTTATCAGACCGC |

[a]Small letters indicate additional bases for introducing digestion sites for *Bam*HI and *Hin*dIII (italicized letters).

[b]Underlined letters indicate the codon of amino acid residues substituted by site-directed mutagenesis.

**Table 2** IDH activity in cell-free extract of *E. coli* DEK2004 transformed with pPpicdM and pPpicdD

| | Growth temperature | | |
|---|---|---|---|
| | 15 °C | 25 °C | 37 °C |
| Monomer-type IDH | 3.7 ± 0.9 | 2.2 ± 0.2 | 2.5 ± 0.2 |
| Dimer-type IDH | 0.11 ± 0.01 | 0.16 ± 0.04 | 0.19 ± 0.03 |

The monomer- and dimer-type IDH activities (U/mg protein) in cell-free extract of *E. coli* DEK2004 transformed with pPpicdM and pPpicdD, respectively, grown at the indicated temperatures were assayed at 40 °C. The values indicate the mean of three independent experiments ± SEM.

**Legend to figures**

**Fig. 1** Physical and restriction map of the genomic DNA fragment containing monomer- and dimer-type IDH genes of *P. psychrophile*. *Arrows* represent the ORFs of monomer- and dimer-type IDH genes.

**Fig. 2** Nucleotide and deduced amino acid sequences of the monomer-type (left) and dimer-type (right) IDH genes of *P. psychrophile*. *Triangles* indicate the transcriptional start points of the respective genes. The underlined sequence is the putative ribosome binding site (SD). The *boxed* nucleotides show putative -35 and -10 sequences of the promoter. The probable stem-loop structure located downstream of the translational stop codon is shown by *two opposite arrows*.

**Fig. 3** Alignment of amino acid sequences of monomer-type IDHs from various bacteria. The deduced amino acid sequence of the monomer-type IDH gene of *P. psychrophila* (P.p) is aligned with those of *C. maris* (C.m; database accession no. BAA03134), *C. psychrerythraea* (C.p; BAE92863) and *A. vinelandii* (A.v; D73443), and those of putative IDH genes from *P. fluorescens* Pf-5 (P.f; AAY93153) and *P. aeruginosa* PAO1 (P.a; AAG06012). The amino acids involved in the binding of isocitrate and metal ion are indicated in *gray boxes*. *White letters* in *black boxes* are amino acids of the binding sites for $NADP^+$. *Arrowheads* indicate the residues substituted in this study.

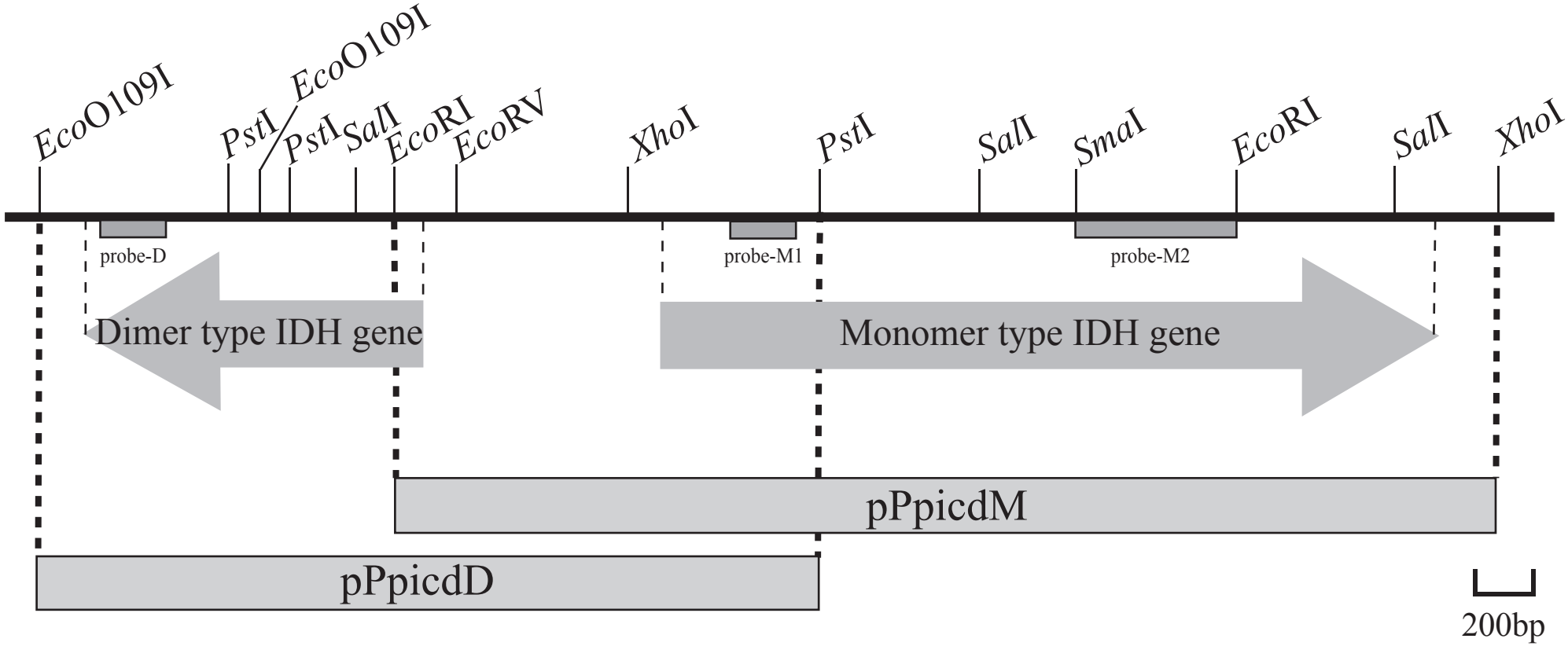
**Fig. 4** Alignment of amino acid sequences of dimer-type IDHs from different bacteria. The deduced amino acid sequence of the dimer-type IDH gene of *P. psychrophila* (P.p) is aligned with those of *C. maris* (C.m; database accession no. BAA03135), *C. psychrerythraea* (C.p; BAE92864) and *E. coli* (E.c; BAB61784), and those of putative IDH genes from *P. fluorescens* Pf-5 (P.f; AAY93152) and *P. aeruginosa* PAO1 (P.a; AAG06011). The amino acids involved in the binding of isocitrate and metal ion are indicated in *gray boxes*. *White letters* in *black boxes* are amino acids of the binding sites for $NADP^+$.

