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Purification and Characterization of a Malic Enzyme from the Ruminal Bacterium *Streptococcus bovis* ATCC 15352 and Cloning and Sequencing of Its Gene

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Malic enzyme (EC 1.1.1.39), which catalyzes L-malate oxidative decarboxylation and pyruvate reductive carboxylation, was purified to homogeneity from *Streptococcus bovis* ATCC 15352, and properties of this enzyme were determined. The 2.9-kb fragment containing the malic enzyme gene was cloned, and the sequence was determined and analyzed. The enzymatic properties of the *S. bovis* malic enzyme were almost identical to those of other malic enzymes previously reported. However, we found that the *S. bovis* malic enzyme catalyzed unknown enzymatic reactions, including reduction of 2-oxoisovalerate, reduction of 2-oxoisocaproate, oxidation of D-2-hydroxyisovalerate, and oxidation of D-2-hydroxyisocaproate. The requirement for cations and the optimum pH of these unique activities were different from the requirement for cations and the optimum pH of the L-malate oxidative decarboxylating activity. A sequence analysis of the cloned fragment revealed the presence of two open reading frames that were 1,299 and 1,170 nucleotides long. The 389-amino-acid polypeptide deduced from the 1,170-nucleotide open reading frame was identified as the malic enzyme; this enzyme exhibited high levels of similarity to malic enzymes of *Bacillus stearothermophilus* and *Haemophilus influenzae* and was also similar to other malic enzymes and the malolactic enzyme of *Lactococcus lactis*.

Carbon dioxide is stimulatory to or is required by *Streptococcus anginosus* (31), *Streptococcus pyogenes* (33), and certain enterococci (8). *Streptococcus bovis* also requires CO_2 for growth in a synthetic medium containing ammonium salts as the sole nitrogen source (45). Incorporation of CO_2 into aspartic acid in *S. bovis* and *Streptococcus faecium* subsp. *durans* has been reported previously (38, 46). Malic enzyme [(*S*)-malate:NAD^+ oxidoreductase (decarboxylating)] catalyzes NAD(P)-dependent L-malate oxidative decarboxylation and NAD(P)H-dependent pyruvate reductive carboxylation (CO_2 fixation), and the malic enzymes have been classified into three groups (EC 1.1.1.38, EC 1.1.1.39, and EC 1.1.1.40) on the basis of their coenzyme specificities and their abilities to catalyze the decarboxylation of oxaloacetate. This type of enzyme was isolated from *Streptococcus faecalis* (27). The participation of malic enzyme in CO_2 fixation has not been well studied with any other bacterial malic enzyme (5, 6, 11, 16, 20, 22, 24, 27, 37, 48) except the malic enzyme of *Micrococcus* sp. (32). In order to reveal the participation of this enzyme in CO_2 fixation, we decided to isolate this enzyme from the ruminal bacterium *S. bovis* and study it.

All of the lactic acid bacteria except *Lactobacillus casei* and *S. faecalis* are known to contain malolactic enzyme instead of malic enzyme; malolactic enzyme converts L-malate to L-lactate directly (6, 27). However, we thought that *Streptococcus* sp. contains malic enzyme, since the results of a preliminary experiment suggested that *Streptococcus* sp. converts L-malate into L-lactate in at least two steps. *S. bovis* ATCC 15352 was available in our laboratory, and we chose this strain as a source of malic enzyme. Strain ATCC 15352 is a UV mutant of parent strain ATCC 15351 (14), which was demonstrated to be a *S. bovis* strain by hybridization analysis (43).

In this paper we describe the purification and the properties of the *S. bovis* malic enzyme, including some unique catalytic properties. We also describe cloning and sequence analysis of the *S. bovis* malic enzyme gene.

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MATERIALS AND METHODS

Materials. NAD^+, $NADP^+$, NADH, and NADPH were purchased from Kojin, Tokyo, Japan. Bovine serum albumin, L-lactate dehydrogenase, D-lactate dehydrogenase, malic enzyme from chicken liver, ATP, ADP, and salmon test DNA were obtained from Sigma Chemical Co., St. Louis, Mo. Alcohol dehydrogenase and isocitrate dehydrogenase were obtained from Oriental Yeast, Tokyo, Japan. D-Malate and DL- and L-2-hydroxyisovalerate were obtained from Aldrich Japan, Tokyo, Japan. DEAE-cellulose, 4-oxovalerate, and *p*-chloromercuribenzoate were obtained from Wako Pure Chemical Industries, Kyoto, Japan. DL-Hydroxyisocaproate and L-hydroxyisocaproate were obtained from Tokyo Kasei Kogyo, Tokyo, Japan. All other chemicals were of the highest grade available.

Bacterial strains and cultivation. *S. bovis* ATCC 15352 was maintained in malic enzyme induction medium (27) containing 2% agar. This medium contained 0.25% (wt/vol) yeast extract, 0.25% (wt/vol) tryptone, 0.5% (wt/vol) K_2HPO_4, 0.4% (wt/vol) sodium L-malate, and 0.1 μg of lipoic acid per ml and was adjusted to pH 7.5 with KOH. Cells were grown in 22 liters of the induction medium at 37°C for 1 day without shaking. The cells were harvested by centrifugation at 5,500 × *g* for 15 min.

Escherichia coli was grown at 37°C in Luria-Bertani medium (34) supplemented with ampicillin (50 to 100 μg/ml) when necessary. Plasmids were constructed and amplified in *E. coli* DH5α (34).

Enzyme activity assay. The oxidative and reductive activities of malic enzyme were determined spectrophotometrically with a Hitachi model 100-50 spectrophotometer on the basis of increases and decreases in the A_{340} of NADH, respectively, at 30°C in 3 ml of reaction mixture. The concentrations of the components in the reaction mixture were not corrected for metal chelate complexes. Each reaction mixture used to assay for L-malate oxidative decarboxylating activity contained 50 mM Tris-HCl buffer (pH 8.2 at 30°C), 0.10 mM $MnSO_4$, 1.0 mM NH_4Cl, 0.5 mM NAD^+, and 5.0 mM neutralized sodium L-malate (27). Each reaction mixture used to assay for pyruvate reductive carboxylating activity contained 50 mM Tris-HCl buffer (pH 6.7 at 30°C), 0.10 mM $MnSO_4$, 1.0 mM NH_4Cl, 0.1 mM NADH, 50 mM $NaHCO_3$, and 20 mM sodium pyruvate, unless

TABLE 1. Purification of malic enzyme from *S. bovis*

Step	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg)[a]	Yield (%)	Purification (fold)
Cell extract	1,074	3,647	3.40	100	1
Ammonium sulfate	237	2,634	11.1	72.2	3.27
Heat treatment	215	3,057	14.2	83.8	4.19
DEAE-cellulose	5.47	527	96.4	14.5	28.4

[a] L-Malate oxidative decarboxylating activity.

indicated otherwise. To make sure that CO_2 was not rapidly lost under these conditions, liquid paraffin was overlaid on each reaction mixture and the cuvette was capped. The reaction mixtures used to assay for 2-oxoisovalerate and 2-oxoisocaproate reductive activities contained 50 mM Tris-HCl buffer (pH 6.7), 1.0 mM 2-oxoisovalerate or 1.0 mM 2-oxoisocaproate, and 0.10 mM NADH, and the reaction mixtures used to assay for D-2-hydroxyisovalerate and D-2-hydroxyisocaproate oxidative activities contained 50 mM glycine–NaOH buffer (pH 9.3), 2.0 mM DL-2-hydroxyisovalerate or 2.0 mM DL-2-hydroxyisocaproate, and 1.0 mM NAD^+. One unit of activity was defined as the amount of enzyme required to reduce 1.0 μmol of NAD^+ per min or to oxidize 1.0 μmol of NADH per min. Specific activity was expressed in units per milligram of protein. The absence of oxaloacetate decarboxylating activity and the absence of L-malate dehydrogenase activity (which catalyzes L-malate oxidation without decarboxylation) were confirmed by a high-performance liquid chromatography (HPLC) analysis of the reaction mixtures and by the methods of Hsu and Lardy (19) and Yoshida and Freese (49), respectively.

