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Purification and Characterization of an α-Haloketone-resistant Formate Dehydrogenase from *Thiobacillus* sp. Strain KNK65MA, and Cloning of the Gene

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***Thiobacillus* sp. strain KNK65MA, which produced an NAD-dependent formate dehydrogenase (FDH) highly resistant to α-haloketones, was newly isolated, i.e., the enzyme showed no loss of activity after a 5-h incubation with α-haloketones, such as ethyl 4-chloro-3-oxobutanoate. The enzyme was also resistant to SH reagents. The enzyme, purified to homogeneity, was a dimer composed of identical subunits. The specific activity was 7.6 u/mg, and the apparent K_m values for formate and NAD^+ were 1.6 and 0.048 mM, respectively. The cloned gene of FDH contained one open reading frame (ORF) of 1206 base pairs, predicted to encode a polypeptide of 401 amino acids, with a calculated molecular weight of 44021; this gene was highly expressed in *E. coli* cells. The deduced amino acid sequence of this FDH had high identity to other bacterial FDHs.**

Key words: NAD-dependent formate dehydrogenase; *Thiobacillus*; α-haloketone-resistance

Optically active compounds have been widely recognized as important synthetic intermediates for pharmaceuticals.[1] Among them, chiral α-halohydrins have been recognized as promising building blocks for asymmetric organic synthesis, i.e., alkyl (*S*)-4-halo-3-hydroxybutanoate and alkyl (*R*)-4-halo-3-hydroxybutanoate have been used as synthetic intermediates of various inhibitors of HMG-CoA reductase[2] and L-carnitine synthesis,[3] respectively. For the preparation of these chemicals, enzymatic reduction has proven to be a useful tool.[4] Although enzymatic reduction required α-haloketones as substrates, these ketones have high reactivity and they inactivate enzymes during this type of reaction. Therefore, it is considered as advantageous if enzymes with high resistance to such substrates can be used. On the other hand, as the enzymatic reduction requires expensive cofactors such as NAD(H) or NADP(H), it is typically coupled to the reaction that regenerates the cofactor.[4–8] Therefore, the enzyme used for the regeneration must also be stable and resistant to α-haloketones.

The use of NAD-dependent FDH,[8–24] which catalyzes the oxidation of formate to carbon dioxide with the reduction of NAD^+ to NADH, is suitable for practical use in the regeneration of the cofactor, because no by-products from this regeneration reaction accumulate in the reaction mixture; therefore, the product is easily isolated, the amount of waste is very low, and the thermodynamic equilibrium of the reaction is favorable. Various NAD-dependent FDHs from plants,[10,11] methylotrophic yeasts,[12–16] and bacteria[17–24] have been investigated. Among them, FDHs from *Candida*,[25] *Pseudomonas*,[26,27] and *Mycobacterium*[24] have been improved, i.e., their resistance to oxidation or thermal inactivation has been increased by site-directed mutagenesis, as enzyme stability is known to be important for practical use. However, no reports have referred to the stability of these FDHs in the presence of α-haloketones.

To obtain FDHs suitable for the economical production of chiral α-halohydrins, we attempted to discover novel FDHs that might show high resistance to α-haloketones. In this report, we discuss the purification and characterization of NAD-dependent FDH derived from newly isolated *Thiobacillus* sp. strain KNK65MA. We also discuss the cloning and expression of the FDH gene in *E. coli*. In this context, the issue of resistance to α-haloketones is also addressed.

Materials and Methods

Chemicals. All chemicals used in this study were the best available commercial products.

