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好冷菌ビブリオＡＢＥ-１株のイソクエン酸脱水素酵素アイソザイムの遺伝子発現調節に関する研究

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ABBREVIATIONS

A, absorbance

BSA, bovine serum albumin

DTT, dithiothoreitol

Hepes, *2*-[*4*-(*2*-Hydroxyethyl)-*1*-piperazinyl] ethanesulfonic Acid

icdI, *Vibrio* sp. strain ABE-I IDH-I gene

icdII, *Vibrio* sp. strain ABE-1 IDH-II gene

IDH, isocitrate dehydrogenase

IDH-I, *Vibrio* sp. strain ABE-1 isocitrate dehydrogenase isozyme-I

IDH-II, *Vibrio* sp. strain ABE-1 isocitrate dehydrogenase isozyme-II

IDH K/P, isocitrate dehydrogenase kinase/phosphatase

LB, Luria broth

MOPS, 3-(*N*-morpholino) propanesulfonic acid

pBS, pBluescript (KS^+ or SK^+)

PMSF, phenylmethylsulfonyl fluoride

PVP, polyvinylpyrrolidone

S, sedimentation coefficient

SSC, saline sodium citrate

SSPE, saline sodium phosphate EDTA

CHAPTER 1

Expression of *Vibrio* sp. strain ABE-1 *icdI* and *icdII* genes in *Eschrichia coli*

SUMMARY

The closely adjacent two genes coding for structurally different isocitrate dehydrogenase isozymes (IDH-I and IDH-II) of a psychrophilic bacterium, *Vibrio* sp. strain ABE-1 were found to be transcribed independently. Northern (RNA) blot analyses of total RNAs from *Vibrio* sp. strain ABE-1 showed that two single bands corresponding 1.4 and 2.4 kb hybridized to the probes prepared from *icdI* and *icdII*, which are the genes encoding IDH-I and IDH-II, respectively. In addition, primer extension experiments located single transcriptional start sites 39 and 97 bp upstream of the start codons of *icdI* and *icdII*, respectively. The expression of the two genes in *Escherichia coli* was examined. At low temperature (15 °C), both IDH-I and IDH-II were expressed and conferred glutamate prototrophic ability on an *E. coli* mutant defective in isocitrate dehydrogenase. The abilities of the isozymes to confer restoration of the *E. coli* mutant to glutamate prototrophy were similar. At high temperature (37 °C), active IDH-I was expressed and conferred restoration of the *E. coli* mutant to glutamate prototrophy, but no functional IDH-II was expressed. Transcription of *icdI* and *icdII* genes

was regulated differently by different environmental conditions. The level of *icdI* mRNA was increased when *E. coli* transformants were cultured in acetate minimal medium, while the level of *icdII* mRNA was increased by lowering the growth temperature for *E. coli* transformants from 37 to 15 °C. The half-lives of *icdI* and *icdII* mRNAs estimated at 15 and 37 °C were almost the same. Thus it was concluded that the increase in the level of *icdII* mRNA at the low-temperature is the result of induction of the gene. In the promoter region of *icdI*, there is a nucleotide sequence homologous to that seen in the acetate-inducible *E. coli ace* operon, while in the promoter region of *icdII*, there is the nucleotide sequence CCAAT, which is a proposed common motif of *E. coli* low-temperature inducible genes. Similar patterns of transcriptional regulation of the two *icd* genes were observed also in *Vibrio* sp. strain ABE-1. However, activity of isocitrate dehydrogenase kinase, which can phosphorylate IDH-I and consequently inactivate the enzymatic activity, was detected in cell lysates of *E. coli* but not of *Vibrio* sp. strain ABE-1.

INTRODUCTION

For survival of almost all organisms, they must adjust their biochemical processes and chemicals of cellular constituents in response to the changes in their environments. In particular, temperature has crucial effects on life of exothermic organisms, such as plants and microorganisms. Bacteria are distributed in a variety of niches on the earth, and some of them can proliferate and survive under 0 °C on one hand, and some of them can grow even at the temperature above 100 °C on the other hand (1, 2). According to the ranges of their growth temperature, bacteria are usually classified into thermophiles, mesophiles, and psychrophiles (3). There have been many biochemical and biophysical studies to elucidate the key mechanisms or chemicals defining the range of growth temperature of individual bacterial species. It is easy to understand that the maximum limit of growth temperature can be certainly determined by the stabilities of essential macromolecules for the bacterial life, indeed, thermostabilities of almost all proteins from thermophiles are much higher than majority of those from mesophiles and psychrophiles. The molecular mechanism that brings about the increased thermostability of the proteins is one of the most challenging problems in the modern biochemistry and biophysics. However, it remains largely unknown what contributes to the growth of psychrophilic bacteria at low temperature such as freezing point of water. There are some suggestions for this without conclusive evidence; 1) keeping the membrane fluidity at low temperature (2, 4), 2) the presence of enzymes

which can catalyze biochemical reactions sufficiently for the needs of proliferation at low temperature (5). Since the bacterial membrane is the important site for many biological processes including energy metabolism, taking up nutrient from environmental medium, and controlling internal environment, the membrane fluidity must be controlled adequately for these processes. Thus many studies have been focused on analysis of the lipid and fatty acid compositions of the bacterial membranes in relation to the changes in environmental temperature. The results of these studies demonstrated that the bacteria which favor to grow in the low-temperature range have comparatively high proportion of unsaturated or short chain fatty acids in their membranous lipids than mesophiles (2). However, when mesophilic bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* are grown at near their minimum limit of the growth temperature, they are able to increase the amount of unsaturated or short chain fatty acids to the similar level as observed in psychrophilic bacteria (6, 7). Biochemical studies on the enzymes of psychrophilic bacteria are very few, and temperature-activity profiles of majority of their enzymes are similar to those of mesophilic enzymes. Although a few enzymes which exhibit high catalytic ability at low-temperature has been reported (2), none of them has been shown to be essential for sustaining the bacterial growth at low-temperature.

Vibrio sp. strain ABE-1, which was isolated from sea water sample on the coast of Abashiri, Hokkaido, is a psychrophilic bacterium with an optimum and maximum growth temperature at 15 °C and about 22 °C,

respectively (8). Minimum limit of this bacterial growth temperature has not been determined yet, however, this bacterium is able to grow well near subzero temperature. Previously, Ochiai et al found that this bacterium has two distinct forms of $NADP^+$-specific isocitrate dehydrogenase (IDH: EC 1. 1. 1. 42) differing in thermostability and salt dependency (9). On the basis of their electrophoretic migration, Ochiai *et al.* designated the isozyme, which moves faster, as IDH-I, and the other as IDH-II, respectively. Particularly interesting difference between the biochemical properties of the two isozymes was observed in their thermal stabilities. Optimum temperature for the enzymatic activity of IDH-I is 50 °C, and thermostability of this isozyme is comparable to those of mesophilic enzymes. On the other hand, optimum temperature for the enzymatic activity of IDH-II is 20 °C, and this isozyme is completely inactivated by the incubation at the temperature above 25 °C. In addition, IDH-II exhibits considerably high enzymatic activity (about 25 % of the optimum level) even at 4 °C. This temperature-activity profile of IDH-II is almost same as the growth characteristic of *Vibrio* sp. strain ABE-1 (8).

The bacterial IDH, which catalyzes the oxidative decarboxylation of D-isocitrate to α-ketoglutarate and carbon dioxide coupling with the reduction of $NADP^+$, has been considered to be a member of tricarboxylic acid cycle, because the most bacteria so far examined have only one, either the dimeric type IDHs composed of about 45 kDa subunits or monomeric type IDHs of about 80 kDa polypeptide. Accordingly, IDH has been suggested to play an important role for the

bacterial life. Judging from the temperature-activity profile of IDH-II of *Vibrio* sp. strain ABE-1, this enzyme has been suggested to have much more important role than IDH-I, however, the coexistence of the isozymes made it difficult to determine conclusively which one is essential for this bacterium. In order to clear the differences between IDH-I and -II on the molecular basis, Ishii et al purified the isozyme and found that their NH_2-terminal amino acid sequences, and immuno-cross reactivities were completely different from each other (10). Furthermore, they demonstrated that IDH-I is a dimeric enzyme composed of 45 kDa subunit, whereas IDH-II is a monomeric enzyme of 80 kDa polypeptide (10, 11). The dimeric type of IDHs have been shown in a variety of bacterial species including *Escherichia coli* (12), *Bacillus stearothermophilus* (13), *Rhodopseudomonas sphaeroides* (14), *Thermus thermophilus* (15), and cyanobacterium *Anabaena* sp. strain PCC 7120 (16), and the genes encoding the enzymes were also cloned and sequenced from some of them (12, 15, 16). Furthermore, the detailed molecular structure of the *E. coli* IDH was elucidated through crystallographic study, and the amino acid residues which interact with isocitrate, coenzyme, and metal ions, were determined (17). Comparisons of the deduced amino acid sequence of the *E. coli* IDH with those of other dimeric IDHs revealed that the above amino acid residues were all conserved in these enzymes (15, 16, 18-23). Because IDH-I of *Vibrio* sp. strain ABE-1. is a dimeric enzyme and show similar immunological cross-reactivity with *E. coli* IDH, this isozyme has been suggested to be an enzyme homologous to the *E. coli* enzyme (24). On

the other hand, the monomeric type of IDHs have been demonstrated in a few bacterial species including *Azotobacter vinelandii* (25), *Vibrio parahaemolyticus* (24), *Rhodomicrobium vannielii* (26), however, there was no available data on the amino acid sequences of these proteins. Ishii et al first cloned and determined the sequence of the gene (*icdII*) coding for a monomeric type of IDH, IDH-II, from *Vibrio* sp. strain ABE-1 (27). At the same time they also succeeded to clone and sequence the gene (*icdI*) encoding the other isozyme, IDH-I (27). As expected, IDH-I, dimeric type of IDH, had a high amino acid sequence identity (74.3 %) to the *E. coli* IDH, however, the deduced amino acid sequence of IDH-II, monomeric type of IDH, was completely different from that of IDH-I (27). In addition, no significant matches were discovered in the DNA or protein data bases, when the sequences of the *icdII* gene and its protein were compared with other sequences (27). These results suggested that IDH-I and -II have evolved from different ancestral origins or diverged from each other at very early step of evolution. The other interesting findings of cloning genes encoding IDH-I and -II isozymes were that the two genes (*icdI* and *icdII*) were closely adjacent with a spacer of 276 bases in the order *icdII-icdI* (27). In this spacer region, 85 bases downstream of the stop codon of *icdII*, there appears to be a ρ-independent, terminator-like structure followed by a promoter, a Shine-Dalgarno region, and the start codon of *icdI* (27). Another promoter and ribosome-binding region are also seen upstream of the initiation codon of *icdII* (27). These structurally characteristics of the genes strongly suggested that the two genes are transcribed

independently, however, the experimental evidences to confirm this were not obtained at that time. In addition, regulatory aspects of the expression of the two genes were still unknown.

In order to clarify the above unsettled problems and evaluate the physiological roles of the two IDH isozymes, I first attempted to determine the transcriptional start sites of the two genes, and then construct expression plasmids in *E. coli* cells to examine how the transcriptions of the two genes are regulated by environmental conditions. As the results, it was confirmed that the two genes are transcribed independently, and the expressions of the genes are differently regulated. Namely, *icdI* was an acetate-inducible gene, while *icdII* was a low-temperature inducible gene.

MATERIALS AND METHODS

Materials. DL-Isocitrate, 2-mercaptoethanol, acrylamide, *N,N'*-methylenebisacrylamide, and agarose LE were obtained from Nacalai Tesque Inc.(Kyoto, Japan). $NADP^+$ was the products of Oriental Yeast (Osaka, Japan). Marker proteins for molecular weight were purchased from Pharmacia. All restriction endonucleases were obtained from Nippon Gene (Toyama, Japan), Toyobo (Osaka, Japan), or New England Biolabs, Inc. (MA, USA). Marker nucleotides for molecular size were products of Nippon Gene (Toyama, Japan). DNA polymerase (Klenow fragment), T4 polynucleotide kinase, ribonuclease inhibitor, a DNA ligation kit, a random primer DNA labeling kit, a DNA Blunting kit, and agarose LO3 were purchased from Takara Shuzo (Kyoto, Japan). [γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from ICN Biomedical Inc. (CA, USA). Rifampin was obtained from Wako Chemicals (Osaka, Japan). Hybond-N^+ and Hybond-C super were products of Amarsham International (England). Hybridization bags were products of GIBCO BRL (MD, USA). A DNA cell (disposable DNA trapping cell) and a dye reagent for protein assay were products of Dai-ichi Pure Chemical Co., (Tokyo, Japan) and Bio-Rad laboratories, Inc. (CA, USA), respectively. All other reagents were of analytical grade.

Bacterial strains, plasmids, and media. A psychrophilic marine bacterium, *Vibrio* sp. strain ABE-1 (*Vibrio* ABE-1) was cultured at 15 °C with vigorously shaking in a nutrient medium containing each 1.0% peptone and meat extract with 0.5 M NaCl or synthetic minimal medium

as previously described (8). The strains of *Escherichia coli* and the plasmids used in this study are shown in Table 1 and Fig. 1. *E. coli* DEK2004, a mutant defective in isocitrate dehydrogenase (a gift from Dr. Peter Thorsness), was used as a host for transformation with *Vibrio* sp. strain ABE-1 *icd* genes. The medium for growth of this strain was Luria broth (LB, 28) or morpholinepropanesulfonic acid (MOPS)-based medium (29) supplemented with 0.5 mM glutamate and 0.5% glucose, 2.0% sodium acetate, or 2% sodium succinate. To determine the restoration of the auxotrophic phenotype of the transformed *E. coli* mutant, glutamate was omitted from the medium. Media for other strains of *E. coli* were supplemented with a trace amount of thiamine (about 10 to 100 nM). When necessary, media were supplemented with 50-100 μg of ampicillin and/or 15 μg of tetracycline per ml. Expression plasmids for *icdI* and *icdII* were constructed as follows: pIS102, the expression plasmid for *icdI*, was constructed by digesting pIS101, which is the pBluescript KS^+ inserting with *Vibrio* sp. strain ABE-1 *icdI*, with *Eco*RV that cleaves at the restriction site present between a putative terminator-like sequence of *icdII* and promoter region of *icdI*, and ligating it to *Eco*RV- and *Xba*I-digested pBluescript KS^+. The expression plasmid for *icdII*, pIS202, which contains the above terminator-like sequence located downstream of *icdII* ORF, was constructed by using the *Xba*I-*Sac*I fragment of λVAI21 (27) and pBluescript KS^+ (Fig. 2). Plasmid pSS512 was constructed by insertion of the 1.9-kbp *Eco*RI-*Hin*dIII fragment of pTK512 (12), which contains the *E. coli icd* gene (a gift from Dr. Peter Thorsness), into

*Eco*RI-*Hin*dIII-digested pBluescript SK^+.

Primer extension analysis. To determine the transcriptional start sites of the *icdI* and *icdII* genes, the following 31- and 32-mer oligonucleotides were chemically synthesized on a Milligen model 7500 or Applied Biosystems model 381A DNA synthesizer as the primers for *icdI* and *icdII*, respectively: 5'-GGGATTGTTAGGTACTGATAACTTA CCGTCG-3', complementary to the internal sequence from bases 51 to 81 downstream from the ATG codon of *icdI*, and 5'-GCTTGAATAATGGG TAATAAAGAATACGTCGC-3', complementary to that (from bases 58 to 89 downstream from the ATG codon) of *icdII* (27). The primers were 5' end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Total RNAs from *Vibrio* sp. strain ABE-1 cells grown in peptone-meat extract (8) were isolated by the method of Case *et al* (30). The RNAs (600 μg) was hybridized with either of the labeled primers. The primer extension reaction (28) was performed by using Rous-associated virus 2 reverse transcriptase (Takara Shuzo Co.), and products were analyzed by electrophoresis on a 6% polyacrylamide sequencing gel using Sequence kit (a sequenase labeled dCTP kit from United States Biochemical, OH, USA).

Northern (RNA) blot analysis. Total RNAs from *Vibrio* sp. strain ABE-1 cells, which were grown in nutrient or synthetic minimum medium containing 2% sodium-acetate or 2% sodium-succinate at different temperatures (0, 5, 10, or 15 °C) were extracted and purified by a single-step RNA isolation method (AGPC method) as described previously (31). Similarly, total RNAs from *E. coli* DEK2004 cells

harboring pIS102 or pIS202, which were grown in LB or MOPS medium supplemented with 0.5% glucose or 2% acetate at 15, 25, or 37 °C, were isolated. To monitor the changes in the level of *E. coli icd* mRNA, RNAs were extracted from *E. coli* K-12 cells grown at 37 °C in MOPS medium supplemented with glucose or acetate (for details, see the legend to Fig. 8). The bacterial cells used for RNAs isolation were in the mid-logarithmic phase of growth (*Vibrio* sp. strain ABE-1; A_{600} = 1.2, *E. coli* transformants; A_{550} = 0.8). The isolated RNAs were separated on agarose gels (1.0 or 1.2%) containing 0.66 M formaldehyde, transferred to nylon membranes (Hybond-N^+), and then hybridized with appropriate radiolabeled DNA fragment. The 343-bp *Sac*I-*Sac*I fragment of *icdI*, the 620-bp *Hin*dIII-*Eco*RI fragment (or the 828-bp *Pst*I fragment) of *icdII*, and the 493-bp *Sal*I-*Pvu*I fragment of *E. coli icd* were used as probes for mRNAs from *icdI*, *icdII*, and *E. coli icd*, respectively (Fig. 2). Blotting protocols and procedures for membrane hybridization were the same as the suitable conditions for Amersham Hybond-N^+ systems blotting in 500 ml of 0.05 M NaOH for 3 h at room temperature; hybridization in total 25 ml solution containing 6.25 ml of 20 X SSPE (3.6 M NaCl, 0.2 M sodium phosphate, and 0.02 M EDTA pH 7.7), 1.25 ml of 100 X Denhardt's solution (2% [w/v] BSA, 2% [w/v] Ficoll, and 2% [w/v] PVP), 1.25 ml of 10% [w/v] SDS, heat-denatured 0.5 ml of 1 mg/ml solution of sonicated Herring sperm DNA, and appropriate radiolabeled probe, for overnight at 65 °C. After hybridization, the membranes were washed successively in SSC (1 X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS) as

follows: 2 X SSC at room temperature for 10 min (repeat), 1 X SSC at 65 °C for 15 min, and 0.1 X SSC at 65 °C for 10 min (repeat). Autoradiography was performed by exposing the membranes to a X-ray film (Fuji RX medical X-ray film) at -80 °C for appropriate time. The amount of mRNA that hybridized with each specific probe was quantified from the autoradiographs by densitometry described in the procedure for measuring mRNA stability.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Conditions for SDS-PAGE were described previously (10) except 7.5 or 8% acrylamide was used. As protein marker, a kit of Pharmacia Biotech containing phosphorylase b from rabbit muscle (94,000 Da), albumin from bovine serum (67,000 Da), ovalbumin from egg white (43,000 Da), carbonic anhydrase from bovine erythrocyte (30,000 Da), trypsin inhibitor from soybeam (20,100 Da), and α-lactalbumin from bovine milk (14,400 Da), was used. After electrophoresis, the separated proteins were stained with Coomassie brilliant blue R-250. And then the gel was used for Western blot analysis, or dried at 80 °C for 2 h for detection of radiolabeled proteins.

Western blot (immunoblot) analysis. The procedure for preparation of antibodies raised against the IDH isozymes were described previously (10). The purified isozymes (0.2 μg) and proteins (10 μg) in cell free extract from an *E. coli* mutant harboring pIS102 or pIS202 were separated by SDS-polyacrylamide (7.5%) gel electrophoresis as described above, transferred to nitrocellulose membranes (Hybond-C), and subjected to Western blot analysis with the enhanced chemiluminescence

(ECL) Western blotting detection system (Amersham) and 5,000-10,000 fold diluted rabbit anti-IDH-I or -II antibody (10).

Estimation of mRNA stability. *E. coli* mutant DEK2004 cells carrying either plasmid containing *icdI* or *icdII* genes were grown in LB at 15 or 37 °C. When the cultures reached the mid-logarithmic phase of growth (A_{550} = 0.8), rifampin was added to 200 μg/ml to block further initiation of transcription, and the cells were allowed to continue to grow at the same temperature. Samples (1.5 ml) were then withdrawn from the cultures at 2-min intervals and the cells were pelleted by centrifuging in a microcentrifuge for 30 sec at 15,000 rpm at 4 °C. After the supernatant was removed, the pellet was subsequently frozen by liquid nitrogen and stored at -80 °C until use. RNA was extracted for Northern blot analysis as described above. Hibridizing bands were quantitated by scanning the most intense part of the bands with a Bio-imaging analyzer BAS2000 (Fuji film, Tokyo, Japan). Graphs of percent value of mRNA remaining were plotted against time after rifampin addition, and the best line was calculated by the least squares method. The half life of the mRNAs was estimated by reading from the graph the time taken to reduce the level of mRNA to 50%.

***In vitro* phosphorylation of IDH-I.** *E. coli* DEK2004 and *Vibrio* sp. strain ABE-1 were cultured in LB and nutrient medium, respectively, and the cell free extracts were prepared by sonic disruption of the cells (9). IDH isozymes were purified from *Vibrio* sp. strain ABE-1 cells as described previously (10). One microliter of the purified IDH-I solution (0.2 μg protein) was transferred to Eppendorf tubes, and 10 μl of

cell free extract (10 μg protein) of *E. coli* DEK2004 or *Vibrio* sp. strain ABE-1 was added. Then, 1 μl of [γ-^{32}P]ATP (0.37 MBq; 167 TBq/mmol) was added, and the mixture was incubated at 37 °C or 15 °C. After appropriate time intervals, 2 μl of 10% SDS, 5 μl of 50% glycerol, and 2 μl of 100 mM sodium phosphate (pH 7.2) were added, and the mixture was boiled for 1 min to terminate the reaction. After the sample was cooled at room temperature, proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and radio-labeled polypeptides were detected by autoradiography.

Enzyme assay. The procedure of preparation of a sonic cell free extract of the bacteria cells was described previously (9). $NADP^+$-IDHs activities were assayed spectrophotometrically at 40 °C for IDH-I, 20 °C for IDH-II, or 37 °C for *E. coli* IDH, in a mixture (final volume 2 ml) containing 33 mM Tris-HCl (pH 8.4 at 20 °C), 250 mM NaCl, 0.67 mM $MnCl_2$, 0.12 mM $NADP^+$, 2 mM DL-isocitrate, and an appropriate amount of enzyme. The reaction was started by the addition of $NADP^+$ or DL-isocitrate, and measured the initial rate of increase in absorbance at 340 nm. One unit of enzyme activity is defined as the amount of enzyme catalyzing the reduction of 1 μmol of $NADP^+$ per min. One unit of the enzyme activity was defined as the amount of the enzyme catalyzing the reduction of 1 μmol of $NADP^+$ per min. Protein concentration was determined by the Bio-Rad protein assay procedure which is based on the method of Bradford (32) with bovine plasma γ-globulin as a standard.

RESULTS

Transcriptional start sites and Northern analyses of *icdI* and *icdII* transcripts in *Vibrio* sp. strain ABE-1. In order to examine whether the two closely adjacent genes *(icdI* and *icdII*) of *Vibrio* sp. strain ABE-1 are transcribed independently, the initiation site of each gene was determined by primer extension analyses. Essentially the same profiles were obtained by using different RNAs isolated from the cells grown on peptone-meat extract, succinate, or acetate. Typical results are shown in Figs. 3 and 4. The initiation sites for the *icdI* and *icdII* genes were found to be G (39 bases upstream of the ATG codon of the *icdI* translational start point) and T (97 bases upstream of the ATG codon of the *icdII* translational start point), respectively (Fig. 3). Therefore, the putative promoter motifs (33) at -35 and -10 for *icdI* and *icdII* are TTTAGT and TACTTT, and TTTATA and AACTAT, respectively (Fig. 4). These results suggest that each of the *icd* genes of *Vibrio* sp. strain ABE-1 is separately expressed.

To confirm further this is true, Northern blots prepared with total RNA isolated from *Vibrio* sp. strain ABE-1 cells grown on various carbon sources were hybridized with either the 343-bp *Sac*I-*Sac*I fragment [internal to *icdI*, at positions 2735 to 3077 from the *Hin*dIII (a) site in (27)] or the 620-bp *Hin*dIII (b) -*Eco*RI fragment [internal to *icdII*, at positions 322 to 941 from *Hin*dIII (a) site in (27)]. Two single bands corresponding to approximately 1.4 kb and 2.4 kb hybridized to the probes prepared from *icdI* and *icdII*, respectively (Fig. 5). No transcript

larger than 2.4 kb was detected. In addition, the estimated sizes of the transcripts were in good agreement with those of the respective ORFs plus their flanking regions. These results indicate again the separate expression of *Vibrio* sp. strain ABE-1 *icd* genes, and the promoter- and terminator-like structure found in 5' and 3' regions of each ORF apparently act as the true promoters and terminators.

The amount of *icdII* mRNA in *Vibrio* sp. strain ABE-1 cells did not change significantly by changing the carbon source in the medium, whereas that of *icdI* increased when acetate was used as the sole source of carbon and energy (Fig. 5, lane *icdI*-Acetate). These results were in agreement with the previous observation that the specific activity of IDH-I was increased in the cells grown in acetate medium (10).

Expression systems of the *icd* genes. For confirmation of expression products, and for study of regulatory mechanisms of expression of the two *icd* genes, it was necessary to construct expression systems. I used *E. coli* DEK2004, a mutant in *icd*, as a host for the expression plasmids, because no mutant defective in *icd* of *Vibrio* sp. strain ABE-1 was available. Expressions of *icdI* and *icdII* in *E. coli* transformants with pIS102 (expression plasmid for *icdI*) or pIS202 (expression plasmid for *icdII*) were determined by measuring IDH activity or by Western blot analysis. For expression of *icdI*, the transformants were cultured at 37 °C, while the culture temperature for the transformants with pIS202 was 15 °C because of the extreme thermolability of IDH-II protein (9). Expression level estimated as the enzymatic activity (1.49 U/mg of protein) of the *icdI* gene in *E. coli* cells,

which were cultured in a peptone broth or glucose minimal medium at 37 °C, was about 10 times higher than the activity in a sonic extract of *Vibrio* sp. strain ABE-1 grown at 15 °C (10). Thermolabile IDH (IDH-II) activity, which was completely inactivated by heating the extract at 30 °C for 10 min (24), was detected in a sonic extract prepared from *icdII*-transformed cells grown at 15 °C, and the level (0.11 U/mg of protein) of the activity was approximately three times lower than the value found in a sonic extract of *Vibrio* sp. strain ABE-1 (10). The addition of isopropyl-β-D-thiogalactoside (IPTG) to the culture medium did not affect the expression levels of the *icd* genes (data not shown). Western blot analysis employing immunoglobulin-G raised against IDH-I or IDH-II (10) demonstrated the presence of a protein band corresponding to the purified IDH-I or IDH-II in a sonic extract prepared from the transformed cells (Fig. 6). In the negative control (a sonic extracts from DEK2004 harboring pBluescript KS^+, no positive band was detected [Fig. 6, lanes pBS(KS^+)].

