pyl-β-thiogalactoside at 18 °C for 20 h. The fusion proteins were purified using a glutathione-Sepharose 4B column (Amersham Biosciences) according to the manufacturer's instructions. The recombinant EhMGL1/2 (rEhMGL1/2) were obtained by digestion of these fusion proteins with PreScission Protease (Amersham Biosciences) in the column, followed by elution from the column and dialysis at 4 °C with 100 mM sodium phosphate buffer, pH 6.8, containing 0.02 mM PLP.

The final purified recombinant EhMGL (rEhMGL) proteins were presumed to contain 10 additional amino acids (GPLGSPEFPG) at the N terminus. The purified enzymes were stored at −80 °C with 30–50% dimethyl sulfoxide until use. No decrease in enzyme activity was observed under these conditions for at least 3 months. Protein concentrations were determined by Coomassie Brilliant Blue assay (Nacalai Tesque, Inc., Kyoto, Japan) with bovine serum albumin as the standard.

Amino Acid Alignments and Phylogenetic Analyses—The sequences of MGL and other members of the γ-subfamily of PLP enzymes showing similarities to the amino acid sequences of EhMGL were obtained from the National Center for Biotechnology Information (NCBI, //www.ncbi.nih.gov/) by using the BLASTP algorithm. The alignment and phylogenetic analyses were performed with ClustalW version 1.81 (18) using the Neighbor-Joining (NJ) method with the Blosum matrix. An unrooted NJ tree composed of the amino acid sequences of 13 MGLs and 10 other members of the γ-subfamily of PLP enzymes from various organisms with two EhCSs (β-family of PLP enzymes) as the outgroup was drawn by Tree View ver.1.6.0 (19). Branch lengths and bootstrap values (1000 replicates) were derived from the NJ analysis. Phylogenetic analyses by the maximum parsimony method (MP) and maximum likelihood method (ML) were also conducted using PROTPARS (PHYLIP version 3.57c, Ref. 20) and ProtML (MOLPHY version 2.3, Ref. 21), respectively.

Subcellular Fractionation of the Crude Extract—The lysate of ~3 × 10^6 *E. histolytica* trophozoites was prepared by two cycles of freezing and thawing in 1 ml of cell lysis buffer: 100 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.02 mM PLP, 1 mM dithiothreitol, and a protease inhibitor mixture (Complete Mini EDTA-free, Roche Applied Science, Tokyo, Japan), and 1 μg/ml of *N*-(3-carboxyoxirane-2-carbonyl)-leucyl-amino(4-guanido)butane (E-64, Sigma). The whole lysate was then centrifuged at 14,000 × *g* in a microcentrifuge tube for 20 min at 4 °C to separate the supernatant (soluble cytosolic fraction) and the pellet (debris, membrane, and nuclear fraction).

Anion Exchange Chromatography of the Native Form MGLs—A supernatant fraction obtained from 2 g (wet weight) of the trophozoite pellet, as described above, was filtered with a 0.45-μm-pore mixed cellulose membrane (Millex-HA, Millipore Corporation, Bedford, MA). The sample buffer was exchanged with buffer A (20 mM Tris-HCl, pH 8.0, 0.02 mM PLP, 1 mM dithiothreitol, 1 mM EDTA, and 0.1 μg/ml of E-64) by using a Centricon Plus-20 (Millipore). A 20-ml sample containing ~100 mg of total protein was loaded on a DEAE-Toyopearl HW-650S column (7.5 × 1.6 cm, 15-ml bed volume, Tosoh, Tokyo, Japan) that was previously equilibrated with buffer A. The column was further washed with ~50 ml of buffer A until the A_{280} dropped below 0.1. The bound proteins were then eluted with a 50-ml linear potassium chloride gradient (0–0.5 M) in buffer A at a flow rate of 0.8 ml/min. All 0.8-ml fractions were concentrated to 0.2 ml with a Centricon YM-10 (Millipore). All procedures were performed at 4 °C. The amount of MGL in each fraction was assessed using the hydrogen sulfide assay and immunoblotting as described below.

Size Exclusion Chromatography of Recombinant and Native EhMGLs—To estimate the molecular mass of the recombinant and native EhMGLs, gel filtration chromatography was performed. Approximately 500 μg of recombinant and 100 μg of partially purified native EhMGL were dialyzed against buffer B (20 mM Tris-HCl, pH 8.0, 0.02 mM PLP, and 0.2 M KCl) and concentrated to 1 ml with the Centricon Plus-20. The concentrated samples were then applied to a column of Toyopearl HW-65S (70 × 1.6 cm, 140-ml bed volume, Tosoh) preequilibrated with buffer B. The recombinant and native MGLs were eluted with buffer B at a flow rate of 0.8 ml/min. The peaks were detected by measuring absorbance at A_{280} (recombinant MGLs) and immunoblotting (native MGLs). The same column was calibrated with blue dextran (2000 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) (Amersham Biosciences).

Immunoblot Anaylsis—Polyclonal antisera against recombinant EhMGL1 and 2 were raised in rabbits by Sigma-Genosys (Hokkaido, Japan). Immunoblot analysis was carried out using a polyvinylidene difluoride (PVDF) membrane as described in (22). The blot membrane was visualized by using alkaline phosphatase conjugate-coupled secondary antibody with NBT/BCIP solution (Roche Applied Science) according to the manufacturer's protocol.

Two-dimensional Polyacrylamide Gel Electrophoresis—First-dimensional electrofocusing of two-dimensional PAGE was performed using Immobiline Drystrip, pH 3–10 NL, 7 cm and IPG Buffer pH 3–10 NL (Amersham Biosciences) according to the manufacturer's protocol. Second dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed on 12% SDS-PAGE gel using prestained SDS-PAGE standards, Broad Range (Bio-Rad Laboratories, Inc., Tokyo, Japan), as a molecular marker.

Enzyme Assays and Kinetic Calculations—The enzymatic activitiy of MGL was monitored by measuring the production of α-keto acid, ammonia, and hydrogen sulfide or methanethiol. The standard MGL reaction was performed in 200 μl of 100 mM sodium phosphate buffer, pH 6.8, a reaction mixture containing 0.02 mM PLP, and 0.1–10 mM of each substrate with appropriate amounts of each enzyme.

The α-keto acid assay was performed as described (23). The MGL reaction was terminated by adding 25 μl of 50% trichloroacetic acid. After the proteins were precipitated by centrifugation at 14,000 × *g* for 5 min at 4 °C, 100 μl of the supernatant was mixed with 200 μl of 0.5 M sodium acetate buffer, pH 5.0, and 80 μl of 0.1% of 3-methyl-2-benzothiazolinone hydrazon hydrochloride, and then incubated at 50 °C for 30 min. After the mixture had cooled to room temperature, absorbance at A_{320} was measured. Pyruvic acid and 2-α-butyric acid were used as standards.

For the detection of ammonia, the nitrogen assay (24) was used. A 50-μl sample of the supernatant, as for the α-keto acid assay, was mixed with 50 μl of Nessler's reagent (Nakalai) and 75 μl of 2 N sodium hydroxide, and then incubated at 25 °C for 15 min. Absorbance at A_{410} was measured. Ammonium sulfate was used as a standard.

The hydrogen sulfide assay was performed as described (25–27). Briefly, the MGL reaction was terminated by adding 20 μl of 20 mM *N,N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 N HCl and 20 μl of 30 mM $FeCl_3$ in 1.2 N HCl. After further incubation in the dark for 20 min, the proteins were precipitated by centrifugation at 14,000 × *g* for 5 min at 4 °C, and then the absorption at OD_{650} of the supernatant was measured to quantitate the formed methylene blue. Na_2S was used as a standard.

The methanethiol assay was performed as described (28) using 5,5′-dithio-bis-(2-nitrobenzoic acid). One-hundred microliters of the sample supernatant were mixed with 1 μl of 100 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) in ethanol, and after 2 min incubation at room temperature, absorbance at A_{412} was measured. L-cysteine was used as a standard.

The cysteine and cystathionine assay was performed as described (29). The ninhydrin reagent was prepared by dissolving 1 g of ninhydrin in 100 ml of glacial acetic acid and adding 33 ml of glacial phosphoric acid. For the determination of cystathionine, 0.2 ml of cystathionine-containing solution was mixed with 0.33 ml of the ninhydrin reagent and boiled at 100 °C for 5 min. The solution was then cooled on ice for 2 min and at room temperature for a further 10 min. Absorbance at A_{455} was measured. Cysteine concentrations were determined using the same protocol except for the measurement of absorbance at A_{560}.

Kinetic parameters were estimated with Lineweaver-Burk plots using Sigma Plot 2000 software (SPSS Inc., Chicago, IL) with the Enzyme Kinetics module (version 6.0, Hulinks, Inc., Tokyo, Japan).

Assay of the Inhibition of rEhMGL by DL-Propargylglycine—An α-keto acid assay (described above) with L-methionine as the substrate was performed to evaluate the inhibitory effects of DL-propargylglycine (PPG) on the activity of rEhMGLs. rEhMGL (5 μg) was preincubated with various concentrations of PPG in the standard MGL reaction mixtures (described above) in the absence of L-methionine at 36 °C. The preincubation time was 5 min for kinetic analyses and 1 to 60 min to characterize the slow binding of this inhibitor. After preincubation, the reaction was initiated by adding an appropriate amount of L-methionine to the reaction mixture.

In Vitro Assessment of Amebicidal Reagents—To assess the amebicidal effect of TFMET, the trophozoites were cultured in the BI-S-33 medium containing various concentrations of TFMET or metronidazole, the therapeutic compound commonly used for amebiasis, as a control. After cultivation at 35.5 °C for 18 h, cell survival was assessed with the cell proliferation reagent WST-1 (Roche Applied Science). Briefly, the trophozoites were seeded on 96-well microtiter plates in 200 μl of BI-S-33 medium at a density of 2 × 10^4 cells per well (1 × 10^5 cells/ml), and the lid was completely sealed with a sterilized adhesive silicon sheet (Corning, New York). After these plates were further incubated at 35.5 °C for 18 h, 20 μl of WST-1 reagent was added to each well and the incubation was continued for 2 more hours. The optical density at A_{445} was measured with that at A_{595} as a reference using a microplate reader (Model 550, Bio-Rad, Tokyo, Japan). The initial density and incubation

A

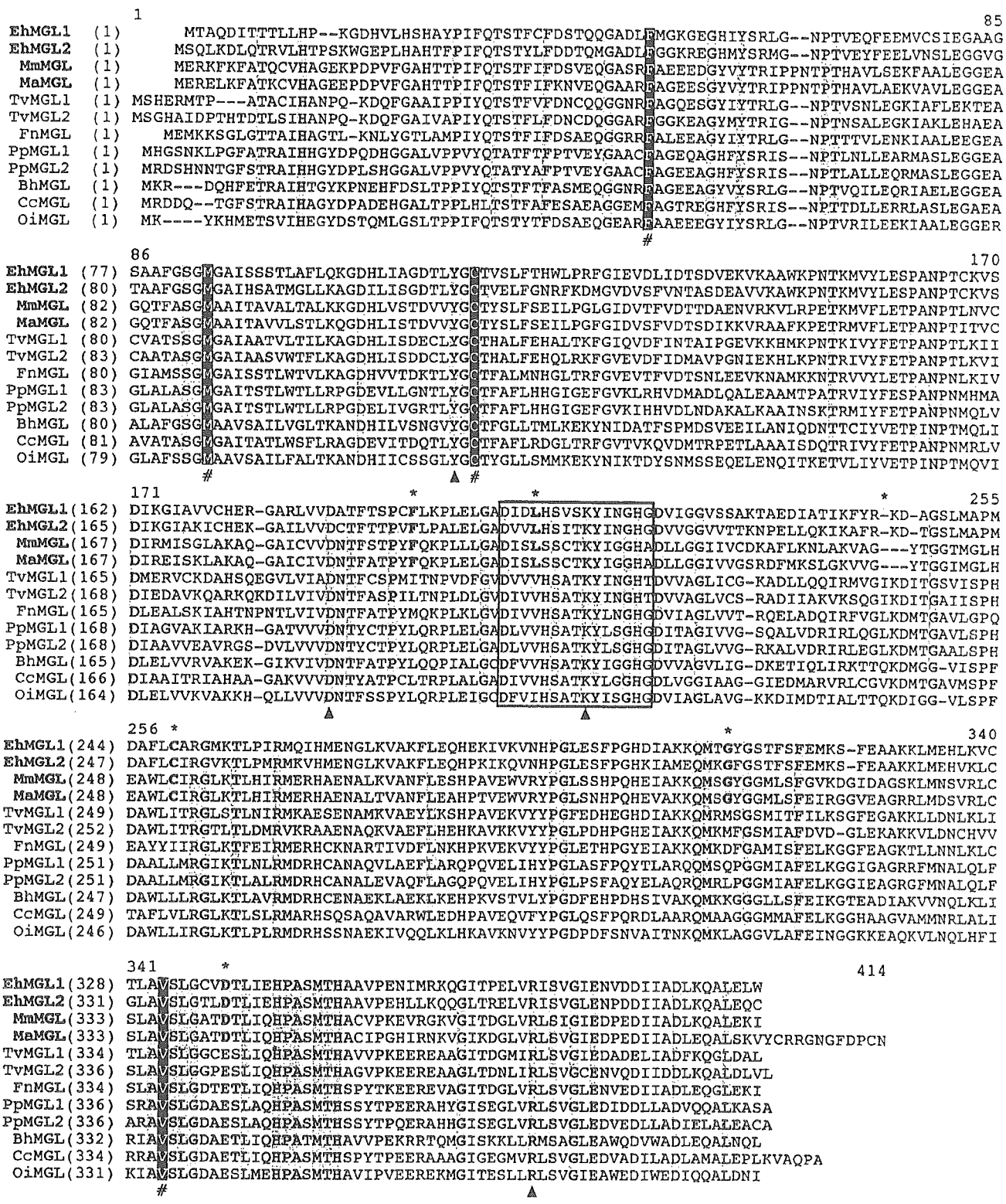

FIG. 2. **Amino acid comparison of MGLs and phylogenetic analysis of γ-subfamily of PLP enzymes.** *A*, comparison of deduced amino acid sequences of MGL from *Entamoeba* and other organisms. Pair-wise alignment was performed as described under "Experimental Procedures". Computer-generated gaps are indicated by *hyphens*. Residues that are conserved among all MGL sequences as well as other members of the γ-subfamily of PLP enzymes are shown *on a gray background*, and "MGL signature-like residues" conserved in MGLs, but not in other members of the γ-subfamily of PLP enzymes, are shown as *reversed letters* on *a black background* and marked with *sharps*. The two amebic and two archaeal MGLs are labeled in *bold*, and their conserved residues are shown on *a gray background* marked with *asterisks*. A region corresponding to the PLP attachment site in cysteine/methionine-metabolizing enzymes (PROSITE accession number: PS00868) is marked with an *open rectangle*. Active-site residues depicted from a crystal structure of PpMGL (33) are indicated with *arrowheads*. Protein accession numbers are shown in Fig. 2*B*. *B*, phylogenetic reconstruction of MGLs and other related PLP enzymes belonging to the γ-subfamily. An unrooted NJ tree, which is representative of the results of three independent methods for phylogenetic reconstruction, is shown. The root is arbitrarily placed using EhCSs (β-family of PLP enzyme) as the outgroup[c], and numbers beside the nodes indicate bootstrap values from 1000 replicates. The horizontal length of each branch is proportional to the estimated number of amino acid substitutions. EhMGL[a] accession numbers are shown in the DDBJ footnote, and other accession numbers are shown in parentheses (NCBI protein data base except [b]Swiss-Prot accession number). Abbreviations are defined in Footnote 2.