Media and culture conditions. For the screening of microorganisms with FDH activity, medium A,

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Abbreviations: FDH, formate dehydrogenase; ORF, open reading frame; PCR, polymerase chain reaction; IPCR, inverted PCR; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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comprised of 6.7 g of Bacto yeast nitrogen base without amino acids (Difco), 0.1 g bonito fillet extract-type CR (Riken Vitamin), 0.1 g yeast extract (Difco), 0.1 g polypeptone (Nippon Seiyaku), 4 g K_2HPO_4, and 8 g methanol, in 1 liter of tap water (pH 9.0), was used. *Thiobacillus* sp. strain KNK65MA was inoculated into 7 ml of medium B, comprised of 10 g glycerol, 10 g tryptone (Difco), 5 g yeast extract, 5 g NaCl, and 20 g methanol, in 1 liter of tap water (pH 7.0), in a test tube, was cultured at 28°C for 2 days with shaking, inoculated into 100 ml of medium A containing 20 g per liter of methanol in a 500-ml shaking flask, and then the mixtures were cultured at 28°C for 7 days with shaking. *E. coli* HB101 carrying pFT002 was cultured in 10 ml of 2YT medium[28] in a test tube at 30°C for 25 h with shaking. The recombinant *E. coli* cells were harvested from 1 ml of cultured broth, suspended in 1 ml of 0.1 M potassium phosphate buffer (pH 7), disrupted by sonication, centrifuged to remove the cell debris, and then assayed. *E. coli* HB101 carrying pFA002, which contained the FDH gene from *Ancylobacter aquaticus* strain KNK607M, was cultured as described previously.[17] *Candida boidinii* ATCC32195 was inoculated into medium C, comprised of 6.7 g Bacto yeast nitrogen base without amino acids, 1 g bonito fillet extract-type CR, 1 g yeast extract, 1 g polypeptone, 2 g KH_2PO_4, 2 g K_2HPO_4, and 8 g methanol, in 1 liter of tap water (pH 6.0), and then the mixture was cultured at 28°C for 72 h with shaking.

Screening methods. The first screening for the isolation of microorganisms with FDH activity was done as follows. A spoonful of soil was suspended in 1 ml of saline, and then 70 μl of the supernatant was used to inoculate 7 ml of medium A in a test tube, which contained methanol as the sole carbon source. After aerobic cultivation at 30°C until growth was detected visually, growth was measured by the absorbance at 600 nm. Then, cells from 1.5 ml of cultured broth were harvested, suspended in 0.5 ml of substrate solution comprised of 100 mM potassium phosphate buffer, 500 mM sodium formate, 1 mM NAD^+, and 1% Triton X-100 (pH 7.0), and were incubated at 30°C for 20 h with shaking. The NADH formed by this method was measured by the increase of absorbance at 340 nm. The cultured broths expressing a high ratio of absorbance at 340 nm per 600 nm were selected. For the second screening, the selected broths were inoculated into medium A, cultured under the same conditions as those described above, and cells from 5 ml of cultured broth were harvested, suspended in 0.5 ml of 100 mM potassium phosphate buffer (pH 7.0), disrupted by sonication, centrifuged to remove the cell debris, and then assayed. The cell-free extracts were mixed with equal amounts of 20 mM ethyl 4-chloro-3-oxobutanoate, incubated for 5 min at 30°C, and then assayed. From the ratio of the activity before and after the incubation with ethyl 4-chloro-3-oxobutanoate, the microorganisms producing FDH with high resistance to ethyl 4-chloro-3-oxobutanoate were selected.