Complementation of *E. coli* mutant defective in IDH by *Vibrio* sp. strain ABE-1 *icd* genes. One (IDH-I) of the two isozymes of *Vibrio* sp. strain ABE-1 IDHs is a homologous enzyme to the *E. coli* IDH, but the other (IDH-II) is a thermolabile enzyme and quite different in amino acid sequence from the *E. coli* enzyme. In order to investigate whether IDH-II is functional in *E. coli* cell, I examined the phenotype of *E. coli* DEK2004, a mutant in IDH deficient, harboring *icdII* gene. Because Thorsness and Koshland reported that *E. coli* DEK2004 shows phenotype of glutamate auxotroph and this phenotype is recovered to the wild type

by transformation with the *E. coli icd* gene (12). Taken into account for the lability of IDH-II above 20 °C, I cultured the mutant harboring either *icdI* or *icdII* gene at high (37 °C) or low (15 °C) temperature (Fig. 7). The mutant harboring vector without the *icd* genes control exhibited clearly phenotypic auxotroph for glutamate at both temperatures, although the glutamate-dependency at low temperature was less than that at high temperature. This difference might be due to the difference in the bacterial growth rates between 15 and 37 °C. Plasmid pIS102 carrying *icdI* gene was able to restore the mutant to glutamate prototrophy at low and high temperatures, whereas plasmid pIS202 carrying *icdII* gene was able to rescue the mutant at only low temperature. These results indicate that *icdII* product, IDH-II, was functional as well as IDH-I in *E. coli* at low temperature. The failure of plasmid pIS202 to restore the *E. coli* mutant to the prototrophy at high temperature might be caused by inactivation of thermolabile IDH-II or impairment at transcriptional/translational level of the gene.

Levels of the transcripts of *icd* genes at low and high temperatures. Next, to investigate the effects of temperature on expression of *Vibrio* sp. strain ABE-1 *icd* genes, I measured the mRNA levels of the genes by Northern blot analyses of RNAs from the *icdI*- and *icdII*-transformed *E. coli* mutants or *Vibrio* sp. strain ABE-1 cells cultured at various temperatures. As shown in Fig. 8, the levels of *icdII* transcript in the *E. coli* mutant harboring pIS202 was markedly increased by lowering the growth temperature from 37 to 15 °C, while that of *icdI* did not change under the same conditions. Increases in the level of the

icdII transcripts were also observed when the growth temperature for *Vibrio* sp. strain ABE-1 was lowered. In *Vibrio* sp. strain ABE-1, the changes in the level of *icdI* transcript were the converse of those in the *icdII* transcripts level. Quantitative estimation by densitometry indicated that there were 12- and 3-fold increases in the amount of *icdII* mRNA in *E. coli* mutants grown at 15 and 25 °C, respectively, compared with the amount in those grown at 37 °C.

As *icdII* was found to be transcribed in the *E. coli* mutant at 37 °C as Fig. 8, I examined whether IDH-II can be synthesized at this temperature (Fig. 9). A protein band cross-reactive with anti-IDH antibody was detected on Western blots of cell extracts from the *E. coli* mutant harboring *icdII* grown at 37 °C (lane: pIS202, 37 °C), but no IDH enzymatic activity could be detected. These results indicate that the failure of pIS202 to restore the *E. coli* mutant to glutamate prototrophy at 37 °C (Fig. 7) was caused by post-translational inactivation of the expressed IDH-II at this temperature.

Stability of *icdI* and *icdII* mRNAs. The level of *icdII* mRNA, but not that of *icdI* mRNA, was changed by the growth temperature (Fig. 8). The difference in the effects of growth temperature on the levels of *icd* mRNAs might have been caused by the different degradation rates of the mRNAs. To compare the stabilites of the mRNAs, cultures of *E. coli* mutants harboring pIS102 or pIS202 were treated with rifampin. As shown in Fig. 10, both of *E. coli* and *Vibrio* sp. strain ABE-1 ceased to grow immediately after the addition of rifampin (200 μg/ml). Rifampin treatment was carried out for different times depending on the

temperature of incubation. Total RNA from the transformants was analyzed by Northern blots probed with fragment either the *icdI* or the *icdII* gene (Fig. 11, A). The relative amounts of mRNA after the addition of rifampin were estimated by densitometry, and the values were plotted as logarithmic percent value of mRNA remaining versus time after rifampin addition (Fig. 11, B). As expected, the half-lives of the *icd* mRNAs at 37 °C were shorter than those at 15 °C; however, there was no significant difference between the half-lives of *icdI* and *icdII* mRNAs at the same temperature. The half-lives of the *icd* mRNAs were 5-8 min and 20-30 seconds at 15 °C and 37 °C, respectively. Therefore, it can be concluded that stability of *icdII* mRNA is almost the same as that of *icdI* mRNA and that the increase in the level of *icdII* mRNA at low temperature is the result of induction of the gene.

Induction of *icdI* gene expression by acetate. It is well known that the *E. coli* IDH is inactivated by phosphorylation at Ser-113 when *E. coli* is cultured in acetate minimal medium (34-36). The amino acid sequence around this serine residue has been found in the deduced amino acid sequences of other IDHs homologous to the *E. coli* enzyme. On the basis of the sequence similarity, these IDHs have been suggested to be similarly regulated by phosphorylation, although no direct evidence for this has been presented (15, 16). But IDH-I is induced in *Vibrio* sp. strain ABE-1 grown in acetate minimal medium (10), nevertheless, the amino acid sequence around Ser-113 in this enzyme is the same as that in *E. coli* enzyme (27). Thus, I speculated two possibilities for the different responses of the *icdI* and *E. coli icd* genes to acetate: first, in the

promoter region of *icdI*, there is a nucleotide sequence homologous to that seen in the acetate-inducible *E. coli ace* operon (37, 38), while no such unique nucleotide sequence is present in the promoter region of the *E. coli icd* gene (12); second is that *Vibrio* sp. strain ABE-1 may not have isocitrate dehydrogenase kinase/phosphatase (IDH-K/P) activity which can phosphorylate serine residue corresponding to Ser-113 in the *E. coli* enzyme (35). To evaluate these possibilities, *E. coli* mutant harboring either pIS102 or pIS202 were grown in glucose medium to mid-logarithmic phase, and the cells were transferred into a medium containing acetate as a sole source of carbon and allowed to continue to grow. At appropriate time intervals after the shift in medium, cells were collected and level of each *icd* mRNA was determined on Northern blots probed with either *icdI* or *icdII* gene fragment (Fig. 12). The level of *icdI* mRNA was increased after the shift from glucose to acetate medium (12-fold after 4 h of the shift), while those of *icdII* and *E. coli icd* mRNA showed almost no change after the shift. These results suggest that a stretch containing the characteristic nucleotide sequence seen in the promoter region of *icdI* is responsible for acetate inducible expression of this gene (Fig. 15, A).

***In vitro* phosphorylation of IDH-I and reduction of the specific activity of the expressed IDH-I in *E. coli*.** Next, to examine whether *Vibrio* sp. strain ABE-1 does not have IDH K/P, I attempted to detect this enzymatic activity by incubating purified IDH-I (0.2 μg protein) with [γ-^{32}P]ATP in the presence of cell-free extract from the *E. coli* DEK2004 or *Vibrio* sp. strain ABE-1. At appropriate time intervals of the

incubation, aliquots of the reaction mixture were subjected to SDS-PAGE for analysis of polypeptides, and radiolabeled polypeptides were detected by exposing the dried gels of SDS-PAGE to a X-ray film (Fig. 13). When the polypeptides on the gels were stained with Coomassie Brilliant Blue R-250, a polypeptide corresponding to IDH-I was detected in the all lanes with exception of lane *E. coli* (cell-free extraction alone from the *E. coli* DEK2004, Left panel). A radiolabeled protein band which corresponds to IDH-I was appeared after 30 min of the incubation of the purified IDH-I with [γ-^{32}P]ATP in the presence of cell-free extract from *E. coli* mutants but not from *Vibrio* sp. strain ABE-1 incubated either at 15 or 37 °C (Right panel). Then, I measured specific activity of IDH-I in the sonic extract prepared from the *E. coli* transformants. When the cells were shifted from glucose- to acetate-minimal medium (Fig. 14), the specific activity was reduced to 45 % (right bar) of that determined in the sonic extract prepared from the glucose grown cells (after 7 h of the shift). These results indicate that IDH-I could be phosphorylated and inactivated by the *E. coli* IDH K/P, but *Vibrio* sp. strain ABE-1 does not have the IDH-kinase activity.

DISCUSSION

Genes encoding IDH from several prokaryotic (12, 15, 16, 27, 39) and eukaryotic (18-23) organisms have been cloned and sequenced. Among IDHs, the *E. coli* IDH is the only enzyme which has been characterized in detail by crystallographic analysis, and it has been used as a model to understand the molecular structure of the other IDHs. Comparison of the deduced amino acid sequences revealed that these IDHs are all more or less homologous to the *E. coli* IDH. These enzymes have a common feature that they are composed of subunits. On the other hand, Ishii *et al* first revealed that the deduced amino acid sequence of IDH-II (monomeric type IDH) of *Vibrio* sp. strain ABE-1 is completely different from those of the IDHs composed of subunits. Recently, genes coding for monomeric type of IDH have been cloned and sequenced from other bacterial sources, *Corynebacterium glutamicum* (39), and *Azotobacter vinelandii* (40). The deduced amino acid sequence of these monomeric type of IDH are highly homologous to that of IDH-II of Vibrio sp. strain ABE-1 (40). Since *Vibrio* sp. strain ABE-1 has another structurally different IDH isozyme (27), IDH-I, which is a dimeric type and highly homologous to the *E. coli* IDH, this raised questions of whether the IDH-II is functional in *E. coli* cells as well as the dimeric enzyme, and what mechanisms control the expressions of the two *icd* genes. In this study, I demonstrated that both *icdI* and *icdII* genes were expressed in *E. coli* cells and the expressed products were functional.

The results of the primer extension analysis (Fig. 3 and 4) and the Northern blot analysis of RNA isolated from *Vibrio* sp. strain ABE-1 (Fig. 5) suggest that the two genes are expressed separately, nevertheless closely adjacent with a spacer of 276 bases in order *icdII-icdI* (27). These results clearly indicate that the putative regions estimated from the nucleotide sequences of the genes are true promoters of each gene. Moreover, confirmations for the expression of the two genes in *E. coli* cells were obtained by Western blot analyses (Fig. 6) and measuring the enzymatic activity. In addition, evidence for that both the enzymes are equally functional in *E. coli* cells were obtained by transforming the *E. coli icd* defective mutant with either plasmid carrying *icdI* or *icdII* gene (Fig. 7). Auxotrophic property of this mutant was restored to glutamate prototrophy by the transformation, although *icdII*-bearing plasmid failed this restoration at 37 °C. As this phenotypic restoration has also been shown by transformation of the mutant with *E. coli icd* (12) or *icd* genes cloned from several other sources (16), it can be concluded that both the enzymes are functional in *E. coli* cells. The failure of the plasmid carrying *icdII* to restore the *E. coli* mutant at a high temperature (37 °C) is in agreement with the finding that IDH-II is completely inactivated both *in vitro* and *in vivo* at temperatures above 20 °C (9).

On the basis of sequence similarity, IDHs can be roughly categorized into two different groups (16, 41). One is composed of $NADP^+$-IDH of eukaryotic origin, including the alfalfa enzyme (22), cytosolic enzyme of rat liver (21), and mitochondrial enzyme of porcine heart (18) and *Saccharomyces cerevisiae* (19). The other is a group composed of

prokaryotic $NADP^+$-IDHs and the NAD^+-IDH subunits IDH1 and IDH2 of *S. cerevisiae* (42, 43). The sequence of IDH-II is far different from those of the above-mentioned two groups, and therefore, this enzyme may form a novel third group together with other homologous monomeric $NADP^+$-IDHs, including the enzymes from *A. vinelandii* (40) and *C. glutamicum* (39). The monomeric IDHs of *R. vannielii* (26) and *V. parahaemolyticus* (24) also belong to this group, because they share common NH_2-terminal amino acid sequence and immuno-cross-reactivity (24).

The amino acid sequence of IDH-I showed high identity (74.3 %) with that of the *E. coli* enzyme (27). Ser-113 in the *E. coli* enzyme (12, 44) has been identified at the site for phosphorylation by IDH kinase/phosphatase when the enzyme was inactivated in the cells grown on acetate as a sole carbon source. This residue was conserved in IDH-I at the same position; however, the specific activity of IDH-I in *Vibrio* sp. strain ABE-1 increased when this bacterium was grown on acetate (10). As seen in Fig. 5, this increase was accomplished at the transcriptional level but not by modification after translation. It is interesting to note that the nucleotide sequence in the *icdI* promoter region resembles that in the *E. coli ace*B promoter region (Fig. 15, A), which has been suggested to interact with IclR (45), a repressor for acetate-inducible *ace* operon. Such a characteristic sequence is not present in the promoter region on *E. coli icd*. The amount of the *icdI* transcript but not of *icdII* was increased when *E. coli* transformant harboring pIS102 or pIS202 were grown in acetate minimal medium (Fig. 12). These results indicate that

icdI and *icdII* were regulated differently at the transcriptional level in *E. coli*. However, the specific activity of IDH-I expressed in *E. coli* cells grown on acetate was reduced to 45% of that in the glucose-grown cells (Fig. 14). As purified IDH-I was phosphorylated by incubating the enzyme with [γ-^{32}P]ATP and cell extract from *E. coli*, but not from *Vibrio* sp. strain ABE-1 (Fig. 13), the expressed IDH-I might be inactivated by phosphorylation at Ser-113, as proposed for the inactivation of *E. coli* IDH (12). These results suggest that a functional IDH kinase/phosphatase (46), which can phosphorylate IDH-I, is absent in *Vibrio* sp. strain ABE-1. The lack of IDH kinase/phosphatase resulted in about a 10-fold increase in the level of IDH-I activity in acetate-grown *Vibrio* sp. strain ABE-1 cells compared with that in succinate-grown cells (27, 47). Since the level of IDH-II is not affected by shifting the carbon source in the medium (Fig. 12), total IDH activity in *Vibrio* sp. strain ABE-1 is increased when the cells are grown on acetate (10, 47). Furthermore, the levels of isocitrate lyase and malate synthase, which are key enzymes for glyoxylate shunt (48), are markedly increased in *Vibrio* sp. strain ABE-1 by culturing the cells on acetate-containing medium (47). As s consequence, glyoxylate shunt would be operative without reducing the rate of the tricarboxylic acid cycle in these cells (Fig. 16). Elucidation of the physiological significance of active operation of the two metabolic pathways for the utilization of acetate in *Vibrio* sp. strain ABE-1 must await further analyses.

On the other hand, the transcriptional level of *icdII* encoding IDH-II, which is a thermolabile monomeric enzyme, was increased by lowering

the growth temperature (Fig. 8). This increase was not due to the instability of the mRNA, because the half-life of *icdII* mRNA estimated at a low (15 °C) or high (37 °C) temperature was essentially the same as that of *icdI* mRNA (Fig. 11). Thus, it can be concluded that *icdII* is a low-temperature-inducible gene. Although the mechanism of activation of *icdII* transcription at a low temperature is not known at present, a nucleotide sequence, 5'-CCAAT-3', which has been proposed to be a common motif of several low-temperature-inducible promoters of *E. coli* (49), is also present in the promoter region of *icdII* (Fig. 15, B). Therefore, the expression of *icdII* at a low temperature in *E. coli* might be activated by binding of the *E. coli* low-temperature enhancer, CS7.4 (50), to the promoter region of the *icdII* gene in *trans*. A similar mechanism may be operative in *Vibrio* sp. strain ABE-1, because the level of *icdII* mRNA was increased by lowering the growth temperature for this bacterium (Fig. 8).

Catalytic efficiency expressed by the ratio of V_{max}/K_m is a kinetic parameter to evaluate the physiological role of enzyme. When the ratio was calculated with K_m values for isocitrate, purified IDH-I and IDH-II gave values of 0.0406 and 1.017, respectively, at 4 °C (9). This suggests that IDH-II is about 25-fold more active than IDH-I at low temperatures. When the ratio was calculated with K_m values for $NADP^+$, IDH-II showed about a 70-fold-higher potential than IDH-I at a low temperature. These features of IDH-I and IDH-II may indicate that IDH-II is an enzyme which has a greater degree of adaptation to low temperatures than IDH-I. Thus, acquisition by *icdII* of the low

temperature inducible promoter might have facilitated the adaptation of *Vibrio* sp. strain ABE-1 to low-temperature growth.

CHAPTER 2

Low-temperature inducible *icdII* gene and its implications in the bacterial adaptation to low-temperature

SUMMARY

The expression in *Escherichia coli* of the *icdII* gene encoding a psychrophilic bacterium, *Vibrio* sp. strain ABE-1, isocitrate dehydrogenase isozyme-II, was found to be regulated by two independent *cis*-elements. One is the suppressor, which was located in the 35 base pair region of 560 bases upstream from the transcriptional start site for *icdII*. The other is a nucleotide sequence, 5'-CCAAT-3', locating at 2 bases upstream of the -35 promoter region of *icdII*, that is the essential factor for the low-temperature dependent induction of *icdII*. By deleting the suppressor, the both transcriptional and translational levels of *icdII* at low temperature (15 °C) was increased about 20-fold. Whereas the expression level of *icdII* at high temperature (37 °C) was not changed by deletion of the suppressor. A *trans*-factor, which specifically binds the suppressor sequence, was detected by gel shift assay using crude extracts prepared from *E. coli* cells cultured at 15 or 37 °C. On the other hand, no such a *trans*-factor was detected in *Vibrio* sp. strain ABE-1. The low-temperature dependent induction of *icdII* was reduced about 10-fold by deleting the CCAAT sequence. While the expression

level of the gene at high temperature (37 °C) was not affected by the deletion. Thus the both sequence, suppressor and CCAAT, act as the *cis*-elements for the transcription of *icdII* only at the low-temperature. The growth rate of *E. coli* transformants at low-temperature was depended on the expression level of *icdII*. For instance, the doubling time of *E. coli* transformants harboring the plasmid carrying the suppressor sequence deletion *icdII* was 6.8 h, and that of those harboring the plasmid carrying the vector control was 11.3 h. Furthermore, *icdII* was found to confer the ability to grow at low-temperature on *E. coli*. Although the *E. coli* cells were not able to form colony on an agar-plate of synthetic medium at 10 °C, the *E. coli* transformants carrying *icdII* could form colonies on the plate when the medium was supplemented with yeast extract. Supplementation with azolectin or casamino acid had no effect on the growth of the transformants.

INTRODUCTION

The heat shock response including cold shock one has well been characterized among prokaryotes and eukaryotes. All organisms examined produce some specific proteins in response to elevated temperatures as well as to a variety of many other stress factors such as anoxia, ethanol, or heavy metal ions (51-56). Up to the present as yet, the heat and cold shock responses in *Escherichia coli* has been well characterized. When cultures of *E. coli* cells are transferred from 30 °C to 42 °C, 17 heat shock proteins are expressed transiently. On the other hand, as for a response to cold temperatures, *E. coli* cell cultures shifted from 37 °C to 10 °C showed the induction of 13 cold shock proteins, which were none of heat shock proteins (57-61). Almost all the cold shock proteins indicated an approximate 10-fold induction of synthesis after the shift to cold temperature. The induction of all of these proteins is initiated during the lag phase of growth that follows the temperature downshift. Some of the cold shock proteins were recently identified proteins CS7.4 (50) and H-NS (63). One of them, CS7.4, the major cold shock protein of *E. coli*, is a hydrophilic protein with a molecular mass of 7,402 Da. The gene for this major cold shock protein has been cloned and sequenced, however, no direct function could be assigned because its interruption was not carried out yet. The initial hypothesis accounting the low-temperature induction that CS7.4 (encoded by *cspA*) protects cells from damage caused by growth of large ice crystals during freezing (58, 60), like low-temperature-induced

proteins, BN115, BN28, and Hsp90 in an eukaryotes, *Brassica napus* (68, 75, 76). While, a DNA binding property has been proposed for CS7.4 because of its strong similarity to conserved regions of eukaryotic DNA binding proteins (62). The major cold shock protein of *E. coli*, CS7.4, was recently found to act as a cold shock transcriptional enhancer that specifically recognizes and binds to the promoter region of the *hns* gene regulating the expression of the nucleoid protein, H-NS (63). However, the exact mechanism by which CS7.4 activates transcription remains unclear. The mechanism of transcriptional enhancement of the *hns* gene expression was suggested by specific recognition at a nucleotide sequence of 5'-CCAAT-3' locating in the promoter region of *hns*. It is expounded that CS7.4 protein binds just in front of the promoter-bound RNA polymerase consequently the expression activity is enhanced. Thus, homology between prokaryotic (CS7.4) and eukaryotic transcription factors (C/EBP family, 77-79) may indicate a novel structural motif facilitating DNA-protein interactions. However, molecular mechanism of the low-temperature-dependent expression of the genes of psychrophilic bacteria has not been reported yet.

In Chapter I, I demonstrated that *icdII* is a low-temperature inducible gene and a sequence of CCAAT is also present in its promoter region. In contrast to mesophilic bacteria such as *E. coli*, psychrophilic bacteria are permanently exposed to low-temperature. Thus they must have adapted much more degree to low-temperature than mesophiles. From the results described in Chapter 1, it can be suggested that acquisition of a set of low-temperature adapted enzyme and low-temperature inducible

promoter is one of the advantageous strategy for survival of psychrophilic bacteria at low-temperature. In order to investigate whether this is true, I analyzed the effects of successive deletion of the upstream noncoding region of the *icdII* on its expression in *E. coli*, and examined whether *E. coli* acquires the ability to grow at low-temperature by transforming with *icdII*.

MATERIALS AND METHODS

Materials. Sources of all restriction endonucleases and marker nucleotides used in this study were the same as described in Chapter 1. AmpliTaq DNA polymerase was the product of Perkin Elmer (CA, USA). A Kilo-Sequence deletion kit, exonuclease III, and mung bean nuclease were purchased from Takara Shuzo (Kyoto, Japan). Mineral oil (light white oil) for PCR and azolectin [L-α-phosphatidylcholine (L-α-Lecithin), type II-S: from soybean] were obtained from Sigma (MO, USA). [γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from ICN Biomedical Inc. (CA, USA). Hybond-N^+ (positively charged nylon membrane) was the product of Amarsham International (England). Hybridization bag was product of GIBCO BRL (MD, USA). Poly-deoxy-inosinic-deoxy-cytidylic-acid [poly d(I-C)]was obtained from Boehringer Mannheim (Germany). Polyethylene glycol mono-*p*-isooctylphenyl ether (Triton X-100) was product of Nacalai tesque Inc. (Kyoto, Japan). All other reagents were of analytical grade.

plasmids. The plasmids used in this study are shown in Fig. 17. pMS26, pMS92, pMSF6, pMSF7, pMSF8, pMS42, and pMS77 were constructed from pIS202. pMS26, pMS92, pMS42, and pMS77 were used for determination of nucleotide sequence of *icdII* upstream noncoding flanking region of 1572 bases in length, while the other plasmids were used to study expression of *icdII* in *E. coli*, The plasmids pMF8C, pMSF8S, pMS42C, and pMS42S were products of PCR as described in PCR of Materials and Methods (Fig. 22)

Deletions of the upstream noncoding region of the *icdII* gene. Overlapping deletions were generated by the method of Henikoff (66), and by using Kilo Sequence Deletion Kit (Takara Shuzo, Kyoto, Japan). Double-stranded template DNA for sequencing was purified from *E. coli* XL1-blue (stratagene) cells harboring with one of the plasmids carrying the deleted gene. The *E. coli* cells were grown in LB for overnight at 37 °C, and DNA was prepared by the method of alkaline lysis and ultra-centrifugation through a CsCl density gradient (28). The DNA samples were dialyzed exhaustively against $T_{10}E_1$ (10 mM Tris-HCl [pH 8.0] containing 1 mM EDTA) and used as templates. Alternatively, some DNA samples were purified with a Wizard minipreps DNA purification system (Promega, WI, USA). All nucleotide sequences were determined by the cycle sequencing method by using -21M13 and M13 reverse dye-primer with ABI 373 DNA Sequencer (Applied Biosystems Inc., CA, USA). The sequences were analyzed and compared by using the computer programs of Genetyx (Software Development Co., Tokyo, Japan).

Northern (RNA) blot analysis. Total RNAs were extracted from *Vibrio* sp. strain ABE-1 cells and *E. coli* DEK2004 cells, which contained each pIS, pMS, and pMSF plasmids. *Vibrio* sp. strain ABE-1 was grown in nutrient medium at 15 °C, while *E. coli* transformants were grown in LB medium at 15, 25, or 37 °C. The extracted RNAs were purified by a single-step RNA isolation method (AGPC method) as described previously (31). Procedures for agarose gel electrophoresis, transfer to membrane, hybridization and washing membranes were the

same as described in Materials and Methods of Chapter 1.

Gel shift assay *Vibrio* sp. strain ABE-1 and *E. coli* DEK2004 harboring pMS77 were cultured in nutrient medium or LB, respectively, as described in Materials and Method of Chapter 1. Sonic extract was prepared as described previously. The crude extract was used to examine *trans*-factor(s) which is able to bind the promoter region of *icdII*. A 91 bp *Dra*I-*Spe*I fragment from the pIS202 was used as a probe (Fig. 20). The probe was 5' end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. DNA-protein binding assay was carried out in the mixture of total volume of 18 µl containing 20 µg of total proteins, 10 µl of solution D (20 mM Hepes-KOH [pH 7.9], 100 mM KCl, 20% glycerol, 0.5 mM DTT, and 0.5 mM PMSF), 2 µl of 10 mM $MgCl_2$, 2 µl of 125 mM spermidine, 3 µg of poly[d(I-C)], and 1 ng of end-labeled probe (ca. 1 kBq, 5-10 kcpm). After incubating the mixture for 1 h at 15 °C, aliquot of the mixture was subjected to 4% polyacrylamide gel electrophoresis in a buffer containing 6.7 mM Tris-HCl (pH 7.9), 1 mM EDTA, and 3.3 mM sodium acetate. Non radiolabeled probe and herring DNA were used as competitor DNA and nonspecific DNA, respectively. Autoradiogram was obtained by drying the gel at 80 °C for 2 h and then exposed to a X-ray film at -80 °C for appropriate time.