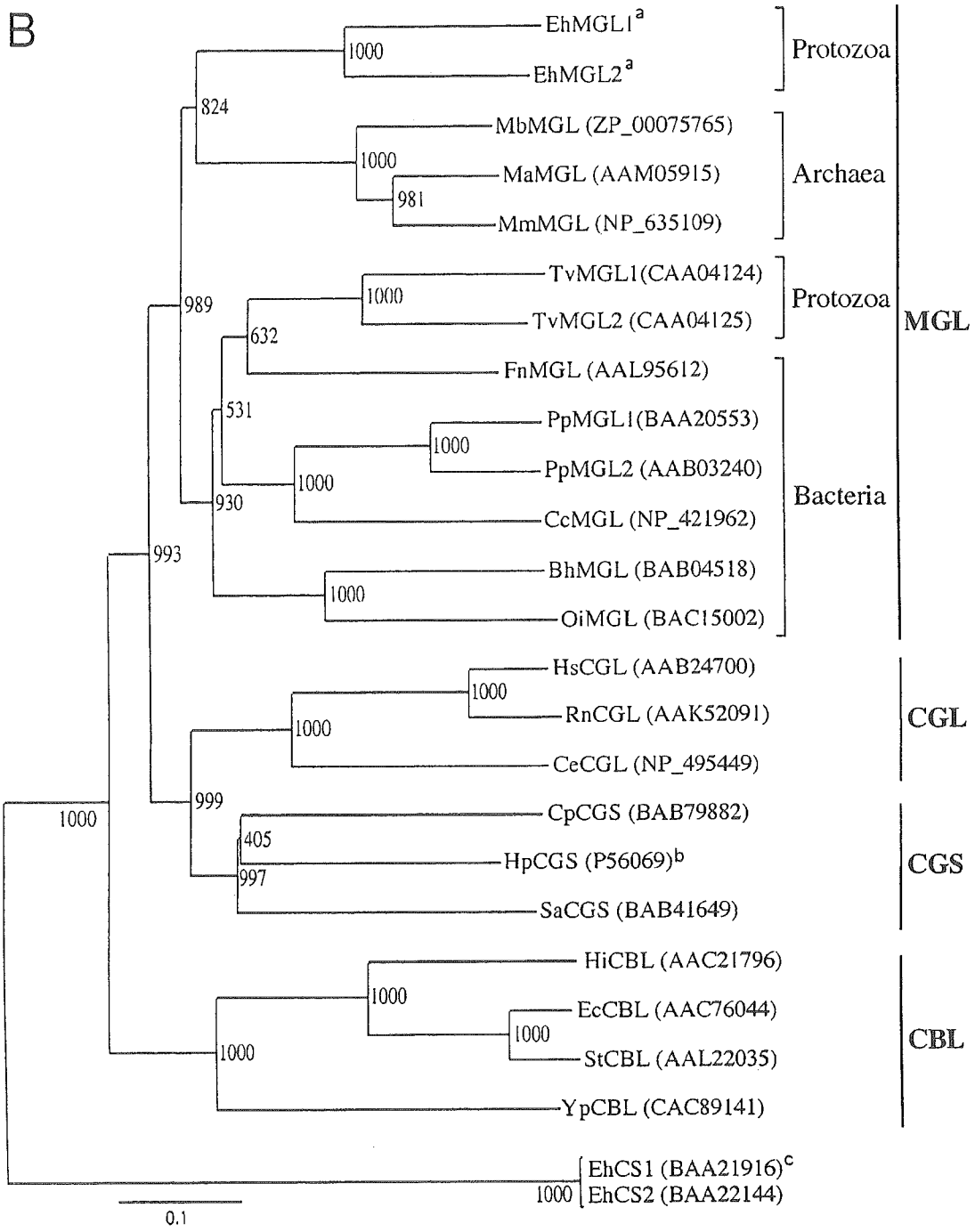

FIG. 2—*continued*

period of the cultures were chosen to maintain the control trophozoites in the late-logarithmic growth phase throughout the experiment, and also to allow the measurement of optical density in the linear portion of the curves (between 4×10^3 to 2.0×10^5 cells/ml).

RESULTS AND DISCUSSION

Data Base Search of the PLP-dependent Enzymes—In an attempt to obtain genes encoding the PLP-dependent enzymes involved in the metabolism of sulfur-containing amino acids, we searched the genome data base for putative proteins that possessed a conserved PLP-binding domain as described under "Experimental Procedures." Two independent contigs were found in the genome data base. These contigs (Contig 315785 and 316820, TIGR) contained two similar but not identical ORFs that encode proteins possessing a region containing the PLP-binding motif of the γ-subfamily of PLP enzymes. Non-coding flanking regions within these contigs also showed significant variations (data not shown). All other contigs or singletons showing significant identity to these two contigs perfectly overlapped them, which is consistent with the notion that these fragments are present as a single copy in the genome.

We also searched for genes containing the PLP-binding site of the β-family of PLP enzymes using both the amebic CS and the yeast and mammalian CBS. However, after eliminating contigs and singletons that contain genes encoding the two CS isotypes described previously (14), no contig or singleton was found to contain this motif. This suggests that *E. histolytica* possesses two uncharacterized genes encoding proteins that belong to the γ-subfamily of PLP enzymes, and lacks the β-family of PLP enzymes (*i.e.* CBS) known to be involved in the

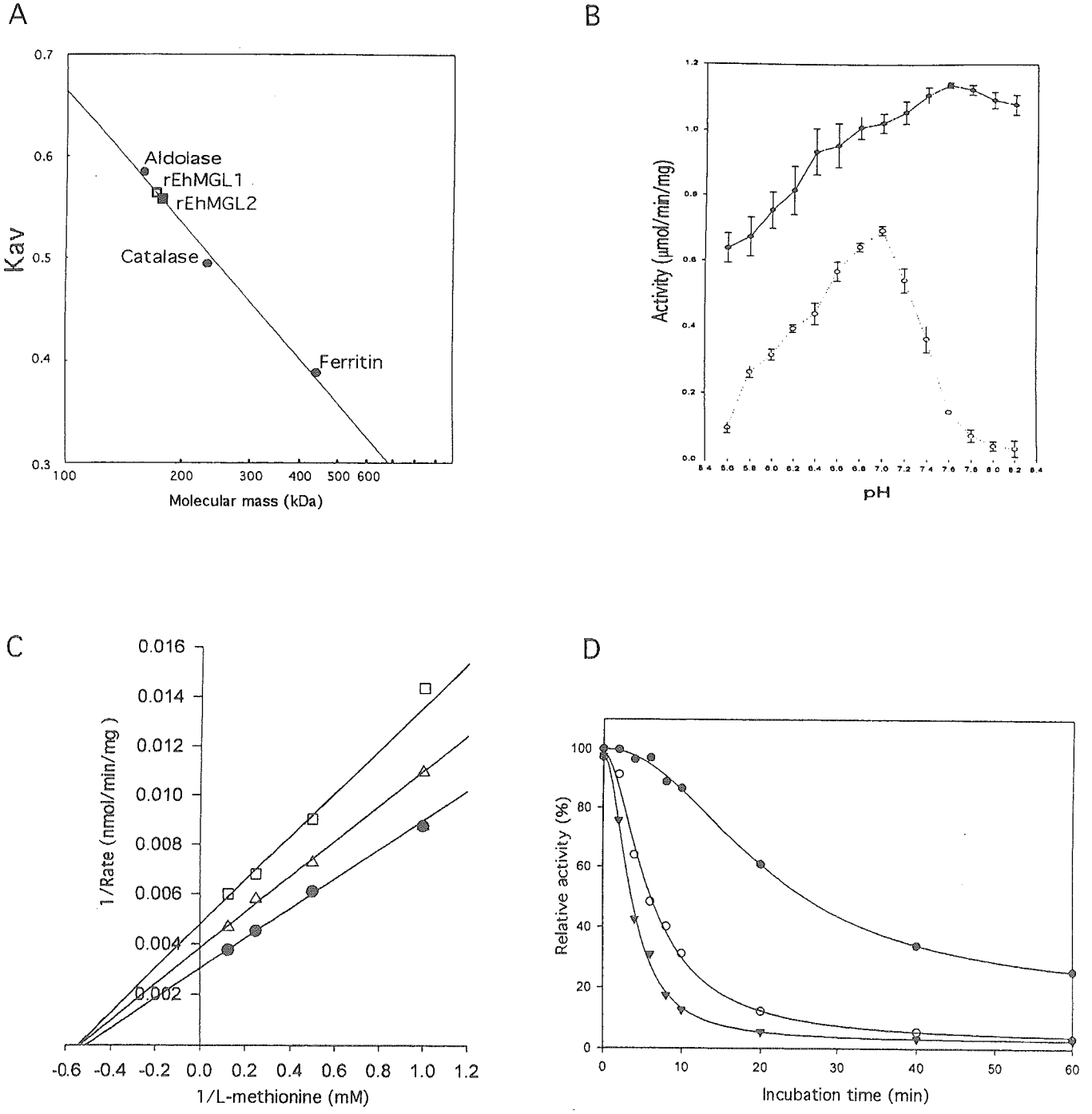

FIG. 3. **Enzymological characteristics of the recombinant EhMGL isotypes.** *A*, Size exclusion chromatography of the recombinant EhMGLs. rEhMGL1 (□) and 2 (■)were applied to a column of Toyopearl HW-65S and detected as described under "Experimental Procedures." Standard calibration of this column was performed with blue dextran (2000 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa). The K_{av} of each protein was calculated using the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$ (V_e, elution volume; V_t, total bed volume; V_0, column void volume). *B*, pH optimum of rEhMGLs. The optimum pH was determined at 37 °C with 50 mM MES buffer (pH 5.6–6.2), MOPS buffer (pH 6.4–7.4), and HEPES buffer (pH 7.4–8.2). Activities of rEhMGL1 (○) and 2 (●) were monitored by hydrogen sulfide assay for 5 min using 5 mM DL-homocysteine and 1 μg of each rEhMGL in a 200 μl reaction mixture as described under "Experimental Procedures." Data shown are means ± S.D. of triplicates. *C*, Lineweaver-Burk double reciprocal plot of MGL showing non-competitive inhibition of rEhMGL2 by DL-propargylglycine (PPG). rEhMGL2 was preincubated with 0 (●), 10 (△), or 20 μM (□) PPG in a reaction mixture without L-methionine at 36 °C for 5 min. Reactions were initiated by adding appropriate concentrations of L-methionine, and pyruvate production was assayed after 5 min. *D*, slow binding inhibition of rEhMGL2 by PPG. rEhMGL2 was preincubated with 0 (●), 10 (○), or 20 μM (▲) PPG in a reaction mixture without L-methionine at 36 °C for each period indicated. Reactions were initiated by adding L-methionine and carried out for 5 min. Percentages of MGL activity relative to the untreated control are shown.

reverse trans-sulfuration pathway in other organisms. We also tentatively concluded that the trans-sulfuration sequences in both the forward and reverse orientation are incomplete since the amebic genome lacks putative genes for CBL, CGS, and CGL (also see below). In addition, the major pathways for cysteine degradation of sulfur amino acids present in mammals, *i.e.* the cysteine sulfinic acid (cysteine dioxygenase as a key enzyme), 4′-phosphopanthetheine (leading to synthesis of coenzyme A and cysteamine), and mitochondrial mercaptopyruvate pathways, are apparently absent in this organism (data

TABLE I
Products of L-methionine degradation by the amebic crude extract and recombinant EhMGLs

Rates of product formation were determined from three independent experiments, and results of one representative experiment are shown. Assays to measure individual products were performed as described under "Experimental Procedures." Reactions were carried out with 10 mM L-methionine at 37 °C for 30 min with 200 μg of crude extract or for 10 min with 3 μg of recombinant enzyme in a 200 μl of reaction mixture. Crude extracts from trophozoites cultured for 48 hrs in BI-S-33 medium supplemented with or without 20 μM PPG were also used. Methanethiol and nitrogen could not be measured when the crude extract was used as a source of MGL because the backgrounds due to endogenous organic and inorganic thiols and nitrogen were not negligible.