Purification of FDH. All procedures were done at 4°C, and all buffers used were potassium phosphate buffer containing 1 mM dithiothreitol and 1 mM EDTA. Step 1: Washed strain KNK65MA cells, collected from 3600 ml of the cultured broth, were suspended in 180 ml of 100 mM buffer (pH 7.0); the cells were then disrupted by sonication. Step 2: The enzyme solution was fractionated with ammonium sulfate (25–60% saturation), and then the precipitate was dissolved in 10 mM buffer (pH 6.5) and dialyzed. Step 3: The enzyme solution was placed on a DEAE-Sepharose column (4.5 × 14 cm), and then eluted with 10 mM buffer (pH 6.5) and 100 mM buffer (pH 6.5). Step 4: Ammonium sulfate was added to the active fractions (24% saturation), and then the solution was placed on a phenyl-Toyopearl 650M (Tosoh) column (2.5 × 20 cm), eluted with 100 mM buffer (pH 6.0) containing 24%-saturated ammonium sulfate, and then eluted with a reduction in ammonium sulfate concentration to 0% saturation. The combined active fractions were concentrated by ammonium sulfate, and then the precipitate was dissolved in 10 mM buffer (pH 6.5) and dialyzed. Step 5: The enzyme solution was placed on a Blue Sepharose 6 Fast Flow (Pharmacia Biotech) column (1.5 × 13 cm), eluted with 10 mM buffer (pH 6.5), and then with an increase in the NaCl concentration to 0.5 M. This operation was done twice, and then the eluted active fractions were concentrated by ultrafiltration. Step 6: The enzyme solution was placed on a Gigapite (Seikagaku Corporation) column (1.5 × 6.5 cm), and then eluted with buffer (pH 6.5); this procedure was increased stepwise in potassium phosphate concentration (2 mM, 50 mM, 70 mM, and 200 mM). This operation was done twice, and then the eluted active fractions were collected and concentrated to 6.0 ml by ultrafiltration.

Measurement of FDH activity. The standard FDH assay was done as follows. The assay mixture, comprised of 1.25 ml of 100 mM potassium phosphate buffer (pH 7.0), 1.5 ml of 1 M sodium formate in 100 mM potassium phosphate buffer (pH 7.0), 150 μl of 100 mM NAD^+, and 100 μl of enzyme solution, was incubated at 30°C, and the increase in absorbance at 340 nm was monitored. One unit of FDH was defined as the amount that catalyzed the reduction of 1 μmol of NAD^+ per min.

The K_m and V_{max} of the purified enzyme were calculated from double-reciprocal Lineweaver-Burk plots. As regards the formate, a standard assay mixture containing various concentrations of formate and

Table 1. Oligonucleotides Used as Primers for PCR

Primer	Sequence
SP1	5′-GCN AAR ATN CTN TGY GT-3′
AP1	5′-GGY TGN GGR AAC CAN ACR TC-3′
SP2	5′-GAG ACG CTG AAG CTG TTC AA-3′
AP2	5′-TAG TGG TCG ATC TTC GGC AGA TCG T-3′
SP3	5′-ACA CTG CAG ATA CGC GCG AGA GGA GAT-3′
AP3	5′-TCT GAA TTC GTT CGC CTG GTC GGT CTG T-3′
SP4	5′-ACG CAT ATG GCG AAA ATA CTT TGC-3′
AP4	5′-AGT CTG CAG TTA GCC GGC CTT CTT GAA-3′

N is indicated as A or G or C or T; R is indicated as A or G; Y is indicated as C or T.

The underlined portions indicate the recognition site of restriction enzymes; *Pst* I site for SP3, *Eco* RI site for AP3, *Nde* I site for SP4, and *Pst* I site for AP4.

5 mM NAD^+ was used; for the NAD^+, a mixture containing various concentrations of NAD^+ and 500 mM formate was used.

Analytical methods. The protein concentration was measured by the method of Lowry *et al.*[29] using bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done using 10% polyacrylamide gels and the methods of Laemmli *et al.*[30] The gel was stained with Coomassie brilliant blue R250. High-performance gel permeation chromatography on a $G3000SW_{XL}$ column (Tosoh) was done to measure the molecular mass of the native enzyme using the protein molecular weight standards kit MS II (Boehringer Ingelheim). The *N*-terminal amino acid sequence of the purified FDH was analyzed by automated Edman degradation using a model 49X Procise (Applied Biosystems).

Oligonucleotides used as primers for PCR. Sequences of the primers for polymerase chain reaction (PCR) are shown in Table 1. Synthetic oligonucleotides were designed as degenerated primers; SP1 corresponding to the *N*-terminal amino acid sequence AKILCV, and AP1 corresponding to the amino acid sequence DVWFPQP, conserved among FDHs of *Aspergillus*, *Neurospora*, *Hansenula*, *Mycobacterium*, and *Pseudomonas*. For inverted PCR (IPCR),[31] SP2 and AP2 primers were used, whereby both SP2 and AP2 corresponded to the partial sequence in the fragment amplified by PCR with the genomic DNA as a template and the degenerated SP1 and AP1 primers. SP3 and AP3 primers corresponded to the partial sequence in the fragment amplified by IPCR. SP4 and AP4 primers corresponded to the deduced *N*-terminal and *C*-terminal region of the FDH, respectively.