Gel electrophoresis and other analytical methods. The purity of the enzyme and the subunit molecular weight were estimated by using a modified Laemmli gel (23). Native gradient polyacrylamide gel electrophoresis (PAGE) was also used to estimate the molecular weight of the native enzyme as recommended by the manufacturer (Pharmacia, Uppsala, Sweden). The molecular weight of the native enzyme was also estimated by gel filtration at room temperature, using HPLC and a TSK-GEL 3,000SW column (7.5 by 600 mm) equilibrated with 100 mM Tris-HCl buffer (pH 7.4 at 4°C) containing 100 mM NaCl at a flow rate of 1.0 ml/min. Samples were detected at 280 nm. A molecular weight calibration kit (Pharmacia) was used as the standard. Isoelectric focusing was carried out at 4°C as described by Manabe et al. (30) to estimate the isoelectric point. The gels were stained with Coomassie brilliant blue R-250. The protein concentration was determined by the method of Lowry et al. (28), with bovine serum albumin as the standard. The N-terminal amino acid sequence of the malic enzyme was determined by automated microsequencing (model 473A apparatus); the Edman degradation reactions were kindly performed by workers at the Tsukuba Research Center of Mitsubishi Petrochemical Co.

Organic acids were analyzed by HPLC at room temperature by using a Shodex Ionpac C811 column (8 by 500 mm) equilibrated with 10 mM $HClO_4$ at a flow rate of 1.0 ml/min. Samples were detected at 209 nm.

Enzyme purification. All purification procedures were carried out at 4°C unless indicated otherwise.

(i) Preparation of cell extract. Wet cells (19.5 g) were suspended in 250 ml of extraction buffer (50 mM Tris-HCl buffer [pH 7.4 at 4°C], 10 mM 2-mercaptoethanol, 1 mM EDTA) and disrupted with an ultrasonic disintegrator (Kaijo Denki, Tokyo, Japan) for 60 min. The debris was removed by centrifugation at 5,500 × *g* for 20 min.

(ii) Ammonium sulfate precipitation. Solid ammonium sulfate (88.5 g) was gradually added to the cell extract (252 ml) to 55% saturation. After the preparation had stood for 7 h, the supernatant was collected by centrifugation at 5,500 × *g* for 20 min and then brought to 75% saturation in the same manner. After this preparation had stood for 14 h, the precipitate was collected by centrifugation at 5,500 × *g* and dissolved in a minimal volume of extraction buffer. This solution was dialyzed overnight against 500 ml of the same buffer.

(iii) Heat treatment. The enzyme solution from the ammonium sulfate precipitation step was divided into 1-ml portions, and each portion was subjected to heat treatment at 62°C for 3.5 min. The precipitate was removed by centrifugation at 11,000 × *g* for 25 min.

(iv) DEAE-cellulose column chromatography. The enzyme solution from the heat treatment step was loaded onto a DEAE-cellulose column (10 by 200 mm) that had been previously equilibrated with extraction buffer. The enzyme was eluted with 51 ml of extraction buffer containing 100 mM NaCl and then with 42 ml of extraction buffer containing 150 mM NaCl at a flow rate of 0.3 ml/min. The fractions containing the enzyme activity were pooled (47.5 ml), and a precipitate was obtained by adding ammonium sulfate to 75% saturation. After the preparation had stood for 12 h, the precipitate was collected by centrifugation at 11,000 × *g* for 25 min and dissolved in a minimal volume of extraction buffer, and the resulting preparation was dialyzed overnight against 100 ml of the same buffer.

Kinetic studies. The activity of the purified malic enzyme was studied as a function of the substrate concentration. Except for the substrate tested, the composition of the assay system was the same as that described above. The kinetic constants (K_m, maximum velocity [V_{max}]) for substrates and the dissociation constants (K_d) for cations were calculated from the Lineweaver-Burk plot. The V_{max} was the maximum molecular concentration of substrate catalyzed by 1.0 μmol of malic enzyme per min. The V_{max} of reductive activity was calculated from the NADH data, since the activity was measured at NADH concentrations that were lower than the K_m value.

Construction and screening of the DNA library. Cells of *S. bovis* (1.8 g, wet weight) grown to the stationary phase were harvested, resuspended in 20 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 100 mg of lysozyme, and incubated for 2 h at 37°C. Then 2 mg of proteinase K was added to the cell solution, and the cell solution was incubated for 1 h at 37°C. After the cells were lysed by adding sodium dodecyl sulfate (SDS) to a final concentration of 1% (wt/vol) and the preparation was incubated for 10 min at 37°C, DNA was extracted by standard methods (4). *S. bovis* DNA was partially digested with *Sau*3AI and fractionated on a 10 to 40% sucrose gradient. Genomic DNA that was 10 to 17 kb long was ligated to cloning vector Charomid 9-36 (Nippon Gene, Toyama, Japan) cleaved with *Bam*HI. The ligation mixture was then packaged by using an in vitro packaging kit (Amersham, Buckinghamshire, England) and introduced into *E. coli* DH5α as recommended by the manufacturer.

Colonies were screened by the modified hybridization method (4), using synthetic oligonucleotide mixtures (5′GA[A/G]CA[A/G]GC[A/C/G/T]AA[A/G]AA[A/G]TT[T/C]GG3′) labeled with [γ-^{32}P]ATP (ICN Biomedicals, Costa Mesa, Calif.). The mixtures were designed by using the NH_2-terminal amino acid sequences of the malic enzyme determined in this study. Each hybridization solution contained 0.9 M NaCl, 50 mM NaH_2PO_4, 5 mM Na_2 EDTA, 0.5% (wt/vol) SDS, 5× Denhardt solution (4), and 2 mg of denatured salmon test DNA per 100 ml. The hybridization temperature was 42°C. A positive clone was purified. Recombinant *E. coli* DH5α was grown in Luria-Bertani medium supplemented with ampicillin to the stationary phase, harvested, resuspended in 5 ml of the extraction buffer, sonicated, and subjected to the malic enzyme assay described above. The cloned DNA was subjected to a Southern blot analysis, which was done by using a DIG DNA labeling and detection kit (Boehringer Mannheim GmbH, Mannheim, Germany) and CSPD (a concentrated chemiluminescent substrate for alkaline phosphatase) (TROPIX, Bedford, Mass.) according to the manufacturers' instructions.