PCR (polymerase chain reaction). To mutate the promoter region of *icdII* gene, I synthesized the mutated gene by PCR method. The DNAs from plasmid pMSF8 and pMS42 were used as templates for the reaction. Two pairs of 30-mer synthetic oligonucleotides were obtained (Takara Shuzo, Kyoto, Japan): the one is for deleting a sequence CCAAT

locating at 2 bases upstream from -35 region (-42 to -38); for this purpose, 5'-AATTTATAGGGTTTGGTAAGTTTTCGAACT-3' at nucleotide positions -37 to -8 from transcriptional start site of *icdII* (Fig. 18) and 5'-CCTACACAATATTTCTAAAAAACACTTAGA-3', complementary to the sequence at nucleotide positions -43 to -72, were synthesized: and the other is for deleting a sequence, which seems to construct a stem-loop like structure, locating at +43 to +86 from transcriptional start site of *icdII*; for this purpose, 5'-GGAATTTCAATGAGCACTGATAACTCAA AA-3' at nucleotide positions +87 to +116 and 5'-CGACCCTCATGTTC GGTAATTGAACAAATC-3', complementary to the sequence at nucleotide positions +42 to +13, were synthesized. These oligonucleotides were used as primer for PCR. Thermal cycler and the program for PCR were used with Gene Amp PCR System 2400 (Perkin Elmer, CA, USA). Fifty μl of the reaction mixture, which contains each 1.75 μl of 10 mM dATP, dCTP, dGTP, and dTTP, each 1 μl of 1 μM downstream primer and upstream primer, and 5 μl of 10 X PCR buffer [500 mM Tris-HCl (pH 9.2), 140 mM $(NH_4)_2SO_4$, 22.5 mM $MgCl_2$] containing 1 ng of template DNA and 3.5 unit of AmpliTaq DNA polymerase, was used for following thermal cycles; denaturation of template at 94 °C for 2 min followed by 10 times repeat of 1st cycle [94 °C for 10 sec, 65 °C for 30 sec, and 68 °C for 5 min], 20 times repeat of 2nd cycle [94 °C for 10 sec, 65 °C for 30 sec, 68 °C for 5 min plus cycle elongation of 20 sec for each cycle. The products were treated by T4 polynucleotide kinase and then self-ligated by Ligation Kit. The ligated products which lost the sequences of CCAAT and stem-loop like

structure were designated as pMSF8C, pMSF8S, and pMS42C, pMS42S, respectively. (Fig. 22). *E. coli* DEK2004 was transformed with either plasmid.

Growth characteristics of *E. coli* transformants. *E. coli* DEK2004 was transformed with each pIS202, pMSF8, pMS42, or pSS512. *E. coli* XL1-Blue transformed with pBluescript KS^+ was used as negative control. The transformants were precultured in LB medium containing 50 μg of ampicillin and 15 μg of tetracycline per ml at 37 °C for overnight, and inoculated to a fresh LB medium and cultured at 15 °C with vigorously shaking. The growth of the cells were monitored by measuring the absorbance at 550 nm. The growth of the *E. coli* transformants on agar-plate was also examined at 10 °C. The transformants were precultured as described above. The precultured cells were inoculated onto an agar-plate of MOPS or LB medium. MOPS medium contained 50 μg/ml of ampicillin, 15 μg/ml of tetracycline, and 100 nM thiamine. Glucose (50 mM) or succinate (100 mM) was used as a sole source of carbon and energy. To examine auxotrophic phenotype of the *E. coli* transformants at 10 °C, MOPS medium was supplemented with each yeast extract (0.05%, w/v, Difco, MI USA), casamino acid (0.2%, w/v, Difco), or azolectin (0.05%, w/v, Sigma). For dispersion of azolectin, Trition X-100 was added to the medium at a final concentration of 0.5% (v/v). The concentrations of bases and vitamins used as a cofactor for the growth of the transformants at 10 °C were as follows: thiamine hydrochloride (Wako pure chemical industries, Ltd., Osaka, Japan), 0.1 mg/l; nicotinic acid amide

(niacinamide, Wako), 0.1 mg/l; riboflavin (Nacalai tesque Inc., Kyoto, Japan), 0.5 mg/l; pyridoxine hydrochloride (Nacalai), 0.1 mg/l; calcium-D(+)-pantothenate (Nacalai), 0.1 mg/l; biotin (Nacalai), 1 μg/l; *p*-aminobenzoic acid (Nacalai), 0.1 mg/l; choline (Nacalai), 2 mg/l; inositol (Wako) 1 mg/l; adenine (Wako), 10 mg/l; xanthine (Nacalai), 10 mg/l; hypoxanthine (Wako), 10 mg/l; guanine (Sigma), 10 mg/l; thimine (Nacalai), 10 mg/l; uracyl (Wako), 10 mg/l; and/or cytosine (Wako), 10 mg/l.

Enzyme assay. The bacterial cells in mid-logarithmic phase of the growth were harvested, and disrupted with a Bronson Sonifier 250 (CT, USA) in the buffer containing 20 mM Tris-HCl [pH 7.5], 250 mM NaCl, 2 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. The supernatant was obtained by centrifugation (10,000 X g, for 20 min, at 4 °C), and used as a crude extract. IDH activity and the protein concentration were determined as described in Materials and Methods of Chapter 1. One unit of the enzyme activity was defined as the amount of the enzyme capable of catalyzing the reduction of 1 μmol of $NADP^+$ per min.

RESULTS

Nucleotide sequence of upstream noncoding region of *icdII*. In order to investigate whether the upstream noncoding region of *icdII* gene is responsible to low-temperature dependent expression of *icdII*, first I determined the nucleotide sequence from -1713 to -140 (corresponding to *Xba*I site and *Hin*dIII site, respectively, in Figs. 1 and 18) base, where the sequence has not been determined yet. The nucleotide sequence of this region is shown in Fig. 18. The G + C content of this region was 36.2 %, and was similar to those of the other sequenced regions of *Vibrio* sp. strain ABE-1. No unique sequence such as forming stem-loop structure, absolute tandem repeat, or reverse repeat was detected in this region. However, it was found that three ORF-like sequences were present in the region of -1334 to -1257, -1232 to -882, and -784 to -203 at nucleotide positions counted from transcriptional start site of *icdII*. Although these ORFs-like are corresponded to the peptides of 26, 117, and 194 amino acid residues, respectively, there found no homologue in the databases of nucleotide sequences and proteins.

Effects of deletions of the upstream noncoding region of *icdII* on the expression. To examine whether *cis*-element(s) acting on the low-temperature dependent expression of the *icdII* is present in the upstream noncoding region of the gene, I deleted the noncoding region successively from 5'-end in various lengths. Effects of deletion on the expression of the gene was determined by measuring the levels of mRNA and enzymatic activity in the transformants grown at different temperatures

(15, 25, and 37 °C). The expression level did not change significantly in the transformants harboring the successive deletion plasmids till pMS92 (Fig. 19, A). In my surprise, a dramatic increase in the level was observed when the transformants harboring deletion plasmid pMS42 were grown at 15 °C. Smaller but significant increase in the expression level was also observed when the transformants were grown at 25 °C, but no change was observed in the cells grown at 37 °C. These results suggest that a sequence containing in the upstream noncoding region of pMS92 may act as *cis*-element to suppress the low-temperature induction of *icdII*. To determine the nucleotide sequence, I prepared the successive deletion plasmids as shown in Fig. 17, and examined the transcriptional level by Northern blot analysis (Fig. 19, B). From these results, the sequence acting as a *cis*-element was found to be comprised of 35 bases of the following nucleotide sequence; 5'-GTTATACCATACGGAGCTTAATTCTTTACGTAACA-3', which is located at the position from -560 to -526 nucleotide residues. I referred this region to as F35-bp region.

Gel shift assay. As a next step towards understanding the low-temperature regulatory expression of *icdII*, I tried to examine whether the *trans*-acting-factor which specifically binds to F35-bp region is present in *E. coli* and *Vibrio* sp. strain ABE-1 cells. A 91 bp *Dra*I-*Spe*I fragment carrying the F35-bp region was used for gel shift assay with the crude extract from *E. coli* or *Vibrio* sp. strain ABE-1. The results are shown in Fig. 21. A single band which resulted from specific binding with the DNA probe was detected when the crude extract of *E. coli* grown at 15

°C was used. Similar observation was obtained by using the crude extract of *E. coli* grown at 25 or 37 °C (data not shown). However, no specific band was detected when the crude extract of *Vibrio* sp. strain ABE-1 was used. These results suggest that a factor(s) which specifically binds to F35-bp region is expressed in *E. coli* in regardless to the growth temperature, while *Vibrio* sp. strain ABE-1 does not have such a factor.

Upstream sequence, CCAAT, is required for low-temperature dependent induction of *icdII*. There are two unique nucleotide sequences in the promoter region of *icdII*. The one is a sequence, CCAAT, locating at 2 bases upstream of the -35 region of *icdII*. This sequence has been suggested to be a common motif of low-temperature inducible genes of *E. coli*. The other is a sequence of 44 bp locating between +43 and +86 nucleotide position. Judged from the characteristics of the sequence, this sequence seems to form a stem-loop like structure. To investigate whether these sequence are responsible to the low-temperature induction of *icdII*, I attempted to construct the expression plasmids that carry the gene from which the above unique sequences were specifically deleted. For this purpose, PCR techniques were used as described in Materials and Methods. The obtained PCR products are schematically represented in Fig. 22. pMSF8C and pMSF8S are the plasmids that they lost the sequences of CCAAT and 44 bp length, respectively. In addition, I constructed the plasmids, pMS42C and pMS42S, that they lost 35 bases from 5'-end of pMSF8C and pMSF8S, respectively. In this deleted region, there is the sequence

which act as a suppressor for the low-temperature-dependent induction of *icdII*. *E. coli* DEK2004 was used as a host for the transformation with these plasmids.

The expression level of the *icdII* gene was examined by Northern blot analyses (Fig. 23) and measuring the enzymatic activity of IDH-II (Table III). The levels of mRNA were greatly changed when the transformants were cultured at 15 °C. The mRNA level of the transformants harboring pMSF8C, which carry the gene specifically deleted CCAAT sequence, was reduced than those of the transformants harboring pMSF8 or pMSF8S, which carry the gene containing CCAAT sequence. Whereas the specific deletion of the sequence, which seems to form stem-loop structure, had no effect on the expression (compare the expression level of the transformants harboring pMSF8 with that of the transformants harboring pMSF8S). The effect of specific deletion of CCAAT sequence on the expression was much more clearly demonstrated when plasmids, pMS42, pMS42C, and pMS42S, which carry the gene containing no suppressor sequence of 35 bp, were used for the transformation. The expression level of the transformants harboring each of these plasmids was increased about 20-fold than that of the transformants harboring each of the corresponding plasmids. However, reduction in the mRNA level was observed again by specific deletion of CCAAT sequence. The pattern of the changes in the specific activity of IDH-II was almost parallel to that of the changes in mRNA level (TableIII). On the other hand, no change was observed in the mRNA level when the transformants were cultured at 37 °C. These results

clearly indicate that the sequence, CCAAT, is essential for the low-temperature dependent expression of *icdII*.

Enhanced low-temperature growth of *E. coli* by *icdII*. Previously I suggested that IDH-II of a psychrophilic bacterium, *Vibrio* sp. strain ABE-1, has a high catalytic activity at low-temperature, because this enzyme showed high V_{max}/K_m values *in vitro* at low-temperature. To evaluate the physiological role of IDH-II *in vivo*, *E. coli* DEK2004 or *E. coli* XL1-Blue was transformed with each plasmid pIS202, pMSF8, or pMS42. As shown in Fig. 22, pMSF8 and pMS42 are the deletion plasmids derived from pIS202. The *icdII* mRNA level in the transformants harboring pMS42 was higher about 20-fold than that in the transformants harboring pMSF8 or pIS202 (Table II), because, in the pMS42, the sequence of 35 bp *cis*-acting as a suppresser for the low-temperature dependent induction of *icdII* was deleted. While the expression level in the transformants harboring pMSF8 was almost same as that in the transformants harboring pIS202. The transformants harboring pMS42 were able to grow much faster (doubling time, 6.8 h) than those harboring vector control or pSS512 carrying *E. coli icd* gene (doubling time, 11.3 h). The growth rate of the transformants harboring pMSF8 showed the intermediary value (doubling time, 9.0 h). It is interesting to note that the growth rate was completely depended on the expression level of *icdII* in the transformants. These results clearly suggest that IDH-II plays an important role and confers the ability to grow at low-temperature on *E. coli*. To investigate further, I examined the growth characteristics of *E. coli* transformants on an agar-plate of

minimum medium at 10 °C, because it was reported that *E. coli* ML30, a representative strain of *E. coli*, can grow at about 7.8 °C on nutrient rich medium (64) but it ceases to grow at 10 °C on minimun medium (61, 65). The results are shown in Table IV and Fig. 25. All the transformants examined in this study could grow on a nutrient-rich medium, nevertheless the time period for the colony formation of the transformants harboring vector control required more several days than those of the transformants containing *icdII*. However, no transformants could grow on a minimum medium containing succinate as a sole source of carbon and energy. When the temperature for the culture was raised from 10 to 15 °C, all the transformants were able to form colonies again on the same medium. This result suggested that some cofactors other than IDH-II was also required for the growth of the transformants at 10 °C. To investigate the growth factors, the succinate minimum medium was supplemented with each casamino acid, azolectin or yeast extract as indicated in Table IV. As expected, the transformants harboring the plasmid carrying *icdII* were able to grow on the succinate plate supplemented with yeast extract but no visible colony was found on the plate inoculated with the transformants containing vector control. Supplementation with azolectin or casamino acid had no effect on the ability of the transformants to form colony. Furthermore, the time period required to form visible colony of the transformants harboring pMS42 was shorter than that of the transformants harboring pMSF8. Because the expression level of *icdII* in the former transformants was higher about 20-fold than that in the latter transformants, it can be

concluded that the ability to form colony at 10 °C is depended on the expression level of *icdII*. These results clearly indicate that IDH-II plays an essentially important role for *E. coli* to grow at 10 °C, and some other steps leading to vitamins or bases are cold-sensitive sites.

DISCUSSION

In the chapter 1, I reported that *icdII* of a psychrophilic bacterium, *Vibrio* sp. strain ABE-1, is a low-temperature inducible gene (82). The presence of low-temperature inducible genes were reported in some other bacteria (49-66) and plants (67-72), and it was known that the low-temperature inducible genes of plants have been related with the freezing and/or dehydration tolerance, while the physiological functions of the bacterial low-temperature inducible gene have been scarcely known exception of *cspA* encoding CS7.4 of *E. coli* (50). Although it is interesting to know the molecular mechanism how psychrophilic bacteria acquired the ability to grow at low-temperature, information on the low-temperature inducible gene of psychrophilic bacteria has not been available except for the *icdII* of *Vibrio* sp. strain ABE-1.

In this study (chapter 2), I attempted to investigate the molecular mechanism of the bacterial adaptation to low-temperature by characterizing the regulatory mechanism of the *icdII*. Because it is well known that transcriptions of many genes are regulated by binding of *trans*-factor(s) on *cis*-acting sequences which are located on the upstream noncoding region of the genes, first I determined the nucleotide sequence of the upstream noncoding region of *icdII* (Fig. 18). The upstream noncoding region was 1713 bp in length. In this entire region, there was no sequence homologous to those reported to act as *cis*-factor for the bacterial genes, although a sequence, CCAAT, which seems to be a common motif of the low-temperature inducible promoter of *E. coli*, was

present at 2 bases up of -35 region. Three ORFs-like sequences corresponding to 26, 117, and 194 amino acid residues, respectively, were found in this region, however, there found no homolog in data bases of nucleotide sequence and proteins. Other unique nucleotide sequence to form stem-loop structure, or promoter was not found. From these results, these ORF-like sequences seem not to have any physiological meaning. Interestingly, the transcriptional level of *icdII* was greatly increased when a deletion plasmid, pMS42, was used for transformation. Because this increase in the transcriptional level was observed only when the transformants were cultured at 15 °C, it can be concluded that the nucleotide sequence acting as a suppressor for the low-temperature dependent induction of *icdII* was present in the deleted region. From the detailed analyses of the successive deletion plasmids, I found that the crucial sequence was comprised of 35 bp and located at -560 to -525 nucleotide positions (Fig. 19, 20). I designated this sequence as F35-bp. This sequence was found to be unique because no significant matches were discovered in the DNA data bases, when this sequence was compared with other sequences of transcription factors of bacterial or vertebrate (73). When the crude extract from *E. coli* cells cultured at 15, 25, or 37 °C was incubated with the radiolabeled DNA probe containing F35-bp, a protein-DNA complex was formed. While there was no specific protein(s), which interact with the DNA probe, in the crude extract from *Vibrio* sp. strain ABE-1. These results suggest that protein(s) which can specifically bind to the F35-bp is expressed in a temperature independent manner in *E. coli*, but not in *Vibrio* sp. strain

ABE-1. Although a precise role of the protein-DNA interaction is not known at the present, the protein(s) may act as a *trans*-factor for the suppression of low-temperature dependent induction of *icdII*. Thus *E. coli*, which favors to grow at around 37 °C (74), tends to suppress the expression of the gene containing low-temperature inducible promoter. These findings raised a interesting question what is the signal for the low-temperature dependent induction. In this respect, it is interesting to note that a nucleotide sequence, CCAAT, which has been recently suggested to be a common motif for low-temperature inducible promoters of *E. coli*, is present 2 bp upstream from the -35 region of *icdII*. The *E. coli* transformants harboring expression plasmid, pMSF8C or pMS42C, from which the CCAAT sequence was specifically deleted, did not respond to the low-temperature induction of *icdII*. These results clearly indicate that the CCAAT sequence acts as a *cis*-element for the low-temperature dependent induction of *icdII*. The CCAAT sequence is known as a cis-element activating transcription of many eukaryotic genes including α1(I) and α2(I) collagen, albumin, major histocompateibility complex II, and β-action (83, 84). Accordingly, this sequence may be one of the widely distributed *cis*-element for regulation of gene expression from prokaryotes to eukaryotes. In *E. coli*, CS7.4, which is a major cold shock protein of *E. coli*, has been suggested to recognize the CCAAT sequence and activate the transcription, however, detailed mechanism of the low-temperature dependent expression of the genes is still obscure. Further work must be done to elucidate mechanism of the low-temperature dependent induction of the genes.

I previously suggested that acquisition of a set of low-temperature inducible promoter and the gene coding for low-temperature adapted enzyme which catalyzes a key step of metabolism is an important event in the bacterial adaptation to low-temperature. Indeed, the findings that *icdII* of *Vibrio* sp. strain ABE-1 conferes the ability to grow at 10 °C on *E. coli* support this hypothesis. Furthermore, the conferred ability was dependent on the expression level of *icdII*. For the growth of *E. coli* on a synthetic medium at 10 °C, supplementation of yeast extract to the medium was also required. These results suggest that the membrane fluidity of *E. coli* at 10 °C is similar state to that at higher temperature, and there are a few cold-sensitive steps which can be rescued by the addition of yeast extract.

Investigation of the molecular mechanism of the bacterial adaptation to low-temperature is just beginning, and there are many problems to be cleared before elucidation of the detailed mechanism, however, it may be concluded that the psychrophilic bacteria were derived from the mesophilic bacteria which could modify a relatively small number of enzymes to exhibit their catalytic activity at low-temperature. This is the great difference from the thermophilic bacteria which had to increase the stabilities of almost all enzymes at high temperature.

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REFERENCES

1. **Ingraham, J. L.** 1962. *In* The Bacteria (I. C. Gunsalus and R. Y. Stranier eds) **Vol. 3**, p. 256-296, Academic Press, London and New York
2. **Russel, N. J.** 1984. Mechanisms of thermal adaptation in bacteria: Blueprint for survival. Trends Biochem. Sci. **9:** 108-112.
3 **Herbert, R. A.** 1986. *In* Microbes in Extreme Environments (R. A. Herbert and G. A. Codd eds) p. 1-23, Academic Press, London and New York
4. **Ratlege, R., and S. G. Wilkinson** eds 1989. Microbial Lipids **Vol. 2.** Academic Press. Departments of Biochemistry and Chemistry, University of Hull, UK.
5. **Fulco, A. J.** 1974. Metabolic alterations of fatty acids. Annu. Rev. Biochem. **43:** 215-241.
6. **Erwin, J., and K. Bloch.** 1964. Biosynthesis of unsaturated fatty acids in microorganisms. Science **143:** 1006-1012.
7. **Fulco, A. J.** 1983. Fatty acid metabolism in bacteria. Prog. Lipid. Res. **22:** 133-160.
8. **Takada, Y., T. Ochiai, H. Okuyama, K. Nishi, and S. Sasaki.** 1979. An obligately psychrophilic bacterium isolated on the Hokkaido coast. J. Gen. Appl. Microbiol. **25**: 11-19.
9. **Ochiai, T., N. Fukunaga, and S. Sasaki.** 1979. Purification and some properties of two NADP-specific isocitrate dehydrogenase from an obligately psychrophilic marine bacterium, *Vibrio* sp. strain

ABE-1. J. Biochem. **86:** 377-384.

10. **Ishii, A., S. Imagawa, N. Fukunaga, S. Sasaki, O. Minowa, Y. Mizuno, and H. Shiokawa.** 1987. Isozymes of isocitrate dehydrogenase from and obligately psychrophilic bacterium, *Vibrio* sp. strain ABE-1: purification, and modulation of activities by growth conditions. J. Biochem. **102:** 1489-1498.
11. **Ochiai. T., N. Fukunaga, and S. Sasaki.** 1984. Two structurally different NADP-specific isocitrate dehydrogenases in an obligately psychrophilic bacterium, *Vibrio* sp. strain ABE-1. J. Gen. Appl. Microbiol. 30: 479-487.
12. **Thorsness, P. E., and D. E. Koshland, Jr.** 1987. Inactivation of isocitrate dehydrogenase by phosphorylation in mediated by the negative charge of the phosphate. J. Biol. Chem. **262:** 10422-10425.
13. **Howard, R. C., and R. R. Becher.** 1970. Isolation and some properties of the triphosphopyridine nucleotide isocitrate dehydrogenase from *Bacillus stearothermphilus*. J. Biol. Chem. **245:** 3186-3194.
14. **Chung, A. E., and J. E. Braginski.** 1972. Isocitrate dehydrogenase from *Rhodopseudomonas sphaeroides*: purification and characterization. Arch. Biochem. Biophys. **153:** 357-367.
15. **Miyazaki, K., H. Eguchi, A. Yamagishi, T. Wakagi, and T. Oshima.** 1992. Molecular cloning of the isocitrate dehydrogenase gene of the extreme thermophile *Thermus thermophilus* HB8. Appl. Environ. Microbiol. **58:** 93-98.

16. **Muro-Pastor, M. I., and F. J. Florencio.** 1994. $NADP^+$-isocitrate dehydrogenase from the cyanobacterium *Anabaena* sp. strain PCC7120: purification and characterization of the enzyme and cloning, sequencing, and disruption of the *icd* gene. J. Bacteriol. **176:** 2719-2726.

17. **Hurley, J. H., A. M. Dean, D. E. Koshland, Jr., and R. M. Stroud.** 1991. Catalytic mechanism of $NADP^+$-dependent isocitrate dehydrogenase: implications from the structures of magnesium-isocitrate and $NADP^+$ complexes. Biochemistry **30:** 8671-8678.

18. **Haselbeck, R. J., R. F. Colman, and L. McAlister-Henn.** 1992. Isolation and sequence of a cDNA encoding porcine mitochondrial NADP-specific isocitrate dehydrogenase. Biochemistry **31:** 6219-6223.

19. **Haselbeck, R. J., and L. McAlister-Henn.** 1991. Isolation, nucleotide sequence, and disruption of the *Saccharomyces cerevisiae* gene encoding mitochondrial NADP(H)-specific isocitrate dehydrogenase. J. Bio. Chem. **266:** 2339-2345

20. **Huh, T., J. Ryu, J. Huh, H. Sung, I. Oh, B. J. Sung, and R. L. Veech.** 1993. Cloning of a cDNA encoding bovine mitochondrial $NADP^+$-specific isocitrate dehydrogenase and structural comparison with its isozymes from different species. Biochem. J. **292:** 705-710.

21. **Jennings, G. T., S. Sechi, P. M. Stevenson, R. C. Tuckey, D. Parmelee, and L. McAlister-Henn.** 1994. Cytosolic $NADP^+$-

dependent isocitrate dehydrogenase: isolation of rat cDNA and study of tissue-specific and developmental expression. of mRNA. J. Biol. Chem. **269:** 23128-23134.

22. **Shorrosh, B. S., and R. A. Dixon.** 1992. Molecular characterization and expression of an isocitrate dehydrogenase from alfalfa (*Medicago sativa* L.). Plant Mol. Biol. **20:** 801-807.
23. **Udvardi, M. K., T. R. McDermott, and M. L. Kahn.** 1993. Isolation and characterization of a cDNA encoding $NADP^+$-specific isocitrate dehydrogenase from soybean (*Glycine max*). Plant Mol. Biol. **21:** 739-752.
24. **Fukunaga, N., S. Imagawa, T. Sahara, A. Ishii, and M. Suzuki.** 1992. Purification and characterization of monomeric isocitrate dehydrogenase with $NADP^+$-specificity from *Vibrio parahaemolyticus* Y4. J. Biochem. 112: 849-855.
25. **Chung, A. E., and J. S. Franzen.** 1969. Oxidized triphosphopyridine nucleotide specific isocitrate dehydrogenase from *Azotobacter vinelandii*: isolation and characterization. Biochemistry **8:** 3175-3184.
26. **Leyland, M. L., and D. Kelly.** 1991. Purification and characterization of a monomeric isocitrate dehydrogenase with dual coenzyme specificity from the photosynthetic bacterium, *Rhodomicrobium vannielii*. Eur. J. Biochem. 202: 85-93.
27. **Ishii, A., M. Suzuki, T. Sahara, Y. Takada, S. Sasaki, and N. Fukunaga.** 1993. Genes encoding two isocitrate dehydrogenase isozymes of a psychrophilic bacterium, *Vibrio* sp. strain ABE-1. J.