Gene cloning. The genomic DNA was prepared from strain KNK65MA by the miniprep method.[32] A 0.95 kbp portion of the FDH gene was amplified by PCR using the genomic DNA as a template with SP1 and AP1 primers. The reaction mixture was composed of each primer, the genomic DNA, the four deoxynucleotides, ExTaq DNA polymerase, and 10x-buffer for ExTaq (Takara).

For the Southern hybridization, genomic DNA (500 ng) digested with each restriction enzyme was transferred onto a Hybond-N^+ filter (Amersham Pharmacia Biotech), as described in the manufacturer's instructions, and then hybridization was done with a probe that was a segment of the FDH gene amplified by PCR using the genomic DNA as a template with SP1 and AP1 primers. Hybridization, preparation of the probe, and detection of the signals were done according to the manufacturer's instructions included with the Gene Images kit (Amersham Pharmacia Biotech).

According to the method of Triglia *et al.*,[31] IPCR was done as follows. The genomic DNA was digested with *Eco* RI, and then self-ligated with T4 DNA ligase. PCR was done using the self-ligated DNA as a template with SP2 and AP2 primers. The reaction mixture was composed of each primer, the self-ligated DNA solution, the four deoxynucleotides, LATaq DNA polymerase, and 2x-GC buffer I for LATaq (Takara). The amplified fragments were used directly for the nucleotide sequencing.

A fragment containing the ORF of the FDH gene was amplified by PCR using the genomic DNA as a template with the SP3 and AP3 primers. The reaction mixture was composed of each primer, the genomic DNA, the four deoxynucleotides, LATaq DNA polymerase, and 2x-GC buffer I for LATaq (Takara). A pFT001 was constructed by cloning the amplified fragment into the *Pst* I-*Eco* RI site of pUC19. The same manipulations, from the amplification to the cloning into pUC19, were done three times, and all of the sequences of the cloned fragments were analyzed. All of the DNA manipulations were done using standard methods, as described by Sambrook *et al.*[28]

Construction of an expression vector. To efficiently express the FDH gene in *E. coli*, a plasmid, pFT002, was constructed. PCR was done using genomic DNA as a template with SP4 and AP4 primers. The amplified fragment was subcloned between *Nde* I and *Pst* I sites in pUCNT, which was constructed as an expression vector by Nanba *et al.*,[33] and sequenced. The resultant plasmid, pFT002, was introduced into *E. coli* HB101.

DNA sequencing. The nucleotide sequences were analyzed by the dideoxy chain termination method[34] with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), using a DNA Sequencing kit, BigDye Terminator V2.5 Cycle Sequencing Ready Reaction (Applied Biosystems). Similarity searches for the deduced amino acid sequence were done with the

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BLAST program of the National Center for Biotechnology Information (NCBI) at the National Library of Medicine. The nucleotide sequence data has been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases; the data has been assigned the accession number AB106890.

Evaluation of resistance to α-haloketones. Cells of *E. coli* HB101 (pFT002), *E. coli* HB101 (pFA002), and *Candida boidinii* ATCC32195 were harvested, suspended in 100 mM potassium phosphate buffer (pH 6.5; one-half volume of the cultured *E. coli* broth, and a one-tenth volume of *Candida*), disrupted by sonication for *E. coli* cells, and by glass bead for the *Candida* cells; the suspensions were then centrifuged to remove the cell debris. The cell-free extracts were incubated in the presence of the α-haloketones shown in Table 3, and then assayed.