Product	Rate of production [nmol/min/mg]			
	Crude extract		Recombinant enzyme	
	BI-S-33	BI-S-33 + 20 μM PPG	rEhMGL1	rEhMGL2
Methanethiol	n.d.[a]	n.d.	524.7	738.7
α-Buryric acid	0.157	0.004	288.7	468.7
Nitrogen	n.d.	n.d.	385.3	793.3

[a] n.d., not determined.

not shown). Therefore, *E. histolytica* must have alternative enzymes involved in the degradation of toxic sulfur-containing amino acids, *e.g.* homocysteine, which is implicated in the well characterized human genetic disease homocysteinuria and its cytotoxicity (30).

Identification and Features of EhMGL Genes and Their Proteins—The nucleotide sequences of the 1170- and 1179-bp ORFs recognized in the contigs described above were homologous to those of the γ-subfamily of PLP-dependent enzymes, including MGL, CBL, CGS, and CGL. These putative genes encode 389- and 392-amino acid polypeptides with predicted molecular masses of 42.3 and 42.7 kDa and predicted pIs of 6.01 and 6.63, respectively. We designated these genes *EhMGL1* and *EhMGL2* since their predicted proteins showed highest similarity to methionine γ-lyase (MGL; EC 4.4.1.11) from the Archaea. The deduced amino acid sequences of EhMGL1 and 2 are 69% identical to each other. The EhMGL1 and 2 proteins showed 44–49/44–48% identity to MGL from three archael species, *i.e. Methanosarcina mazei* (MmMGL)[2] *Methanosarcina acetivorans* (MaMGL), and *Methanosarcina barkeri* (MbMGL), respectively. EhMGL1 and 2 also showed 39–46/40–43% identity to MGL from bacteria, *i.e. Fusobacterium nucleatum* (FnMGL, 46/43%) and *Pseudomonas putida* (PpMGL1, 39/40%), and 45/43% identity to MGL from another eukaryotic protozoan parasite, *i.e. T. vaginalis* (TvMGL1). In addition, EhMGL1 and 2 showed 39/41% identity to CGS from *Helicobacter pylori* (HpCGS), 37/36% identity to human CGL (HsCGL), and 26/28% identity to *E. coli* CBL (EcCBL).

Comparison of the deduced amino acid sequences of these MGLs as well as 18 other PLP enzymes (Fig. 2*A*, only MGLs are shown) revealed conserved amino acids as well as residues unique to MGLs. Phe^{44}, Met^{84}, Cys^{110}, and Val^{331} (amino acid numbers are based on EhMGL1) were conserved among all MGLs and absent in all other PLP enzymes. Conservation of Cys^{110} was previously reported for MGL from *P. putida* (31) and *T. vaginalis* (32). Amino acid residues implicated in substrate binding and catalysis from the crystal structure of MGL from *P. putida* (Tyr^{108}, Asp^{180}, Lys^{205}, Arg^{367}) (33) were also conserved in EhMGLs, although these residues were also shared by other PLP-dependent enzymes.

Considering these MGL-specific residues, the *MmMGL* and *MbMGL* genes, which were initially deposited in the data base as CGS (NP_635109) and a hypothetical protein (NZ_AAAR-01001136), respectively, likely encode MGL since all the important residues that were shown to be unique to and shared by biochemically characterized MGLs from other organisms, including the amebic MGL, were completely conserved in these 2 sequences. In addition, amino acid residues of Phe^{188}, Leu^{200}, Cys^{248}, Gly^{302}, and Asp^{337}, and a deletion (at position 232–233) were uniquely conserved among the two EhMGL isozymes and all the archaeal MGLs.

Phylogenetic Analyses of EhMGLs—Phylogenetic reconstruction of the 23 protein sequences that belong to the γ-subfamily of PLP enzymes from a variety of organisms, together with two EhCS isotypes (β-family of PLP enzymes) as the outgroup, was performed with the NJ, MP, and ML methods as described under "Experimental Procedures." These analyses (only the result of the NJ method is shown in Fig. 2*B*) revealed that PLP enzymes involved in sulfur amino acid metabolism were clearly divided into four distinct groups, *i.e.* MGL, CGL, CGS, and CBL, which was supported by high bootstrap proportions (98.9–100%). In the MGL clade, a monophyletic relationship among the MGLs from *E. histolytica* and three Archaea, *i.e. M. acetivorans*, *M. mazei*, and *M. barkeri*, was confirmed (bootstrap proportion 82.4%), while bacterial MGLs and TvMGLs formed an independent clade (93.0%). Therefore, MGL appeared to be subdivided into the *Entamoeba-Archaea* and the *Trichomonas-Bacteria* groups. TvMGLs and EhMGLs did not form a statistically supported clade with any of the three independent analytical methods (data not shown). That is, despite a predicted close relationship between these two protozoan organisms based on several biological and biochemical characteristics (*e.g.* anaerobic metabolism and a lack of mitochondria and the glutathione system), they do not likely share a common ancestor for their MGLs.

Evolutionary Distribution of MGL—The presence of an MGL gene or its encoded protein has been demonstrated in only a fraction of bacteria, including *Clostridium sporogenes* (34), *P. putida* (= *ovalis*) (35), *Pseudomonas taetrolenz* (36), *Bacillus halodurans* (37), *Aeromonas sp.* (38), *Citrobacter intermedius* (39), and *Brevibacterium linenese* (40), and only two eukaryotic organisms, *T. vaginalis* (41) and *E. histolytica* (this study). Coombs *et al.* (41, 42) also reported that MGL activity was not detected in crude extracts from the other anaerobic protozoan parasites *Entamoeba invadens, Trichomonas fetus, Trichomitus batrachorum,* and *Giardia lamblia*, suggesting that the presence of MGL is not directly associated with anaerobic metabolism. In addition, our search for a putative MGL gene in 23 archaeal genome databases available at NCBI revealed that only 3 Archaea, *M. mazei*, *M. acetivorans,* and *M. barkeri*, possess orthologous genes. We were also unable to find a MGL ortholog in other eukaryotes including yeasts, fungi, slime mold, and higher eukaryotes. This unique distribution of MGL strongly supports the premise that two distinct MGL subgroups have been horizontally transferred, *i.e.* from a subgroup of Archaea to *E. histolytica* and from a subgroup of bacteria to *T. vaginalis*.

Molecular and Structural Characterization of Recombinant EhMGL Isoenzymes—In order to understand the biochemical properties of the two EhMGL isoenzymes, recombinant proteins were produced. The recombinant proteins (rEhMGL1 and 2) were assessed to be >95% pure with Coomassie-stained

[2] Names of organisms: Eh, *Entamoeba histolytica*; Mm, *Methanosarcina mazei Goe1*; Tv, *Trichomonas vaginalis*; Fn, *Fusobacterium nucleatum subsp. C2A nucleatum ATCC 25586*; Ma, *Methanosarcina acetivorans*; Mb, *Methanosarcina barkeri*; Pp, *Pseudomonas putida*; Hs, *Homo sapiens*; Ec, *Escherichia coli K12*; Bh, *Bacillus halodurans*; Cc, *Caulobacter crescentus CB15*; Oi, *Oceanobacillus iheyensis*; Rn, *Rattus norvegicus*; Ce, *Caenorhabditis elegans*; Cp, *Clostridium perfringens*; Hp, *Helicobacter pylori*; Sa, *Staphylococcus aureus subsp. aureus N315*; Hi, *Haemophilus influenzae Rd*; St, *Salmonella typhimurium LT2*; Yp, *Yersinia pestis*.

TABLE II
Relative activities and kinetic properties of rEhMGLs

Relative activities and kinetic parameters were determined by using α-keto acid or nitrogen assays. Reactions were performed for 10 min at 36°C with 5 mM (for relative activities) or 0.2-10 mM (for kinetics) of each substrate and an appropriate amount of enzyme. Kinetic parameters were determined by α-keto acid assay for all the substrates except DL-homocysteine and *O*-acetyl-L-serine, which were monitored by nitrogen assay. For L-methionine, both assays were used. Experiments were repeated three times, and results of one representative experiment are shown (means ± S.D.). To determine kinetic constants, at least 7 different substrate concentrations were used. Lineweaver-Burk plots were obtained to calculate parameters using Sigma Plot 2000 software (SPSS Inc., Chicago, IL) with the Enzyme Kinetics module (version 6.0, Hulinks, Inc., Tokyo, Japan).

Substrate	Relative activity		K_m		V_{max}	
	rEhMGL1	rEhMGL2	rEhMGL1	rEhMGL2	rEhMGL1	rEhMGL2
	%		mM		μmol/min/mg	
L-Methionine	100.0	181.6	0.94 ± 0.16	1.90 ± 0.15	0.36 ± 0.20	0.44 ± 0.30
DL-Homocysteine	112.2	294.7	3.40 ± 0.67	1.87 ± 0.77	0.98 ± 0.11	1.31 ± 0.24
L-Cystathionine	<10	<10	ND[a]	ND	ND	ND
L-Cysteine	19.7	160.1	n.d.[b]	2.30 ± 0.10	n.d.	1.26 ± 0.81
O-Acetyl L-serine	11.1	33.8	n.d.	0.89 ± 0.23	n.d.	0.085 ± 0.05

[a] ND, not detectable.
[b] n.d., not determined.

SDS-PAGE gel (data not shown). The apparent molecular masses of rEhMGL1 and 2 (Fig. 4*A*, immunoblots using antibodies against rEhMGL1 and 2 are shown) agreed well with the predicted values (43.3 and 43.7 kDa, respectively, with 10 extra amino acids attached at the N terminus). Two-dimensional PAGE (Fig. 4*C*, *upper two panels*; also see below) showed that these rEhMGL isoenzymes had pIs consistent with those calculated (5.9 and 6.5, respectively). Gel filtration chromatography showed that the molecular mass of the native forms of rEhMGL1 and 2 was 171–177 kDa (Fig. 3*A*), indicating that both rEhMGL proteins form a homotetramer.

Enzymological Characterization of EhMGL Isoenzymes—Both rEhMGL1 and 2 catalyzed α-, γ- or α-, β-elimination of L-methionine, DL-homocysteine, L-cysteine, and OAS, but not L-cystathionine, to form α-keto acid, ammonia, and hydrogen sulfide (from cysteine), methanethiol (from methionine) or acetate (from OAS) (Tables I and II). The specific activity of rEhMGL1 and 2 (*e.g.* 0.36 and 0.44 μmol/min/mg toward L-methionine, respectively) was significantly lower than that of recombinant MGLs from other organisms (*e.g.* rPpMGL, 45.3 μmol/min/mg (43) and rTvMGL1/2, 10.4 ± 0.31/0.67 ± 0.05 μmol/min/mg (32), while the K_ms for substrates were comparable (0.9–3.4 mM) (Table II). Although EhMGL1 and 2 showed moderate homology in their amino acid sequences to EcCBL, HsCGL and HpCGS (shown above), L-cystathionine degradation by either rEhMGL1 or rEhMGL2 was negligible.

Marked differences in substrate specificity and specific activity exist between EhMGL isotypes. rEhMGL1 preferentially degraded L-methionine and DL-homocysteine and showed less activity toward cysteine and OAS, whereas rEhMGL2 catalyzed the degradation of these four amino acids with a comparable efficiency. That is, the ratio of rEhMGL1 activity toward L-cysteine to that toward L-methionine was 0.20 whereas that of rEhMGL2 was 0.88. In addition, rEhMGL2 generally showed 1.8- to 8-fold higher level of specific activity than rEhMGL1, independent of substrates (Table II).

Inter-isotype differences in substrate specificity were previously reported for two MGL isozymes from *T. vaginalis* (32). rTvMGL1 was shown to possess a broader substrate range than rTvMGL2; rTvMGL1 prefers methionine whereas rTvMGL2 is able to utilize methionine, homocysteine, OAS, and cysteine at comparable levels (*e.g.* the ratio of activity toward L-cysteine to that toward L-methionine was 0.58 for rTvMGL1 whereas for rTvMGL2 it was 1.58). The most striking difference between the amebic and trichomonal MGLs was that the latter have a strong preference toward homocysteine (more than 30-fold higher activity for homocysteine than both methionine and cysteine). Reactivity toward L-cystathionine was absent in both the amebic and trichomonal MGLs. This is in good contrast to a recombinant *Pseudomonas* MGL (43). It should be noted that the recombinant TvMGLs and a native form *P. putida* MGL (44) also lacked reactivity for L-cystathionine, which may suggest that reactivity for L-cystathionine is easily lost during purification or in case of ectopic expression using the bacterial system.

The pH optima for the two EhMGL isoenzymes were also significantly different (Fig. 3*B*). Such isotype-dependent differences in the optimum pH have not previously been described for MGLs in other organisms. This, together with the fact that the two EhMGL isotypes show only 69% identity and have distinct pI values, indicates that they may interact with different proteins and also may be localized in distinct subcellular compartments. Immunolocalization of each MGL isotype in amebic transformants expressing epitope-tagged EhMGL1 and 2, which is now underway, should help to further clarify these possibilities.

Next, in order to verify the stoichiometry of the reactions catalyzed by rEhMGLs, individual products of L-methionine catabolism, *i.e.* methanethiol, α-butyric acid, and ammonia, were measured. Approximately equal amounts (within a range of 2-fold) of these compounds were detected (Table I), verifying that rEhMGLs possess comparable α- and γ-lyase activities against methionine.