**Fig. 5** Northern blot analysis of monomer- and dimer-type IDH genes of *P. psychrophile*. Total RNA (10 μg) is isolated from the *P. psychrophila* cells grown at the indicated temperatures on LB medium supplemented with 25 mM sodium acetate (+ace) or none.

**Fig. 6** Effect of temperature on activity (**A**) and thermostability (**B**) of His-tagged IDHs. ♦, Monomer-type IDH; ■, Dimer-type IDH. In **B**, residual activity after incubation for 10 min at the indicated temperatures is represented as a percentage of that without the incubation.

**Fig. 7** Effect of temperature on activity (**A**) and thermostability (**B**) of wild and mutated monomeric IDHs. Wild-type and mutant IDHs (E55K, H158N, P490E, E595K and P709A) are indicated by *closed diamond* (♦), *closed circle* (●), *closed triangle* (▲), *open circle* (○), *open triangle* (Δ) and *open square* (□), respectively. In **B**, residual activity after incubation for 10 min at the indicated temperatures is represented as a percentage of that without the incubation.

**Fig. 8** Location of IDH isozyme genes on the chromosome DNA in several bacteria. Relative configuration of IDH genes of *C. maris*, *C. psychrerythraea* strain NRC1004, *P. aeruginosa* strain PAO1, *P. fluorescens* strain Pf0-1, *P. putida* strain KT2440, *P. psychrophila*, and *Burholderia cenocepacia* strain MC0-3 (*Burkholderia cepacia* complex) on their chromosomes are shown. Locational data for IDH genes from *C. maris* and *C. psychrerythraea* strain NRC1004 have been reported previously (Ishii et al 1987; Maki et al 2006). Information for IDH genes in other bacteria were obtained from Genome Information Broker (GIB) in DNA Data Bank of Japan (DDBJ) (http://gib.nig.ac.jp).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PstI
EcoO109I
PstI
SalI
EcoRI
EcoRV
XhoI
PstI
SalI
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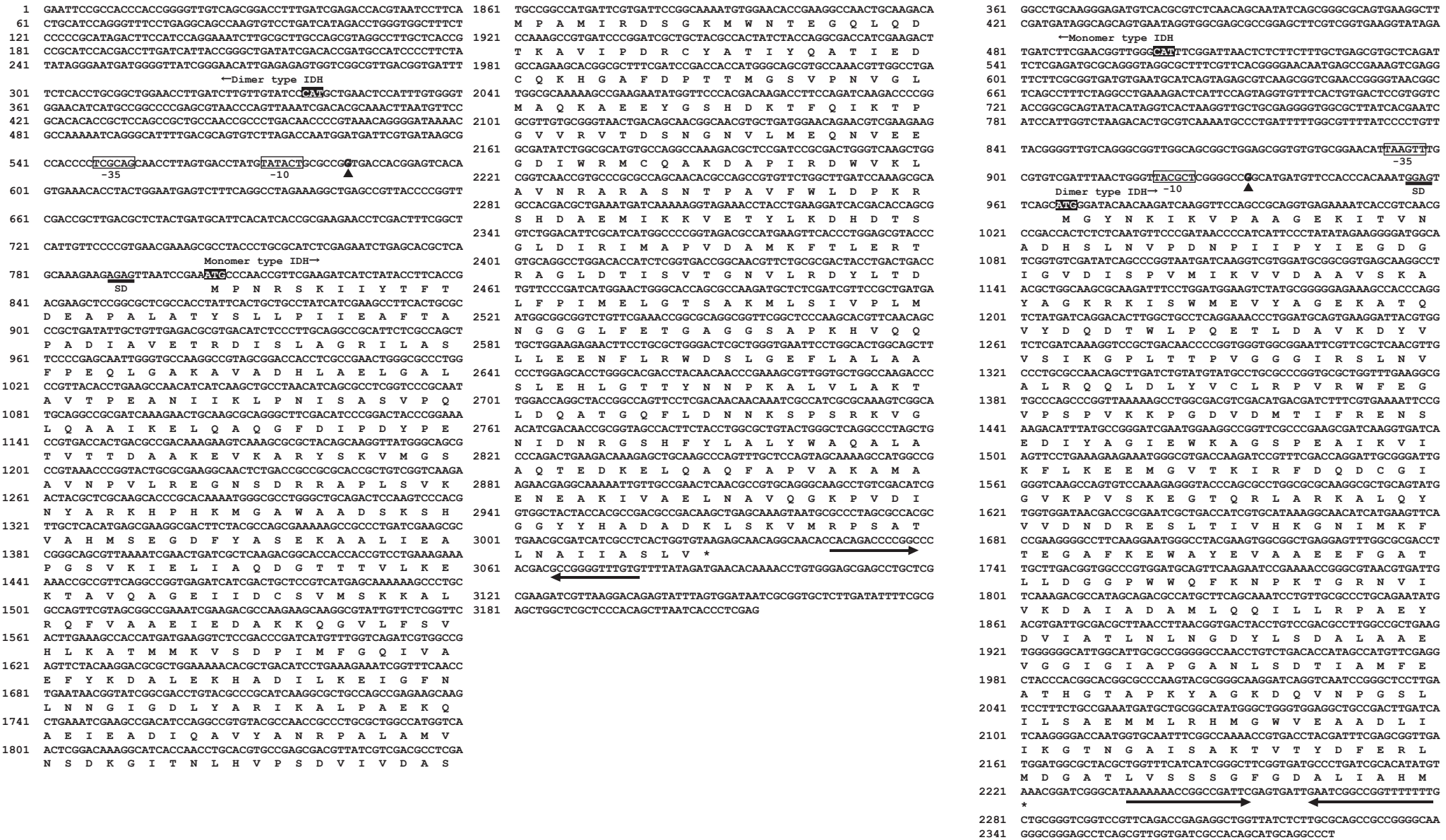
SalI
XhoI
probe-D
probe-M1
probe-M2
Dimer type IDH gene
Monomer type IDH gene
pPpicdM
pPpicdD
200bp