Results and Discussion

Isolation of microorganisms that produce FDH with high resistance to α-haloketones

The strains producing FDH with high resistance to ethyl 4-chloro-3-oxobutanoate were isolated from a total of 507 soil samples after enrichment culture with methanol as the sole carbon source. Among these strains, KNK65MA had the highest resistance to ethyl 4-chloro-3-oxobutanoate, and no loss in FDH activity was observed under the conditions of the second screening described in Materials and Methods. The strain expressed 0.41 units of FDH activity per ml of the culture broth, and was identified as *Thiobacillus* sp., the FDH of which has been reported previously.[35-39]

Table 2. Purification of FDH from *Thiobacillus* sp. Strain KNK65MA

Step	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Yield (%)
Crude extract	1040	3570	0.291	100
Ammonium sulfate	895	2040	0.439	86.1
DEAE-Sepharose	673	323	2.08	64.7
Phenyl-Toyopearl	166	30.7	5.41	16.0
Blue-Sepharose	107	15.9	6.73	10.3
Gigapite	63.4	8.42	7.53	6.10

Purification and properties of FDH

The FDH was purified about 26-fold (Table 2). The purified enzyme was a single protein band of 44 kDa on SDS-PAGE. The relative molecular mass of the enzyme was estimated to be about 90 kDa, based on gel permeation chromatography. These results demonstrated that the enzyme is composed of two identical subunits.

The enzyme was most active at pH 5.6, and showed relatively high activity within a wide pH range (Fig. 1(A)). The activity was measured at various temperatures, as shown in Fig. 1(B), and reached a maximum at about 58°C. The stability of the FDH rendered it suitable for practical use, as the enzyme was stable within a wide pH range (Fig. 2(A)). Furthermore, it was stable at 50°C or lower (Fig. 2(B)).

The apparent K_m values for formate and NAD^+ were 1.6 and 0.048 mM, respectively, which were lower than those of the other FDHs, with the exception of the respective *Paracoccus* FDH (0.036 mM for NAD^+)[21] and *Pseudomonas oxalaticus* FDH (0.14 mM for formate) values.[18] The lower K_m values of the enzyme were favorable for the cofactor regeneration system because of the higher activity of

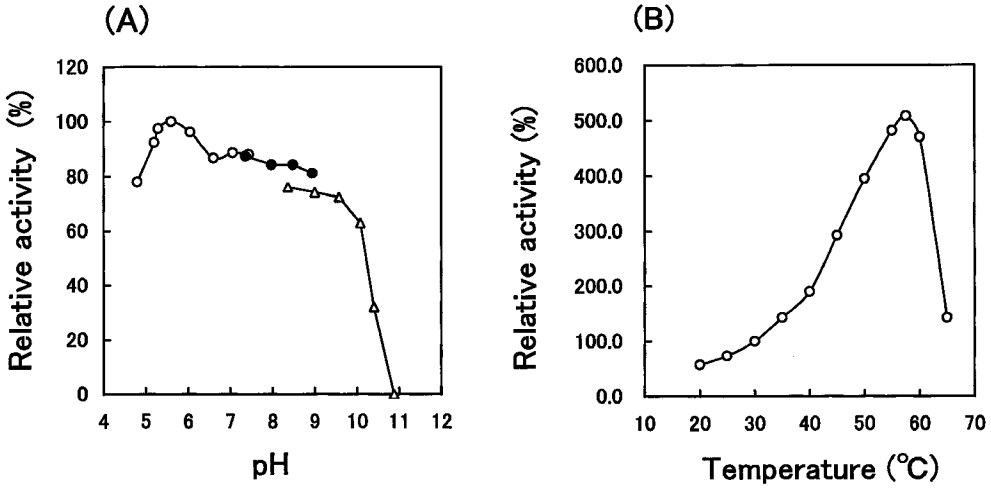

Fig. 1. Effects of pH (A) and Temperature (B) on the Activity of FDH.