We also examined whether EhMGL catalyzed the formation of cystathionine from cysteine and, as a source of the homocysteine moiety, *O*-acetyl-L-homoserine or *O*-succinyl-L-homoserine (45), in a reaction known to be catalyzed by cystathionine γ-synthase. However, neither rEhMGL1 nor 2 catalyzed these reactions (data not shown); instead, both enzymes used L-cysteine as substrate for α-, β-elimination. Thus, given our failure to find CBS, CGL, CGS, and CBL homologs in the genome data base, we concluded that, unlike other organisms, *E. histolytica* lacks several key enzymes and their genes involved in the forward and reverse trans-sulfuration reactions.

Inhibition of MGL by PPG—To better understand the biochemical characteristics of EhMGL, we evaluated effects of DL-propargylglycine (PPG), a potent inhibitor of the γ-subfamily of PLP-dependent enzymes (46, 47), on the recombinant EhMGLs. Activity of both rEhMGL1 and 2 was inhibited by PPG in an irreversible and slow-binding manner (Fig. 3, *C* and *D*) with an apparent K_i of 35 μM (for rEhMGL2) with 5 min of preincubation, which agreed well with a previous report on human CGL (47).

We further assessed the effect of PPG on the parasite's MGL in cultures (Table I). The MGL activity of trophozoites was almost completely inhibited (97.5%) when they were cultivated in the BI-S-33 medium supplemented with 20 μM PPG, while control CS activity was not affected by PPG; control CS activity

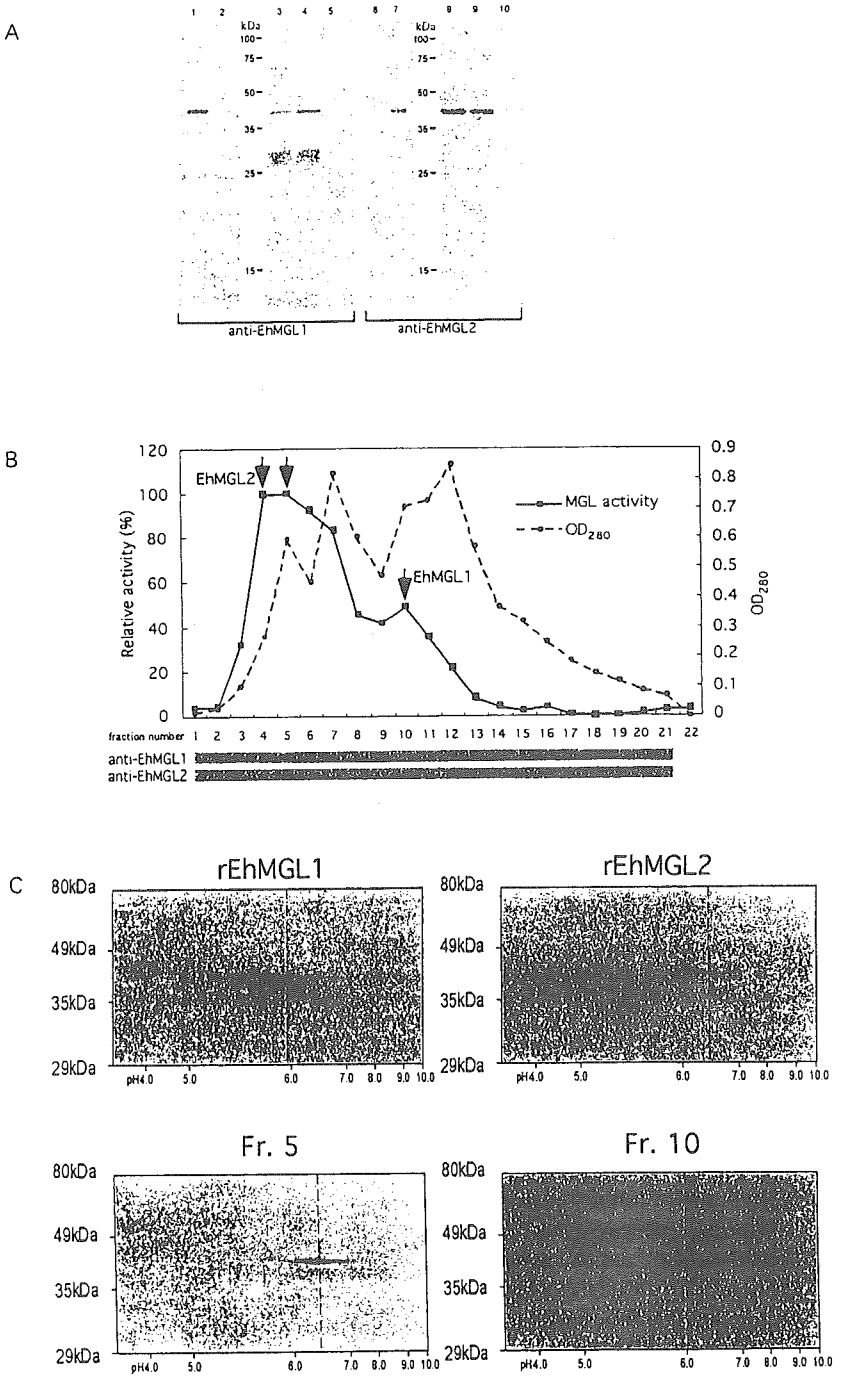

FIG. 4. **Immunoblot analyses of the native and recombinant EhMGL.** *A*, immunoblot analysis showing relative abundance and subcellular localization of two isotypes of EhMGLs. Recombinant EhMGL1 (*lanes 1* and *6*, 5 ng), recombinant EhMGL2 (*lanes 2* and *7*, 5 ng), a whole trophozoite lysate (*lanes 3* and *8*, 20 μg), a soluble fraction (*lanes 4* and *9*, 20 μg), and a pellet fraction (*lanes 5* and *1*, 20 μg) were electrophoresed on 10% SDS-PAGE gel, and subjected to immunoblot analyses with either anti-EhMGL1 (*lanes 1–5*) or anti-EhMGL2 (*lanes 6–10*) antibody as described under "Experimental Procedures." *B*, elution profile of the native EhMGL1 and EhMGL2 obtained by DEAE anion exchange chromatography. *Upper panel* shows MGL activities and A_{280} of individual fractions. *Arrows* indicate fractions in which EhMGLs were recognized with the antisera. *Middle* and *lower panels* show immunoblots of each fraction with anti-EhMGL1 (*middle*) and anti-EhMGL2 (*lower*) antibodies. *C*, two-dimensional PAGE analyses of the native and recombinant EhMGLs. *Upper panels*, one-hundred nanograms of rEhMGL1 or rEhMGL2 was subjected to two-dimensional PAGE and immunoblot analysis. *Lower panels*, two major MGL-containing peak fractions that were eluted from the DEAE column (*frs. 5* and *10*) were subjected to two-dimensional PAGE, followed by immunoblot analyses with the anti-EhMGL1 (*fr. 10*) or anti-EhMGL2 (*fr. 5*) antibody.

from the trophozoites cultured without PPG (72.8 nmol/min/mg) was comparable to that with PPG (69.4 nmol/min/mg). Interestingly, growth inhibition of trophozoites was negligible under these conditions (data not shown). Further, the growth inhibition was less than 5% even at higher PPG concentrations (up to 0.5 mM) (Fig. 5*B*).

These results suggest that MGL may not be essential for the amebae cultured *in vitro* using rich media. Considering the fact that this parasite possesses only incomplete methionine-cysteine conversion (*i.e.* trans-sulfuration) pathways and also lacks the cysteine degradation pathways present in other organisms, *e.g.* mammals, as described above, it is not understood how toxic sulfur-containing amino acids are degraded in the absence of MGL. A trace amount of MGL, together with other unidentified enzymes, may compensate for the decrease of MGL activity. It is also conceivable that the production of α-keto acids, *i.e.* pyruvate and butyrate, by MGL may not be essential in amebae in a nutrient-rich environment despite the fact that these products are used to form acetyl-CoA and α-propionic acid in a reaction catalyzed by pyruvate:ferredoxin oxidoreductase, and thus play a critical role in energy production in anaerobic protozoa (48). Furthermore, the other products of MGL, *i.e.* methanethiol and hydrogen sulfide, which have been implicated in the pathogenesis of oral microorganisms (49, 50), may not be required for *in vitro* growth of the amebic trophozoites. We are currently testing whether MGL shows more detrimental effects on amebae in nutrient-limited xenic cultures and also in animal intestine and liver models.

Characterization of Native Form EhMGL Isotypes in E. histolytica Trophozoites—Immunoblot analysis of the fractionated trophozoite lysate using specific antibody against rEhMGL1

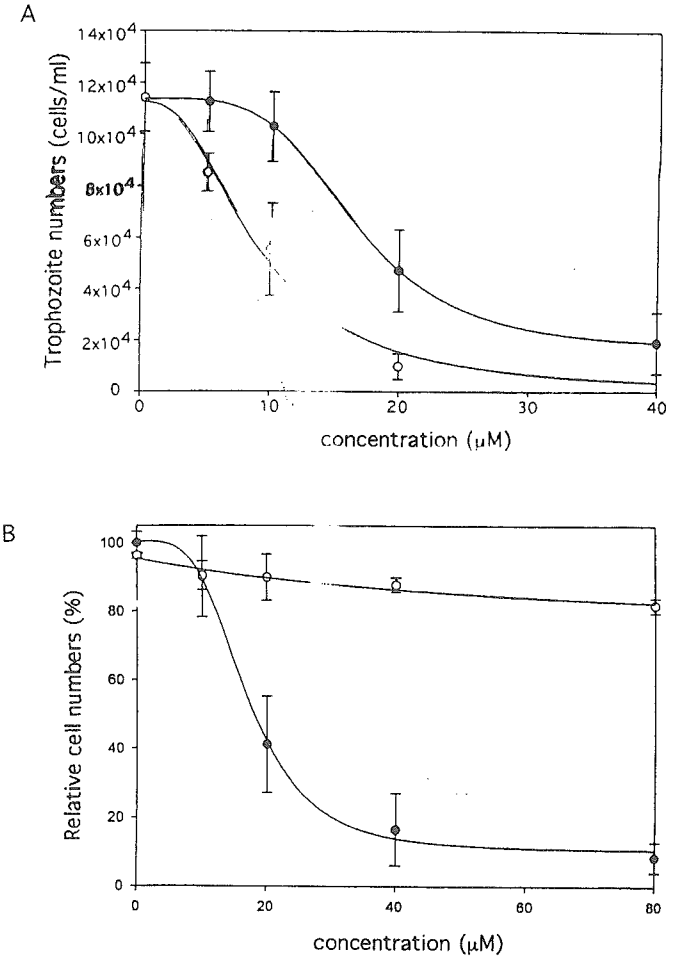

FIG. 5. **Effects of TFMET on the *E. histolytica* trophozoites.** *A*, cytotoxic effects of TFMET and metronidazole on the *E. histolytica* trophozoites. Trophozoites (1×10^5 cells/ml) were cultured with various concentrations of TFMET (●) and metronidazole (○) in BI-S-33 for 18 h. Numbers of live cells were assessed as described under "Experimental Procedures." Data shown are means ± S.D. of four independent experiments. *B*, amebicidal effect of TFMET was abolished by PPG. The trophozoites were cultivated with various concentrations of TFMET with (○) or without (●) 0.5 mM PPG. Percentages of the trophozoites relative to the untreated control are shown. Data shown are means ± S.D. derived from four independent experiments.

or rEhMGL2 showed that both of the native EhMGL isotypes were predominantly present in the cytosol fraction of the *E. histolytica* trophozoites (Fig. 4*A*). A quantitative estimate using densitometric measurements of native EhMGL in the trophozoite lysate with recombinant EhMGLs as controls revealed that EhMGL1 and 2 constitute ~0.05–0.1% of the total soluble protein of the cell (Fig. 4*A*, data for estimation not shown), which agreed well with the activity in the crude extract (Table I).

Fractionation of the whole trophozoite lysate by anion exchange chromatography, followed by the measurement of MGL activity in each fraction, revealed two major peaks possessing MGL activity (Fig. 4*A*, fractions *(frs.) 4–7* and *9–11*). Immunoblot analyses using anti-EhMGL1 and 2 antibodies identified the first and second peaks as EhMGL2 and EhMGL1, respectively. To correlate the recombinant and native form EhMGLs further, we subjected concentrated samples corresponding to the first and second DEAE peaks (frs. 5 and 10) to two-dimensional gel electrophoresis and immunoblotting. We identified a single spot for each native EhMGL isotype on a two-dimensional gel with measured pIs that agreed well with theoretical pIs (6.01 for rEhMGL1, 6.66 for rEhMGL2). We also examined, by size exclusion chromatography, the subunit structure of these native EhMGL isotypes obtained from the DEAE columns. The apparent molecular mass of the native EhMGL1 and 2 was determined to be ~170 kDa (data not shown). These results suggest, based on the size of the EhMGL monomers observed on SDS-PAGE and two-dimensional gels, that the native EhMGL1 and EhMGL2, similar to the recombinant proteins, also form a homotetramer. This contrasts well to a native MGL from *T. vaginalis*, which was suggested to form a heterotetramer (*i.e.* 2 molecules each of MGL1 and MGL2) (32).

Toxic Effects of TFMET on the Amebic Trophozoites—To exploit MGL as a potential target to develop a new therapeutic against the ameba, we tested if the methinonine analog trifluoromethionine (TFMET) shows an inhibitory effect on trophozoite growth. TFMET, a fluorine substitution-containing analog of methionine, is presumed to be catabolized by MGL to form α-keto butyrate, ammonia, and trifluoromethanethiol. Trifluoromethanethiol is non-enzymatically converted to carbonothionic difluoride (CSF_2), a potent cross-linker of primary amine groups (51).