**Fig. 1**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-35
-10
Monomer type IDH→
SD
←Monomer type IDH
Dimer type IDH→ -10
-35
SD

Fig. 2

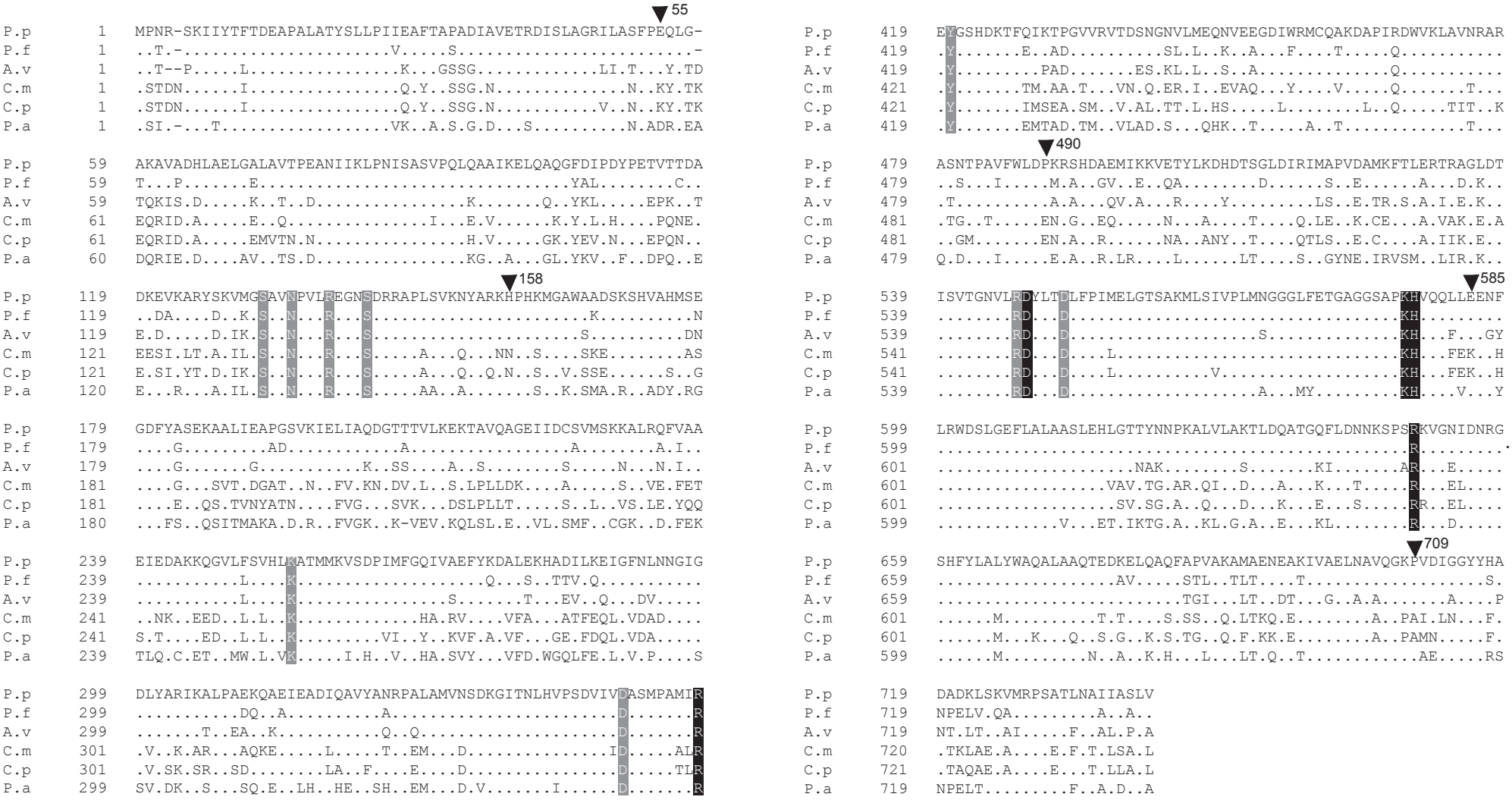



Fig. 3

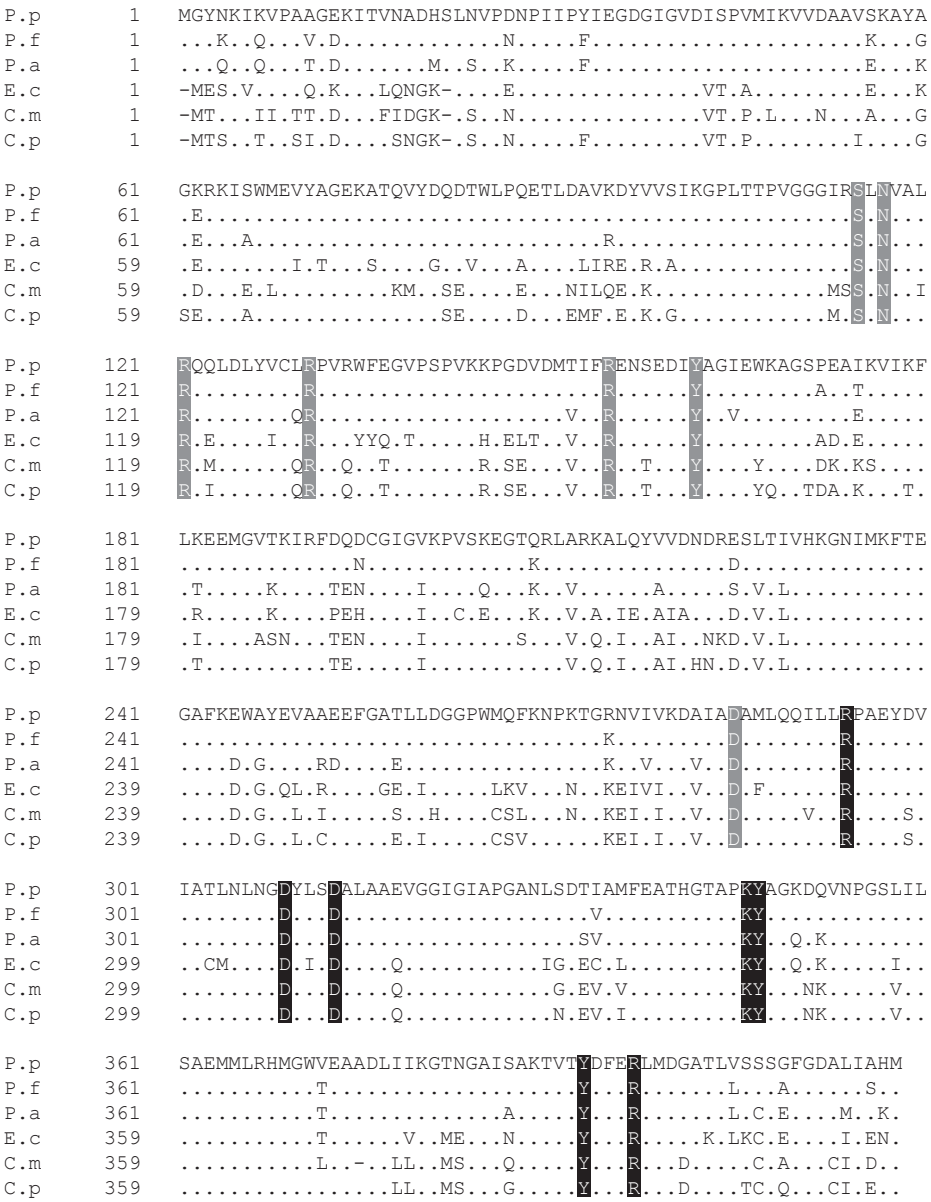

P.p 1 MGYNKIKVPAAGEKITVNADHSLNVPDNPIIPYIEGDGIGVDISPVMIKVVDAAVSKAYA
P.f 1 ...K..Q...V.D.....N.....F.....K...G
P.a 1 ...Q..Q...T.D.....M..S..K.....F.....E...K
E.c 1 -MES.V....Q.K...LQNGK-....E.....VT.A.....E...K
C.m 1 -MT...II.TT.D...FIDGK-.S..N.....VT.P.L...N...A...G
C.p 1 -MTS..T..SI.D....SNGK-.S..N.....F.....VT.P.....I....G
P.p 61 GKRKISWMEVYAGEKATQVYDQDTWLPQETLDAVKDYVVSIKGPLTTPVGGGIRSLNVAL
P.f 61 .E.....S.N...
P.a 61 .E...A.....R.....S.N...
E.c 59 .E.....I.T...S....G..V...A....LIRE.R.A.....S.N...
C.m 59 .D...E.L.....KM..SE....E...NILQE.K.....MSS.N..I
C.p 59 SE...A.....SE....D...EMF.E.K.G.....M.S.N...
P.p 121 RQQLDLYVCLRPVRWFEGVPSPVKKPGDVDMTIFRENSEDIYAGIEWKAGSPEAIKVIKF
P.f 121 R.....R.....R.....Y.....A..T.....
P.a 121 R.....QR.....V..R.....Y..V.....E.....
E.c 119 R.E....I..R...YYQ.T.....H.ELT..V..R.....Y.....AD.E.....
C.m 119 R.M.....QR..Q..T.....R.SE...V..R..T...Y....Y....DK.KS....
C.p 119 R.I.....QR..Q..T.....R.SE...V..R..T...Y....YQ..TDA.K...T.
P.p 181 LKEEMGVTKIRFDQDCGIGVKPVSKEGTQRLARKALQYVVDNDRESLTIVHKGNIMKFTE