Bacteriol. **175:** 6873-6880.

28. **Sambrook, J., E. F. Fritsch, and J. Keller.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
29. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture media for enterobacteria. J. Bacteriol. **119:** 736-749.
30. **Case, C. C., S. M. Roels, J. E. Gonzalez, E. L. Simons, and R. W. Simons.** 1988. Analysis of the promoters and transcript involved in IS10 anti-sense RNA control. Gene **72:** 219-236.
31. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:** 156-159.
32. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitiation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248-254.
33. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13:** 319-353.
34. **Bennett, P. M., and W. H. Holms.** 1975. Reversible inactivation of the isocitrate dehydrogenase of *Escherichia coli* ML308 during growth on acetate. J. Gen. Microbiol. **87:** 37-51.
35. **Hurley, J. H., A. M. Dean, D. E. Koshland, Jr., and R. M. Stroud.** 1990. Regulation of an enzyme by phosphorylation at the active site. Science **249:** 1012-1016.
36. **Hurley, J. H., P. E. Thorsness, V. Ramalingam, N. H. Helmers,**

D. E. Koshland, Jr., and R. M. Stroud. 1989. Structure of a bacterial enzyme regulated by phosphorylation, isocitrate dehydrogenase. Proc. Natl. Acad. Sci. USA **86:** 8635-8639.

37. **Claude, J. C., D. Negre, A. Galinier, B. Duclos, G. Perriere, and A. J. Cozzone.** 1991. regulation of the acetate operon in *Escherichia coli*: purification and functional characterization of the IclR repressor. EMBO J. **10:** 675-679.

38. **Sunnarborg, A., D. Klumpp, T. Chung, and D. C. LaPorte.** 1990. Regulation of the glyoxylate bypass operon: cloning and characterization of *iclR*. J. Bacteriol. **172:** 2642-2649.

39. **Eikmanns, B. J., D. Rittmann, and H. Sahm.** 1995. Cloning, sequence analysis, expression, and inactivation of the *Corynebacterium glutamicum icd* gene encoding isocitrate dehydrogenase and biochemical characterization of the enzyme. J. Bacteriol. **177:** 774-782.

40. **Sahara, T., M. Suzuki, J. Tsuruha, K. Abe, Y. Takada, and N. Fukunaga.** 投稿中

41. **Chen, R. D., and P. Gadal.** 1990. Structure, function and regulation of NAD and NADP dependent isocitrate dehydrogenases in higher plants and in other organisms. Plant Physiol. Biochem. **28:** 411-427.

42. **Cupp, J. R., and L. McAlister-Henn.** 1992. Cloning and characterization of the gene encoding the IDH1 subunit of NAD^+-dependent isocitrate dehydrogenase from *Saccharomyces cerevisiae*. J. Biol. Chem. **267:** 16417-16423.

43. **Cupp, J. R., and L. McAlister-Henn.** 1991. NAD^+-dependent isocitrate dehydrogenase: cloning, nucleotide sequence, and disruption of the *idh2* gene from *Saccharomyces cerevisiae*. J. Biol. Chem. **266:** 22199-22205.

44. **Borthwich, A. C., W. H. Holms, and H. G. Nimmo.** 1984. Amino acid sequence round the site of phosphorylation in isocitrate dehydrogenase from *Escherichia coli*. FEBS Lett. **174:** 112-115.

45. **Negre, D., J. C. Claude, A. Galinier, P. Sauve, and A. J. Cozzone.** 1992. Specific interactions between the IclR repressor of the acetate operon of *Escherichia coli* and its operator. J. Mol. Biol. **228:** 23-19.

46. **Mimmo, G. A., and H. G. Nimmo.** 1984. The regulatory properties of isocitrate dehydrogenase phosphatase from *Escherichia coli* ML308. Eur. J. Biochem. **141:** 409-414.

47. **Ochiai, T., S. Yoshida, N. Fukunaga, and S. Sasaki.** 1988. growth and acetate utilization by a psychrophilic bacterium, *Vibrio* sp. strain ABE-1. J. Fac. Sci., Hokkaido Univ. Ser. V **14:** 115-124.

48. **LaPorte, D. C., P. E. Thorsness, and D. E. Koshland, Jr.** 1985. Compensatory phosphorylation of isocitrate dehydrogenase: a mechanism for adaptation to the intracellular environment. J. Biol. Chem. **260:** 10563-10568.

49. **Qoronfleh, M. W., C. Debouck, and T. Maniatis.** 1992. Identification and characterization of novel low-temperature-inducilble promoters of *Escherichia coli*. J. Bacteriol. **174:** 7902-7909.

50. **Jones, P. G., R. Krah, S. R. Tafuri, and A. P. Wolffe.** 1992. DNA gyrase, CS7.4, and the cold shock response in *Escherichia coli*. J. Bacteriol. **174:** 5798-5802.

51. **Craig, E. A., and C. A. Gross.** 1991. Is hsp70 the cellular thermometer? Trends Biochem. Sci. **16:** 135-140.

52. **Lindquist, S., and E. A. Craig.** 1988. The heat-shock proteins. Annu. Rev. Genet. **22:** 613-677.

53. **Miller, B. S., T. E. Kennedy, and U. N. Streips.** 1991. Molecular characterization of specific heat shock proteins of *Bacillus subtilis*. Curr. Microbiol. **22:** 231-236.

54. **Neidhardt, F. C., R. A. Van Boegelen, and V. Vaughn.** 1984. The genetics and regulation of heat shock proteins. Annu. Rev. Genet. **18:** 295-329.

55. **Streips, U. N., and F. W. Polio.** 1985. Heat shock proteins in bacilli. J. Bacteriol. **162:** 434-437.

56. **Wu, L., and N. E. Welker.** 1991. Temperature-induced protein synthesis in *Bacillus stearothermophilus* NUB36. J. Bacteriol. **173:** 4889-4892.

57. **Broeze, R. J., C. J. Solomon, and D. H. Pope.** 1978. Effects of low temperature on in vivo and in vitro protein synthesis in *Escherichia coli* and *Pseudomonas fluoresence*. J. Bacteriol. **134:** 861-874.

58. **Goldstein, J., N. S. Pollit, and M. Inouye.** 1990. Major cold shock protein of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87:** 283-287.

59. **Inouye, M.** 1991. Minor codon, cold shock protein, and protein folding, p. 95-104. *In* Perspectives on cellular regulation: from bacteria to cancer. Wiley-Less, Inc., New York.

60. **Jones, P. G., M. Cashel, G. Glaser, and F. C. Neidhardt.** 1992. function of a relaxed-like state following temperature down-shifts in *Escherichia coli*. J. Bacteriol. **174:** 3903-3914.

61. **Jones, P. G., R. A. Van Bogelen, and F. C. Nerdhardt.** 1987. Induction of proteins in response to low temperatures in *Escherichia coli*. J. Bacteriol. **169:** 2092-2095.

62. **Wistow, G.** 1990. Cold shock and DNA binding. Nature (London) **344:** 823-824.

63. **La Teana, A., A. Brandi, M. Falconi, R. Spurio, C. L. Pon, and C. O. Gualerzi.** 1991. Identification of a cold shock transcriptional enhancer of the *Escherichia coli* gene encoding nucleoid protein H-NS. Proc. Natl. Acad. Sci. USA **88:** 10907-10911.

64. **Shaw, M. K., A. G. Marr, and J. L. Ingraham.** 1971. Determination of the minimum temperature for growth of *Escherichia coli*. J. Bacteriol. **105:** 683-684.

65. **Ng, H., J. L. Ingraham, and A. G. Marr.** 1962. Damage and derepression in *Escherichia coli* resulting from growth at low temperatures. J. Bacteriol. **84:** 331-339.

66. **Henikoff, S.** 1987. Unidirectional digestion with exonuclease III in DNA sequence analysis. Methods Enzymol. **155:** 156-165.

67. **Singh, J. and A. Laroche.** 1988. Freezing tolerance in plants: a

biochemical overview. Biochem. Cell Biol. **66:** 650-657.

68. **Weretilnyk, E., W. Orr, T. White, B. Iu, and J. Singh.** 1993. Characterization of three related low-temperature-regulated cDNAs from winter *Brassica napus*. Plant Physiol. **101:** 171-177.
69. **Alberdi, M., and L. Cocuera.** 1991. Cold acclimation on plants. Phytochemistry **30:** 3177-3184.
70. **Chauvin, L., M. Houde, and F. Sarhan.** 1993. A leaf-specific gene stimulated by light during wheat acclimation to low temperature. Plant Mol. Biol. **23:** 255-265.
71. **Gilmour, S., N. Artus, and M. Thomashow.** 1992. cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. Plant Mol. Biol. **18:** 13-21.
72. **Laberge, S., Y. Castonguay, and L. Vezina.** 1993. New cold- and drought regulated gene from *Medicago sativa*. Plant Physiol. **101:** 1411-1412.
73. **Faisst, S., and S. Meyer.** 1992. Compilation of vertebrate-encoded transcription factors. Nucleic Acid Res. **19:** 3-26.
74. **Herendeen, S. L., R. A. Van Bogelen, and F. C. Neidhardt.** 1979. Levels of major proteins of *Escherichia coli* during growth at different temperatures. J. Bacteriol. **139:** 185-194.
75. **Krishna, P., M Sacco, J. F. Cherutti, and S. Hill.** 1995. Cold-induced accumulation of hsp90 transcripts in *Brassica napus*. Plant Physiol. **107:** 915-923.
76. **Boothe, J. G., M. D. de Beus, and A. M. Johnson-Flanagan.** 1995. Expression of a low-temperature induced protein in *Brassica*

napus. Plant Physiol. **108:** 795-803.

77. **Pang, J. H., and K. Y. Chen.** 1993. A specific CCAAT-binding protein, CBP/tk, may be involved in the regulation of thymidine kinase gene expression in human IMR-90 diploid fibroblasts during senescence. J. Biol. Chem. **268:** 2909-2916.

78. **Park, E. A., A. L. Gurney, S. E. Nizielski, P. Hakimi, Z. Cao, A. Moorman, and R. W. Hanson.** 1993. Relative roles of CCAAT/Enhancer-binding protein b and cAMP regulatory element-binding protein in controlling transcription of the gene for phosphoenolpyruvate carboxykinase (GTP). J. Biol. Chem. **268:** 613-619.

79. **Legraverend, C., P. Antonson, P. Flodby, and K. G. Xanthopoulos.** 1993. High level activity of the mouse CCAAT/enhancer binding protein (C/EBPα) gene promoter involves autoregulation and several ubiquitous transcription factors. Nucleic Acids Res. **21:** 1735-1742.

80. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. Bio. Techniques **4:** 376-379.

81. **Ishii, S., K. Kuroki, and F. Imamoto.** 1984. Met-tRNA-f2 gene in the reader region of the *nusA* operon in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81:** 409-413.

82. **Suzuki, M., T. Sahara, J. Tsuruha, Y. Takada, and N. Fukunaga.** 1995. Differential expression in *Escherichia coli* of

the *Vibrio* sp. strain ABE-1 *icdI* and *icdII* genes of encoding structurally different isocitrate dehydrogenase isozymes. J. Bacteriol. **177:** 2138-2142.

83. **Hooft van Huijsduijnen, R., X-Y. Li, D. Black, H. Matthes, C. Benoist, and D. Mathis.** 1990. Co-evolution from yeast to mouse: cDNA cloning of the two NF-Y (CP-1/CBP) subunits. EMBO J. **9:** 3119-3127.

84. **Maity, S. N., P. T. Golumbek, G. Karsenty, and B. De-Grombrugghe.** 1988. Selective activation of transcription by a novel CCAAT binding factor. Science **241:** 582-585.

TABLE I. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)	Reference or source
Vibrio ABE-1	a psychrophilic bacterium	(8)
E. coli		
K-12	wild type	
XL1-Blue	*recA*1, *lac*$^-$, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44	Stratagene
	*relA*1, (F' *proAB*, *lacIq*, *lacZ*ΔM15, Tn10)	(80)
DEK2004	*icd*$^-$, *trp*$^-$, *recA*$^-$, and tetracycline resistant	(12)
Plasmids		
pBluescript KS$^+$ or SK$^+$	*ori* ColE1, and ampicillin resistant	Stratagene
pIS102	*Vibrio* ABE-1 *icdI* gene cloned into the *Eco*RV-*Xba*I sites of pBluescript KS$^+$	This study
pIS202	*Vibrio* ABE-1 *icdII* gene cloned into the *Xba*I-*Sac*I sites of pBluescript KS$^+$	This study
pSS512	*E.coli icd* gene derived from pTK512 (12) was cloned into the *Eco*RI-*Hin*dIII sites of pBluescript SK$^+$.	This study
pMS and pMSF series	*icdII*-upstream deletion plasmids derived from pIS202	This study

TABLE II. Effect of the deletion of *icdII*-upstream flanking region on the expression level of IDH-II activity in *E. coli* DEK2004.

Deletion plasmid	Specific activity (units/mg of protein)
pIS202	0.125
pMS26	0.129
pMS92	0.119
pMSF6	0.101
pMSF7	0.119
pMSF8	0.112
pMS42	2.01
pMS77	2.86

Temperature for assay of IDH-II activity was 20 °C, and specific activity was expressed as means of three experimental values. Crude extracts were prepared from the mid-logarithmic phase cells of *E. coli* DEK2004 harboring deletion plasmid of pIS202 (a series of pMS). The transformants were cultured in LB media at 15 °C.

TABLE III. Effects of the deletion of CCAAT and stem-loop like region from *icdII*-promoter region on the expression level of IDH-II activity in *E. coli* DEK2004.

Plasmid	Specific activity (units/mg of protein)
pMSF8	0.112
pMSF8C	0.0355
pMSF8S	0.125
pMS42	2.01
pMS42C	0.277
pMS42S	2.18

Temperature for assay of IDH-II activity was 20 °C, and specific activity was expressed as means of three experimental values. Crude extracts were prepared from the mid-logarithmic phase cells of *E. coli* DEK2004 harboring each plasmid of PCR products. The transformants were cultured in LB media at 15 °C.

TABLE IV. Growth of *E. coli* transformants

	Temperature (°C)					
	10	10	10	10	10	15
Medium	LB	MOPS-succinate	MOPS-succinate	MOPS-succinate	MOPS-succinate	MOPS succinate
Strain/plasmid			yeast extract (0.05%)	azolectin (0.05%)	casamino acid (0.2%)	
XL1-Blue/pMSF8	++	-	+	-	-	++
DEK2004/pMSF8	++	-	+	-	-	++
XL1-Blue/pMS42	++	-	++	-	-	++
DEK2004/pMS42	++	-	++	-	-	++
XL1-Blue/pBS(KS^+)	+	-	-	-	-	+
DEK2004/pBS(KS^+)	+	-	-	-	-	-

The cells precultured in a LB medium at 15 °C for 2 days were inoculated onto a agar plate. The plates were incubated at the indicated temperature for 2 weeks. ++, the colonies were visible within 6 days; +, the colonies were not visible till 9 days after the inoculation. 100 mM succinate were added to media as a sole carbon source.

LEGENDS TO FIGURES

Fig. 1. Mapping of the genomic clones containing the *icdI* and *icdII* genes. Arrows represent the open reading frames of the *icdI* and *icdII* genes. Some restriction sites are distinguished by letters indicated in parentheses. The restriction fragments used in construction of plasmids (pIS102 and pIS202) are shown in lines below the restriction maps.

Fig. 2. Restriction map of recombinant plasmids pIS102, pIS202, and pSS512. pIS102, pIS202, and pSS512 are the plasmids carrying *icdI*, *icdII*, and *E. coli icd* genes, respectively. They all express resistance to ampicillin. Boxes inside the circles indicate the inserted genes fragments in pBluescript. Bars indicate restriction endonuclease cleavage sites used in cloning and preparing fragments of probes as described in Materials and Methods. Amp^r, ampicillin resistance; ORF, open reading frame; *ori*, f1 phage and colE1 origin.

Fig. 3. Primer extension analysis. Primer extension products employing primers complementary to *icdI* and *icdII* are shown in lanes 1 and 6, respectively. RNA (600 μg) used in these experiments was obtained from *Vibrio* sp. strain ABE-1 cells grown in peptone-meat extract medium. The sequencing ladders of the DNAs obtained by using the same primers are shown in lanes 2 to 5 and 7 to 10. Arrows indicate the transcriptional initiation sites.

Fig. 4. Transcriptional start points of *icdI* and *icdII*. Upper panel and lower panel show nucleotide sequences of upstream of the *icdI* and *icdII* genes, respectively. Transcriptional starting points were indicated by arrows (+1). Putative promoter sequences, putative ribosome-binding sites, and translational starting points, are underlined (-35 and -10), doubly underlined, and boxed (ATG), respectively.

Fig. 5. Effects of acetate on transcriptional level of *icdI* and *icdII* genes in *Vibrio* sp. strain ABE-1. Total RNAs (10 μg per lane) extracted from *Vibrio* sp. strain ABE-1 cells grown on peptone-meat extract (lane 1), succinate (lane 2), or acetate (lane 3) were electrophoresed in 1.5 % agarose gel containing 0.66 M formaldehyde. After blotting the RNAs onto a nylon membrane, hybridization was performed by using the radio labeled *Sac*I-*Sac*I DNA fragment of *icdI* (A) or the *Hin*dIII-*Eco*RI DNA fragment of *icdII* (B) as described in Materials and Methods. Position of 23S and 16S rRNAs are shown between the panels.

Fig. 6. Western blot analysis of polypeptides present in the sonic extract prepared from *E. coli* cells transformed with a plasmid bearing either *icdI* or *icdII*. (Left) Purified IDH-I, 0.2 μg of purified IDH-I protein; pBS(KS^+), pBluescript KS^+-transformed cell control (10 μg of protein); pIS102, pIS102-transformed cells (10 μg of protein). (Right) Purified IDH-II, 0.2 μg of purified IDH-II protein; pBS(KS^+), pBluescript KS^+-transformed cell control (10 μg of protein); pIS202, pIS202-transformed cells (10 μg of protein). Anti-IDH-I and anti-IDH-

II antibodies were used for left panel and right panel, respectively. The cells used at left and right panels cultured at 37 °C and 15 °C, respectively. Molecular mass standards are shown to the left of the panel.

Fig. 7. Effects of temperatures on the complementation of *E. coli* IDH defective mutant by *Vibrio* sp. strain ABE-1 *icdI* or *icdII*. *E. coli* DEK2004 (IDH defective mutant) harboring pBluescript KS^+ (A, D), pIS102 (B, C), or pIS202 (C, F) were cultured in MOPS medium in the presence (●) or absence (○) of 0.5 mM glutamate at 15 °C (A, B, and C) or 37 °C (D, E, and F). Preculture of the all bacterial strains was carried out in LB medium for overnight at 37 °C. The growth of the cells were monitored by measuring absorbance at 550 nm.

Fig. 8. Effects of temperatures on the transcripts of *Vibrio* sp. strain ABE-1 *icd* genes in *E. coli*. The upper panels show Northern blots of RNAs probed with a radiolabeled *icdI* fragment, and the lower panels show blots of RNAs probed with a radiolabeled *icdII* fragment. (Left) *E. coli* DEK2004 harboring either pIS102 (upper panel) or pIS202 (lower panel) was cultured at different temperatures as indicated, and total RNA was extracted and analyzed. 6 and 20 μg of the *E. coli* RNAs were used for detection of *icdI* and *icdII* mRNAs, respectively. (Right) Levels of *icdI* and *icdII* mRNAs in *Vibrio* sp. strain ABE-1 RNAs (3 μg) extracted from cells grown at different temperatures as indicated.

Fig. 9. Effects of temperatures on the levels of IDH-II protein in *E. coli*.

Western blot analysis was carried out with following samples: 20 μg of polypeptides in the sonic extracts prepared from *E. coli* DEK2004 cells harboring pIS202 (lane pIS202) or pBluescript SK^+ (lane pBS) cultured at 15 °C or 37 °C, and 0.2 μg of purified IDH-II. After separation of polypeptides by SDS-polyacrylamide gel (7.5 %) electrophoresis, the polypeptides were transferred to a Hybond C super (a nitrocellulose membrane), and subjected to Western blot analysis using with anti-IDH-II antibody and ECL detection system (Amersham).

Fig. 10. Effects of rifampin on the growth of *Vibrio* sp. strain ABE-1 and *E. coli* DEK2004. *Vibrio* sp. strain ABE-1 (*Vibrio* ABE-1, left panel) was cultured in a nutrient medium at 15 °C, while *E. coli* DEK2004 (right panel) was grown in LB medium at 37 °C. When the bacterial growth reached to logarithmic phase (*Vibrio* ABE-1: for 12 h, A_{600}=0.7, *E. coli* DEK2004: for 3 h, A_{550}=0.4), rifampin was added at concentration of 200 μg/ml to block further initiation of transcription (arrows of each panel). The bacterial growth was monitored by measuring turbidity at 600 nm (for *Vibrio* sp. strain ABE-1) or 550 nm (for *E. coli*). Closed circles and open circles indicate the bacterial growth in the presence or absence of rifampin, respectively.

Fig. 11. Stabilities of *icdI* and *icdII* mRNA. A: Northern blots of mRNAs; upper and lower panels show the stabilites of *icdI* and *icdII* mRNAs, respectively. RNAs were isolated from the cells at the indicated times after treatment with rifampin (200 μg/ml) as described in

Materials and Methods. RNAs (3 μg for *icdI* mRNA and 20 μg of *icdII* mRNA) from *E. coli* DEK2004 harboring either pIS102 (upper panels) or pIS202 (lower panels) and grown at 15 or 37°C were subjected to Northern blot analysis. LB medium was used for cultivation of the bacterial cells. Probes for *icd*-mRNAs were the same as those described in the legend to Fig. 5. *Vibrio* ABE-1, RNA (3 μg) from *Vibrio* sp. strain ABE-1 control. B: Plots of mRNA levels against time after addition of refampin; intensity (In %) of radiolabel in hybridizing bands of *icdI*(●), and *icdII* (○) mRNAs, at 15 and 37 °C, respectively. Rifampin was added at 0 time.

Fig. 12. Induction of *icdI* expression in *E. coli* by acetate. *E. coli* DEK2004 harboring either pIS102 or pIS202 was cultured at 37 °C in MOPS medium supplemented with 0.5 % glucose for 4 h; then cells collected from half of the culture were transferred to MOPS medium supplemented with 2 % acetate and were allowed to continue to grow. RNAs were isolated from the cells before and after the shift of medium and analyzed on Northern blots probed with a radio-labeled *icdI* gene fragment as described in Materials and Methods. *E. coli* K-12 was used as a control to monitor the levels of *E. coli icd* mRNA. (Left) RNAs from cells grown in glucose-MOPS medium for 2 and 4 h (left to right, respectively). (Right) RNAs from the cells 0, 0.5, 1, and 2 h after the shift to acetate (left to right, respectively).

Fig. 13. *In vitro* phosphorylation of purified IDH-I. Purified IDH-I

incubated with [γ-^{32}P]ATP in the presence of cell-free extract from *E. coli* DEK2004 or *Vibrio* sp. strain ABE-1 was analyzed by SDS-PAGE and autoradiogram. Details of the reaction conditions for the phosphorylation were described in Materials and Methods. Left panel and right panel are SDS-PAGE stained with Coomassie Brilliant Blue and autoradiogram, respectively. IDH-I, purified IDH-I (0.2 μg); Marker, marker proteins; *E. coli*, cell-free extract of *E. coli* DEK2004; *E. coli* + IDH-I, purified IDH-I incubated with [γ-^{32}P]ATP in the presence of cell free extract from *E. coli* DEK2004 for 1, 10, 30, or 60 min at 37°C. *V.* ABE-1 + IDH-I, purified IDH-I incubated with [γ-^{32}P]ATP in the presence of cell-free extract from *Vibrio* sp. strain ABE-1 for 60 min at 37 or 15 °C. A star show the position of radio-labeled IDH-I.

Fig. 14. Inactivation of IDH-I in *E. coli* cells grown in acetate. *E. coli* DEK2004 harboring either pSS512 (*E. coli icd*) or pIS102 (*icdI*) were cultured at 37 °C in 100 ml of MOPS medium supplemented with 0.5% glucose for 4 h; then cells collected from 1 ml of the culture were transferred into 100 ml of a fresh MOPS medium supplemented with 2% acetate, and were allowed to continue to grow for 7 h. Specific activities of the enzymatic activity were determined with using sonic extracts prepared from cells cultured in glucose (left bar) and acetate (right bar) medium, respectively.

Fig. 15. Alignment of the sequences of promoter of *icdI* and *icdII*. Panel A, alignment of the sequences of the promoter regions of *icdI* and

ace-operon (*ace*B) of *E. coli* (45). The identical nucleotide residues are indicated by asterisks. Panel B, alignment of the sequences of promoter regions of *icdII* and cold shock genes of *E. coli* [*csp*A (58), *nus*A (81), pWQ11f (49), *hns* (63), and pWQ3e (49)]. The 5'-CCAAT-3', which has been proposed for a common motif of csp genes, are boxed. The -35 and -10 regions and the transcriptional starting points are indicated with underlines and +1, respectively.

Fig. 16. Different responses of isocitrate dehydrogenation step to acetate utilization in *Vibrio* sp. strain ABE-1 and *E. coli*. Left; *Vibrio* sp. strain ABE-1, IDH-I, isocitrate dehydrogenase isozyme-I; ICL, isocitrate lyase; MS, malate synthase. Right; *E. coli*, IDH-K/P, isocitrate dehydrogenase kinase/phosphatase. Abbreviations for other enzymes are the same in *Vibrio* ABE-1.

Fig. 17. Insert DNAs of successive deletion plasmids of pIS202. pIS202 is the expression plasmids for *Vibrio* sp. strain ABE-1 *icdII* gene. All insert DNAs were ligated in the vector, pBluescript KS^+ at appropriate restriction endonuclease cleavage sites. Successive deletion plasmids (pMS, pMSF) were constructed from pIS202 as described in Materials and Methods. ORF and number are the open reading frame of *icdII* and nucleotide positions from transcription start site (arrows), respectively.

Fig. 18. Nucleotide sequence of *icdII* upstream noncoding region.