TFMET caused significant growth inhibition in the trophozoites at concentrations as low as 20 μM. In addition, TFMET showed not only a growth inhibitory effect, but also a notable cytolytic effect on the trophozoites under the same condition; *e.g.* trophozoites cultivated in the presence of 20 μM of TFMET were completely lysed within 72 h. The IC_{50} of TFMET (for the growth inhibition) was determined to be 18 μM, which is slightly higher than that of the most commonly used antiamebic drug metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole], which showed an IC_{50} of 7 μM under the same conditions (Fig. 5*A*). This amebicidal effect of TFMET was completely abolished when the trophozoites were co-incubated with 0.5 mM of PPG, an inhibitor of EhMGL (Fig. 5*B*). Under the same conditions, PPG did not abolish the growth inhibition by metronidazole (data not shown). These results strongly support the premise that catabolism of TFMET by MGL is a part of the cytotoxic mechanism of TFMET in the trophozoites. We should also note that the activity of CS, which is the major PLP-dependent enzyme of this parasite, is not inhibited by up to 100 μM PPG (data not shown), further supporting the premise that MGL is a major target of TFMET. The cytotoxic effect of TFMET was also reported for *T. vaginalis* (52).

Finally, the lack of MGL in higher eukaryotes including humans also highlights this enzyme as a suitable and attractive target for the development of a novel chemotherapeutic agent against amebiasis. Combining conventional metronidazole and a new potent drug, *e.g.* TFMET, for the chemotherapy of amebiasis patients should prevent the rise of metronidazole resistance (53).

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Geographic Diversity among Genotypes of *Entamoeba histolytica* Field Isolates

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It has been known that only 5 to 10% of those infected with *Entamoeba histolytica* develop symptomatic disease. However, the parasite and the host factors that determine the onset of disease remain undetermined. Molecular typing by using polymorphic genetic loci has been proven to aid in the close examination of the population structure of *E. histolytica* field isolates in nature. In the present study, we analyzed the genetic polymorphisms of two noncoding loci (locus 1-2 and locus 5-6) and two protein-coding loci (chitinase and serine-rich *E. histolytica* protein [SREHP]) among 79 isolates obtained from different geographic regions, mainly Japan, Thailand, and Bangladesh. When the genotypes of the four loci were combined for all isolates that we have analyzed so far (overlapping isolates from mass infection events were excluded), a total of 53 different genotypes were observed among 63 isolates. The most remarkable and extensive variations among the four loci was found in the SREHP locus; i.e., 34 different genotypes were observed among 52 isolates. These results demonstrate that *E. histolytica* has an extremely complex genetic structure independent of geographic location. Our results also show that, despite the proposed transmission of other sexually transmitted diseases, including human immunodeficiency virus infection, from Thailand to Japan, the spectra of the genotypes of the *E. histolytica* isolates from these two countries are distinct, suggesting that the major *E. histolytica* strains prevalent in Japan at present were likely introduced from countries other than Thailand. Although the genetic polymorphism of the SREHP locus was previously suggested to be closely associated with the clinical presentation, e.g., colitis or dysentery and liver abscess, no association between the clinical presentation and the SREHP genotype at either the nucleotide or the predicted amino acid level was demonstrated.

Entamoeba histolytica is the causative agent of an estimated 40 million to 50 million cases of amebic colitis and liver abscess and is responsible for up to 100,000 deaths worldwide each year (6, 28, 33, 41). It has generally been granted that a majority of individuals infected with *E. histolytica* do not develop symptomatic disease (2, 11, 12, 15, 17, 18, 27). In recent cohort studies in Bangladesh, only about 3% of the *E. histolytica*-infected children developed symptoms attributable to amebic dysentery (16, 32). However, the parasite and the host factors that determine the onset of disease, i.e., whether or not amebae initiate tissue invasion and thus cause symptoms, remain undetermined (3, 41). A high degree of heterogeneity in virulence has been demonstrated previously. Interstrain variations in the adhesion of *E. histolytica* trophozoites to human epithelium have been demonstrated for two *E. histolytica* strains (1, 10), in which underrepresentation of the 35-kDa light subunit of the Gal-GalNAc lectin in the avirulent Rahman strain was shown to be correlated with a lack of cytopathic activity (1). Variations in cysteine proteinase expression between highly virulent and avirulent strains were also reported (23). In addition, marked differences in the levels of lipophosphoglycan-like and lipophosphopeptidoglycan molecules were demonstrated between virulent and avirulent strains of *E. histolytica* (24). Interstrain variations in the ability to produce liver abscesses in both gerbils and hamsters are also known. These interstrain variations in in vitro and in vivo virulence have prompted the World Health Organization's expert committee to recommend reinforced efforts through molecular epidemiological studies to determine whether some subgroups of *E. histolytica* are more likely than others to cause invasive disease (41). Another very puzzling question is why certain groups of infected individuals develop extraintestinal amebiasis without showing apparent intestinal symptoms. This observation also appears to be partially explained by interstrain variations in parasite virulence, i.e., tissue and organ tropisms, and host immune backgrounds, as suggested elsewhere (32). DNA typing of polymorphic genetic loci, recently developed by others (2, 7, 13, 43), helped us to closely examine the polymorphic structures of *E. histolytica* field isolates. While a majority of polymorphic genetic loci lack a correlation with virulent (or avirulent) phenotypes, Ayeh-Kumi et al. (2) recently showed that the serine-rich *E. histolytica* protein (SREHP) genotypes of clinical isolates from patients with liver abscesses were distinct from those of clinical isolates from patients with colitis and dysentery in Bangladesh, suggesting that an association between the SREHP genotypes (35) and clinical presentation may exist (2). Extensive genetic polymorphisms in both noncoding and cod-

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TABLE 1. Background and genotypes of the *E. histolytica* isolates used in this study

No.	Isolate	Isolation		Clinical diagnosis	Serology result[a]	Zymodeme	DNA origin	Type		Chitinase	SREHP
		Location	Date					Locus 1-2	Locus 5-6		
1	TM19	Thailand	1987	Dysentery and colitis	+	II	Xenic	D	A6/Cv	C	12
2	TM20	Thailand	1987	Dysentery and colitis	+	II	Xenic	D	A6/Cv	C	12
3	TM21	Thailand	1987	Dysentery and colitis	+	II	Xenic	B	A6/Cv	C	6
4	TM23	Thailand	1987	Colitis	+	II	Xenic	D	A11v	A	5
5	TM24	Thailand	1987	Dysentery and colitis	+	II	Xenic	I	A7	C	8/16
6	TM25	Thailand	1987	Colitis	+	II	Xenic	D	A11v/A9	A/C	7
7	TM27	Thailand	1987	Colitis	+	II	Xenic	K	A5	C	14
8	TM28	Thailand	1987	Dysentery and colitis	+	II	Xenic	D	A7/Cv	C	9
9	TM29	Thailand	1987	Dysentery and colitis	+	II	Xenic	D	A7/Cv	C	ND
10	TM35	Thailand	NA[b]	ALA	+	ND[c]	Pus	B	A7	E	Irr[d]
11	TM36	Thailand	NA	ALA	+	ND	Pus	B	A6/Cv	C	Irr
12	TM37	Thailand	NA	ALA	+	ND	Pus	L	A7	B	Irr
13	TM51	Thailand	1992	ALA	+	II	Axenic	B	A6/Cv	C	3/F
14	TM53	Thailand	1988	Colitis	+	II	Xenic	D	A7/Cv	C	10/L
15	TM54	Thailand	1988	Colitis	+	II	Xenic	D	A7/Cv	C	10/L
16	TM55	Thailand	1988	Colitis	+	II	Xenic	D	A7/Cv	C	10/L
17	TM58	Thailand	1989	Colitis	+	II	Xenic	D	A7/Cv	C	10/L
18	TM59	Thailand	1989	Dysentery and colitis	+	II	Xenic	D	A7	C	G
18	TM60	Thailand	1989	Colitis	+	II	Xenic	B	A7	E	G
20	TM61	Thailand	1989	Colitis	+	II	Xenic	B	A7	C	G
21	TM62	Thailand	1989	Dysentery and colitis	+	II	Xenic	B	A7	E	G
22	TM63	Thailand	1989	Dysentery and colitis	+	II	Xenic	L	A6/Cv	C	G
23	TM64	Thailand	1989	Colitis	+	II	Xenic	L	A6/C7	C	10/L
24	TM65	Thailand	1989	Dysentery and colitis	+	II	Xenic	D	A9v/A7	E	10/L
25	TM67	Thailand	1989	Colitis	+	II	Xenic	D	A7/Cv	C/F	10/L
26	TM83	Thailand	2001	Dysentery and colitis	+	II	Xenic	D	A7/Cv	C	11
27	TM84	Thailand	2001	Colitis	+	II	Pus	D	A7	C/F	L
28	TM40	Bangladesh	2000	NA	+	ND	Xenic	D	A6	E	15
29	TM41	Bangladesh	2000	NA	+	ND	Xenic	D	A5	C	Mix[e]
30	TM42	Bangladesh	2001	NA	+	ND	Xenic	D	A6	E	13
31	TM43	Bangladesh	2001	NA	−	ND	Xenic	D	A9	C	13
32	TM44	Bangladesh	2001	NA	+	ND	Xenic	D	A8/A5	C	1/4
33	KU6	Ghana	1994	Asymptomatic	+	II	Xenic	L	Cv	C	F
34	KU12	Cambodia	1995	Asymptomatic	+	II	Xenic	D	A5	C	2/17/18
35	PK1	Indonesia	2002	Colitis	ND	ND	Stool	M	A9v/A7	G	H
36	KU33	Institution E[f]	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
37	KU34	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
38	KU35	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
39	KU36	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
40	KU37	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
41	KU38	Institution E	June, 2002	Asymptomatic	−	II	Xenic	F	A5v/Cv	C	K
42	KU39	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
43	KU40	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
44	KU41	Institution E	June, 2002	Asymptomatic	+	VII	Xenic	F	A5v/Cv	C	K
45	KU42	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K

[a] The gel diffusion precipitin test and enzyme-linked immunosorbent assay were used for serology.
[b] NA, not available.
[c] ND, not determined.
[d] Irr, irrelevant PCR fragments.
[e] Mixed, likely a mixture judged by sequencing.
[f] Institution E is located in Yamagata Prefecture, Japan.

ing loci, including the SREHP locus, were previously demonstrated among *E. histolytica* isolates obtained from two social populations (mentally handicapped individuals and homosexual men) in a limited geographic area (domestic cases only in Japan) (14). In the present study, we extend our previous study to answer three specific questions: (i) how polymorphic are the Southeast Asian *E. histolytica* isolates? (ii) how similar or dissimilar are the genotypes of the Southeast Asian strains in comparison to those of the Japanese strains? and (iii) does a correlation exist between the genotypes of the isolates and the clinical presentations that they cause? The results of the present study not only support the previous finding of extensive genetic diversity among *E. histolytica* isolates (2, 7, 13, 14, 43, 44) but also fail to demonstrate a notable association between SREHP genotypes and clinical presentation or geographic origin.

MATERIALS AND METHODS

Clinical specimens. A total of 79 *E. histolytica* isolates, including 45 that were newly isolated, were analyzed in this study (Table 1). Thirty-four strains reported previously (14) were also used in the present study for comparison. Among the 45 new isolates, 10 isolates were obtained from stool samples from asymptomatic but seropositive individuals, with one exception, in an institution for mentally handicapped individuals in Yamagata Prefecture, Japan. Twenty-seven isolates were collected from either stool or liver aspirates from patients who visited outpatient clinics of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Five specimens were kindly provided by Rashidul Haque, Dhaka, Bangladesh, through Mahidol University. Three additional strains were

also isolated from stool samples from two Japanese workers who previously worked in Ghana and Cambodia and a domestic patient from Manado, Indonesia. Four patients had amebic liver abscesses (ALAs), and 24 patients had amebic dysentery and/or colitis. Twelve patients did not show any notable symptoms and thus were considered asymptomatic cyst passers. Identification of individual isolates as *E. histolytica* and not *Entamoeba dispar* was verified as described previously (14) by PCR with *E. histolytica*- and *E. dispar*-specific oligonucleotide primers (see below). A past or present history of invasive amebiasis was verified for 40 patients by serology by the gel diffusion precipitation test (26) and enzyme-linked immunosorbent assay (36). The clinical status of patients infected with five isolates (isolates TM40 to TM44) were not determined. All clinical specimens were collected after informed consent was obtained from the patients.

Cultivation. Xenic and axenic in vitro cultures were established by using Robinson's medium and BI-S-33 medium, respectively, as described previously (9, 29). Most xenic and axenic strains were cryopreserved by the method of Diamond (8) after xenic and axenic cultures were established and were revived 1 to 3 months prior to the present study to minimize possible changes, if any, in the genotypes.