P.f 181 .....N.....K.....D.....
P.a 181 .T.....K....TEN....I....Q...K..V.....A.....S.V.L.....
E.c 179 .R.....K....PEH....I..C.E...K..V.A.IE.AIA...D.V.L.....
C.m 179 .I....ASN...TEN....I.....S...V.Q.I..AI..NKD.V.L.....
C.p 179 .T.....TE.....I.....V.Q.I..AI.HN.D.V.L.....
P.p 241 GAFKEWAYEVAAEEFGATLLDGGPWMQFKNPKTGRNVIVKDAIADAMLQQILLRPAEYDV
P.f 241 .....K.....D.....R.....
P.a 241 ....D.G....RD....E.....K..V...V..D.....R.....
E.c 239 ....D.G.QL.R....GE.I.....LKV...N..KEIVI..V..D.F.....R.....
C.m 239 ....D.G..L.I.....S..H....CSL...N..KEI.I..V..D.....V..R....S.
C.p 239 ....D.G..L.C.....E.I.....CSV.....KEI.I..V..D.....R....S.
P.p 301 IATLNLNGDYLSDALAAEVGGIGIAPGANLSDTIAMFEATHGTAPKYAGKDQVNPGSLIL
P.f 301 .....D...D.....V.....KY.....
P.a 301 .....D...D.....SV.....KY..Q.K.....
E.c 299 ..CM....D.I.D....Q.....IG.EC.L.....KY..Q.K.....I..
C.m 299 .....D...D....Q.....G.EV.V.....KY...NK.....V..
C.p 299 .....D...D....Q.....N.EV.I.....KY...NK.....V..
P.p 361 SAEMMLRHMGWVEAADLIIKGTNGAISAKTVTYDFERLMDGATLVSSSGFGDALIAHM
P.f 361 .....T.....Y...R.....L...A.....S..
P.a 361 .....T.....A.....Y...R.....L.C.E....M..K.
E.c 359 .....T.....V..ME...N.....Y...R.....K.LKC.E....I.EN.
C.m 359 .....L..-..LL..MS...Q.....Y...R...D.....C.A...CI.D..
C.p 359 .....LL..MS...G.....Y...R...D....TC.Q...CI.E..