Nucleotide sequence from *Xba*I site to the translational start site (ATG) of the *icdII* is presented. An arrowhead indicates the transcriptional starting point (+1). SD and Met are the putative ribosome-binding site (GGAA) and the transcriptional start site (ATG), respectively. The possible promoter sequence is indicated by -35 and -10. The sequence which may acts as a *cis*-element is doubly underlined. The sequences, CCAAT and a probable region to form stem-loop like structure are boxed.

Fig. 19. Effects of successive deletion on the expression levels of the *icdII* gene at low-temperature. Northern blots of RNAs (10 μg/lane) from *E. coli* DEK2004 transformed with successive deletion plasmids are shown. Radiolabeled *Pst*I fragment of *icdII* was used as a probe. Transformants were cultured in LB medium at indicated temperatures. *Vibrio* ABE-1, RNA (2 μg) from *Vibrio* sp. strain ABE-1 control.

Fig. 20. Nucleotide sequences of the deleted regions of the plasmids, pMSF6, pMSF7, pMSF8, and pMS42. 5'-terminally deleted plasmid are shown with the 5'-end point of each deletion construct indicated by bars under the nucleotide sequence. The recognition sites of restriction enzymes, *Dra*I and *Spe*I, which were used to prepare the probe of gel shift assay (Fig. 21), are doubly underlined.

Fig. 21. Gel shift assay. A 91 bp *Dra*I-*Spe*I fragment of the pIS202, including a sequence of 35 bp (Fig. 19), was used as a probe. The probe was 5' end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Left and right panel indicate the DNA probe-protein binding used with crude extract from *E. coli* DEK2004 harboring pMS77 and *Vibrio* sp. strain ABE-1, respectively. The transformants were cultured at 15 °C in LB medium. Lane D, labeled probe only (1 ng: ca 1 kBq, 5-10 kcpm); lane D + P, probe DNA plus crude extracts (20 μg) of *E. coli* (left) or *V.* ABE-1 (light), D + P + C; add cold (non-labeled) 91 bp probe (1 μg) as a competitor to D + P system, lane D + P + N; add sonicated herring DNA (1 μg) as non specific DNA to D + P system. After the incubation of the reaction mixture for 1 h at 15 °C, all samples were run on the 4 % acrylamide gel. And the gel was then dried at 80 °C for 2 h and exposed to X-ray film at -80 °C for appropriate time.

Fig. 22. Constructs of pMSF8C, pMSF8S, pMS42C, and pMS42S generated by PCR method Numbers indicate relative nucleotide position from the transcriptional start site. 35-bp, CCAAT, TCS, stem-loop, and ORF are the sequences corresponding to a *cis*-suppresser element, 5'-CCAAT-3', transcriptional start site, stem-loop like region, and open reading frame of *icdII*, respectively.

Fig. 23. Effects of deletion of a sequence of CCAAT or a stem loop like region from the promoter region on the expression levels of the *icdII* gene at low temperature. Northern blots of RNAs (10 μg/lane) from *E. coli* DEK2004 transformed with each pMSF8C, pMSF8S, pMS42C, and pMS42S (Fig. 22) are shown. Radio labeled *Pst*I fragment of *icdII* was used as probe. Transformants were cultured in LB medium at indicated

temperatures.

Fig. 24. Enhancement of low-temperature growth of *E. coli* DEK2004 by transforming with deletion plasmid, pMS42. *E. coli* DEK2004 or XL1-blue was used as a host for the transformation. *E. coli* DEK2004 cells were transformed with pMS42 (○), pMSF8 (△), pIS202 (▲), and pSS512 (□). *E. coli* XL1-Blue transformed with pBluescript KS^+ (■). Precultured cells in a LB medium for overnight at 37 °C was inoculated in a fresh medium (LB) and cultured at 15 °C. The bacterial growth was monitored by measuring absorbance at 550 nm.

Fig. 25. Growth of *E. coli* transformants at 10 °C. *E. coli* XL1-Blue transformed with the indicated plasmid were inoculated onto MOPS-succinate plate and incubated at 10 or 15 °C as described in Materials and Methods. The pictures were taken after 2 weeks of the inoculation. For details, see the Table IV.

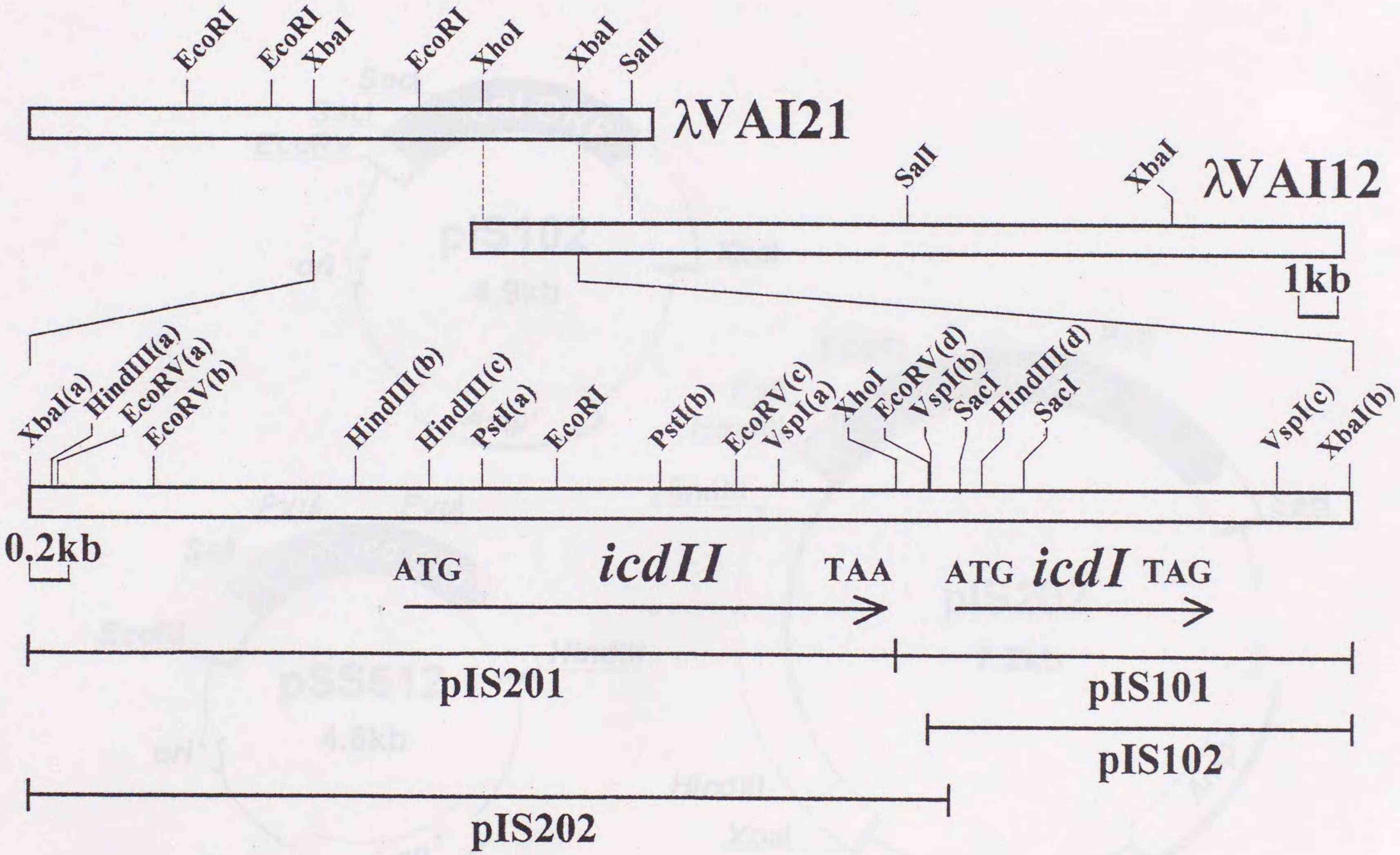

EcoRI
EcoRI
XbaI
EcoRI
XhoI
XbaI
SalI
λVAI21
SalI
XbaI
λVAI12
1kb
XbaI(a)
HindIII(a)
EcoRV(a)
EcoRV(b)
HindIII(b)
HindIII(c)
PstI(a)
EcoRI
PstI(b)
EcoRV(c)
VspI(a)
XhoI
EcoRV(d)
VspI(b)
SacI
HindIII(d)
SacI
VspI(c)
XbaI(b)
0.2kb
ATG
icdII
TAA
ATG
icdI
TAG
pIS201
pIS101
pIS102
pIS202

Figure 1

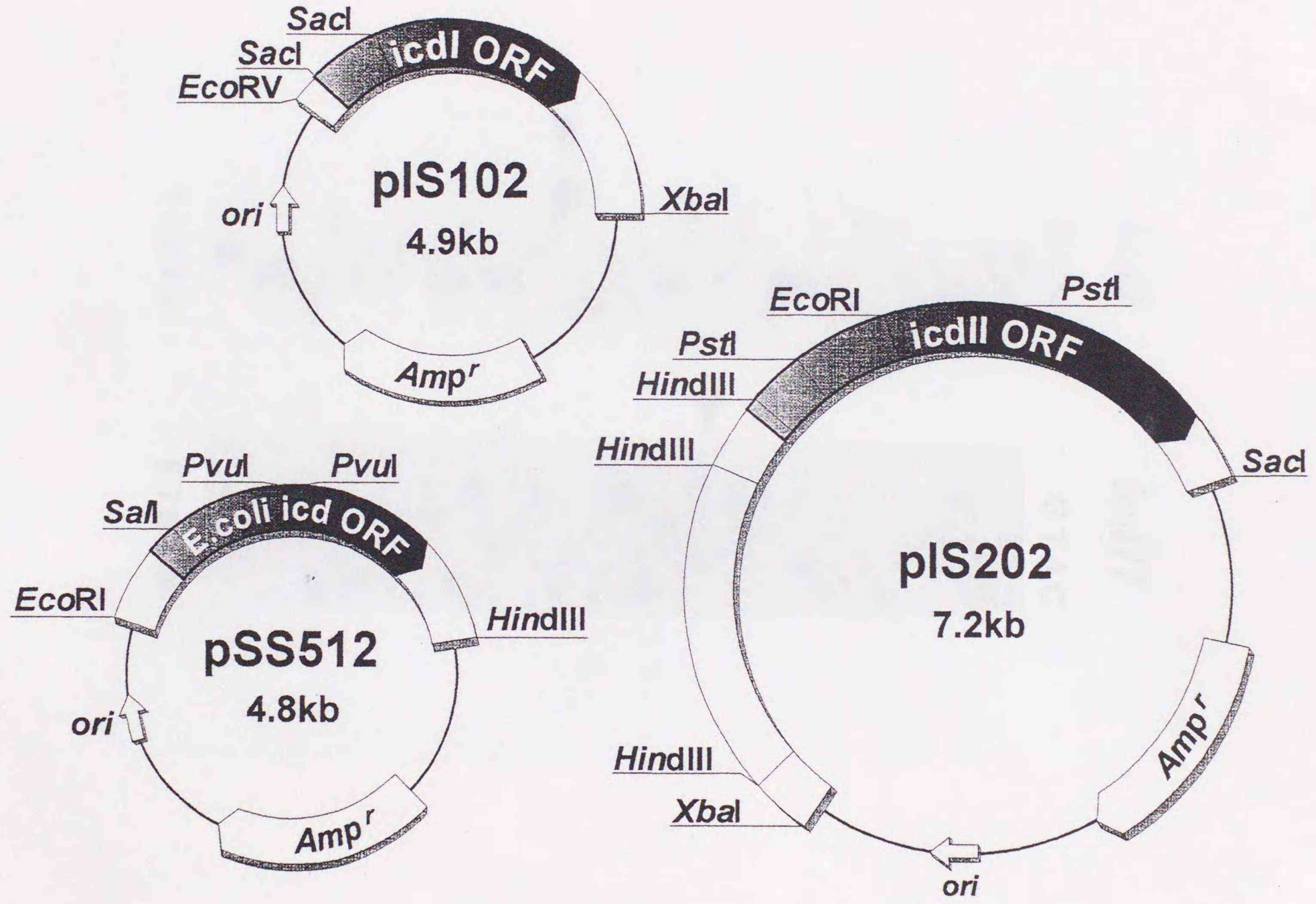

SacI
SacI
EcoRV
icdI ORF
pIS102
4.9kb
ori
XbaI
Amp^r
EcoRI
PstI
PstI
icdII ORF
HindIII
HindIII
SacI
pIS202
7.2kb
HindIII
XbaI
Amp^r
ori
PvuI
PvuI
SalI
E.coli icd ORF
EcoRI
HindIII
pSS512
4.8kb
ori
Amp^r

Figure 2

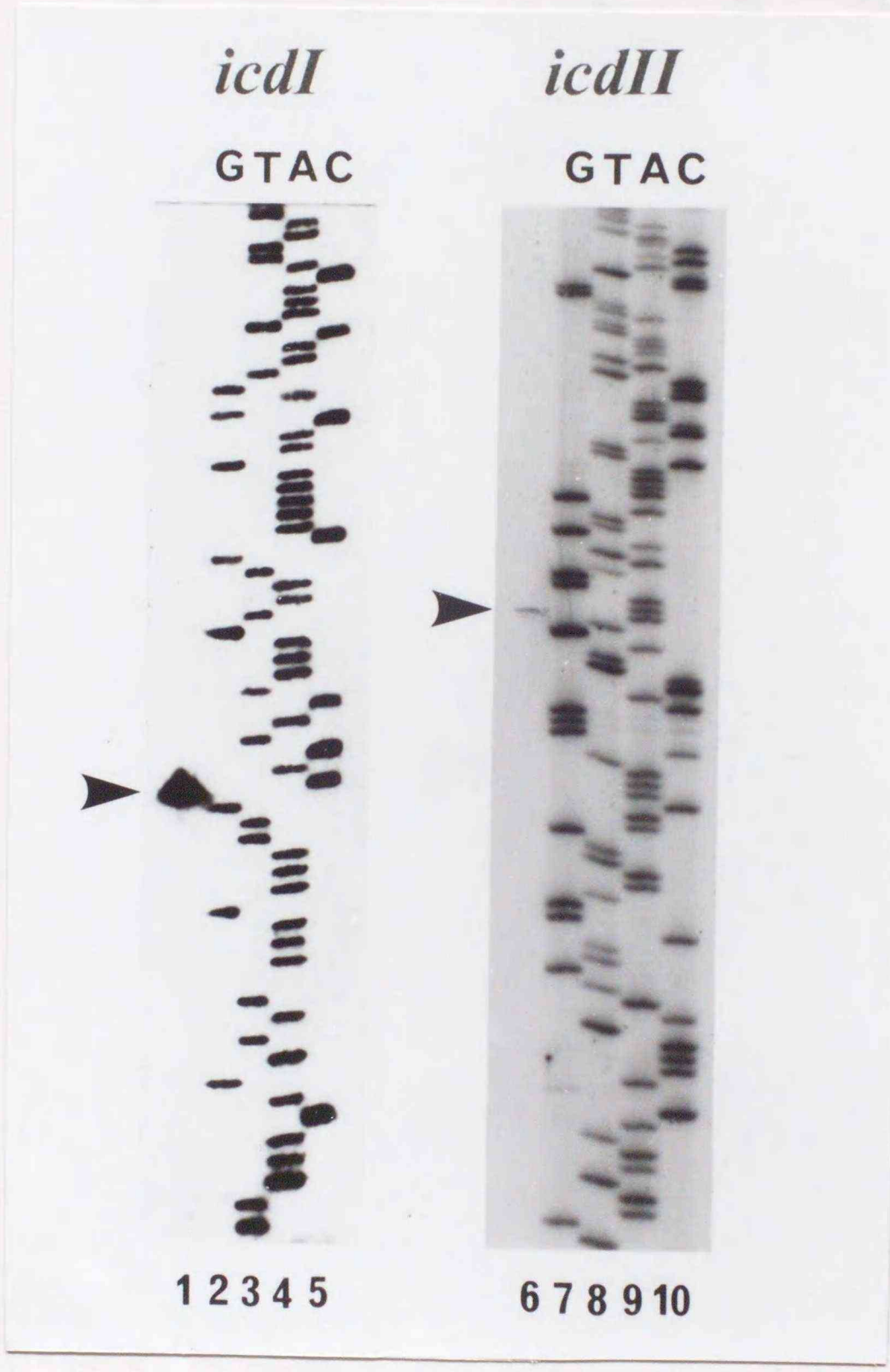


Figure 3

Transcriptional starting points of the *icdI* and *icdII* genes.

icdI

TATCACTAATTAATGTTTAGTTACTCGTTCTTTTTGCATTACTTTAGTAGTGCAAT
-35 -10 +1

TTCTTTTATATCTGTTTAATGTGGAGCTCGCCCTATG
+40

icdII

TGTGTAGGCCAATAATTTATAGGGTTTGGTAAGTTTTCTAACTATACCTTTACTAA
-35 -10 +1

GGTGCCCGATTTGTTCAATTACCGAACATGAGGGTCGTATTATTCAGAGTAGAATC

ACCGGAGCTTTTAGCTAATTAAAATAGGAATTTCAATG
+98

Figure 4

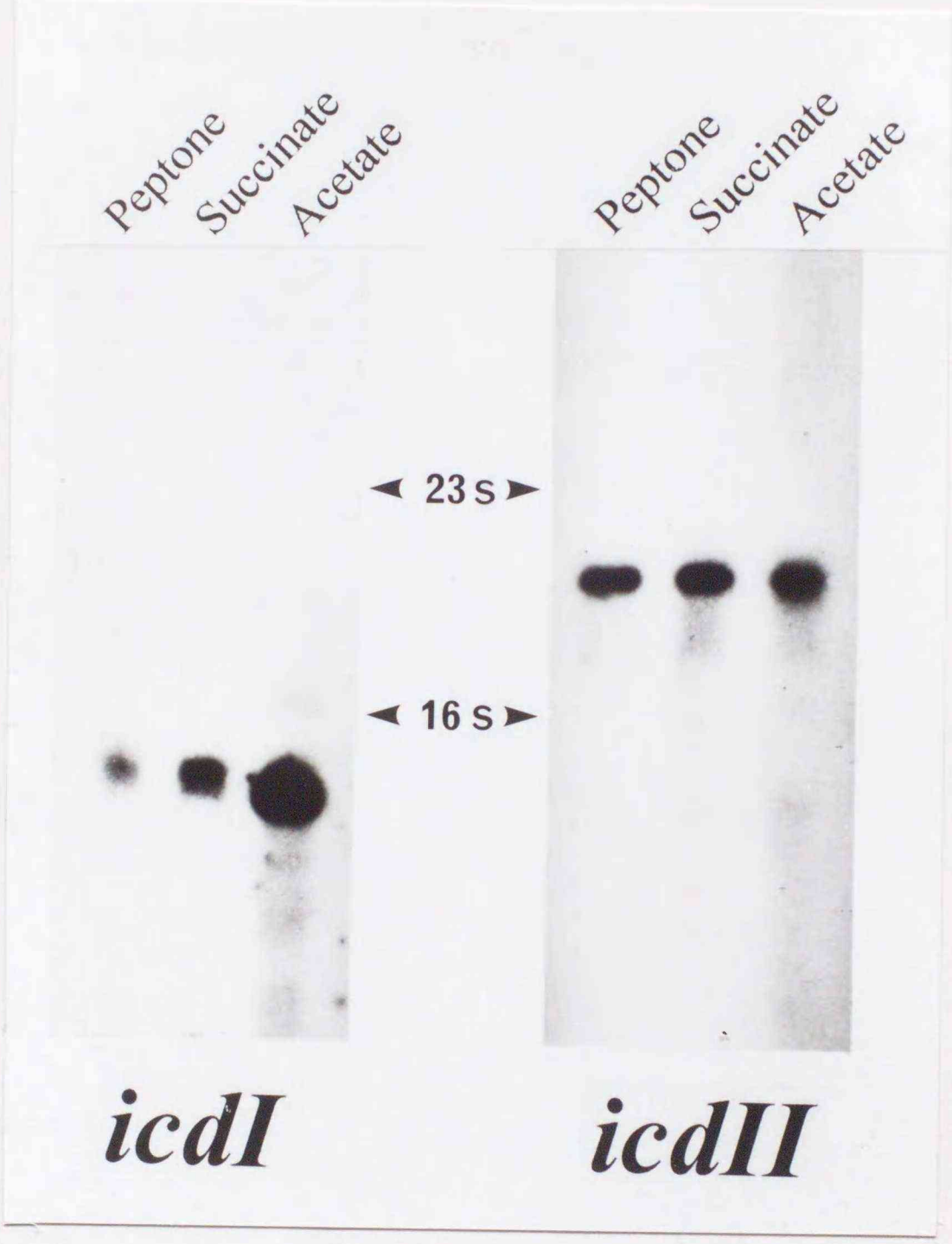

Peptone
Succinate
Acetate
Peptone
Succinate
Acetate
◄ 23 s ►
◄ 16 s ►
icdI
icdII

Figure 5

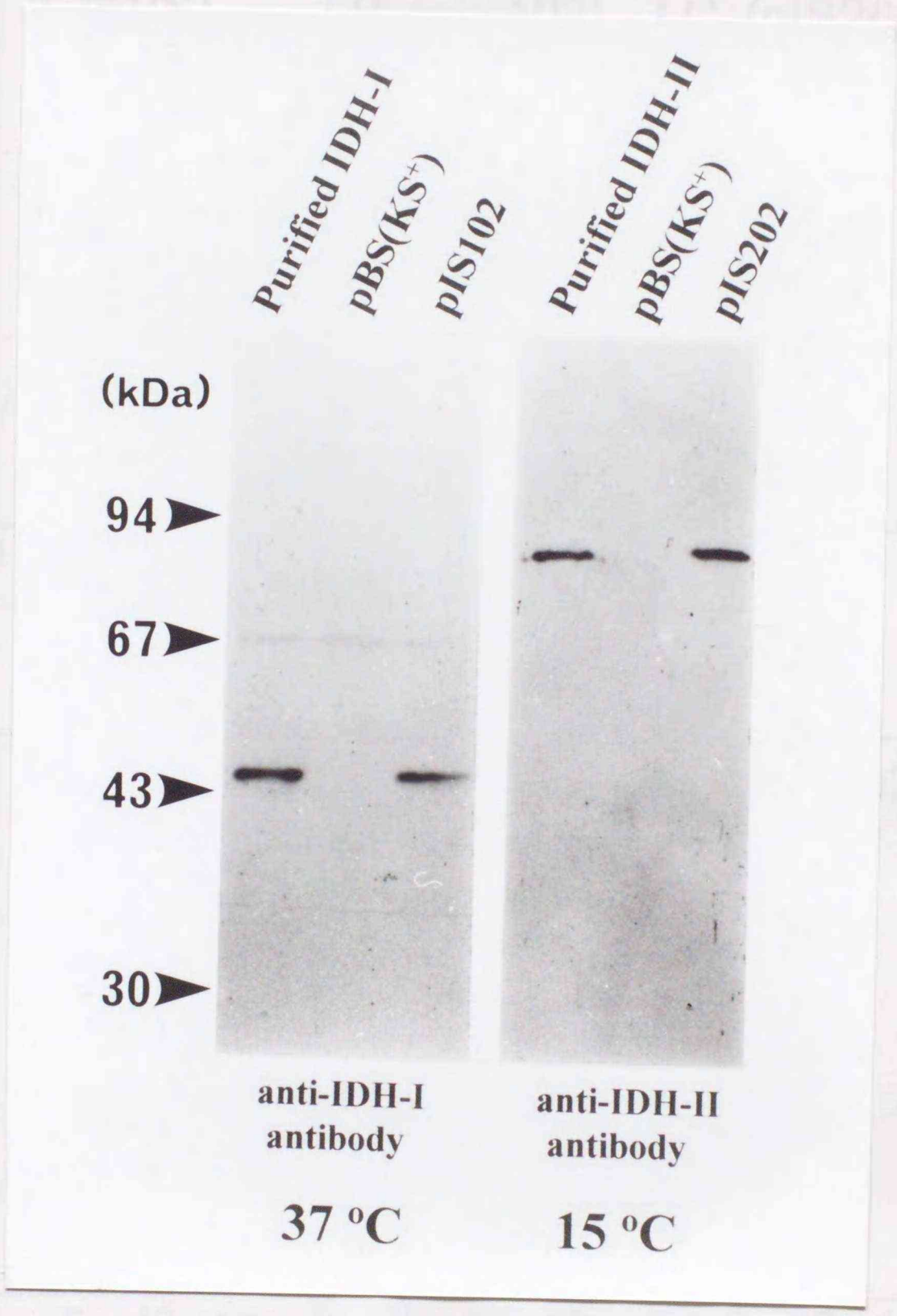

Purified IDH-I
pBS(KS+)
pIS102
Purified IDH-II
pBS(KS+)
pIS202
(kDa)
94
67
43
30
anti-IDH-I antibody
anti-IDH-II antibody
37 °C
15 °C