(A) Enzyme activity was assayed under the standard assay conditions using 0.063 units of purified FDH, with the exception that the following buffers were used at 100 mM: potassium phosphate (pH 5–8), reaction mixtures (pH 4.8–7.4), (○); Tris-HCl (pH 7.5–9), reaction mixtures (pH 7.4–8.9), (●); carbonate (pH 9–11.5), reaction mixtures (pH 8.4–10.9), (△). The relative activity was the percentage of the activity at pH 5.6. (B) Assays were done at various temperatures for 5 min under standard assay conditions using 0.013 units of purified FDH. The relative activity was the percentage of the activity at 30°C.

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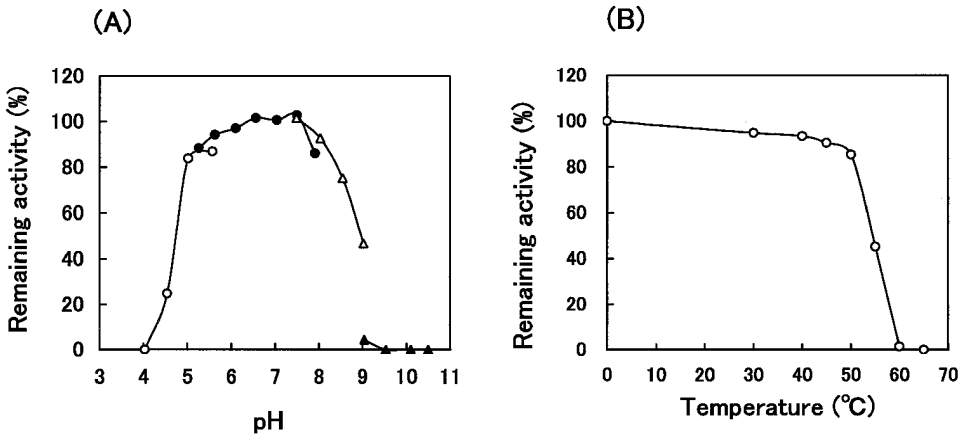

Fig. 2. Effects of pH (A) and Temperature (B) on the Stability of the FDH.

(A) 0.16 units of FDH were incubated at 30°C for 22 h in 0.25 ml of the following buffers: 50 mM of sodium acetate, pH 4.0–5.6 (○); 100 mM of potassium phosphate, pH 5.3–7.9 (●); 100 mM of Tris-HCl, pH 7.5–9.0 (△); 100 mM of carbonate, pH 9.0–10.5 (▲). After incubation, the remaining activity was assayed under the standard assay conditions, and was expressed as the percentage of the activity before the incubation. (B) 0.082 units of FDH were incubated at different temperatures for 10 min in 130 μl of 100 mM potassium phosphate, pH 7.0, and then the remaining activity was assayed under the standard assay conditions, and was expressed as the percentage of the activity after the incubation at 0°C.

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the enzyme, which was observed even at lower concentrations of the substrate.

Using a variety of carboxylic acids, aldehydes, and alcohols as substrates, the activity of the enzyme was examined. Reduction of NAD^+ to NADH was detected only when formate was used as a substrate for concomitant oxidation. The following compounds were judged to be inactive as substrates because no formation of NADH was detected: methanol, formaldehyde, ethanol, acetaldehyde, acetic acid, glyoxylic acid, 1-propanol, propionaldehyde, propionic acid, and glycerol. The enzyme was specific to formate, the same as the other FDHs.[12,13,15,19,20] Some electron acceptors were also investigated as cofactors, with formate as an electron donor. The enzyme was most active in the presence of NAD^+, and the activity in the presence of $NADP^+$ was 4.2% of that in the presence of NAD^+. However, the following compounds were not reduced: ferricyanide, nitro blue tetrazolium, phenazine methosulfate, benzyl viologen, and 2,6-dichloroindophenol.