DNA preparation, PCR, and sequence analysis. Total genomic DNA from trophozoites and/or cysts was purified from either cultured trophozoites or clinical specimens as described previously (14). Identification of *E. histolytica* and exclusion of *E. dispar* were verified by PCR with two sets of primers (primers Hsp1 and Hsp2 for *E. histolytica* and primers Dsp1 and Dsp2 for *E. dispar*) under the conditions described previously (44). Individual *E. histolytica* isolates were classified by PCR amplification of four previously described loci, i.e., locus 1-2 and locus 5-6 (43) and the chitinase and SREHP loci (13), by using four sets of oligonucleotides under the PCR conditions described previously (14), except that an annealing temperature of 50°C was used for all four loci. Loci 1-2 and 5-6 are present as tandemly linked multicopies within a >20-kb region (43) and contain tRNA genes (C. G. Clark, personal communication). No polymorphism in the nucleotide sequences was found among individual repeat units in the genome database (data not shown), which is consistent with the finding that PCR fragments containing these loci are homogeneous. Chitinase and SREHP are each apparently present as a single copy per haploid genome; only one copy of chitinase and SREHP each was found in the HM1 genome database. Therefore, although the ploidy of *E. histolytica* has not been determined, each of these genetic markers can be considered to be present as a single copy (per haploid genome). PCR products containing these loci were directly sequenced with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction II kit (PE Applied Biosystems, Foster City, Calif.) on an ABI PRISM 310 Genetic Analyzer. In cases in which multiple (more than one) bands were recognized after separation by 2% agarose gel electrophoresis, each DNA fragment was excised and sequenced separately, as described previously (14). The expected frequency of mutations with HotStar *Taq*DNA polymerase (Qiagen, Tokyo, Japan) was 2×10^{-5} (data not shown). We also tried to minimize the cycle numbers to avoid the accumulation of PCR products, which is known to increase the chance of introduction of mutations. Thus, when the lengths of the PCR fragments amplified in this study are considered (120 to 490 bp), the chance that mutations were introduced by PCR was negligible. The sequences obtained were manually edited and aligned by using DNASIS (version 3.7; Hitachi, Yokohama, Japan).

Restriction length polymorphism (RFLP) analysis of SREHP locus. Approximately 0.1 μg of the SREHP PCR products was digested with 3 U of *Alu*I (Takara, Tokyo, Japan) in a volume of 20 μl at 37°C for 2 to 16 h. About 5 μl of the *Alu*I-digested material was electrophoresed in 12% polyacrylamide gels (30). To visualize the DNA, the gels were stained by use of a silver staining kit (Pharmacia Biotech, Tokyo, Japan).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in the present work have been submitted to the GenBank/EMBL/DDBJ database under accession numbers AB096653 to AB096676.

RESULTS

High-resolution genotyping of *E. histolytica* field isolates and identification of new genotypes. It was previously shown that the levels of genetic polymorphism of the four polymorphic genetic loci mentioned above among *E. histolytica* isolates in Japan are extremely high (14). However, it is unknown if genetic polymorphisms also exist among the amebic isolates within the areas of developing countries where *E. histolytica* is endemic, e.g., Southeast Asian countries, and, if so, to what extent. Thus, we conducted high-resolution genotyping of these polymorphic loci for a large number of the *E. histolytica* isolates obtained from the area of endemicity. We attempted to answer the following questions: (i) how polymorphic are the Southeast Asian *E. histolytica* strains? (ii) how similar or dissimilar are the genotypes of the Southeast Asian ameba strains in comparison to those of the Japanese strains? and (iii) does any correlation exist between the genotypes of the isolates and the clinical presentations that they cause? We chose Thai isolates for analysis since it has been demonstrated that some of the human immunodeficiency virus (HIV) strains present in Japan were imported from Thailand (5, 20, 40). We amplified the four loci by PCR and sequenced individual fragments from 45 clinical isolates (27 isolates from Thailand; 5 isolates from Bangladesh; 10 isolates from Japan; and 1 isolate each from Cambodia, Indonesia, and Ghana). The profiles of the PCR fragments on agarose gels are shown in Fig. 1A for representative isolates (only data for new genotypes of SREHP are shown; see reference 14 for the previously identified genotypes). After sequencing, we identified among these 45 isolates 3 novel genotypes for locus 1-2 (genotypes K, L, and M), 2 novel genotypes for locus 5-6 (genotypes A11v and A9v), 1 novel genotype for chitinase (genotype G), and 18 novel genotypes for SREHP (genotypes 1 to 18) (a schematic diagram of all SREHP genotypes only is shown in Fig. 2; those of the other loci are not shown; all the sequence information was deposited in the GenBank/EMBL/DDBJ database). When these data are combined with previous data (14), we have identified among our 79 isolates and 4 previously reported isolates (13, 39) 13 different genotypes in locus 1-2, 15 different genotypes in locus 5-6, 9 different genotypes in the chitinase locus (data not shown), and 37 different genotypes in the SREHP locus (Fig. 2). The deduced peptide sequences of the chitinase and SREHP loci were also analyzed. The total number of SREHP genotypes based on the predicted amino acid sequences (31) was only slightly smaller than the number of SREHP genotypes based on the nucleotide sequences of individual PCR fragments (37) (data not shown), whereas the total number of chitinase genotypes was identical between the nucleotide and the predicted amino acid sequences. Among 27 Thai isolates, we identified 5, 7, 6, and 13 distinct genotypes for locus 1-2, locus 5-6, the chitinase locus, and the SREHP locus, respectively (4 isolates were excluded from the analysis of the SREHP locus for the reason explained in footnotes *c* and *d* of Table 1), suggesting that the extent of polymorphism is comparable between the isolates from Thailand and those from the Japanese homosexual men.

Heterozygosity of chitinase and SREHP. Although the chitinase locus was previously found to be homozygous, with one exception (type A/C) (14), we have found three additional isolates with two distinct chitinase genes (previously identified as genotype A/C and a new genotype, C/F). The demonstration of double SREHP genotypes in several isolates (i.e., isolates TM24, TM44, TM51 to TM58, and TM64 to TM67) strongly argues for the heterozygosity of this gene, as suggested previously (2, 13, 14). However, one isolate (isolate KU12) showed triple SREHP fragments on agarose gel electrophoresis corresponding to genotypes 2, 17, and 18.

Intergeographic differences in distributions of genotypes of each polymorphic locus. To examine the similarities and dif-

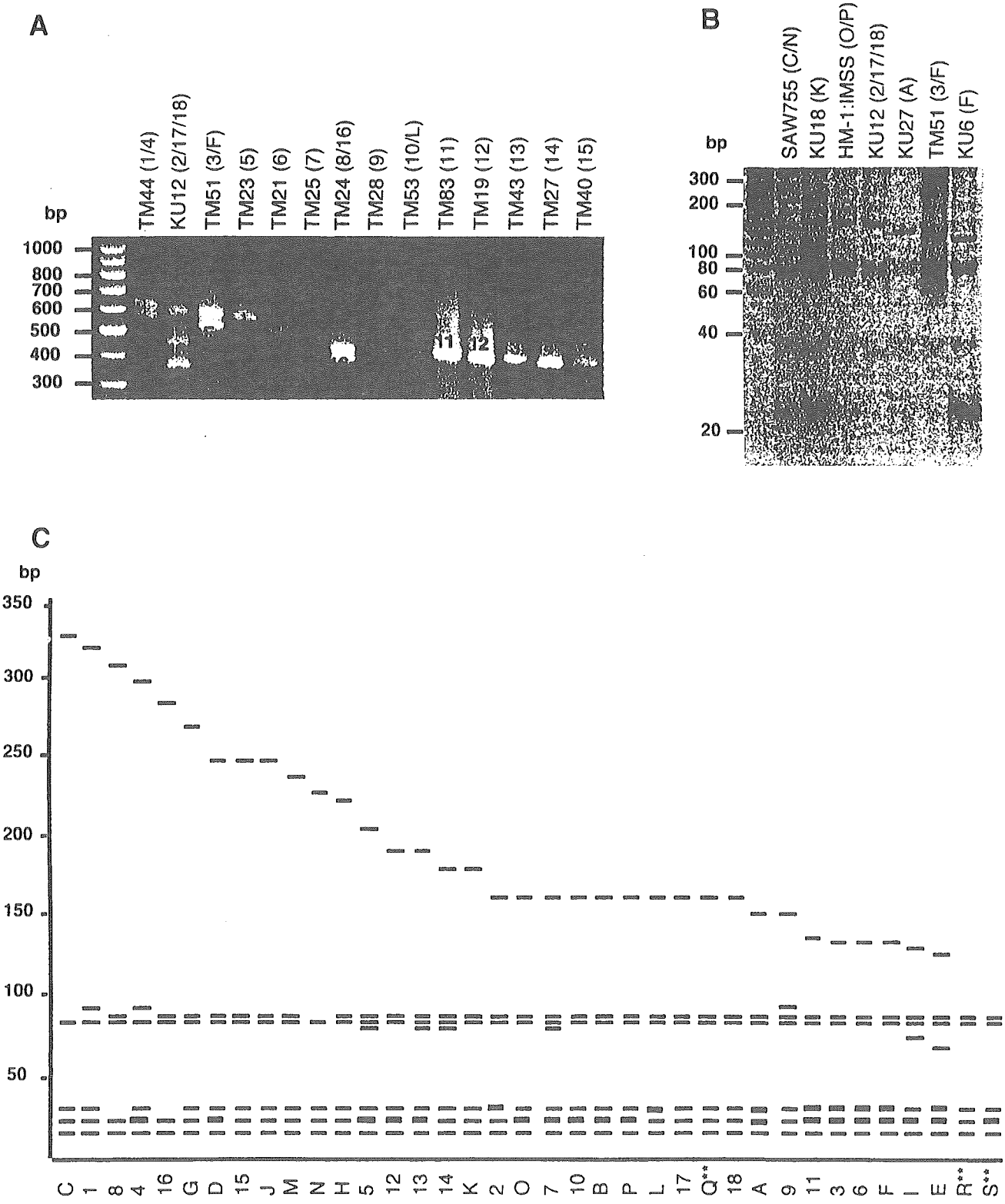
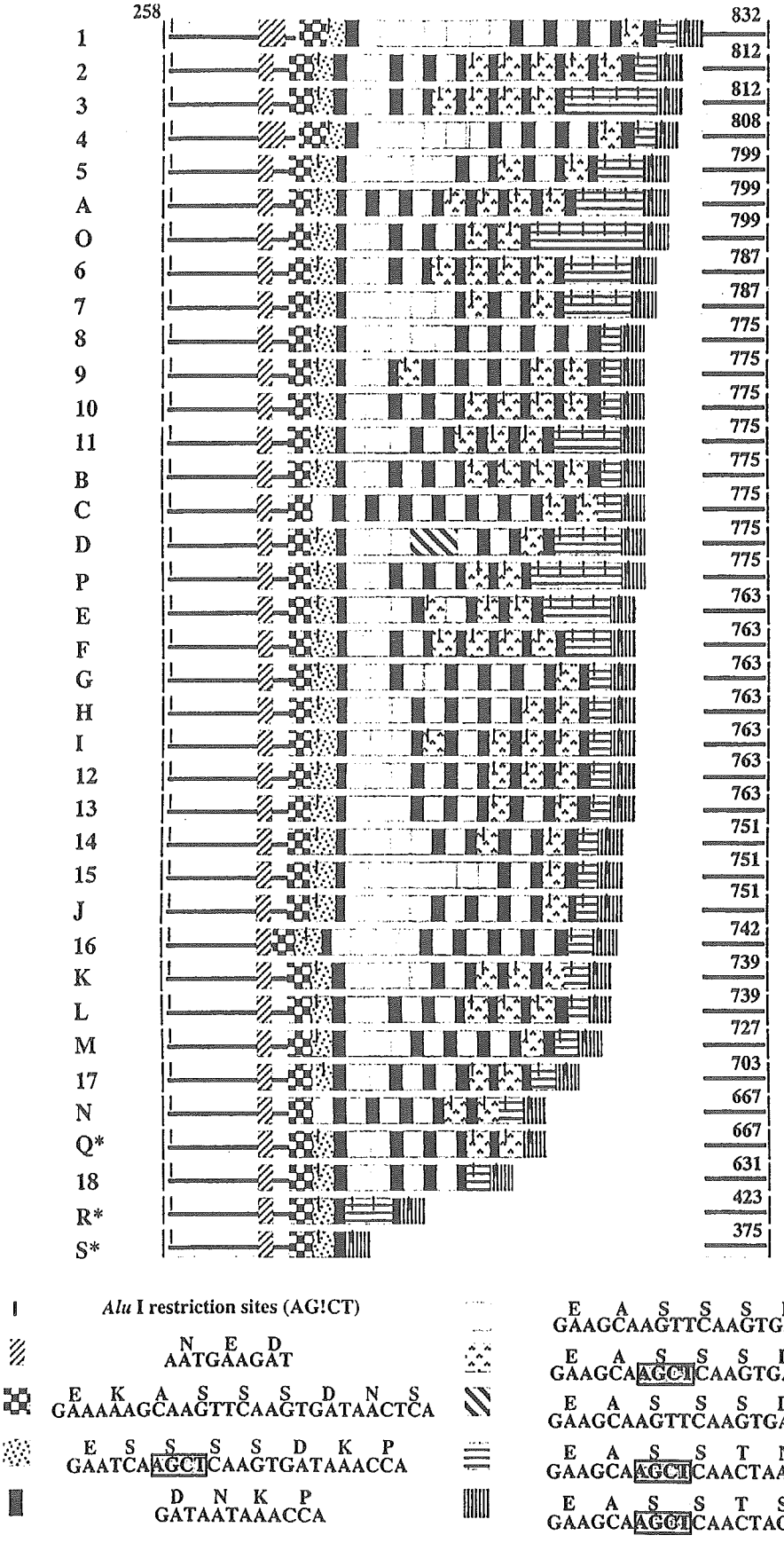


FIG. 1. Profiles of SREHP fragments amplified by PCR from clinical specimens and electrophoresed on agarose gels. All genotypes reported previously (14) and reported by Ghosh et al. (13) are given letter designations (A to S [14; this study]), and new genotypes designated in the present study are given number designations (1 to 18) in this and the other figures. (A) Agarose gel electrophoresis of undigested SREHP PCR fragments from representative *E. histolytica* isolates. Only the results for representative isolates that belong to each genotype (presented in parentheses) are shown. (B) Polyacrylamide gel electrophoresis of PCR-amplified and *Alu*I-digested SREHP fragments from selected isolates. (C) Schematic representation of *Alu*I digests of all genotypes. Note that the individual genotype is designated for each DNA fragment. Double asterisks indicate that more information can be found in reference 13.