Figure 6

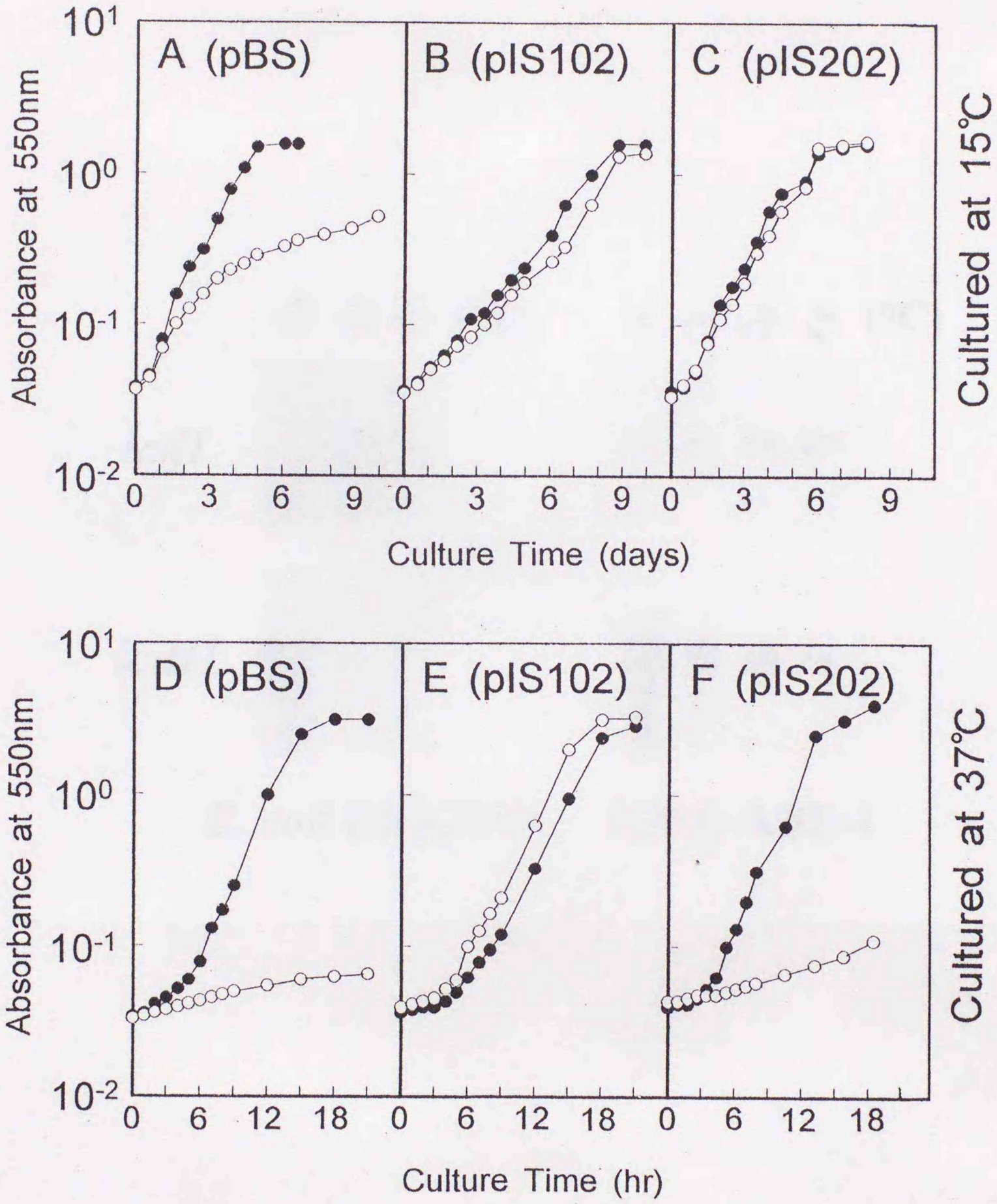

A (pBS)
B (pIS102)
C (pIS202)
Absorbance at 550nm
10^1
10^0
10^{-1}
10^{-2}
0 3 6 9
Cultured at 15°C
Culture Time (days)
D (pBS)
E (pIS102)
F (pIS202)
Absorbance at 550nm
0 6 12 18
Cultured at 37°C
Culture Time (hr)

Figure 7

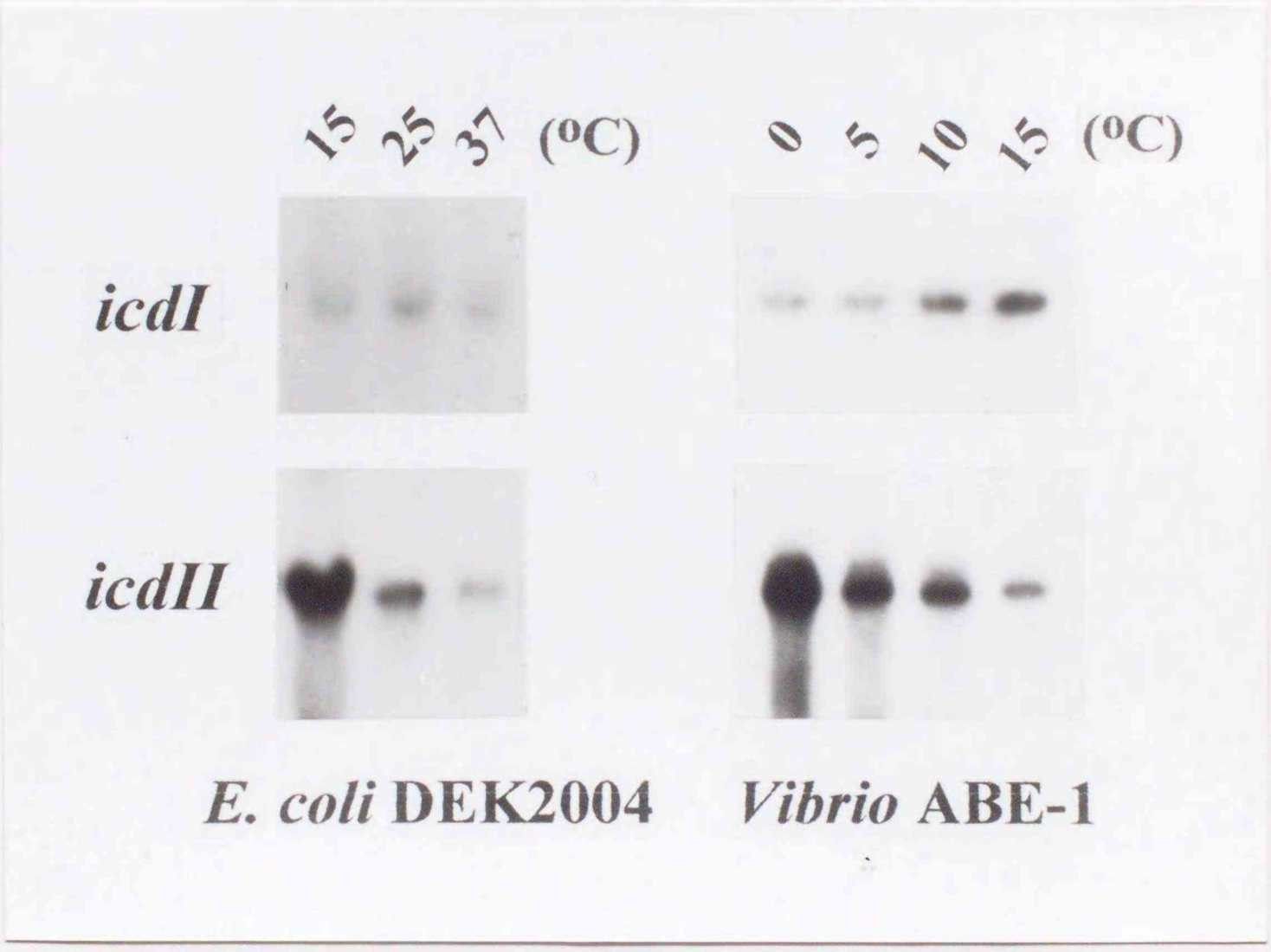


Figure 8

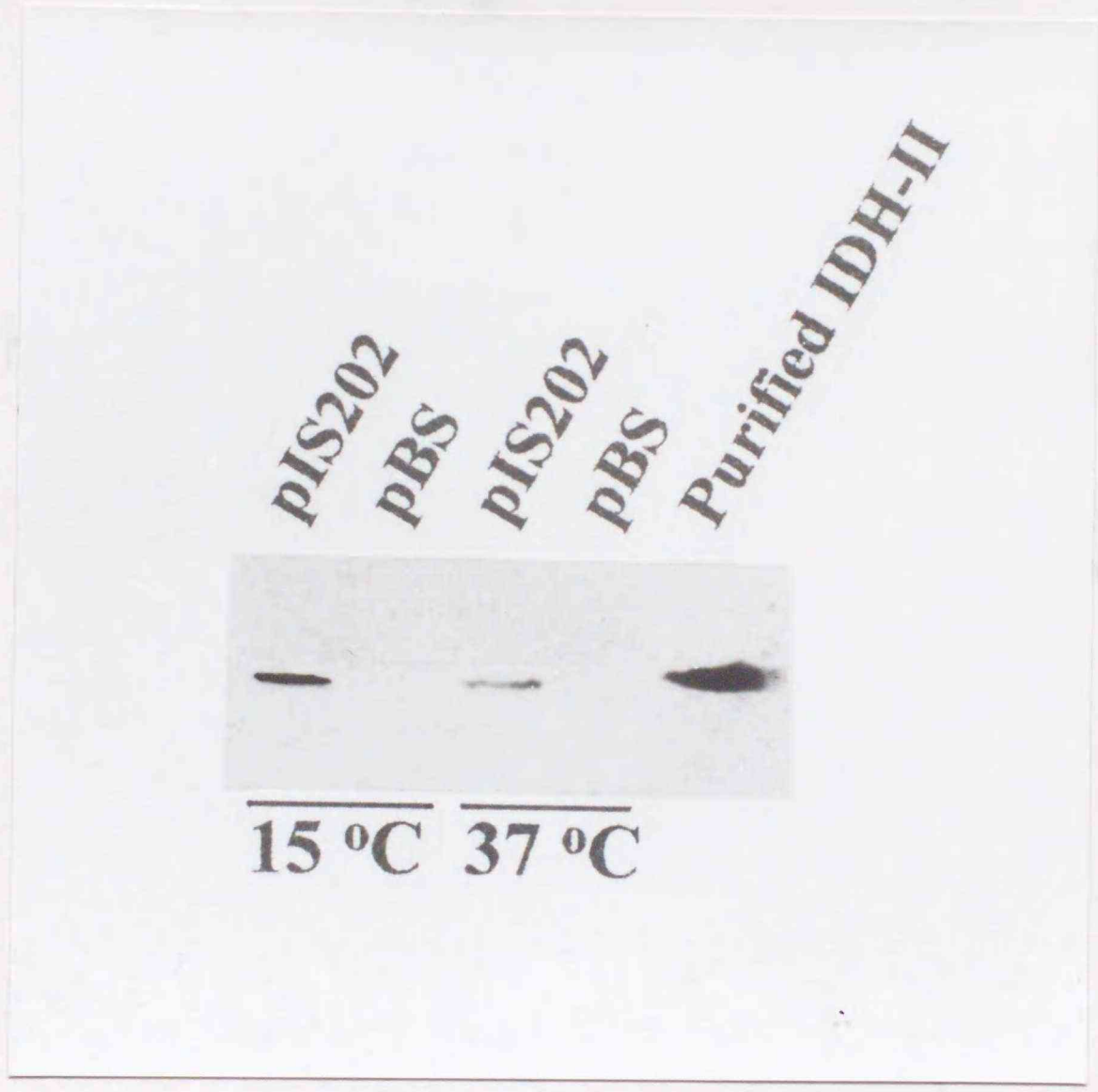


Figure 9

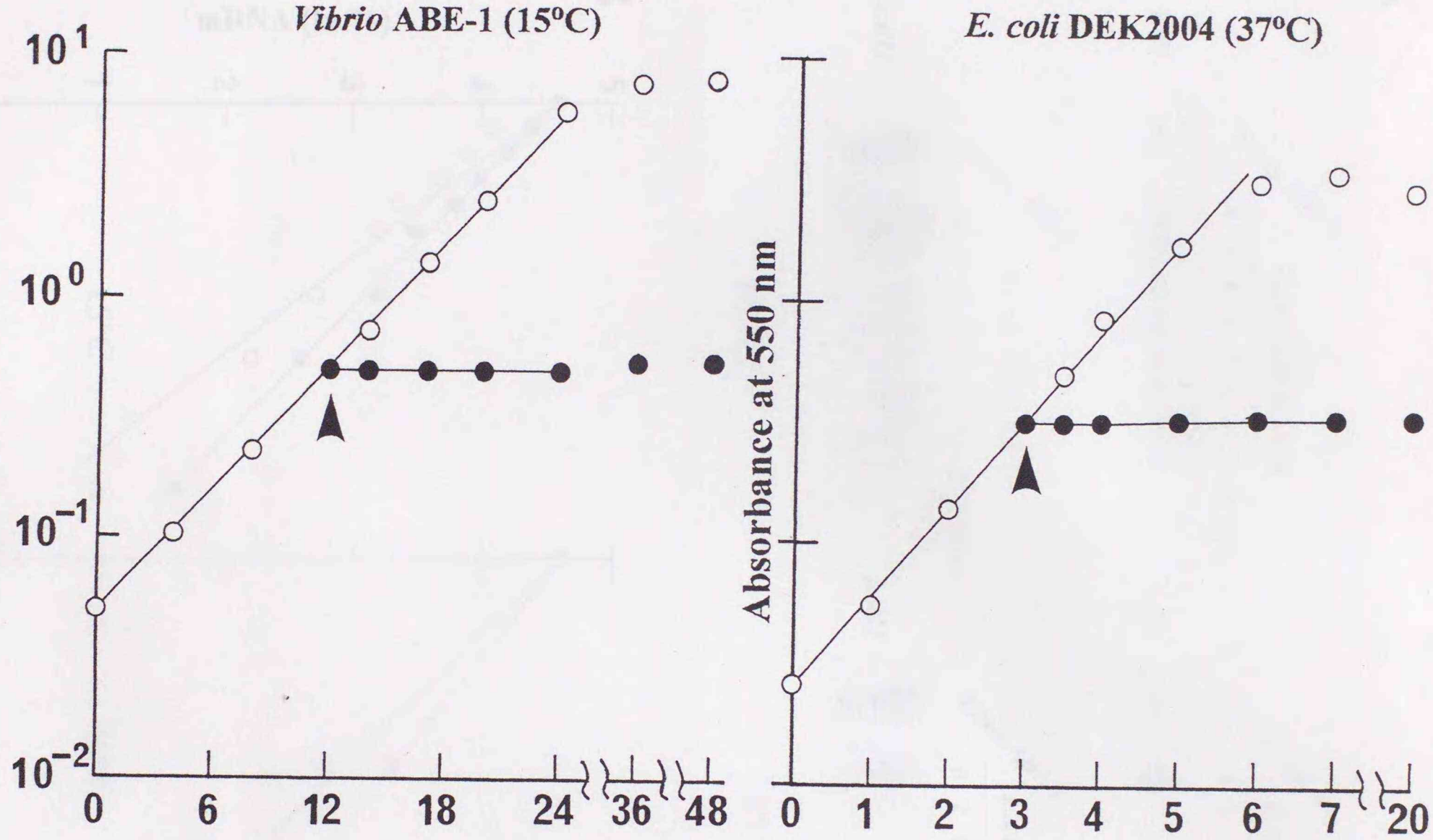
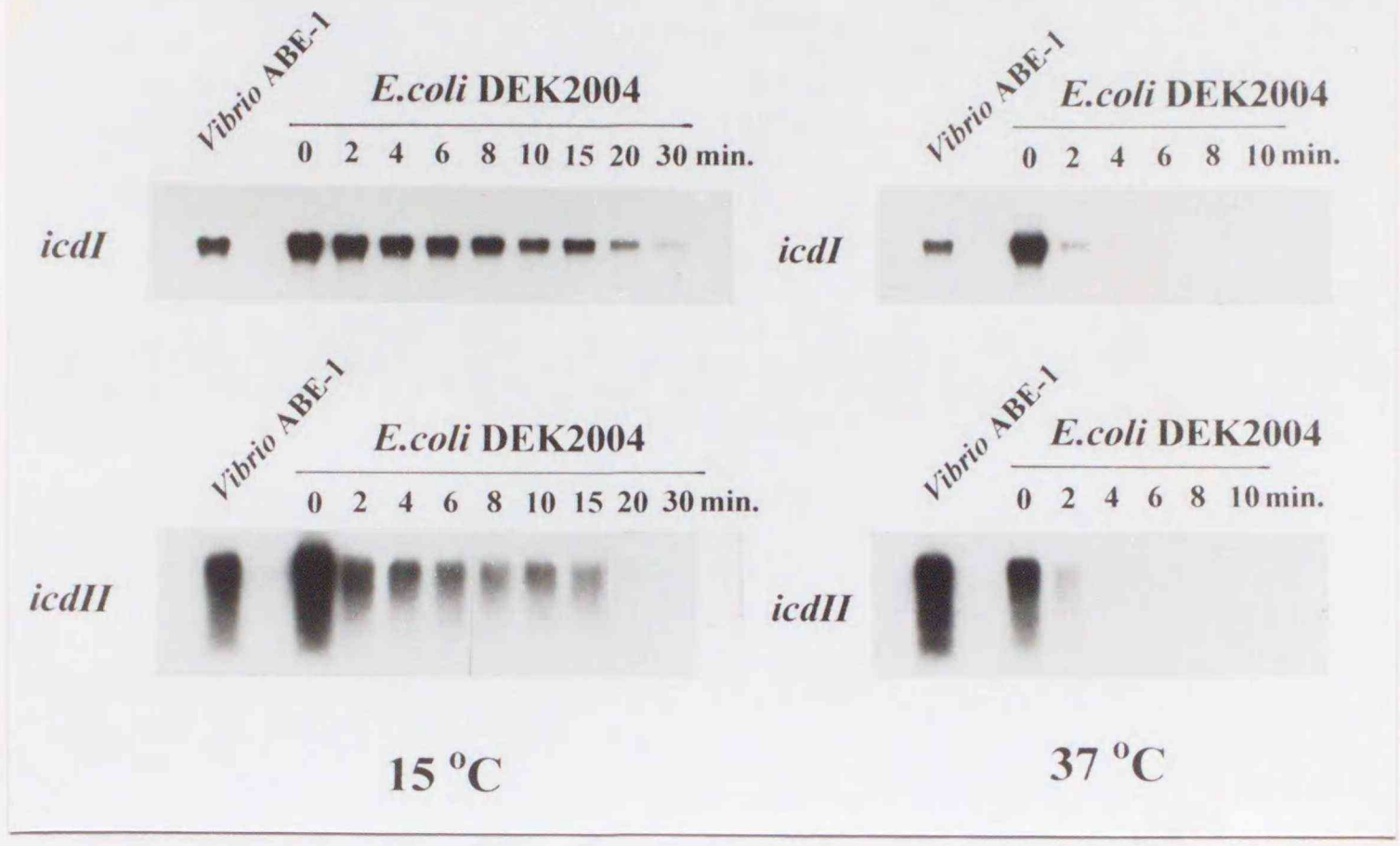

Vibrio ABE-1 (15°C)
E. coli DEK2004 (37°C)
Absorbance at 600 nm
Absorbance at 550 nm
10^1
10^0
10^{-1}
10^{-2}
0
6
12
18
24
36
48
0
1
2
3
4
5
6
7
20
Culture Time (h)

Figure 10

A


Vibrio ABE-1
E.coli DEK2004
0 2 4 6 8 10 15 20 30 min.
icdI
Vibrio ABE-1
E.coli DEK2004
0 2 4 6 8 10 min.
icdI
Vibrio ABE-1
E.coli DEK2004
0 2 4 6 8 10 15 20 30 min.
icdII
Vibrio ABE-1
E.coli DEK2004
0 2 4 6 8 10 min.
icdII
15 °C
37 °C

B

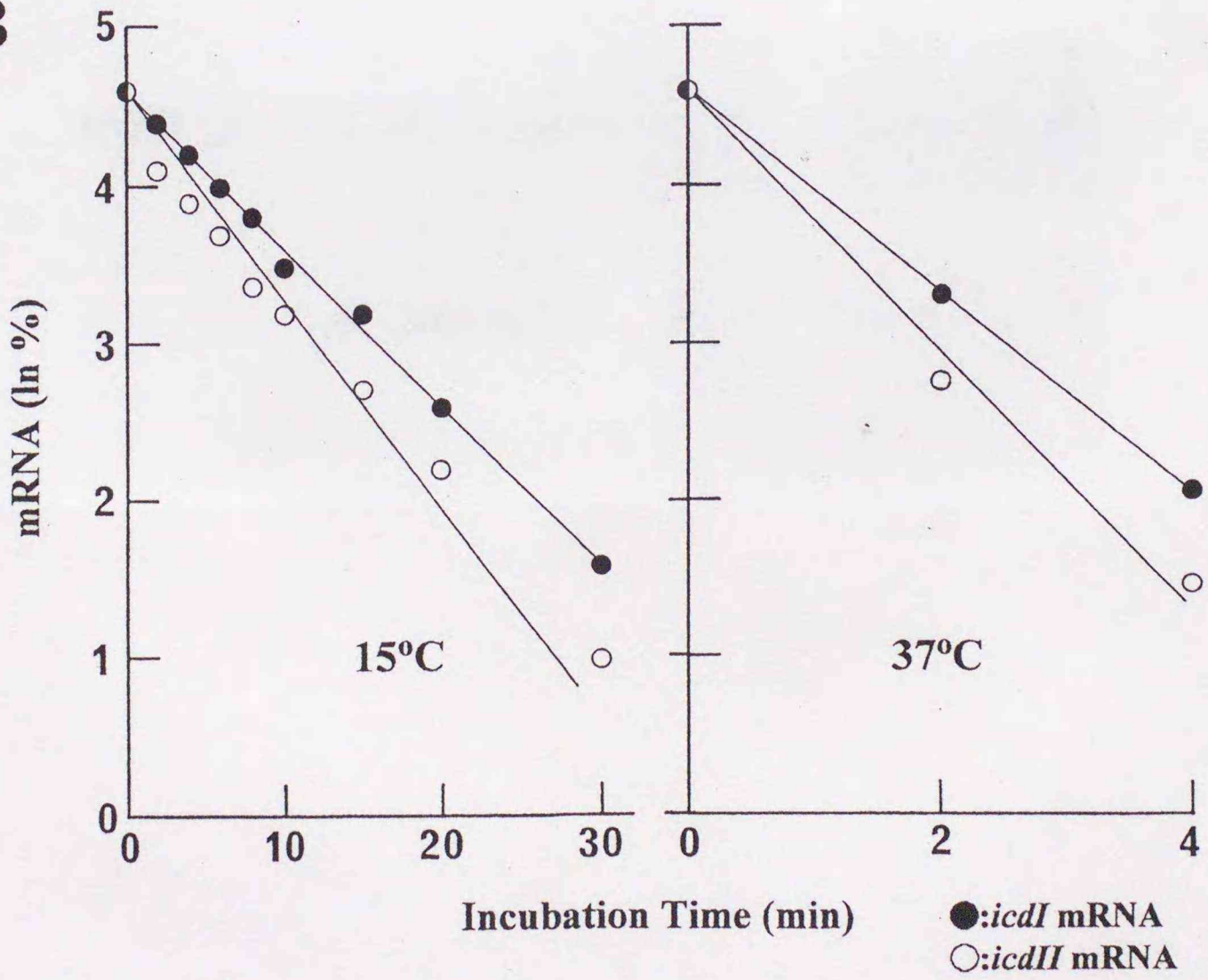

mRNA (ln %)
5
4
3
2
1
0
0
10
20
30
15°C
0
2
4
37°C
Incubation Time (min)
●:icdI mRNA
○:icdII mRNA

Figure 11

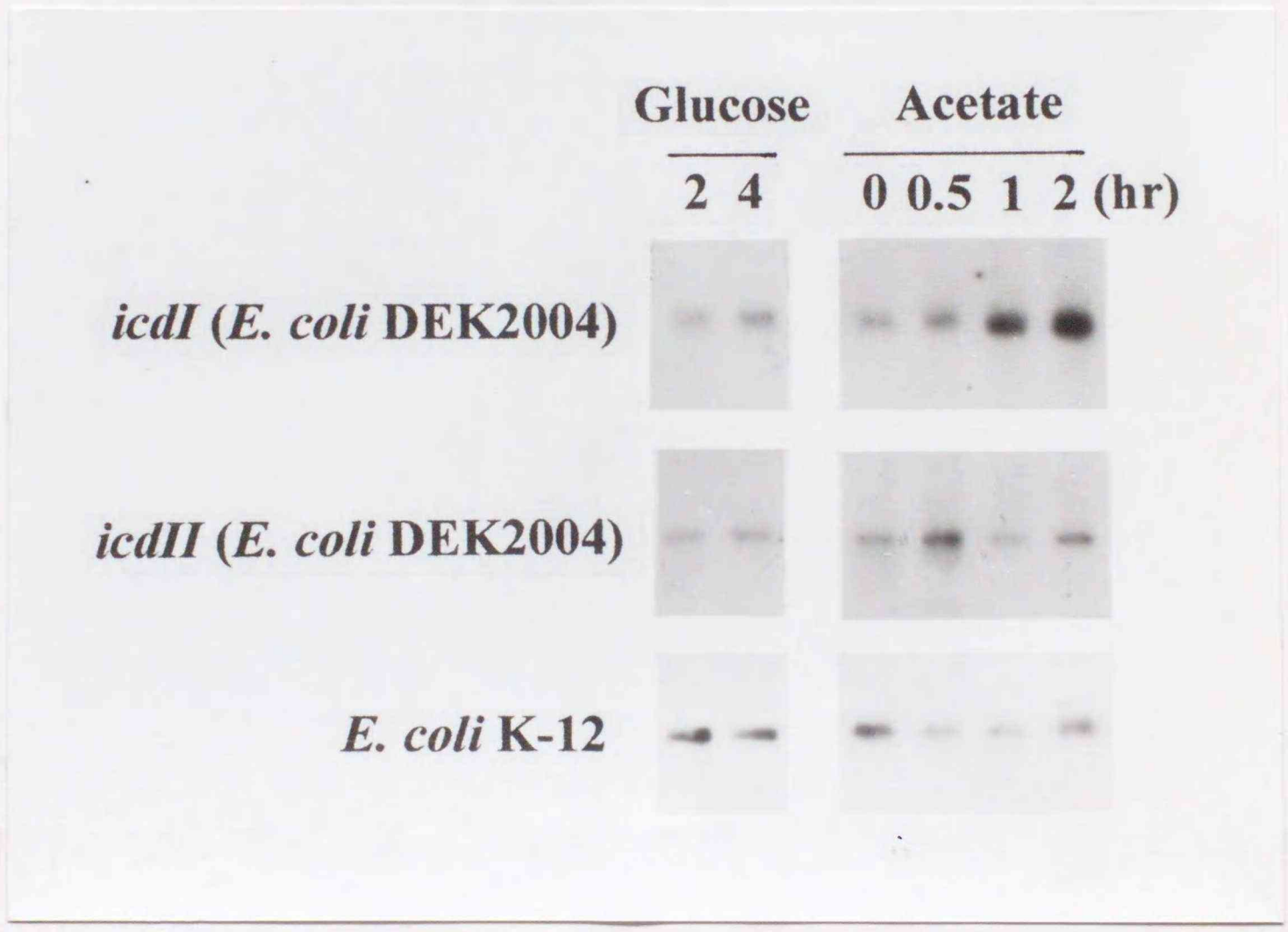

Glucose
Acetate
2 4
0 0.5 1 2 (hr)
icdI (E. coli DEK2004)
icdII (E. coli DEK2004)
E. coli K-12