Enzyme activity was assayed under the standard conditions in the presence of various compounds. The enzyme was inhibited by NaN_3 (1 mM) and Hg^{2+} (1 mM) with 100% inhibition, and slightly inhibited by *p*-chloromercuribenzoic acid (0.2 mM) and Cu^{2+} (0.2 mM) with 10.7% and 13.6% inhibition, respectively. *N*-ethyl maleimide (1 mM) and the metal chelators, EDTA (1 mM) and *o*-phenanthroline (0.2 mM), were not associated with any inhibition of enzyme activity. Resistance to SH reagents such as *p*-chloromercuribenzoic acid and *N*-ethyl maleimide was one of the characteristic properties of this FDH, compared to *Ancylobacter* FDH, which lacked this property.[17]

Cloning and sequencing of the gene for FDH, and comparison of the deduced amino acid sequence with those of other FDHs

Southern hybridization was done using a portion of the FDH gene as a probe, and the genomic DNA digested by *Eco*RI gave a single band of about 4.5 kbp upon agarose gel electrophoresis. Based on the results, IPCR was done using the genomic DNA self-ligated at the *Eco*RI site as a template. Using primers corresponding to the outside of the ORF, and using the genomic DNA as a template, PCR was done; then, a fragment containing the ORF of the FDH gene was amplified. A pFT001 was constructed by insertion of 1275 bp of the amplified *Pst*I-*Eco*RI fragment into pUC19, and then the nucleotide sequence of the insert was analyzed. The same steps were done three times, and all sequences of the obtained clones were identical.

From the DNA sequence submitted to the nucleotide sequence database, the ORF consisted of 1206 bases with a starting triplet, ATG, at position 32 and an ending triplet, TGA, at position 1235. The ORF was predicted to encode a polypeptide of 401 amino acids, with a calculated molecular weight of 44,021. The deduced *N*-terminal amino acid sequence coincided with the sequence of the purified FDH from strain KNK65MA (30 amino acids, not including the fifth amino acid, were identified.), except for the existence of an *N*-terminal methionine. The alignment of the deduced amino acid sequence of this FDH with those of other FDHs is shown in Fig. 3. The FDH of strain KNK65MA had high identity with the other known bacterial FDHs. The identities with the enzymes from *Ancylobacter*,[17] *Pseudomonas*,[19,22] *Mycobacterium*,[24] *Moraxella*,[20] and *Paracoccus*[21,23] were 89.8%, 92.0%, 91.5%, 84.8%, and 82.5%,

Fig. 3. Alignment of the Amino Acid Sequences of Various FDHs.

1, FDH of *Thiobacillus* sp. strain KNK65MA; 2, FDH of *Ancylobacter aquaticus* strain KNK607M;[17] 3, FDH of *Pseudomonas*, sp. 101;[19,22] 4, FDH of *Mycobacterium vaccae* N10;[24] 5, FDH of *Moraxella*, sp.;[20] 6, FDH of *Paracoccus* sp. 12-A;[21,23] 7, FDH of *Candida boidinii*;[25] 8, FDH-like protein of *Aspergillus nidulans*;[40] 9, FDH of *Solanum tuberosum*.[11] The conserved amino acids are marked in black.

respectively. Enzymes from eucaryotes, such as those from yeast (*Candida boidinii*),[25] fungi (*Aspergillus nidulans*),[40] and plants (potato, *Solanum tuberosum*),[11] showed lower identities (49.4%, 53.0%, and 51.7%, respectively). Using the expression plasmid pFT002, the FDH gene was efficiently expressed. The activity of *E. coli* HB101 carrying pFT002 was 11.3 units per ml of the cultured broth, which amounted to an approximately 28-fold increase over the activity of strain KNK65MA.

Resistance to α-haloketones

As shown in Table 3, the FDH of strain KNK65MA, the enzyme produced by recombinant *E. coli*, was highly resistant to inactivation by α-haloketones, except for the case of 4-bromo-3-oxobutano-

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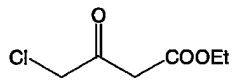
Table 3. Resistance of FDH to α-Haloketones

Compound	Remaining activity of FDH (%)		
	Thiobacillus sp. strain KNK65MA	*Ancylobacter aquaticus* strain KNK607M	*Candida boidinii* ATCC32195
None	101.4	101.4	101.3
Ethyl 4-chloro-3-oxobutanoate	100.7	20.1	14.3
Ethyl 4-bromo-3-oxobutanoate	24.1	0	0
1-chloro-2-oxopropane	100.0	46.2	38.2
Ethyl 2-chloro-3-oxobutanoate	100.0	2.5	5.4
2, 3′-dichloroacetophenone	97.9	27.0	7.4
2-chloro-1-(3-pyridyl)-ethanone	90.1	6.1	1.5