Alu I restriction sites (AG!CT)

N E D
AATGAAGAT

E K A S S S D N S
GAAAAAGCAAGTTCAAGTGATAACTCA

E S S S S D K P
GAATCAAGCTCAAGTGATAAACCA

D N K P
GATAATAAACCA

E A S S S D K P
GAAGCAAGTTCAAGTGATAAACCA

E A S S S D K P
GAAGCAAGCTCAAGTGATAAACCA

E A S S S D K S
GAAGCAAGTTCAAGTGATAAATCA

E A S S T N K P
GAAGCAAGCTCAACTAATAAACCA

E A S S T S N S
GAAGCAAGCTCAACTAGTAATTCA

ferences among the isolates from Japan, Thailand, and other countries, the distributions of the genotypes of the four loci for all 79 isolates analyzed in our previous and present studies, together with those of four strains reported by others (13, 43), were examined (Fig. 3). We excluded 16 of 21 Japanese isolates from mentally handicapped individuals from these analyses because the mass infections were likely attributable to single strains (14), and therefore, inclusion of these genotypes would likely bias the outcomes of the analyses; e.g., locus 1-2 genotype F, locus 5-6 genotype A5v/Cv, chitinase locus genotype C, and SREHP locus genotype K were found in 16 isolates from mentally handicapped individuals. Marked differences in the histograms of locus 1-2, locus 5-6, and the SREHP locus were readily recognized, whereas the distributions of the chitinase locus genotypes were similar among the three groups of isolates. Some differences were very striking; e.g., genotype B of locus 1-2 represents a dominant type among the Japanese isolates (about 40%), while genotype D of locus 1-2 is dominant among the isolates from Thailand and other countries (about 50%) (Fig. 3A). Marked differences in histograms were found not only in a homozygous locus, i.e., locus 1-2, but also in a heterozygous noncoding locus, i.e., locus 5-6. A histogram showing the frequencies of locus 5-6 (Fig. 3B) showed that locus 5-6 genotype A5v or A7 was detected in about 20% of the Japanese isolates, whereas genotypes A6/Cv, A7, and A7/Cv were dominant (about 30%) among the Thai isolates. The results were almost similar when the allelic types, but not combinations of alleles, of locus 5-6 were compared (data not shown). Locus 5-6 allelic genotypes A5, A5v, and A7 were dominant and were found in about 60% of the Japanese isolates, while allelic type A7 was dominant (45%) among the Thai isolates. In contrast to the notable differences in the genotype distributions of loci 1-2 and 5-6, the chitinase locus genotypes showed similar distributions among the three groups (Fig. 3C). The extent of genetic variation of the SREHP locus is much higher than those of the other three loci (Fig. 3D); only one SREHP locus genotype (genotype 7) was shared by the Japanese and Thai isolates.

RFLP analysis of *Alu*I digests of the SREHP locus. Ayeh-Kumi et al. (2) recently demonstrated, using RFLP analysis of the *Alu*I digests of the SREHP PCR fragments (7), polymorphic patterns among clinical isolates from Bangladesh. They also reported that the majority (92%) of isolates from liver abscesses showed patterns distinct from those of the intestinal isolates. On the basis of these data, they proposed that particular SREHP locus genotypes and RFLP patterns may be closely associated with virulence. To further test this hypothesis, we first conducted a computational RFLP analysis based on the nucleotide sequences of the SREHP loci of all isolates that we obtained (Fig. 1C; only representative patterns are shown). Notable differences in the RFLP patterns were seen among these genotypes; the number of the patterns, however, decreased significantly compared to the number obtained by genotyping based on nucleotide sequences (n = 34 to 24 patterns) (Fig. 2). We also found that the histograms of the RFLP patterns between Japanese and Thai isolates differed significantly (data not shown), which was similar to the observation for the comparisons at the nucleotide level. However, we were unable to find any RFLP patterns that correlated with clinical presentations (e.g., ALA, colitis, or cyst carrier), the backgrounds of the patients (e.g., homosexual men or mentally handicapped individuals), or geographic origin. These computational RFLP analyses of the SREHP locus were also verified by *Alu*I digestion and polyacrylamide gel electrophoresis analyses of the PCR fragments from several representative isolates (Fig. 1B).

DISCUSSION

Using high-resolution genotyping based on the nucleotide sequences of four polymorphic loci of *E. histolytica*, we were able to demonstrate that this parasite from an area of endemicity in Southeast Asia has an extremely polymorphic genetic structure; e.g., 21 different combinations of genotypes were found among the 27 isolates obtained from Thailand. In combination with previous results (14), 53 combinations of genotypes were observed among 63 isolates. (Note that 16 isolates from institutions for mentally handicapped individuals [e.g., KU13, KU19 to KU22, KU28, and KU29 [14] and KU34 to KU42] were excluded for the reason described in Results.) This, together with previous work (14), in which an extensive polymorphism of the amebic strains from Japanese homosexual men was shown, reinforces the premise that *E. histolytica* has an extremely complex genetic structure independent of geographic location.

On the basis of the close social and economic relationship between Japan and Thailand and the fact that (i) sexual intercourse between homosexual men is closely associated with both HIV and amebic infections and (ii) comparison of genotypes between Thai and Japanese HIV strains indicates that a proportion of Japanese HIV strains were imported from Thailand (19, 21, 25, 37, 40), we hypothesized that the Japanese and Thai *E. histolytica* isolates might reveal a similar spectrum of genotypes that is indicative of similarities in the population structures of the *E. histolytica* strains between the two countries. However, our results appeared to argue against this hypothesis. Although notable similarities in the genotypes of locus 1-2, locus 5-6, and the chitinase locus were found between the Japanese and Thai isolates, polymorphisms in the SREHP locus have been found to be very extensive: only one of the Thai isolates showed an SREHP genotype identical to that of the Japanese isolates. When the genotypes of all four loci were combined, none of the Thai isolates had genotypes identical to those of the Japanese strains. Extensive polymor-

FIG. 2. Schematic representation of the polymorphisms in the repeat-containing region of the SREHP gene demonstrated among all isolates analyzed in the previous (14) and present studies and reference strains. *Alu*I restriction sites are depicted by vertical lines. The numbers shown correspond to the nucleotides of strain HM1 (accession no. M80910). Gaps were manually introduced to optimize alignments. Conserved regions are highlighted with gray rectangles. The nucleotide and deduced amino acid sequences of tri-, tetra-, octa-, and nonapeptide repeats are shown below. Also note that a previously unidentified tripeptide repeat unit is also included here. Asterisks next to the genotypes indicate that more information can be found in reference 13.

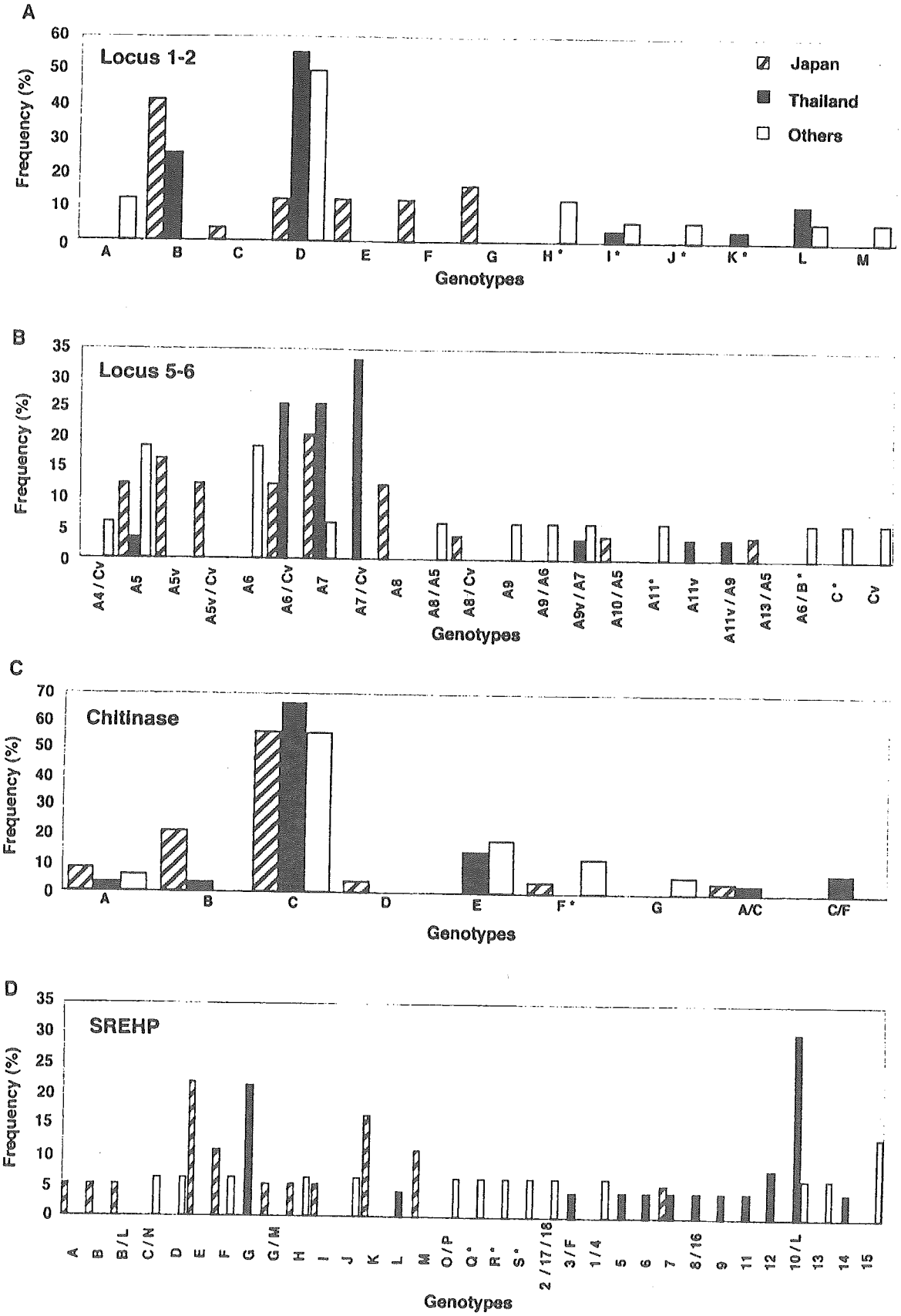


FIG. 3. Histograms showing genotype distributions of locus 1-2 (A), locus 5-6 (B), the chitinase locus (C), and the SREHP locus (D) from representative isolates from Japan, Thailand, and other countries. Only results for representative isolates of each genotype are shown. Asterisks indicate that more information can be found in references 13 and 43. Note that 16 of 21 Japanese isolates obtained from mentally handicapped individuals, which showed identical genotypes for all four loci, were excluded from these analyses (as explained in Results). Also note that 11 isolates showing either mixed or irrelevant PCR bands as judged by sequencing were excluded from analysis of the SREHP locus.

phism of the SREHP locus at the nucleotide level was shown previously (14); e.g., six distinct genotypes were found among 11 isolates from Japanese male homosexual men. Although genetic polymorphisms in a restricted geographic location in the area of endemicity have been reported by Ayeh-Kumi et al. (2), it is conceivable that those investigators underestimated the degree of polymorphism due to a lack of resolution of their analytical methods (PCR amplification of only the SREHP locus, followed by RFLP analysis on agarose gels). On the basis of the nucleotide sequences of all SREHP fragments and computational analyses of virtual RFLPs, we found that several SREHP genotypes would have been indistinguishable by RFLP analysis (e.g., genotypes D, 15, and J and genotypes 10, B, P, L, 17, Q, and 18; Fig. 1C). We should also mention that a histogram of the SREHP genotypes of the Thai isolates was also significantly different from that of the isolates from neighboring countries, including Bangladesh and Indonesia (Fig. 3D), although the number of isolates from those countries was too small to draw a definitive conclusion.

A high degree of genetic polymorphism in the repeat-containing region of SREHP raised a number of questions regarding the function of this protein and its association with the virulence and pathophysiology of *E. histolytica*. One of the most obvious questions is why the degree of polymorphism of SREHP is higher than those of the chitinase and noncoding loci. Although Ayeh-Kumi et al. (2) suggested that certain SREHP genotypes are more likely associated with ALA, this premise was not supported in the present study. We did not find any particular SREHP type, at either the nucleotide sequence level or the predicted amino acid sequence level, in association with clinical presentation. This is not likely due to either a lack of resolution of their analytical methods or differences in geographic backgrounds; the heterogeneity of RFLP patterns that Ayeh-Kumi et al. (2) reported (34 distinct patterns among 54 isolates) was comparable to the polymorphism of the SREHP locus at the nucleotide level that we report here. It has previously been demonstrated (31, 34) that SREHP is highly immunogenic because it possesses a number of conserved epitopes and that more than 80% of the individuals with ALA possess antibodies to SREHP, highlighting this protein as an important vaccine candidate. Together with the remarkable polymorphisms within the repeat-containing region of SREHP, as shown in this and other studies, these data strongly suggest that this polymorphism likely has a biological role, including immune evasion, as suggested elsewhere (34, 45, 46). However, the nucleotide and amino acid polymorphisms of the SREHP locus are too extensive to discuss the biological significance of these polymorphisms and their constraints on SREHP as a functional protein.