**Fig. 4**

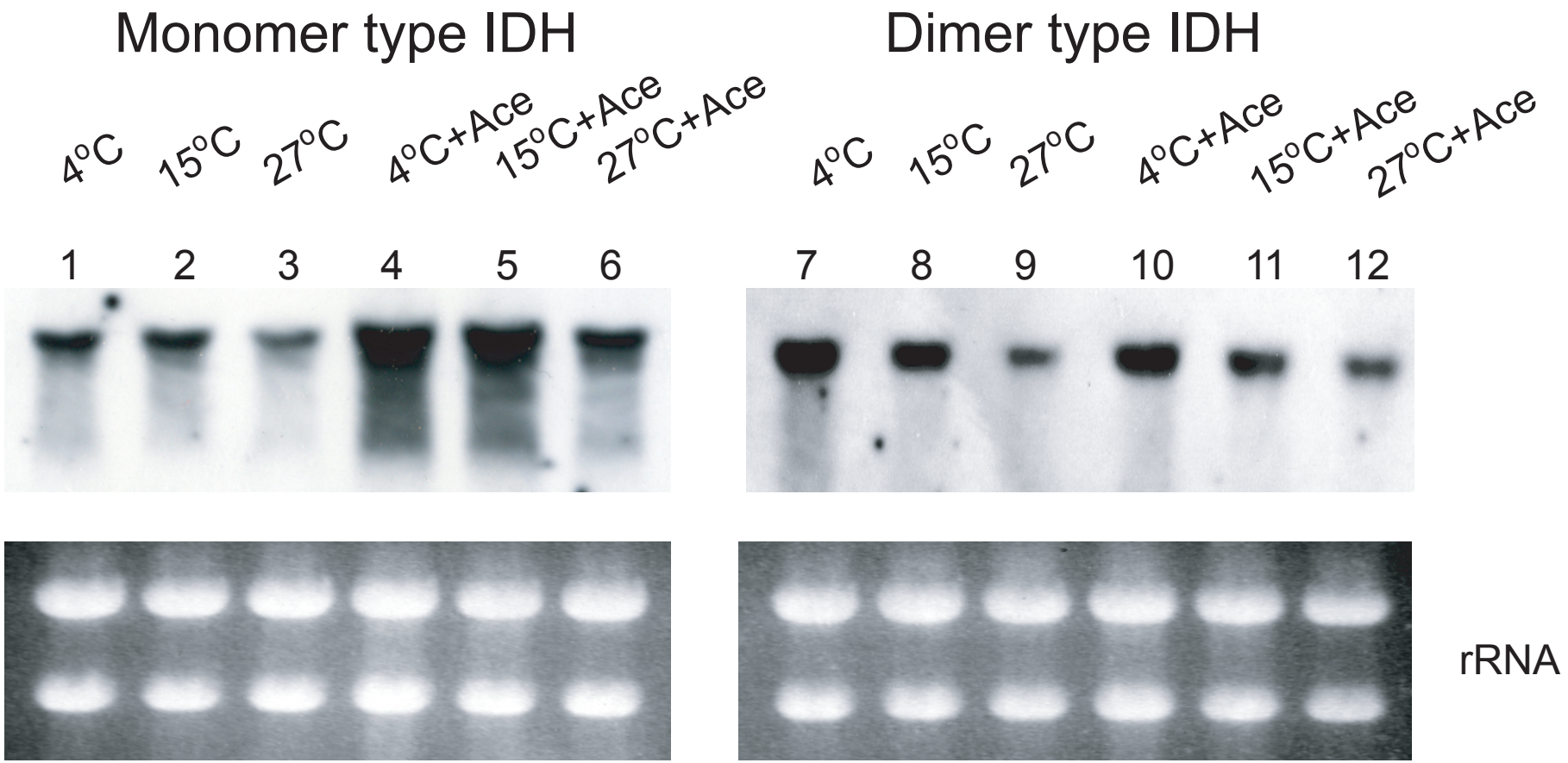
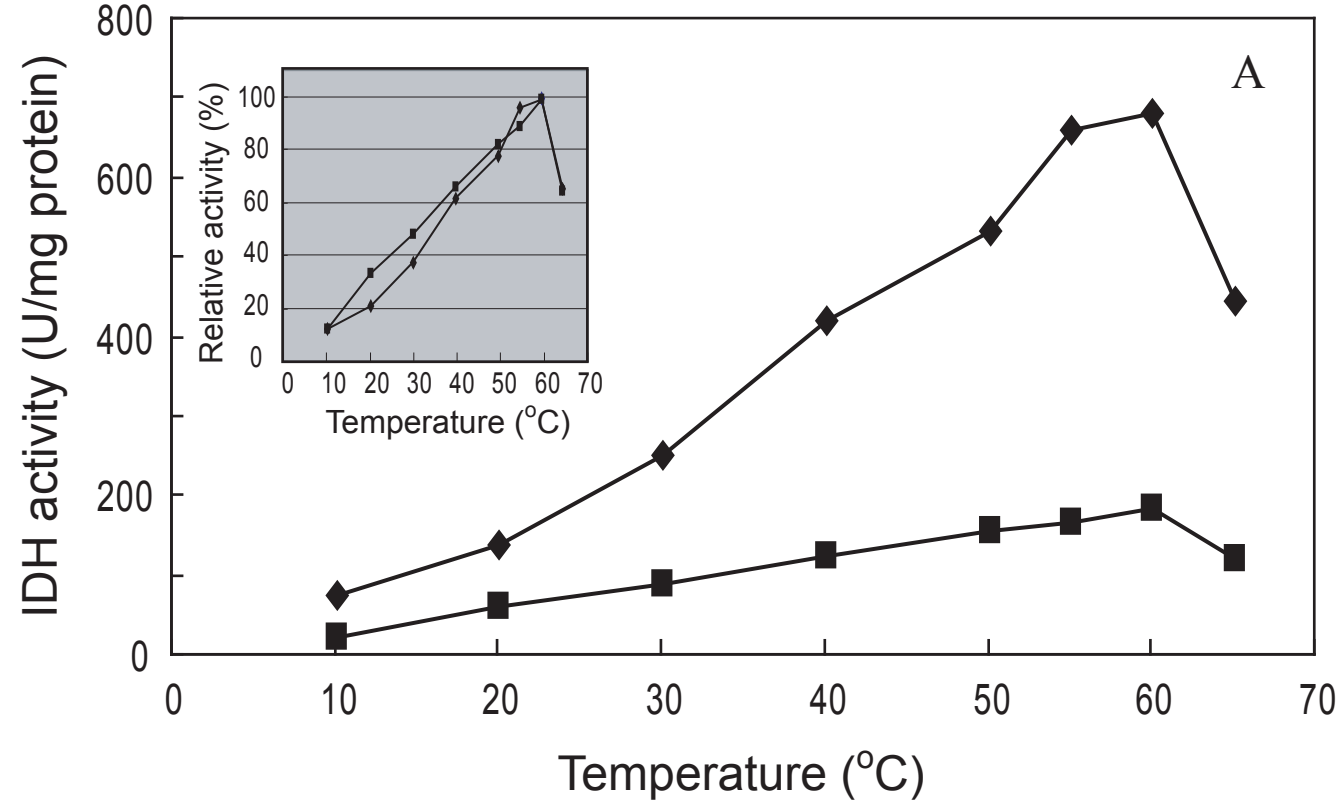