Sequence analysis. The complete nucleotide sequence of the cloned fragment was determined from both strands by the dideoxynucleotide chain termination method, using a model 373A DNA sequencer (Applied Biosystems/Perkin-Elmer, Foster City, Calif.). Plasmids containing the nested deletions were produced by using a deletion kit (Kilo-Sequence; Takara, Kyoto, Japan), and these plasmids were used to determine the sequence. Nucleotide sequence data were analyzed with the GENETYX program (Software Development Co., Tokyo, Japan). Homology comparisons with database sequences were performed by the Blast (1) and Fasta (35) network services.

Nucleotide sequence accession numbers. The sequence data determined in this study have been deposited in the GenBank database under accession numbers U35658 (for the open reading frame 1 [ORF-1] gene) and U35659 (for the *S. bovis* malic enzyme gene).

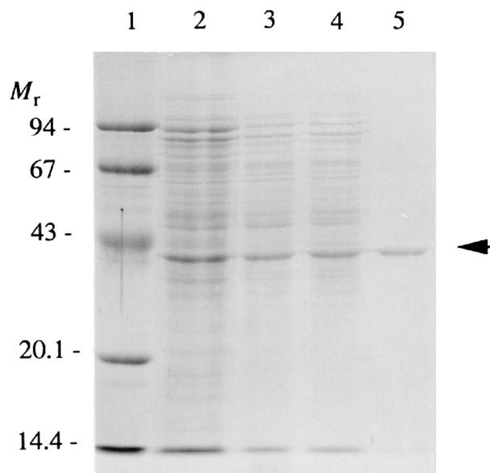

FIG. 1. SDS-PAGE of the malic enzyme from *S. bovis* at various steps of purification. Lane 1, molecular weight markers; lane 2, cell extract; lane 3, preparation after ammonium sulfate precipitation; lane 4, preparation after heat treatment; lane 5, preparation after elution from DEAE-cellulose column. Lanes 1 through 5 contained 10, 85, 26, 20, and 3 μg of protein, respectively. The numbers on the left indicate molecular weights (10^3).

TABLE 2. Effects of L-malate analogs, L-malate metabolite-related compounds, and SH reagents on L-malate oxidative decarboxylating activity

Compound[a]	Relative activity (%)
None	100
Fumarate (5 mM)	88
L-Aspartate (5 mM)	94
L-Tartrate (5 mM)	118
D-Malate (5 mM)	83
Oxaloacetate (5 mM)[b]	18
L-Threonine (5 mM)	98
L-Lactate (5 mM)	88
Glucose (1 mM)	91
Fructose 1,6-bisphosphate (1 mM)	84
Pyruvate (1 mM)	91
Pyruvate (1 mM) + $NaHCO_3$ (1 mM)	91
ATP (1 mM)[b]	10
ATP (0.1 mM)[b]	63
ADP (1 mM)[b]	51
DTT (1 mM)	100
PCMB (0.1 mM)	92
PCMB (1 mM)	0
PCMB (1 mM) + DTT (1 mM)	56
$HgCl_2$ (1 mM)	0
$HgCl_2$ (1 mM) + DTT (1 mM)	ND[c]
Monoiodoacetate (1 mM)	93

[a] DTT, dithiothreitol; PCMB, *p*-chloromercuribenzoate.
[b] Oxaloacetate, ATP, and ADP were neutralized with NaOH.
[c] ND, activity could not be determined spectrophotometrically.

RESULTS

Purification, molecular properties, and stability of the malic enzyme. *S. bovis* ATCC 15352 synthesized malic enzyme when it was maintained and cultivated in malic enzyme induction medium. The induced malic enzyme was isolated by the simple three-step procedure described in Materials and Methods. The procedure summarized in Table 1 led to a 28-fold purification and a yield of 14.5%. The enzyme was purified to apparent homogeneity (Fig. 1). The molecular weight of the subunit was estimated to be 40,000. The results of native gradient PAGE and gel filtration indicated that the molecular weight of the native enzyme was 80,000 and 82,000, respectively. Taken together, these data suggested that the enzyme was a homodimer. The isoelectric point of the enzyme was determined to be 4.8. The amino acid sequence of the 23 N-terminal amino acids was determined to be ATKDVKELAIEQAKKFGG KLEVT.

The stability of the enzyme was studied by monitoring the L-malate oxidative decarboxylating activity. When the enzyme was kept at different pH values at 4°C for 30 h, it was stable at pH 6 to 9. Inactivation occurred rapidly at pH values above 11 and occurred gradually at pH values below 6. When the enzyme was incubated in extraction buffer for 10 min at various temperatures, it was stable at 0 to 60°C, but it was inactivated rapidly at temperatures above 60°C and was completely inactivated at 75°C. During 1 month of storage at 4°C, the enzyme was stable in extraction buffer and in extraction buffer lacking 2-mercaptoethanol, retaining 90 and 78% of the original activity, respectively. However, it was unstable in extraction buffer lacking both 2-mercaptoethanol and EDTA and in extraction buffer lacking only EDTA, retaining 24 and 12% of the original activity, respectively. Thus, 1.0 mM EDTA had a stabilizing effect on the enzyme, and this effect was increased by adding 10 mM 2-mercaptoethanol, but 10 mM 2-mercaptoethanol alone did not have a stabilizing effect.

Properties of the L-malate oxidative decarboxylating activity and the pyruvate reductive carboxylating activity. The *S. bovis* malic enzyme catalyzed L-malate oxidative decarboxylation but did not exhibit L-malate dehydrogenase activity or oxaloacetate decarboxylating activity. $NADP^+$ served as a coenzyme to a lesser extent than NAD^+. The kinetic constants (K_m value and V_{max}) for NAD^+ and $NADP^+$ were as follows: for NAD^+, 0.083 mM and 2,720 mM min^{-1} μmol^{-1}, respectively; and for $NADP^+$, 0.70 mM and 300 mM min^{-1} μmol^{-1}, respectively. Therefore, the correct designation for the purified *S. bovis* malic enzyme is EC 1.1.1.39. The optimum pH was 7.8. The optimum temperature was determined to be 55°C by using HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-NaOH buffer. Of 10 substrates of other dehydrogenases (D-malate, fumarate, L-tartarate, L-lactate, DL-isocitrate, DL-glycerate, 6-phospho-D-gluconate, ethanol, glycerol, and L-threonine), only D-malate was oxidized by this enzyme. The D-malate oxidative activity was about 12.8% of the L-malate oxidative decarboxylating activity.