Figure 12

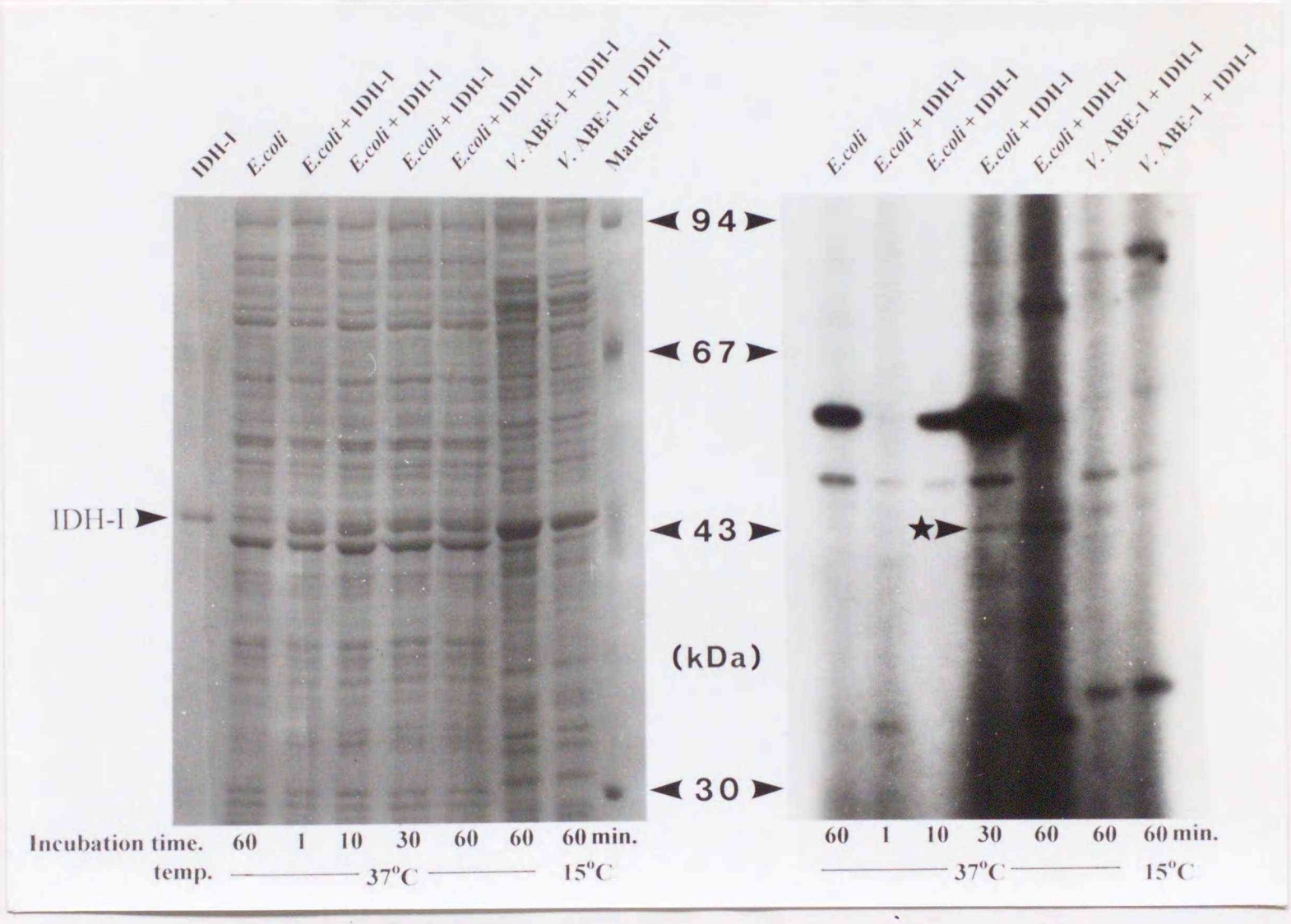

IDH-I
E.coli
E.coli + IDH-I
E.coli + IDH-I
E.coli + IDH-I
E.coli + IDH-I
V. ABE-1 + IDH-I
V. ABE-1 + IDH-I
Marker
94
67
43
30
(kDa)
IDH-I
Incubation time. 60 1 10 30 60 60 60 min.
temp. 37°C 15°C
E.coli
E.coli + IDH-I
E.coli + IDH-I
E.coli + IDH-I
E.coli + IDH-I
V. ABE-1 + IDH-I
V. ABE-1 + IDH-I
60 1 10 30 60 60 60 min.
37°C 15°C

Figure 13

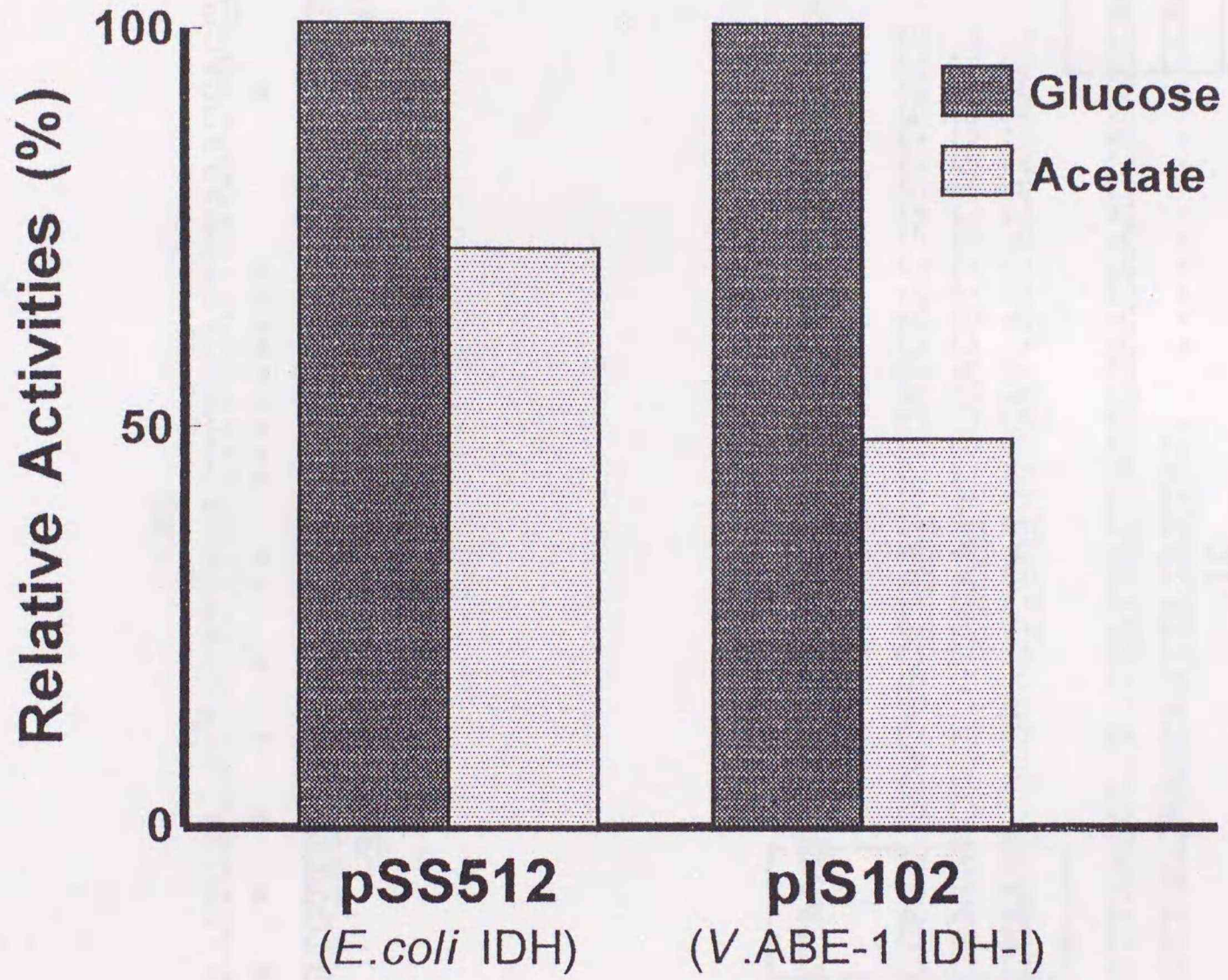

100
50
0
Relative Activities (%)
Glucose
Acetate
pSS512
(E.coli IDH)
pIS102
(V.ABE-1 IDH-I)

Figure 14

A

```
                                -54                      -35                      -10      +1
icdI                              TAATTAAAATGGAAATTGTTTTTGATTTTGCATTTTAAATGAGTAGTCTTAGTTG
                                  *******  *  *  *  *  **  *********        *
aceB                              TAATTAATGTTTAGTTACTCGTTCTTTTTGCATTACTTTAGTAGT
                                -45         -35                      -10     +1
```

B

```
                                -50                -35                          -10       +1
icdII                             TGTGTAGGCCAATAATTTATAGGGTTTGGTAAGTTTTCTAACTATACCTTT

cspA                            TTGCATCA.CCCGCCAAT.GCGTGGCTTAATGCACATCAACGGTTTGACG
nusA                            TTGCATTTTTTACCCAAAACG.A.GTAGAATTTGCCACGTTTCAGGCGC.
pWQ11f                          TTGCATGAG.AAGCCAATA.GCGGGCTTTATGCGGAT.AGCACAACAAC.

hns                             TTGCACAACTGAATTTAAGGC.T.CTATTACTACCCCAACAAACCACCCCAATATAAGTTTGAGA
pWQ3e                           TTGCAGTGATCGGGTTGGCAAATAGTTATATTAC..............CCAATTATGCGAAGAAG
                                 -35                          -10
```

Figure 15

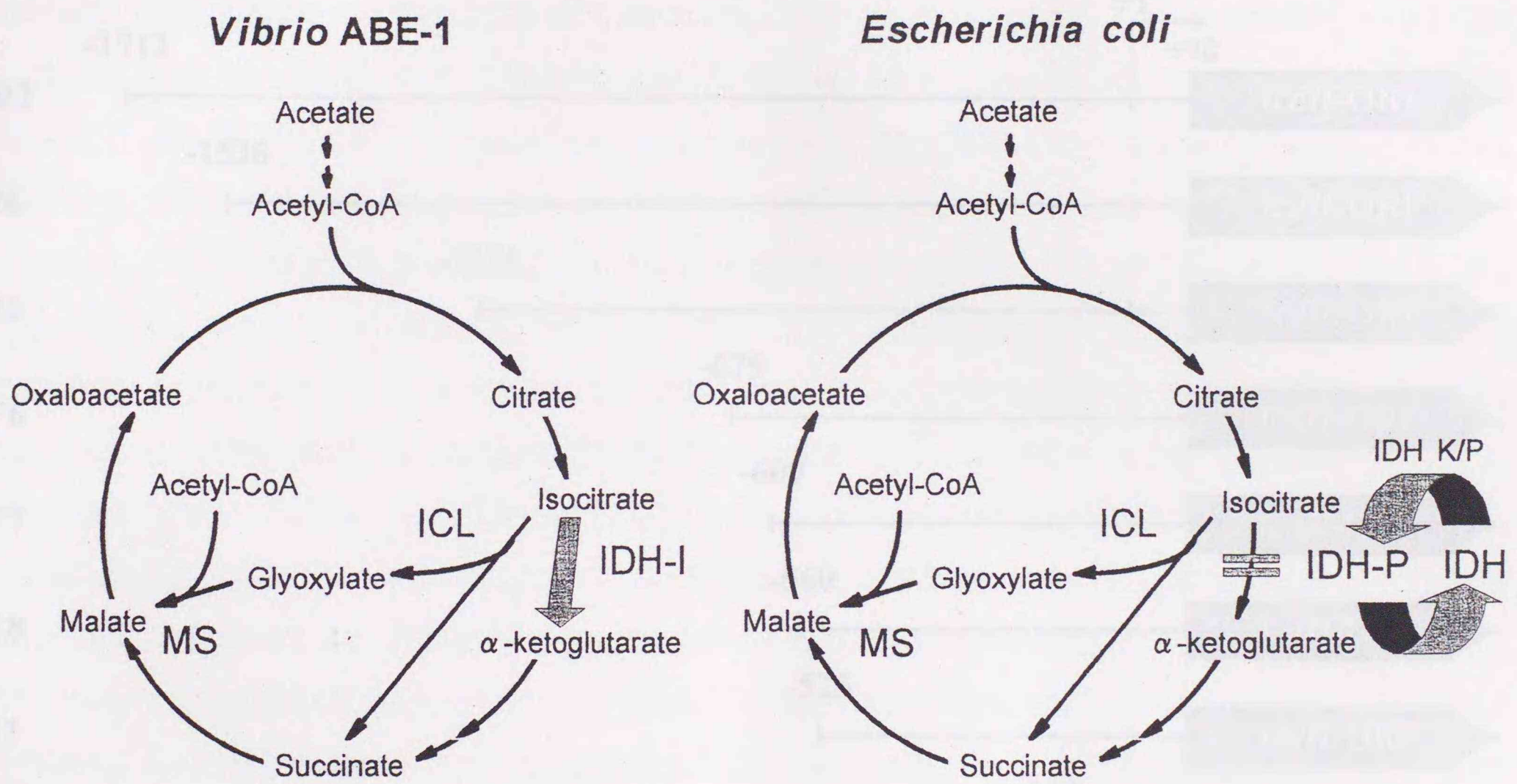

Vibrio ABE-1
Acetate
Acetyl-CoA
Oxaloacetate
Citrate
Acetyl-CoA
Isocitrate
ICL
IDH-I
Glyoxylate
Malate
MS
α-ketoglutarate
Succinate
Escherichia coli
Acetate
Acetyl-CoA
Oxaloacetate
Citrate
IDH K/P
Acetyl-CoA
Isocitrate
ICL
IDH-P
IDH
Glyoxylate
Malate
MS
α-ketoglutarate
Succinate

Figure 16

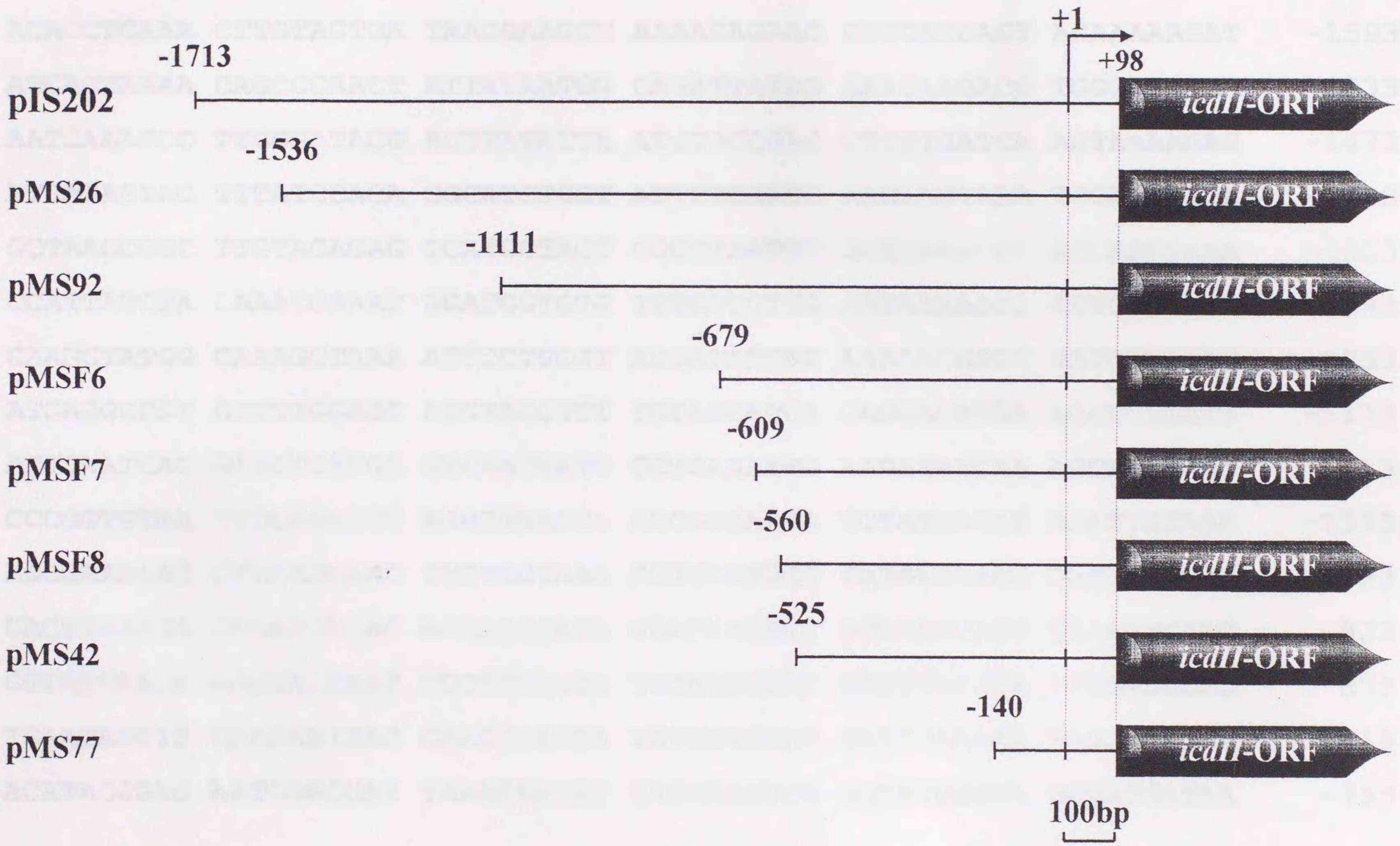

+1
-1713
+98
pIS202
icdII-ORF
-1536
pMS26
icdII-ORF
-1111
pMS92
icdII-ORF
-679
pMSF6
icdII-ORF
-609
pMSF7
icdII-ORF
-560
pMSF8
icdII-ORF
-525
pMS42
icdII-ORF
-140
pMS77
icdII-ORF
100bp

Figure 17

TCTAGAGATA TCGACCGACC AGGTAAAATA GACCCTATTT TTAGTCCTTA TGAAGAAATA -1653

ACACCTGAAA CTTGTAGTGA TAACGAAGCT AAAACAGAAG CTTCATCAGT AGAAAAAGAT -1593

ATCACTAAAA CAGCCCAACT ATTATAATGG CAGATTATAG AAACAAGACG TGGGAAATGA -1533

AATCAAAGCC TTGGGATACG ACTTATATTA ATTTGCCGAC CTTTTGATCA AGTAAAAGAG -1473

ATCAAAGTAC TTTATCCACA CGCATCTCGT ATTTTCCATC AAGAAGTAAA TCCAACATCA -1413

GCTAAGCCGC TTGTACAGAG TCATGGTACT CGCCTAATTT GGTTAAACCC ACCAGCTAAA -1353

CCATTATCTA CAAATGAAAT GGATGGTGTG TTTGGTCTTG AATATAAGCC TGTCCACACC -1293

CAAGCTATGG CAAAGCTAAA ATTCCTGCGT ACGATATGAT AAAAACGTCT TATCAATATC -1233

ATGAGGGGGT GCTTTGGAGG ATGTACGTTT TGTAGCATCA CAGGACATGA AGGTCGTATT -1173

ATTCAATCAC GTTCTCATGA GTCAATTATT GCTGAAATTG AAGATATCAA AGTAAAAGTT -1113

CCCGGTGTAA TTCCAGATTT AGGCGGACCA ACCGCCAATA TGTATCACCT AAATTGTAAA -1053

AGCGAAAAAG CTGAAGCAAC TTGTCGTAAA CCTTCATGCG TATGGCCAAC CATTTGTGGC -993

CACTTAGATA CCGATCACAC ACCAACGATA GAACTATACC GTAAAGCACG TAAAGTTAAG -933

GGTATTAAGA AGGTACTAAT CGCTTCGGGG TGTACGCTAC GATTTGGCTA TTGAACACCC -873

TGAATATGTT CAAGAATTAG CAACCCATCA TGTGGGTGGT TATTTAAAAA TAGCACCTGA -813

ACATACCGAG AATGGGCCAT TAAATAATAT GATGAAGCCA GGTATGGGTA GCTATGATAA -753

Figure 18

ATTCAAAGAG ATGTTTGATC ATTACTCAAA ACTTGCTGGT AAAAAACAAT ATTTAATTCC -693

TTATTTTATC TCTGCTCATC CAGGTACTAC CGATAGAGAT ATGGTAAATT TAGCTTTATG -633

GCTAAAAGAA AATAACTTTA AATTGGATCA AGTGCAAAAC TTTTATCCTT CGCCATTAGC -573

AAATGCAACA ACGTTATACC ATACGGAGCT TAATTCTTTA CGTAACATTA CTAGTAAAAA -513

CTTAGCAAAA GAAGATGGTA AAATTACAGT GCCTAAAGGC GCTATTCAAC GACGTTTGCA -453

CAAAGCGATT TTACGTTACC ATGATCCAGT TAATTGGCCA CAAGTTCGTC AGGCATTGAC -393

AAAAATGGGA TTAAGTAAAC TTATTGGCTC TGCGCCAAAT TGCCTAGTGC CTAAAGAAAC -333

ACGTGCTGAG CAAAATTCAA CCAATAGAAA TAGACAAGGT AAAAATAACT CTGCTGGTAA -273

TATAACTTCA CCAGGTCAAC AAAGAGCTAA ATCTGGTTAT GGGAAAAAAC CAACTAATGG -213

AAAACAGGCT TAACCCGCTT TAGTGATAAT CAGTTTAAAA AGAAAAAGTA ATCCGAGAGC -153

AAGTTTAGTA TTAAGCTTGC TTATATAATT TAGAAATATC ACTTAATTTT TTATAGCATC -93

CTACAGTAAA TATTTTCATT TCTAAGTGTT TTTTAGAAAT ATTGTGTAGG CCAATAATTT -33

ATAGGGTTTG GTAAGTTTTC TAACTATACC TTTACTAAGG TGCCCGATTT GTTCAATTAC +28

CGAACATGAG GGTCGTATTA TTCAGAGTAG AATCACCGGA GCTTTTAGCT AATTAAAATA +88

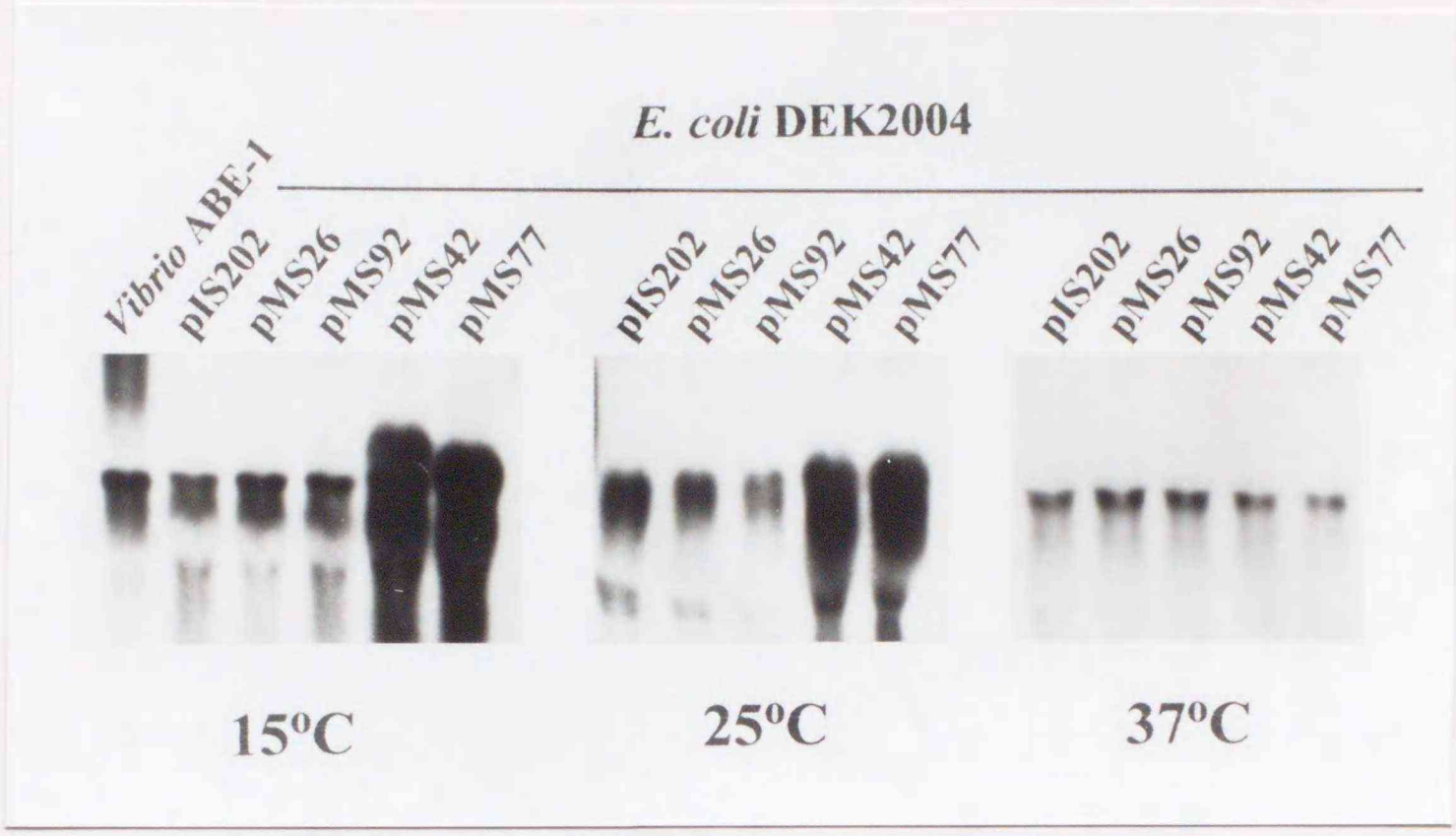
GGAATTTCAA TG +100

SD MET.