To verify the stability of the genotype observed for each isolate, we examined the nucleotide sequences of the four loci from four different isolates (isolates KU14, KU18, and KU26 [14] and isolate KU36) using xenic cultures, monoxenic cultures (cultivation with *Crithidia fasciculata*), and axenic cultures. We found no change in the genotypes of any of the four loci in these four strains (data not shown), indicating, together with previous findings (7, 44), that the nucleotide sequences of these loci are stable under a variety of conditions, e.g., long-term cultivation, axenization, cell cloning, and animal passage.

We should also note that the genotypes of the isolates obtained from institution E (locus 1-2, genotype F; locus 5-6, genotype A5v/Cv; the chitinase locus, genotype C; and the SREHP locus, genotype K) are identical to those of isolates from two other institutions for mentally handicapped individuals (institutions B and C) (14). These isolates were obtained from independent mass infection events at remote geographic locations (Kanagawa, Shizuoka, and Yamagata Prefectures in Japan, approximately 540 km apart) at different times (1994, 2000, and 2002). This finding further supports the premise that the genotypes of the *E. histolytica* isolates are stable after human transmission.

We also present further evidence of the heterozygosity of the chitinase and SREHP loci. The presence of multiple isoenzymes showing distinct affinities for substrates and the inhibitor allosamidin was demonstrated in *Entamoeba invadens* (38), posing the question of why only a small proportion (5%) of *E. histolytica* isolates possess multiple chitinase isoenzymes. The presence of multiple chitinase isoenzymes may be beneficial for the ameba since a broader substrate range may be covered by isoenzymes possessing distinct properties, as shown for two isoforms from *Serratia marcescens* (4), *E. invadens* (38), and *Plasmodium gallinaceum* (39). In contrast to chitinase genes, the SREHP locus was found to be heterozygous in approximately 29% of all isolates, suggesting the biological significance of heterozygosity in this gene. The presence of the triple SREHP genes in isolate KU12 cannot be due to a mixed culture or cross contamination since (i) none of these three bands were found in the other isolates and (ii) none of the other loci, i.e., locus 1-2, locus 5-6, and the chitinase locus, showed mixed patterns. This is inconsistent with the previous finding indicating that the SREHP gene appears to be present in a single copy (22). Thus, this isolate may represent a triploid or aneuploid, although the ploidy of reference strain HM1 was previously suggested to be at least four (42).

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Bacterial Expression of a Human Monoclonal Antibody-Alkaline Phosphatase Conjugate Specific for *Entamoeba histolytica*

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We previously produced human monoclonal antibody Fab fragments specific to *Entamoeba histolytica* in *Escherichia coli*. In order to use these Fab fragments for diagnostic purposes, an expression vector to produce a fusion protein of Fab and alkaline phosphatase (PhoA) in *E. coli* was designed and constructed. The *E. coli* PhoA gene was fused to the 3′ terminus of the gene encoding the heavy-chain Fd region. The kappa and Fd genes from a previously prepared antibody clone, CP33, which is specific for the 260-kDa lectin of *E. histolytica*, were used as human antibody genes. When the fusion protein of CP33 and PhoA was incubated with paraformaldehyde-fixed trophozoites of *E. histolytica* and developed with a substrate, the trophozoites appeared to be stained. These results demonstrate the feasibility of bacterial expression of a human monoclonal antibody-PhoA conjugate specific for *E. histolytica* and that the antibody can be used to detect *E. histolytica* antigen without the use of chemically conjugated secondary antibodies.

Amebiasis caused by infection with *Entamoeba histolytica* is one of the most important parasitic diseases not only in developing countries but also in developed countries. It has been estimated that 50 million people develop amebic colitis and extraintestinal abscesses, resulting in 40,000 to 100,000 deaths annually (3). Laboratory diagnosis of intestinal amebiasis is usually based on the microscopic detection of the organism in stool samples. However, nonpathogenic commensal *Entamoeba dispar*, which is morphologically identical with but genetically distinct from *E. histolytica*, has been identified recently as a separate species (9). Since treatment of *E. dispar* infection is not required, accurate diagnostic tools to discriminate between the two species are needed (3).

The application of monoclonal antibodies (MAbs) is one of several strategies for specific and sensitive diagnoses of infectious diseases. A number of MAbs which react specifically with *E. histolytica* or *E. dispar* have been produced by hybridoma technology (14, 16, 19–21). It has been reported that some MAbs were useful for detecting *E. histolytica* antigen in fecal and serum samples by sandwich enzyme-linked immunosorbent assay (1, 2, 11, 12). Recently, a new technology to produce a Fab fragment or single-chain Fv fragment in *Escherichia coli* has been established (4, 6, 15). The construction of vectors for the production of Fab in *E. coli* has also been reported (22, 24). When mouse immunoglobulin genes derived from a hybridoma producing *E. histolytica*-specific MAbs were expressed in this system, the specificity of the recombinant mouse Fab was comparable to that of the parent antibody (22). More recently, recombinant human MAb Fab fragments specific for *E. histolytica* have also been prepared from peripheral lymphocytes of a patient with an amebic liver abscess and of an asymptomatic cyst carrier (8, 18, 23). In order to use these human Fabs for diagnostic purposes, we report here the bacterial expression of a human Fab-alkaline phosphatase (PhoA) conjugate specific for *E. histolytica*.

The phagemid vector pRPLS/Fab1 (24) was digested with restriction enzymes *Not*I and *Eco*RI. A synthetic DNA linker consisting of two oligonucleotides (5′-GGCCGCAGGTGGC GGAGGTTCTGGTGGCGGAGGTTCTGGTGGCGGAGG TTCTAGACTCGAGTAAG-3′ and 5′-AATTCTTACTCGAG TCTAGAACCTCCGCCACCAGAACCTCCGCCACCAG AACCTCCGCCACCTGC-3′) was inserted into the *Not*I/*Eco*RI site of pRPLS/Fab1, thus creating a 15-mer Gly4Ser linker and recognition site for *Xba*I and *Xho*I. The resulting phagemid, pFab1-L, was digested with restriction enzymes *Xba*I and *Xho*I. To clone the *E. coli* PhoA gene, *E. coli* XL1-Blue was alkali lysed, neutralized, and subjected to PCR amplification. Two synthetic primers (5′-CCTCTAGAGGTACCCCAGAAATGCCTGTT CTAGAAA-3′ and 5′-GGCTCGAGTTTAAGCCCCAGAG CGGC-3′) were used to amplify 1.45 kb of the PhoA gene (17). The amplified gene was digested with *Xba*I and *Xho*I and subcloned into the *Xba*I/*Xho*I site of pFab1-L, resulting in phagemid pFab1-PhoA. This phagemid, pFab1-PhoA, was digested with *Xho*I and *Eco*RI. A synthetic DNA linker consisting of two oligonucleotides (5′-TCGAGGGTGGCGGAGGTTCT CATCACCATCACCATCACTAAG-3′ and 5′-AATTCATGG TGATGGTGATGGTGATGAGAACCTCCGCCACCC-3′) was inserted into the *Xho*I/*Eco*RI site of pFab1-PhoA, thus creating a 15-mer Gly_4Ser linker and a His_6 tag. The resulting plasmid was named pFab1-PhoA-H (Fig. 1).

As the source of human immunoglobulin genes, the kappa and Fd genes from a previously prepared antibody clone, CP33 (23), which is specific for the 260-kDa lectin of *E. histolytica*, were used for production of the fusion protein. The DNA fragment containing light- and heavy-chain genes was obtained by *Nhe*I/*Not*I digestion of pFab1-His2. The fragment was ligated with pFab1-PhoA-H and then introduced into compe-

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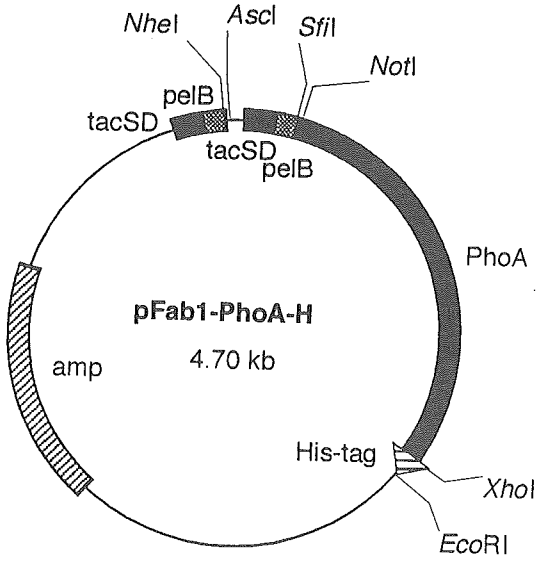

FIG. 1. Structure of plasmid vector pFab1-PhoA-H used for expression of the fusion protein of Fab and alkaline phosphatase. Genes encoding the light chain and the Fd region of the heavy chain are ligated into the *Nhe*I/*Asc*I and *Sfi*I/*Not*I sites, respectively. tacSD, tac promoter Shine-Dalgarno sequence; pelB, signal sequence of pectate lyase of *Erwinia carotovora*; PhoA, gene for alkaline phosphatase; His-tag, gene for hexahistidine tag; amp, gene for ampicillin resistance.

tent *E. coli* JM109. The bacteria were spread on Luria-Bertani plates containing 50 μg of ampicillin per ml, and the vector with the inserts was selected. The positive clone was cultured in 1 liter of super broth (30 g of tryptone, 20 g of yeast extract, 10 g of MOPS [morpholinepropanesulfonic acid] per liter [pH 7]) containing ampicillin at 37°C until an optical density at 600 nm of 0.5 was achieved. Isopropyl-β-D-thiogalactopyranoside was added to the cultures to a final concentration of 100 μM, and the cultures were then incubated at 30°C for 12 h to achieve optimal expression. The bacteria were pelleted by centrifugation at 6,000 × *g* for 20 min, suspended in 20 ml of phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride, and then sonicated. The lysates were centrifuged at 12,000 × *g* for 30 min, and the supernatant was filtered through 0.2-μm-pore-size syringe filters (Iwaki, Tokyo, Japan). Purification of the fusion protein from the supernatant was performed by affinity chromatography with His•Bind resin (Novagen, Madison, Wis.) in accordance with the manufacturer's instructions. Purified fusion protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (22). Western immunoblot analysis was also performed as previously described (22). The horseradish peroxidase (HRP)-conjugated goat immunoglobulin G (IgG) fraction specific to the human kappa chain (Organon Teknica, Durham, N.C.) and HRP-conjugated rabbit IgG fraction specific to alkaline phosphatase (Rockland, Gilbertsville, Pa.) were used for detection.

Approximately 2 × 10^5 trophozoites of *E. histolytica* HM-1:IMSS cultured axenically in BI-S-33 medium (10) were incubated on acetone-washed coverslips at 37°C for 30 min. The trophozoites were fixed with 4% paraformaldehyde in PBS for 30 min and then washed three times with PBS. After blocking with 5% bovine serum albumin was conducted for 15 min, the cells were incubated with the recombinant protein (50 μg/ml) for 30 min. After the cells were washed with PBS, development was conducted with a Vector red alkaline phosphatase substrate kit I (Vector Laboratories, Burlingame, Calif.) for 30 min in accordance with the manufacturer's instructions. Microscopic observation of the cells was performed under bright-field and fluorescent conditions by using a Nikon (Tokyo, Japan) XF-EFD2 fluorescence microscope.

SDS-PAGE analysis of the purified fusion protein of CP33 and PhoA revealed the expected sizes of two bands with apparent molecular masses of 25 and 75 kDa, although minor bands with apparent molecular masses of 50 kDa were also present (Fig. 2A). With Western immunoblot analysis, the 25-kDa band was recognized by an anti-human kappa chain goat antibody (Fig. 2B, lane 1). On the other hand, the 75-kDa band was detected by an anti-PhoA rabbit antibody, indicating that the molecule was a fusion protein of the Fd fragment and PhoA (Fig. 2B, lane 2). When the fusion protein of Fab-PhoA was incubated with paraformaldehyde-fixed trophozoites of *E. histolytica* and developed with the substrate, the surfaces of the trophozoites were stained clearly under both bright-field and fluorescent conditions (Fig. 3).

Recombinant human antibodies have been developed re-

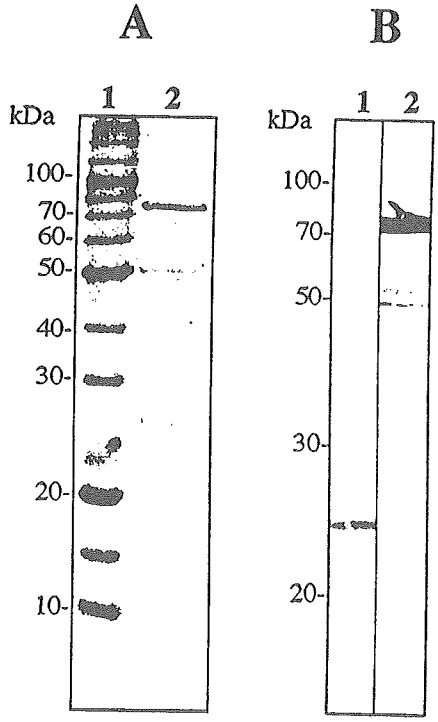

FIG. 2. SDS-PAGE (A) and Western immunoblot (B) analyses of a purified fusion protein of human Fab CP33 and alkaline phosphatase. (A) Two micrograms of the protein was subjected to analysis in 10% gel under reducing conditions and then stained with Coomassie brilliant blue. Lane 1, molecular size markers (BenchMark protein ladder; Life Technologies, Gaithersburg, Md.); lane 2, purified CP33-PhoA. Numbers to the left indicate molecular masses of the markers (in kilodaltons). (B) Protein bands were transferred to a polyvinylidene difluoride membrane. Lane 1 was treated with HRP-labeled anti-human kappa chain goat antibody. Lane 2 was treated with HRP-labeled anti-PhoA rabbit antibody.