When 0.05 mM Mn^{2+} was replaced by another divalent cation (Mg^{2+}, Co^{2+}, Ni^{2+}, Sr^{2+}, Ca^{2+}, Cu^{2+}, Hg^{2+}, Cd^{2+}, Ba^{2+}, Zn^{2+}, Fe^{2+}, or Sn^{2+}) at a concentration of 1.0 mM, 82.3% of the original activity was observed with Mg^{2+}, 72.7% of the original activity was observed with Co^{2+}, and 24.7% of the original activity was observed with Ni^{2+}. Sr^{2+} and Ca^{2+} gave 0.63 and 0.50% of the original activity, respectively. The effects of Fe^{2+} and Sn^{2+} could not be detected spectrophotometrically. Other divalent cations gave no activity. Thus, the enzyme had a requirement for Mn^{2+} and Mg^{2+}. Moreover, the Mn^{2+} saturation profile exhibited a marked sigmoidicity. The activity was increased by adding monovalent cations to the standard reaction mixture without ${NH_4}^+$ by 28.1-fold and 9.5-fold in the presence of 1.0 mM ${NH_4}^+$ and 1.0 mM K^+, respectively, while Na^+ and Li^+ had no effect on the activity. The K_d values were as follows: Mg^{2+}, 0.19 mM; ${NH_4}^+$, 0.23 mM; and K^+, 3.48 mM. The activity was inhibited when oxaloacetate, ATP, ADP, *p*-chloromercuribenzoate, $HgCl_2$, and EDTA were added to the standard reaction mixture (Table 2). The inhibitory effects of *p*-chloromercuribenzoate and $HgCl_2$ and the recovery of activity when dithiothreitol was added indicated that a sulfhydryl group was necessary for activity.

The *S. bovis* malic enzyme catalyzed pyruvate reductive carboxylation. The optimum pH was 7.5. NADPH served as a coenzyme to a much lesser extent than NADH (the activity with NADPH was 3.3% of the activity with NADH). The V_{max} for the carboxylating activity was 34% of the V_{max} for L-malate oxidative decarboxylating activity. The kinetic constants for these activities are shown in Table 3.

The *S. bovis* malic enzyme did not catalyze carboxylation of the pyruvate analogs, 2-oxobutyrate, 2-oxovalerate, 2-oxocaproate, 2-oxoisovalerate, 2-oxoisocaproate, 4-oxovalerate, and phosphoenolpyruvate. However, when 2-oxoisovalerate and

TABLE 3. Kinetic constants of the *S. bovis* malic enzyme

Activity	V_{max} (mM min^{-1} μmol^{-1})	K_m (mM)	V_{max}/K_m (min^{-1} μmol^{-1})
L-Malate decarboxylation	2,720	0.63	4,320
Pyruvate carboxylation	930	11.4	82
D-2-Hydroxyisovalerate oxidation	17	0.42	41
D-2-Hydroxyisocaproate oxidation	31	0.21	150
2-Oxoisovalerate reduction	150	0.12	1,260
2-Oxoisocaproate reduction	470	0.12	4,000
$NaHCO_3$		25.3	37

2-oxoisocaproate were used as substrates in a reaction mixture lacking $NaHCO_3$, a significant decrease in A_{340} was observed. Thus, this enzyme may catalyze the reduction of 2-oxoisovalerate and the reduction of 2-oxoisocaproate.

Properties of the 2-oxoisovalerate and 2-oxoisocaproate reductive activity and the D-2-hydroxyisovalerate and D-2-hydroxyisocaproate oxidative activity. The results of the HPLC analysis (Fig. 2 and 3) confirmed that the *S. bovis* malic enzyme catalyzed the reduction of 2-oxoisovalerate and the reduction of 2-oxoisocaproate in the presence of NADH and the oxidation of D-2-hydroxyisovalerate and the oxidation of D-2-hydroxyisocaproate (but not the oxidation of the L isomers) in the presence of NAD^+ (Fig. 4). NADPH was not used as a coenzyme.

The optimum pH for both the 2-oxoisovalerate reductive activity and the 2-oxoisocaproate reductive activity was 6.6, and the optimum pH for the 2-hydroxyisocaproate oxidative activity was 9.5. In contrast to the L-malate oxidative decarboxylating activity, the enzyme did not require Mn^{2+} and Mg^{2+} to catalyze these reactions, and the reductive activity did not increase in the presence of NH_4^+. Six other commercially available dehydrogenases (L-lactate dehydrogenase [EC 1.1.1.27] from rabbit muscle, D-lactate dehydrogenase [EC 1.1.1.28] from *Staphylococcus epidermidis*, isocitrate dehydrogenase [EC 1.1.1.42] from yeast cells, malic enzyme [EC 1.1.1.40] from chicken liver, alcohol dehydrogenase [EC 1.1.1.1] from yeast cells, and L-glutamate dehydrogenase [EC 1.4.1.3] from bovine liver) were examined, and the D-lactate dehydrogenase and the isocitrate dehydrogenase exhibited 10.2 and 0.2% of the 2-oxoisocaproate reductive activity, compared with the L-malate oxidative decarboxylating activity of the *S. bovis* malic enzyme. Malic enzyme from chicken liver did not exhibit reductive activity. Kinetic constants for these activities are shown in Table 3.

Cloning and sequence of the malic enzyme gene. A total of about 25,000 colonies were screened, and only one positive clone which expressed malic enzyme was obtained. This positive clone harbored the recombinant vector Charomid 9-36, which contained the 4.6-kb insertion of *S. bovis* genomic DNA. The 4.6-kb fragment was subjected to a restriction analysis and subcloned in pUC18, resulting in plasmid pSK1 (Fig. 5A). A 0.66-kb *Dra*III-*Dra*III fragment was produced from the 4.6-kb fragment and was used as a probe for Southern blot analysis, and then the fragment was confirmed to be derived from *S. bovis*. A 2.9-kb *Pvu*II-*Eco*RI fragment was subcloned in pUC18, resulting in plasmid pSK2. The 2.9-kb *Pvu*II-*Eco*RI fragment in pSK2 was deleted from the *Pvu*II site to the *Eco*RI site, resulting in deleted plasmid pSK2. Cell extracts of *E. coli* DH5α transformants containing pUC18, pSK1, pSK2, and deleted plasmid pSK2 were prepared, and specific activities were determined. The cell extract of the pUC18 transformant did not exhibit malic enzyme activity; however, the cell extract of the pSK1 transformant exhibited greater malic enzyme activity than the cell extract of the pSK2 transformant (Fig. 5A). Addition of 0.4% L-malate did not affect the expression of the malic enzyme in *E. coli* DH5α.

To delete the 2.9-kb *Pvu*II-*Eco*RI fragment in the opposite direction, the fragment was subcloned in the pBluescriptII cloning vector. The entire nucleotide sequence of the 2.9-kb *Pvu*II-*Eco*RI fragment was determined by using these deleted fragments. The results of a computer analysis showed that there were two open reading frames (ORFs) which could be transcribed in the same direction, a 1,299-nucleotide ORF (ORF-1) and a 1,170-nucleotide ORF (ORF-2). A deletion analysis, a homology analysis, and the deduced NH_2-terminal amino acid sequence revealed that ORF-2 encoded the malic enzyme (Fig. 5). A possible Shine-Dalgarno sequence (AGG AGG) (10) was observed, but no typical promoter region was found between ORF-1 and ORF-2 (Fig. 5B). A possible terminator sequence, consisting of a palindrome followed by oligothymidine, was found 14 nucleotides downstream from the putative TAA termination codon of ORF-2 (Fig. 5C).