Monomer type IDH
Dimer type IDH
4°C
15°C
27°C
4°C+Ace
15°C+Ace
27°C+Ace
1
2
3
4
5
6
7
8
9
10
11
12
rRNA

**Fig. 5**


A
IDH activity (U/mg protein)
800
600
400
200
0
0
10
20
30
40
50
60
70
Temperature (°C)
Relative activity (%)
100
80
60
40
20
0
0 10 20 30 40 50 60 70
Temperature (°C)

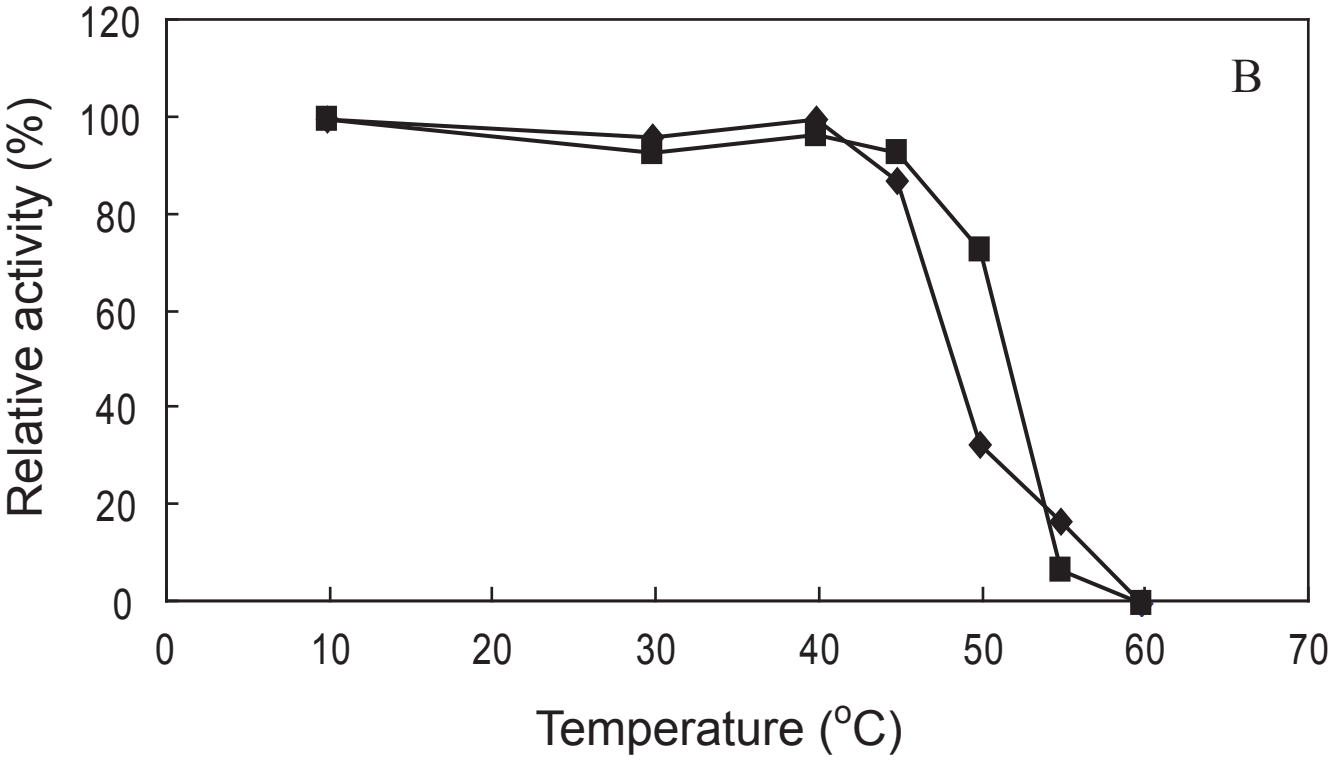
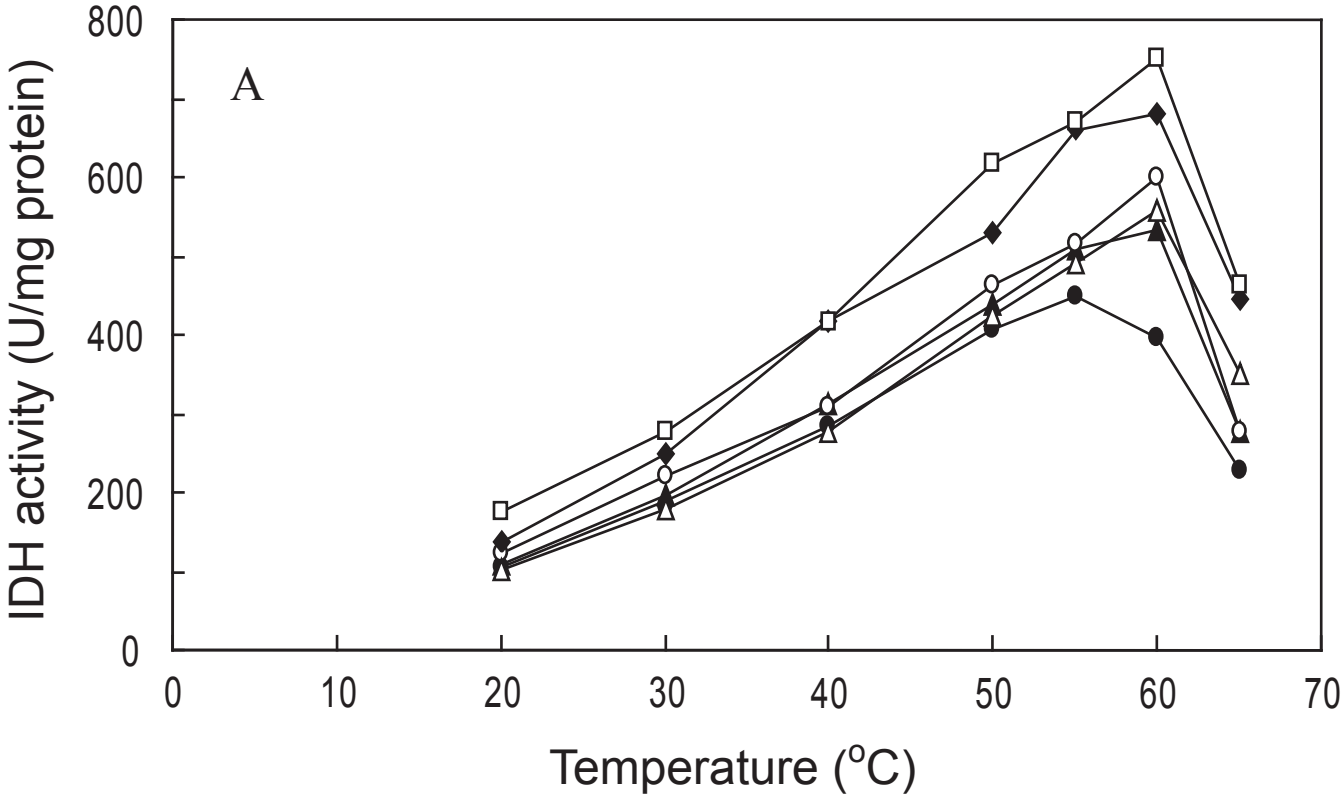

B
Relative activity (%)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Temperature (°C)

**Fig. 6**


A
IDH activity (U/mg protein)
800
600
400
200
0
0
10
20
30
40
50
60
70
Temperature (°C)