The cell-free extracts prepared as described in Materials and Methods were mixed with an equal amount of 20 mM α-haloketones dissolved in 100 mM potassium phosphate buffer (pH 6.5), incubated for 5 h at 30°C, and then assayed. The resistance to α-haloketones was expressed as a percentage of the remaining activity after the incubation compared to that before the incubation.



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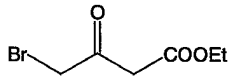
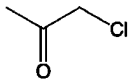
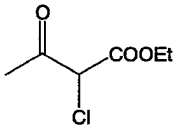


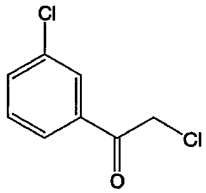
Ethyl 4-bromo-3-oxobutanoate



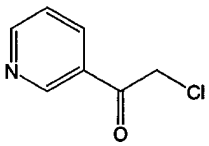
1-chloro-2-oxopropane



Ethyl 2-chloro-3-oxobutanoate



2, 3'-dichloroacetophenone



2-chloro-1-(3-pyridyl)-ethanone

ate. Compared to the results with the enzyme, the FDHs of *Ancylobacter*[17] and *Candida*[25] were inactivated to a much greater extent by α-haloketones. The higher stability of the strain KNK65MA FDH was unique to this type of FDH; this feature proved important for the regeneration of the cofactor in the enzymatic conversion of α-haloketones to chiral α-halohydrins, which are important synthetic intermediates for pharmaceuticals.

One of the reasons for the observed resistance to α-haloketones may have been the replacement of the Cys residues of the FDH, which is not the case with the other FDHs, as shown in Fig. 3. Because α-haloketones are considered to react with nucleophilic functional groups such as the SH group,[41,42] and therefore α-haloketones may react with the free SH groups of the enzyme and may thereby inactivate it. Existence of the free SH groups in *Pseudomonas* FDH has been suggested previously.[8] Replacement of Cys residues may in this manner reduce the reactivity between the enzyme and α-haloketones. Other bacterial FDHs[17-24] contain seven Cys residues at the 6th, 146th, 183rd, 249th, 256th, 289th, and 355th positions; however, in the case of strain KNK65MA, Cys residues at the 256th and the 289th positions are replaced by Val and Ala, respectively. Furthermore, the FDH of strain KNK65MA was more resistant to SH reagents than that of *Ancylobacter*,[17] suggesting that the reactive SH groups on the enzyme were decreased in number. In addition, the mutant FDH of *Pseudomonas* containing a Cys256Ser or a Cys256Met replacement have been reported to have increased resistance to inactivation by oxidation and SH reagents.[26] These findings suggest the importance of the 256th residue being Val, in the case of the FDH of strain KNK65MA, in order to achieve this type of resistance. On the other hand, *Candida* FDH[25] contains only two Cys residues, at the 23rd and 262nd positions, which correspond to the 53rd and 289th positions of bacterial FDHs, respectively. Slusarczyk *et al.*[25] have reported that the Cys23 (the 53rd position in bacterial FDH) of the unstable wild-type *Candida* FDH was replaced by Ser by site-directed mutagenesis; in that study, the stability in terms of oxidative stress was increased. Based on this report, the 53rd residue of bacterial FDHs being Ser, as is the case in the FDH of strain KNK65MA, is also important for stability. In addition to the potential effects of the 53rd and the 256th residues, there may be other explanations for the observed resistance to α-haloketones, such as an effect of the 289th residue, or the influence of other non-identical amino acid residues.

In conclusion, we obtained α-haloketone-resistant FDH, which was found to be suitable for the economical production of chiral α-halohydrins, when used as an enzyme for cofactor regeneration.

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