The G+C contents of the 2.9-kb fragment, ORF-1, and ORF-2 were 38.2, 38.3, and 42.3 mol%, respectively. ORF-2 could be translated into a polypeptide containing 389 amino acid residues (the *S. bovis* malic enzyme) with a predicted molecular weight of 41,600 and a predicted isoelectric point of 5.1; these values were in agreement with the molecular weight and isoelectric point estimated by using SDS-PAGE (Fig. 1) and isoelectric focusing, respectively. This polypeptide contained 57.1% hydrophobic amino acid residues, 17.0% neutral amino acid residues, and 26.0% hydrophilic amino acid residues.

Homology analysis of the sequences. Global levels of similarity between the *S. bovis* malic enzyme sequence and database protein sequences were calculated with the Fasta (35) and Blast (1) network services. As expected, the *S. bovis* malic enzyme exhibited similarities to other malic enzymes, especially those of the bacteria *Bacillus stearothermophilus* (22) and *Haemophilus influenzae* (12), and less similarity to the *E. coli* malic enzyme (29). The Blast search assigned a high score to the malolactic enzyme of *Lactococcus lactis* (2, 9). A high score was also assigned to a mox protein (whose function was unknown) of *Mycobacterium leprae* (GenBank accession number, MLU15180).

Furthermore, the Blast search revealed four conserved regions in the malic enzymes of *S. bovis*, other bacteria, yeasts, plants, and mammals and the malolactic enzyme of *Lactococcus lactis* (Fig. 6). The Blast search also revealed that other proteins unrelated to the malic enzyme possessed these conserved regions. Kidney bean cinnamyl alcohol dehydrogenase (41) possessed these four regions, adhesin AP65-1 of *Trichomonas vaginalis* (GenBank accession number, TVU18346 1) had regions A, C, and D and the COOH-terminal half of region B, and dihydrolipoamide dehydrogenase of *Acholeplasma laidlawii* (40) and succinyl coenzyme A synthetase homologous protein P36 of *Dictyostelium discoideum* (3) had the COOH-terminal half of region B.

DISCUSSION

Malic enzyme was purified from *S. bovis* ATCC 15352, and its properties were determined. We speculated that malic enzyme catalyzed the CO_2 fixation reaction in *S. bovis*; the results of our kinetic studies, however, suggest that this enzyme scarcely catalyzes the carboxylation reaction in *S. bovis*.

The M_r of the subunit of the *S. bovis* malic enzyme (40,000) was the same as the M_r reported for the subunit of the malic enzyme of *Clostridium thermocellum* (24), was similar to the M_rs reported for the subunits of the enzymes of *Sulfolobus solfataricus*, *Lactobacillus casei*, and *B. stearothermophilus* (49,000 ± 1,500, 37,000, and 50,000, respectively) (5, 6, 22), but was lower than the M_rs reported for the subunits of the enzymes of *E. coli*, *Pseudomonas diminuta*, human liver, and a roundworm, *Ascaris suum* (60,000 to 70,000) (13, 37, 48, 50). The malic enzyme of *S. bovis* is a homodimer. The enzymes of *Sulfolobus solfataricus* and a tapeworm, *Hymenolepis diminuta*, are also homodimers (5, 25), while the NAD-dependent malic enzyme of *E. coli* and the malic enzymes of *C. thermocellum*, *Ascaris suum*, *B. stearothermophilus*, and human liver are homotetramers (13, 22, 24, 48, 50), the NADP-dependent malic enzyme of *E. coli* is a homohexamer (20), and the *P. diminuta*