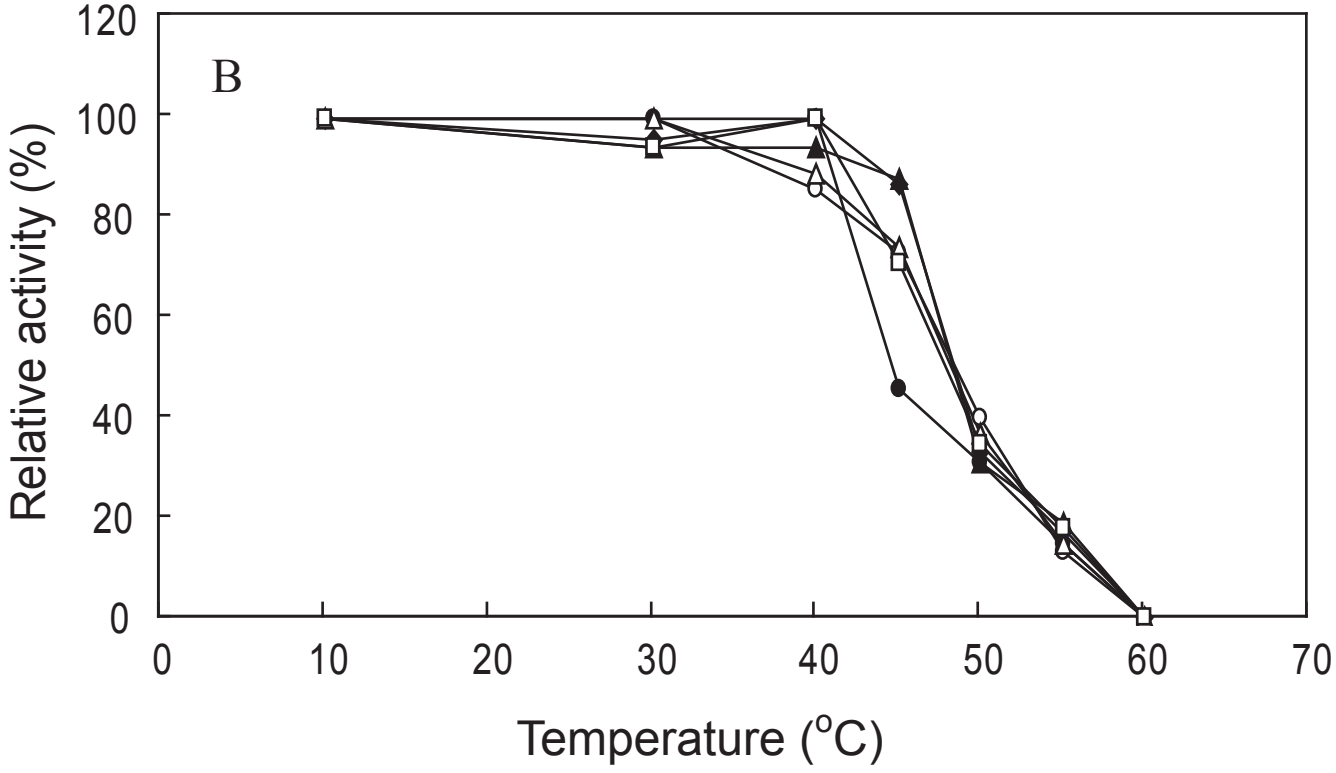

B
Relative activity (%)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Temperature (°C)

**Fig. 7**

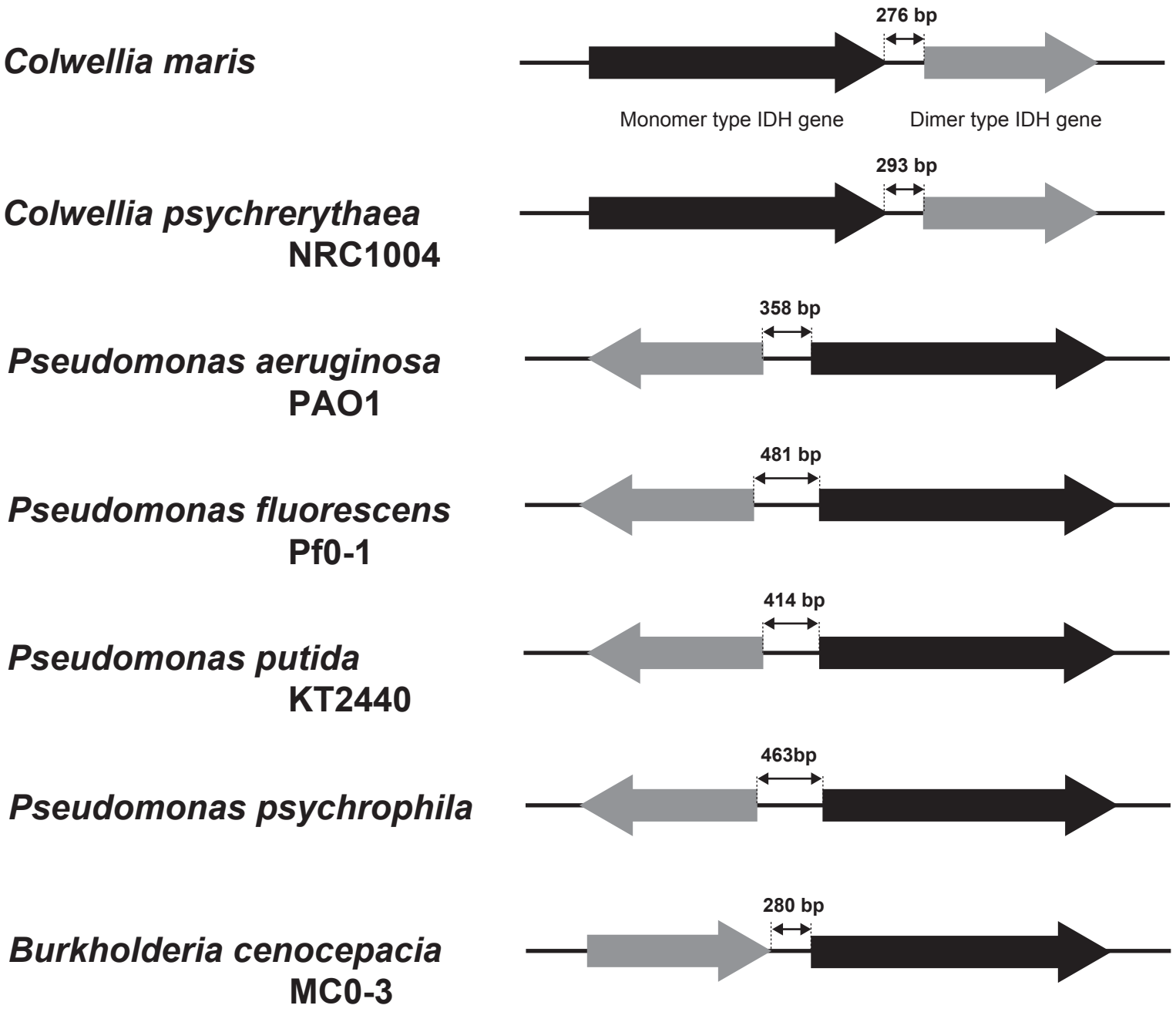

Colwellia maris
276 bp
Monomer type IDH gene
Dimer type IDH gene
Colwellia psychrerythaea
NRC1004
293 bp
Pseudomonas aeruginosa
PAO1
358 bp
Pseudomonas fluorescens
Pf0-1
481 bp
Pseudomonas putida
KT2440
414 bp
Pseudomonas psychrophila
463bp
Burkholderia cenocepacia
MC0-3
280 bp

**Fig. 8**