−35 −10 +1

Figure 18-*Continued.*

A


E. coli DEK2004
Vibrio ABE-1
pIS202
pMS26
pMS92
pMS42
pMS77
pIS202
pMS26
pMS92
pMS42
pMS77
pIS202
pMS26
pMS92
pMS42
pMS77
15°C
25°C
37°C

B

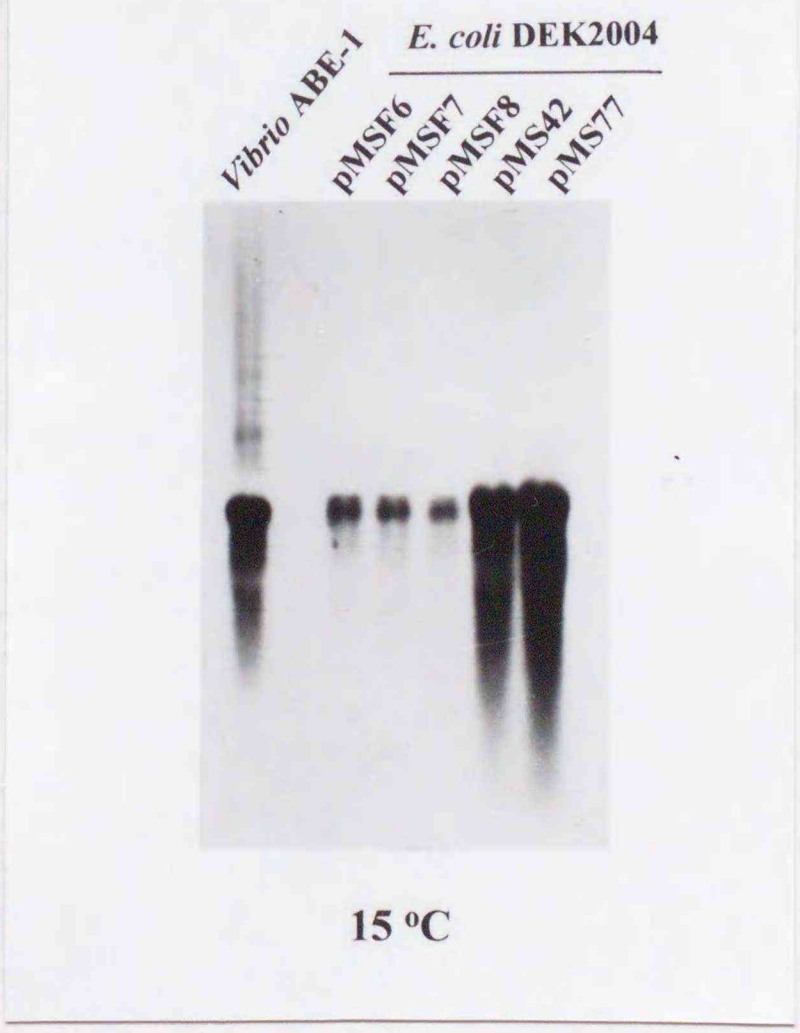

E. coli DEK2004
Vibrio ABE-1
pMSF6
pMSF7
pMSF8
pMS42
pMS77
15 °C

Figure 19

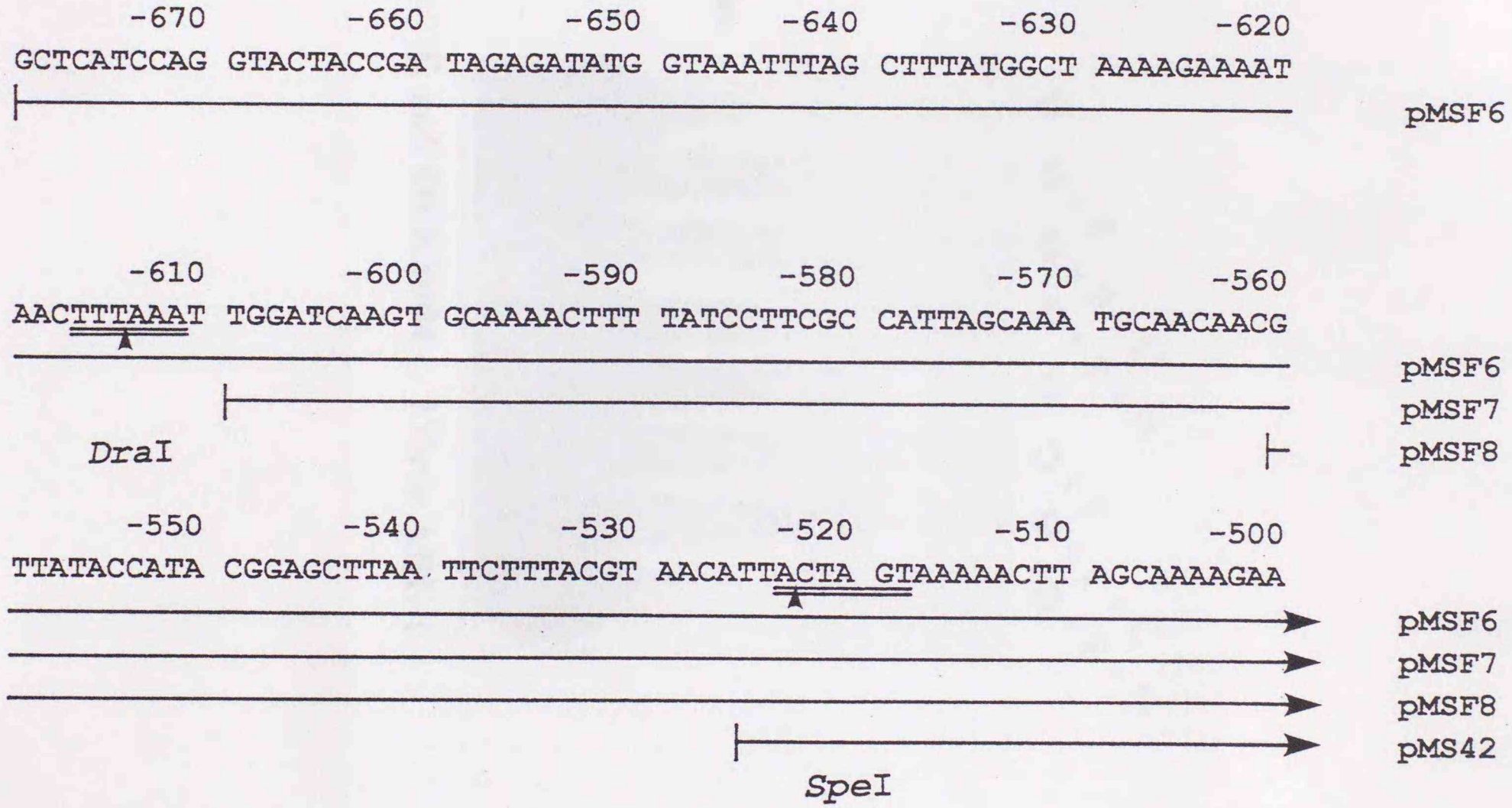

-670 -660 -650 -640 -630 -620
GCTCATCCAG GTACTACCGA TAGAGATATG GTAAATTTAG CTTTATGGCT AAAAGAAAAT
pMSF6
-610 -600 -590 -580 -570 -560
AACTTTAAAT TGGATCAAGT GCAAAACTTT TATCCTTCGC CATTAGCAAA TGCAACAACG
pMSF6
pMSF7
DraI
pMSF8
-550 -540 -530 -520 -510 -500
TTATACCATA CGGAGCTTAA TTCTTTACGT AACATTACTA GTAAAAACTT AGCAAAAGAA
pMSF6
pMSF7
pMSF8
pMS42
SpeI

Figure 20

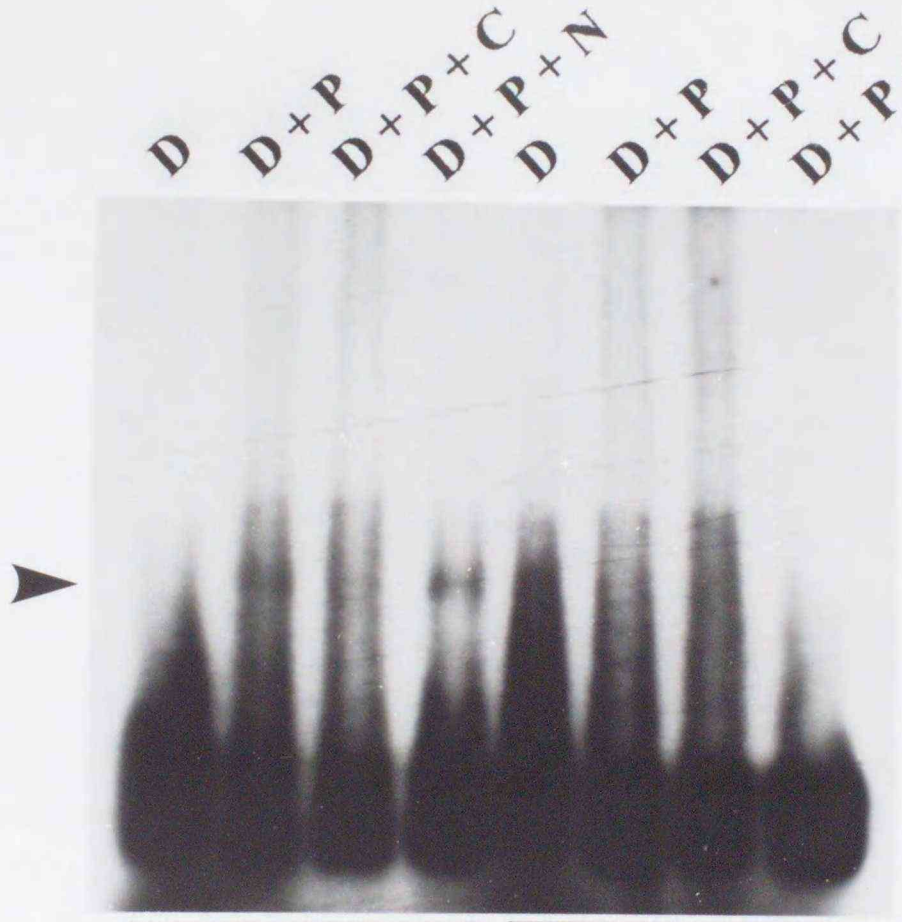


Figure 21

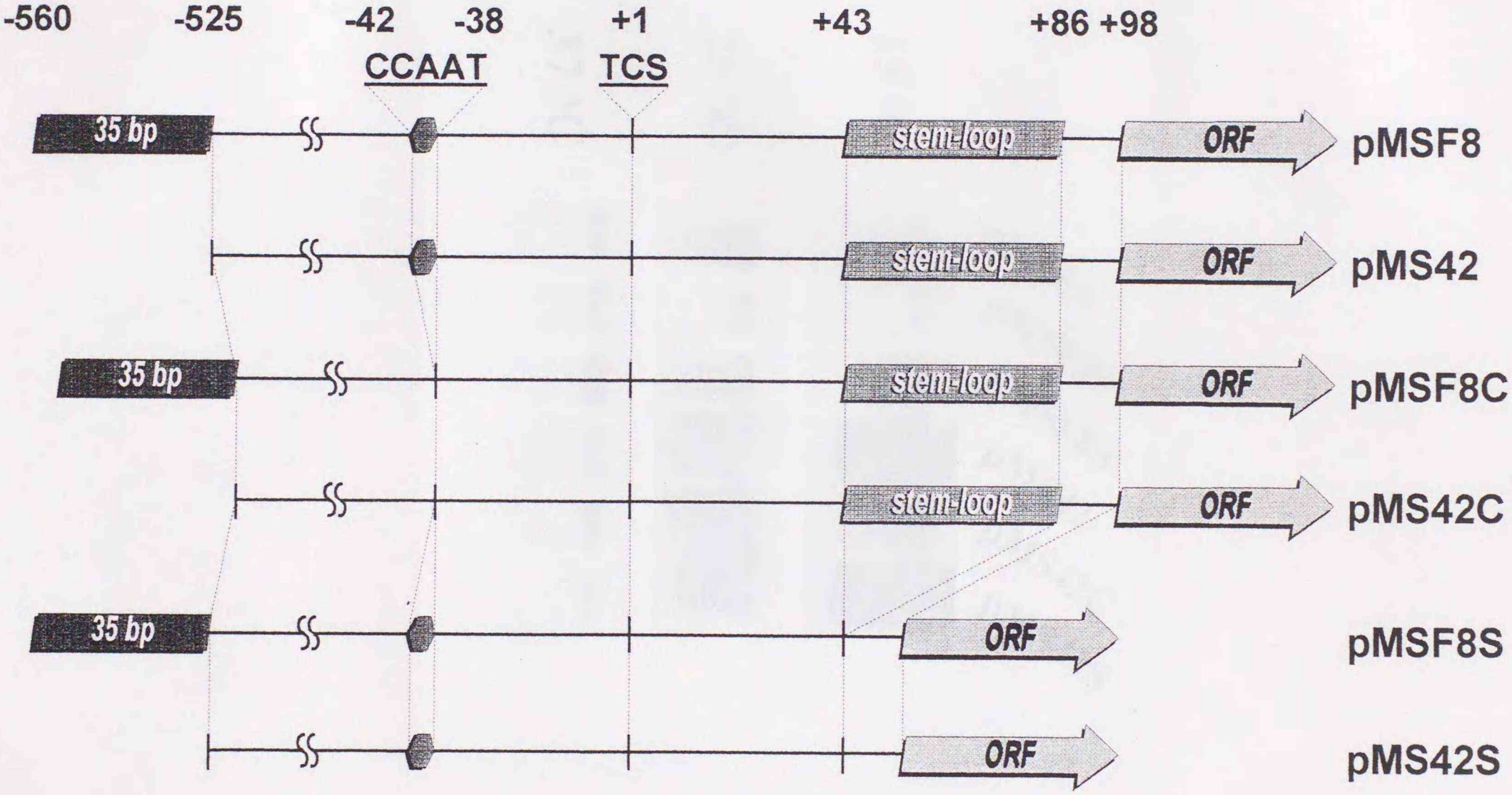

-560
-525
-42
-38
+1
+43
+86
+98
CCAAT
TCS
35 bp
stem-loop
ORF
pMSF8
pMS42
pMSF8C
pMS42C
pMSF8S
pMS42S

Figure 22

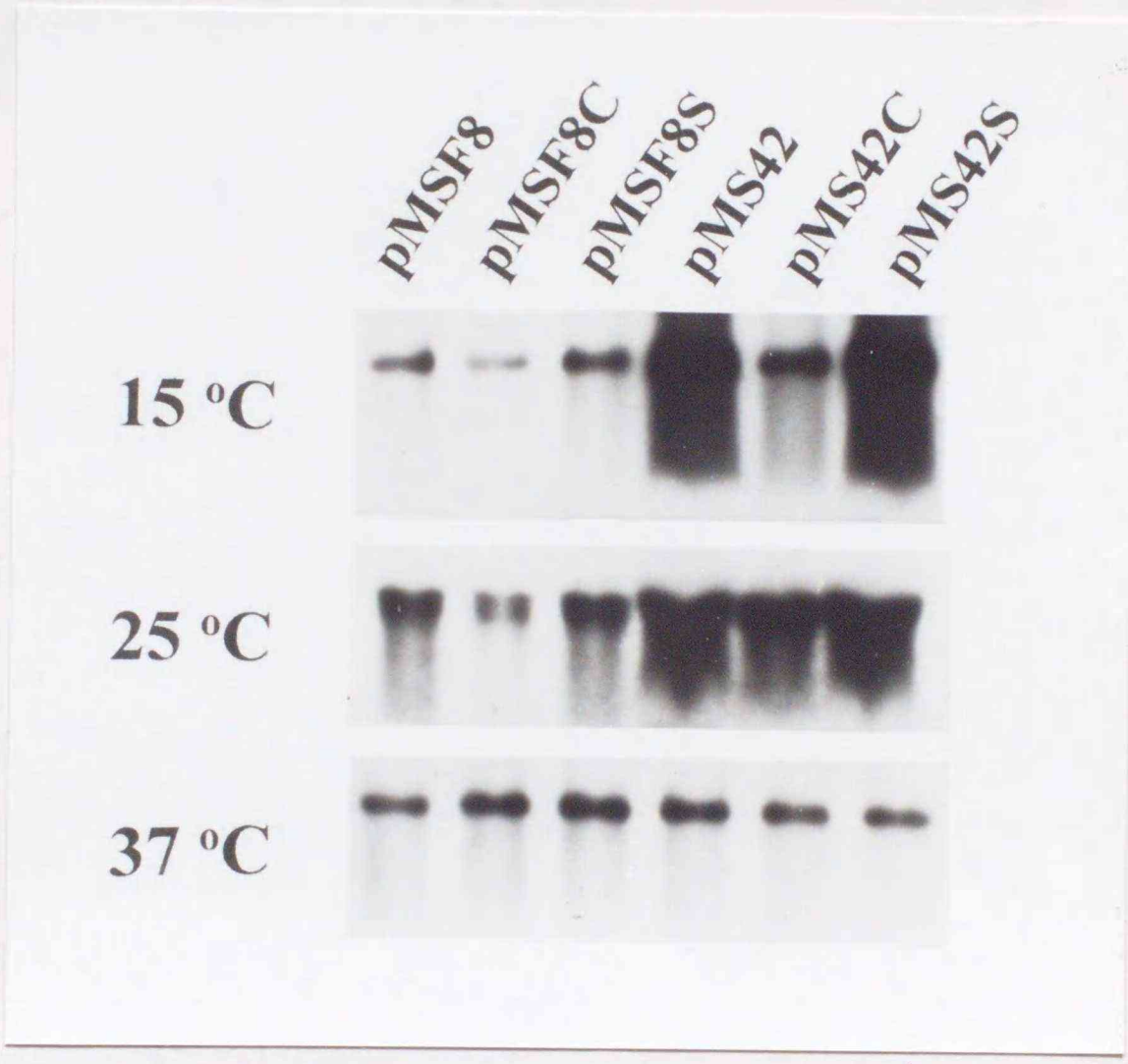


Figure 23

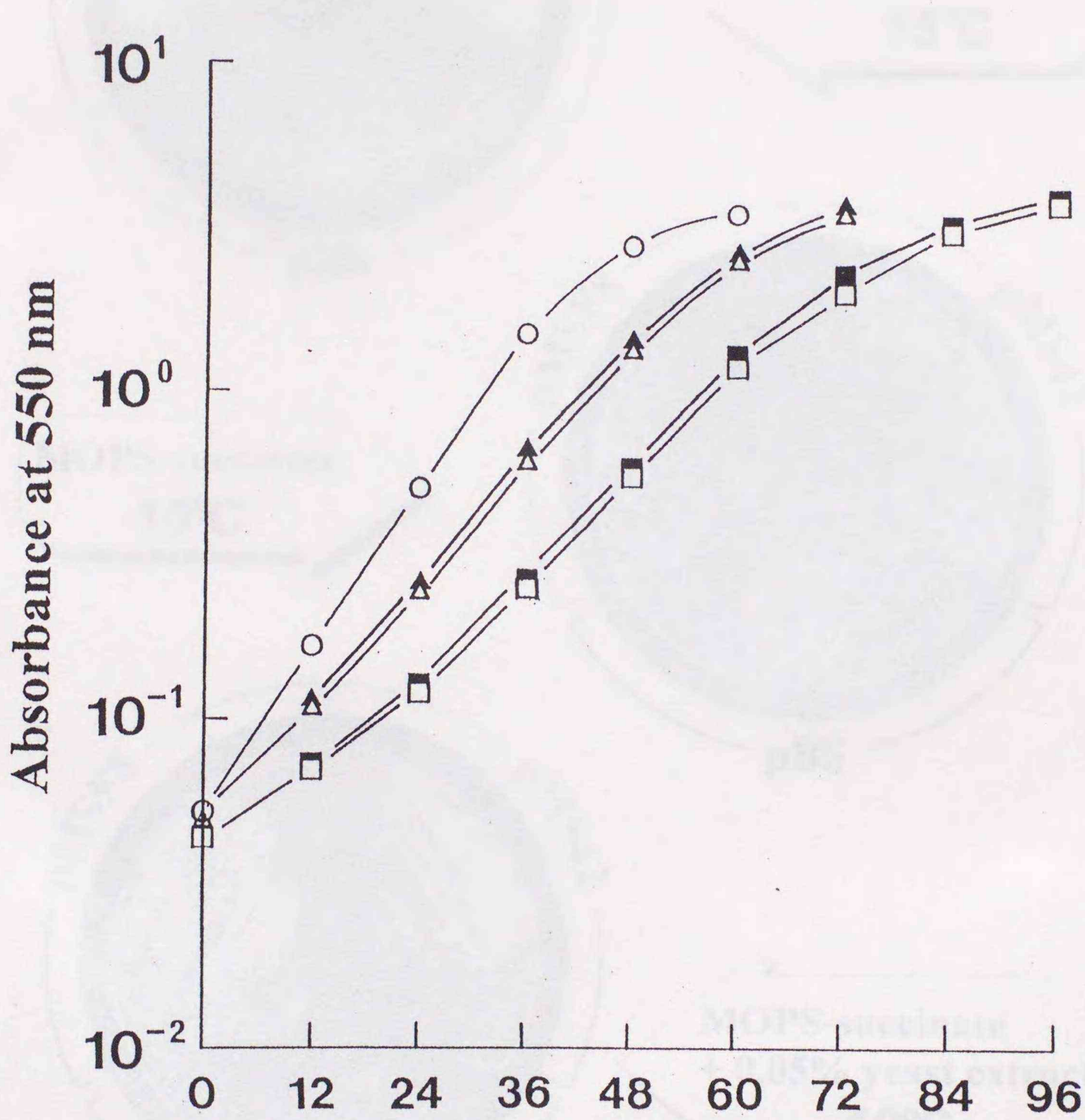
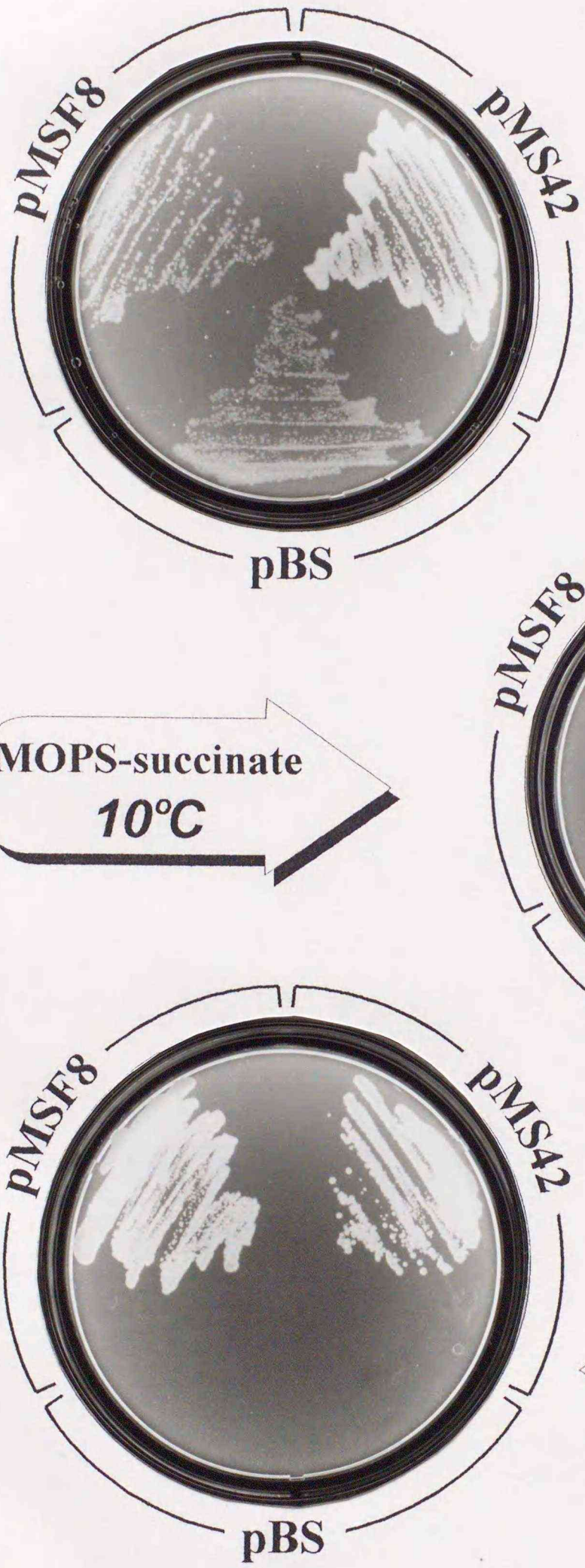
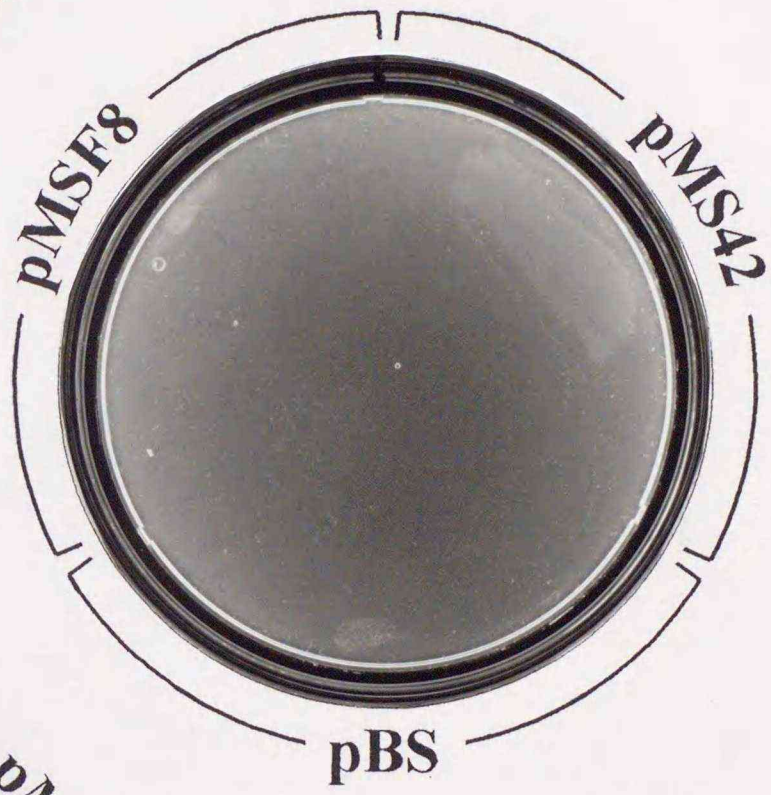

Absorbance at 550 nm
10^{1}
10^{0}
10^{-1}
10^{-2}
0
12
24
36
48
60
72
84
96
Culture Time (h)

Figure 24



MOPS-succinate
15°C



MOPS-succinate
10°C

MOPS-succinate
+ 0.05% yeast extract
10°C

***E. coli* XL1-Blue**

Figure 25

inches 1 2 3 4 5 6 7 8

cm 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Kodak Color Control Patches

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Blue Cyan Green Yellow Red Magenta White 3/Color Black

Kodak Gray Scale

C Y M

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A 1 2 3 4 5 6 **M** 8 9 10 11 12 13 14 15 **B** 17 18 19