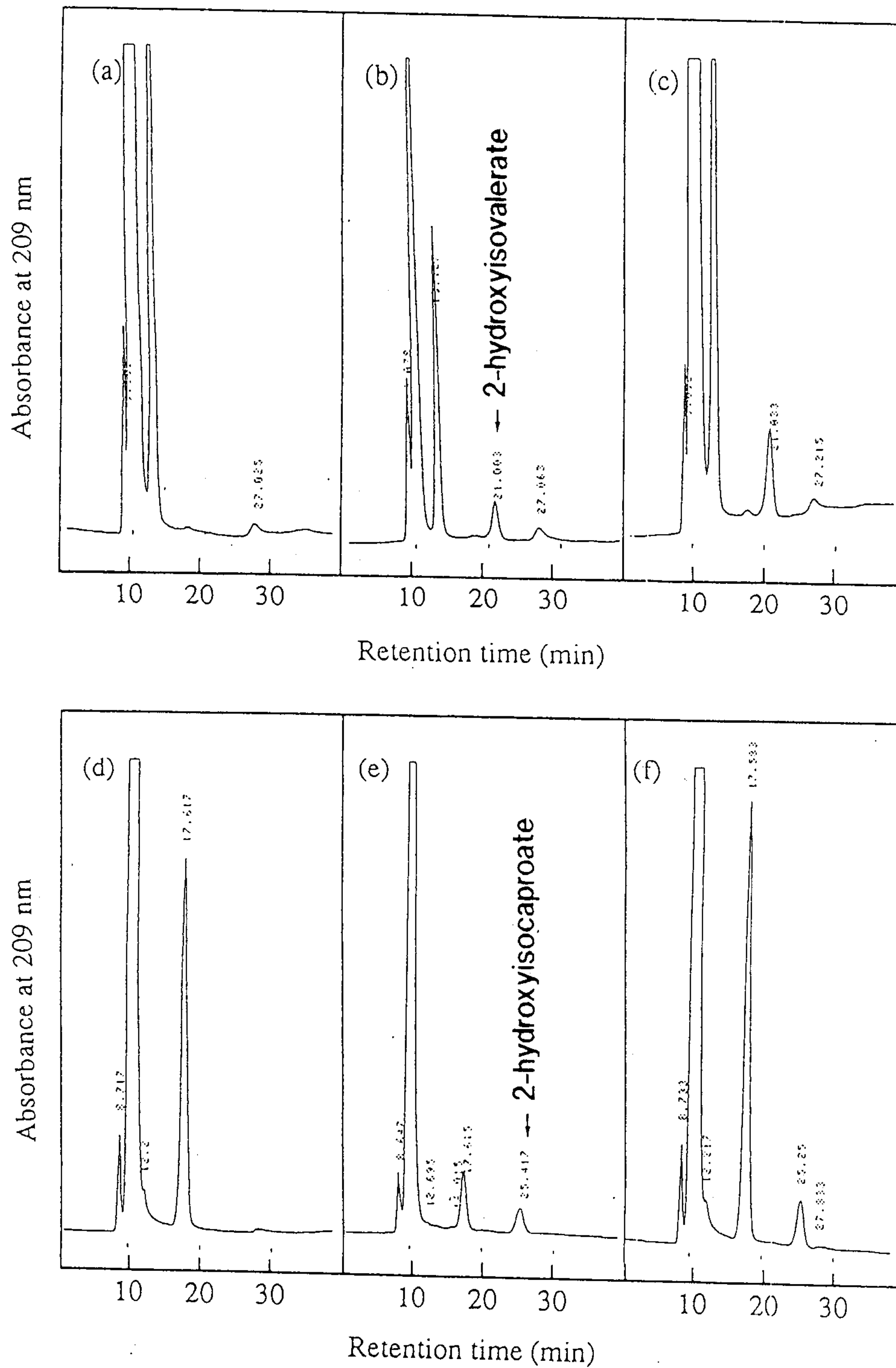

FIG. 2. HPLC profiles on a Shodex Ionpak C811 column (8 by 500 mm) of 2-oxoisovalerate and 2-oxoisocaproate reduction reaction mixtures catalyzed by the *S. bovis* malic enzyme. The peak at 21 min in panel b and the peak at 25 min in panel e correspond to the products of the reduction reactions (2-hydroxyisovalerate and 2-hydroxyisocaproate, respectively). (a and b) HPLC profiles of the zero-time and 77-min 2-oxoisovalerate reduction reaction mixtures, respectively. (c) HPLC profile of the zero-time reaction mixture containing authentic DL-2-hydroxyisovalerate. The peaks at 21 min in panels b and c correspond to 2-hydroxyisovalerate. (d and e) HPLC profiles of the zero-time and the 80-min 2-oxoisocaproate reduction reaction mixtures, respectively. (f) HPLC profile of the zero-time reaction mixture containing authentic DL-2-hydroxyisocaproate. The peaks at 25 min in panels e and f correspond to 2-hydroxyisocaproate. The reactions were terminated by adding 1 N HCl.

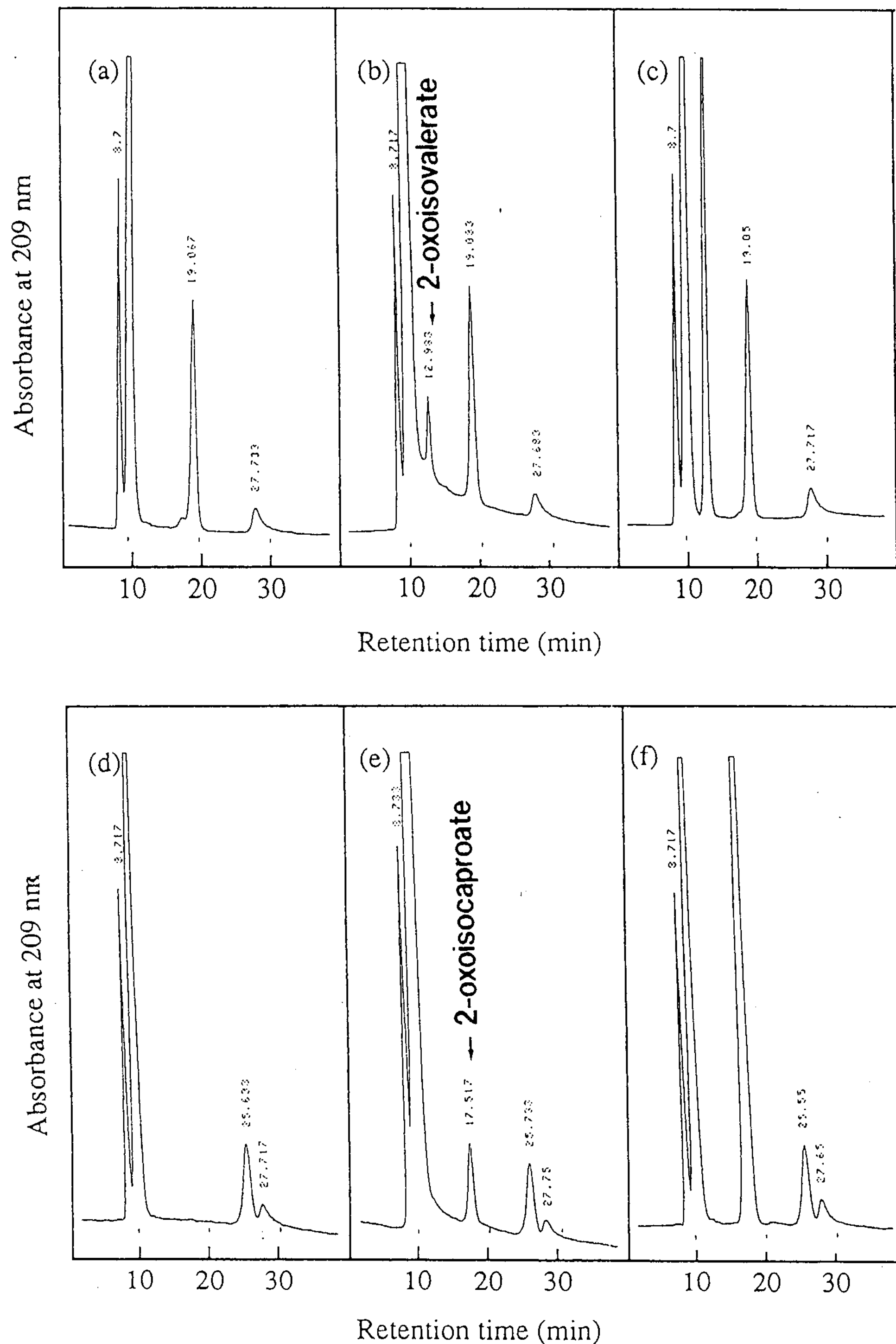

FIG. 3. HPLC profiles on a Shodex Ionpak C811 column (8 by 500 mm) of 2-hydroxyisovalerate and 2-hydroxyisocaproate oxidation reaction mixtures catalyzed by the *S. bovis* malic enzyme. The peak at 13 min in panel b and the peak at 17.5 min in panel e correspond to the products of the oxidation reactions (2-oxoisovalerate and 2-oxoisocaproate, respectively). (a and b) HPLC profiles of the zero-time and 240-min D-2-hydroxyisovalerate oxidation reaction mixtures, respectively. (c) HPLC profile of the zero-time reaction mixture containing authentic 2-oxoisovalerate. The peaks at 13 min in panels b and c correspond to 2-oxoisovalerate. (d and e) HPLC profiles of the zero-time and 330-min D-2-hydroxyisocaproate oxidation reaction mixtures, respectively. (f) HPLC profile of the zero-time reaction mixture containing authentic oxoisocaproate. The peaks at 17.5 min in panels e and f correspond to 2-oxoisocaproate. The reactions were terminated by adding 1 N HCl.

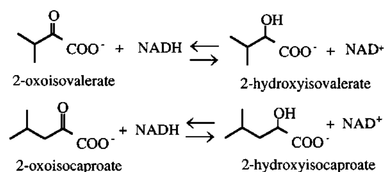

FIG. 4. Reduction of 2-oxoisovalerate and 2-oxoisocaproate and oxidation of D-2-hydroxyisovalerate and D-2-hydroxyisocaproate catalyzed by the *S. bovis* malic enzyme.

enzyme is a homodecamer (37). The subunit molecular weights of all of the bacterial malic enzymes except the malic enzyme of *E. coli* were lower than the subunit molecular weights of the enzymes of higher organisms.

The general catalytic features of the *S. bovis* malic enzyme appeared to be quite similar to the general catalytic features of many other malic enzymes reported previously. The optimum pH of the L-malate oxidative decarboxylating activity of this enzyme was 7.8, which is similar to the optimum pH values of other malic enzymes. The optimum pH of the pyruvate reductive carboxylating activity of this enzyme was 7.5, while the optimum pH of the *B. stearothermophilus* enzyme was 6.0 (22). The optimum temperature of this enzyme was 55°C, which is similar to the values obtained for the *Lactobacillus casei* enzyme (60°C) (6), the *B. stearothermophilus* enzyme (55°C) (22), and the *P. diminuta* enzyme (50°C) (37) but is lower than the value obtained for the *Sulfolobus solfataricus* enzyme (85°C) (5). On the basis of the optimum temperature and the experimental finding that incubation at 60°C for 10 min did not inactivate the malic enzyme from *S. bovis*, this enzyme seems to be heat stable.

The *S. bovis* malic enzyme required divalent cations to catalyze L-malate oxidative decarboxylation. Moreover, this activity was increased by adding K^+ or NH_4^+, like the activities of other malic enzymes. The K_d values of this enzyme for K^+ and NH_4^+ were 3.48 and 0.23 mM, respectively. This suggests that activation of the enzyme by NH_4^+ may have a physiological function. Lamed and Zeikus speculated that NH_4^+ activation of the enzyme is a physiological signal which turns on NADH generation when NH_4^+ is available for biosynthesis (24). The results of the SH reagent inhibition studies suggest that the SH group is necessary for activity, as reported previously for the enzymes of *C. thermocellum* (24) and *E. coli* (48).

We found that the *S. bovis* malic enzyme could catalyze reduction of 2-oxoisovalerate, reduction of 2-oxoisocaproate, oxidation of D-2-hydroxyisovalerate, and oxidation of D-2-hydroxyisocaproate, but not oxidation of the L isomers. The properties of these catalytic activities, such as the lack of a metal ion requirement and the optimum pH, were quite different from the properties of the L-malate oxidative decarboxylating activity. We believe, however, that the lack of a metal ion requirement for these nondecarboxylating activities was not unreasonable, since it is thought that the role of a metal ion in the oxidative decarboxylation of L-malate by malic enzymes is to polarize the carbonyl group of the oxaloacetate intermediate during decarboxylation (15, 21). The physiological roles of these catalytic activities are unknown. 2-Oxoisovalerate and 2-oxoisocaproate are known to be metabolic intermediates of leucine and valine, respectively. These compounds are dehydrogenated by 2-oxoisovalerate dehydrogenase (EC 1.2.4.4) in the leucine-valine degradation pathway, or they are transaminated by branched-chain amino acid aminotransferase (EC 2.6.1.42) in the leucine-valine biosynthesis pathway. The K_m

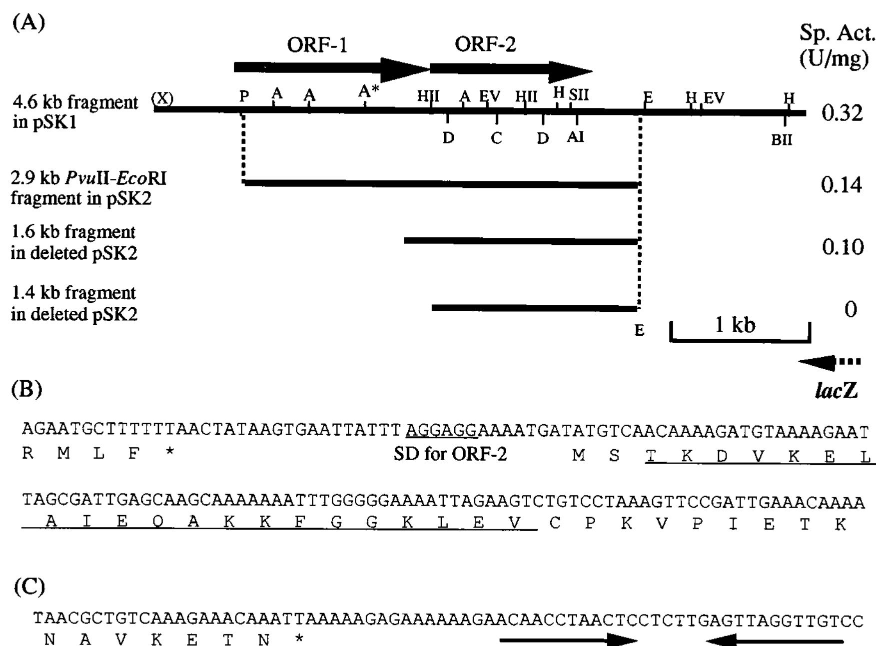

FIG. 5. (A) Cloned 4.6-kb DNA fragment and the result of deletion analysis. Abbreviations for restriction sites: P, *Pvu*II; A, *Ava*II; HII, *Hin*cII; D, *Dra*III; EV, *Eco*RV; C, *Cla*I; H, *Hin*dIII; SII, *Sac*II; AI, *Ava*I; E, *Eco*RI; BII, *Bgl*II. The *Ava*II site indicated by an asterisk was not digested by *Ava*II, although an *Ava*II recognition site exists. Sp. Act., specific activities of the malic enzyme in cell extracts of *E. coli* DH5α transformed with pSK2, pSK1, and deleted pSK2. The solid arrows indicate the locations, sizes, and transcriptional directions of ORF-1 and ORF-2 (the malic enzyme structural gene). The dotted arrow indicates the transcriptional direction of the *lacZ* gene of pUC18 on pSK1 and pSK2. (B) Nucleotide sequence of the 5′-terminal region of ORF-2. (C) Nucleotide sequence of the 3′-terminal region of ORF-2. Part of the ORF is indicated by translation into amino acids. The underlined amino acids are the amino acids determined from purified malic enzyme of *S. bovis*. The putative Shine-Dalgarno sequence (SD) for ORF-2 is underlined. The asterisks indicate termination codons. The arrows indicate a sequence capable of forming a stem-loop structure.

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             Region A                              Region B
S.bov. 69 VISDGSAVLGLGNIG 83---159 FHDDQHGTAIVVLAALYNSLKLINKKIEDI-
B.ste.144 VVSDGTAVLGLGDIG 158--234 FHDDQHGTAIVVLAGLLNALKIVDKKLEDI-
H.inf. 69 VISNGTAVLGLGNIG 83---159 FHDDQHGTAIISAAAIINSLRIVGKKIEDV-
L.lac.139 VVTDAEGILGIGDWG 153--256 FNDDIQGTGIVVLGGIFGSLDITGEKLTDQ-
Yeast.235 VVSDSEGILGIGDQG 249--351 FNDDIQGTGAVVMASLIAALKHTNRDLKDT-
Maize.231 VTTDGERILGLGDLG 245--348 FNDDIQGTASVVLAGLLAALKMVGGTLAEQ-
HumMt.159 VTTDGERILGLGDLG 173--276 FNDDIQGTAAVALAGLLAAQKVISKPISEH-
Pigeo.138 VVTDGERILGLGDLG 152--255 FNDDIQGTASVAVAGLLAALRITKNRLSDH-
          *+++   +**+*+ *          *+**  **  +    +  +  +    + +

        (Region B)          Region C              Region D
S.bov.-HVVINGGGSAGLSI 202--279 EQPVIFAMANP 289--316 QINNVLAFPGIFR-
B.ste.-KVVLTGIGAAGIAC 277--354 RDPIVFAMANP 364--391 QINNVLCFPGIFR-
H.inf.-RLVASGAGAASIAC 202--279 AHPIILALANP 289--317 QVNNVLCFPFIFR-
L.lac.-VYLCYGGGSAGAGI 298--394 ERPVIFPISNP 404--445 QANNSLIHPGLGL-
Yeast.-RVLIYGAGSAGLGI 394--490 PRPIIFPLSNP 500--537 ENNNCYSFPGIGL-
Maize.-TYLFLGAGEAGTGI 391--483 ERPIIFSLSNP 493--534 QSNNAYIFPGLGL-
HumMt.-KILFLGAGEAALGI 319--412 ERPVIFALSNP 422--464 QGNNVYIFPGVAL-
Pigeo.-TVLFQGAGEAALGI 298--388 NKPIIFALSNP 398--440 QGNNSYVFPGVAL-
         +  * * *                *++  ++**            + **    * +

           (Region D)
S.bov.-GALDARAKKITIEMQIAAAKGIAKL353
B.ste.-GALDCRAREINEEMKLAAAKAIASV428
H.inf.-GALDVGATTINEEMKRAAVYAIADL354
L.lac.-GMLASEAKLLTDEMIGAAAHSLSGL482
Yeast.-GAVLSRATTITDKMISAAVDQLAEL574
Maize.-GLVISGAVRVHEDMLLAASKALADQ571
HumMt.-AVILCNTRHISDSVFLEAAKALTSQ501
Pigeo.-GVISCGLKHIGDDVFLTTAEVIAQE477
         +     +   +       +
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FIG. 6. Amino acid sequences conserved in enzymes that are similar to the *S. bovis* malic enzyme. The asterisks indicate identical residues. The similar residues detected by the Blast search are indicated by plus signs. Abbreviations: S.bov., B.ste., H.inf., Yeast., Maize., HumMt., and Pigeo., malic enzymes of *S. bovis*, *B. stearothermophilus* (22), *H. influenzae* (12), *Saccharomyces cerevisiae* (Swiss-Prot accession code, MAOX YEAST), maize chloroplasts (36), human mitochondria (26), and pigeon livers (7), respectively; L.lac., malolactic enzyme of *Lactococcus lactis* (2, 9).

values for 2-oxoisovalerate and 2-oxoisocaproate, which were lower than the value for L-malate, suggest that these activities may function physiologically. Deamination of reduced amino acids, in particular deamination of branched-chain amino acids, was inhibited by an increase in the ratio of NADH to NAD in rumen microorganisms (17). The relationship of this inhibition to malic enzyme activities, including branched-chain oxoacid reductive activity, is of interest. We have not succeeded in determining the optical activity of the product of this reduction. Considering that the enzyme oxidized D branched-chain hydroxy acids, we suppose that the products are D branched-chain hydroxy acids.

We also succeeded in isolating the DNA fragment containing the malic enzyme gene from the *S. bovis* genomic library and determined its sequence. In this fragment two ORFs were located in the same direction, which was opposite the direction of the *lacZ* promoter in pUC18. A sequence analysis indicated that ORF-1 and ORF-2 are cotranscribed. The results of the expression study suggest that a better-functioning promoter in *E. coli* DH5α is located upstream of ORF-1, since the malic enzyme was expressed better in the pSK1 transformant than in the pSK2 and deleted pSK2 transformants (Fig. 5A). Some promoter sequences were also detected in ORF-1. We suppose that the malic enzyme expression in the pSK2 and deleted pSK2 transformants is due to the promoter sequences in ORF-1.

Homology research revealed that the *S. bovis* malic enzyme did not exhibit similarities to CO_2 fixation enzymes except malic enzymes and other enzymes related to branched-chain keto acids. A homology analysis of different malic enzymes and malolactic enzyme revealed four highly conserved regions (Fig. 6). Three of these regions (all of the regions except region D) were previously identified by Viljoen et al. (39). A consensus sequence, hn-G-G--G---n--n--------n-n, which serves as a $\beta\alpha\beta$ fold for the binding of ADP moieties, was previously identified by Wierenga et al. (44), and this sequence was found in region A and the COOH-terminal half of region B; this consensus sequence has a characteristic arrangement of glycine (G), nonpolar (n), and hydrophilic (h) residues at certain crucial positions for a compact $\beta\alpha\beta$ fold. In the *S. bovis* malic enzyme sequence, the consensus sequences were as follows: 73 DG--V-G-G--G---A--V 91 and 189 HV-I-G-G--G---T--F 206. It has been suggested that the NH_2-terminal half of region B (residues 159 to 183 in the *S. bovis* malic enzyme) is the putative Mn^{2+}-binding site, and one of the putative Mn^{2+}-binding ligands was supposed to be Asp (42) (Asp-162 in the *S. bovis* malic enzyme). Although that fact that an SH group is important for *S. bovis* malic enzyme activity was demonstrated by the SH reagent inhibition study (Table 1), only two cysteine residues were found in the *S. bovis* malic enzyme sequence (Cys-24 and Cys-143). Cys-143 was also conserved in the *B. stearothermophilus* and *H. influenzae* enzymes, while it was replaced with a histidine residue in the enzymes of human mitochondria, pigeons, and maize and with serine and lysine residues in the malolactic enzyme of *Lactococcus lactis* and the malic enzyme of *Saccharomyces cerevisiae*, respectively. Cys-24 was not conserved. Hsu et al. reported that Cys-99 in the duck liver malic enzyme was important for the binding of L-malate and divalent metal ions (18). Cys-99 in the duck liver malic enzyme corresponded to Ser-47 in the *S. bovis* malic enzyme. Kidney bean cinnamyl alcohol dehydrogenase (41) had all four highly conserved regions, adhesin AP65-1 of *T. vaginalis* (GenBank accession number, TVU18346 1) had regions A, C, and D and the COOH-terminal half of region B, and dihydrolipoamide dehydrogenase of *Acholeplasma laidlawii* (40) and succinyl coenzyme A synthetase homologous protein P36 of *D. discoideum* (3) had the COOH-terminal half of region B. These facts suggest that these proteins contain the $\beta\alpha\beta$ fold structure for binding of ADP moieties. The presence of the NH_2-terminal half of region B in cinnamyl alcohol dehydrogenase indicates that this enzyme requires a divalent cation. In fact, the presence of zinc in this enzyme has been reported previously (47). The fact that L-malate is not similar to cinnamyl alcohol and the fact that there is little available information concerning AP65-1, however, prevent us from speculating on the role of regions C and D.

Analyses of ORF-1 upstream of the malic enzyme gene and of its product are in progress.

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