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Cloning and Expression of a Putative Alcohol Dehydrogenase Gene of *Entamoeba histolytica* and Its Application to Immunological Examination

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To clone and express the genes encoding major antigens of *Entamoeba histolytica*, we constructed a λgt11 cDNA library for *E. histolytica* HM1:IMSS and screened it with pooled sera from patients with amoebiasis. A 1,223-bp cDNA was cloned (clone 1223), and its nucleotide sequence was determined. The amino acid sequence predicted to be encoded by the open reading frame of clone 1223 consisted of 396 residues and showed 32.5 and 32.3% homology to the NADH-dependent butanol dehydrogenases I and II (bdhA and bdhB) of *Clostridium acetobutylicum*, respectively. In addition, 29 of the 34 consensus positions of bdhA and bdhB were also well conserved in clone 1223. The recombinant protein expressed from clone 1223 had an estimated molecular mass of 43.5 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The antigenicity and specificity of the recombinant protein were evaluated by an enzyme-linked immunosorbent assay using sera obtained from two clinical groups of patients with amoebiasis and a group of healthy controls. The recombinant protein had potent and specific antigenicity. In all, 53 serum samples (88.3%) from 60 patients with amoebiasis were positive for immunoglobulin G antibody against the recombinant protein, with a mean optical density value of 0.42. In contrast, 53 of 54 healthy control serum samples were negative, with only 1 positive serum sample showing the lower optical density value. These results suggested that clone 1223 is promising in terms of providing a useful antigen for the accurate serodiagnosis of amoebiasis and that the gene encodes a putative alcohol dehydrogenase of *E. histolytica*.

Infection with invasive trophozoites of *Entamoeba histolytica* causes amoebic colitis followed by bloody dysentery or liver abscess. The prevalence of amoebiasis is particularly high in developing countries, including India and countries in southeast Asia and Africa. About 10% of the world's population is infected with *E. histolytica*, and more than 40,000 people die of amoebiasis every year (11, 22). In western countries, amoebiasis has been emerging as a sexually transmitted disease in high-risk groups, such as homosexuals (8, 10, 24). Contaminated-hand-to-mouth infection has been also identified in mental hospitals, where most of the patients with nosocomial amoebiasis are asymptomatic cyst carriers or are seropositive (9, 14).

In the laboratory diagnosis of amoebiasis, immunological examinations are as important as microscopic detection of trophozoites or cysts (5). Usually crude cell lysates prepared from axenic cultures of trophozoites have been used as antigens for various serodiagnostic tests. Since preparations contain massive amounts of antigenic cellular components common to eukaryotic cells, cross-reactions giving false-positive results are inevitable to some extent. To resolve this difficulty by increasing the specificity and sensitivity of the test, the antigenic composition of the pathogen used to monitor specific antibody responses closely related to the disease process of amoebiasis should be addressed first. The most suitable strategy involves the cloning and expression of genes encoding the specific antigen and the subsequent antigenic evaluation of the expression products by using sera from patients with amoebiasis. In this context, several groups have cloned and expressed the genes encoding the cell surface antigens of *E. histolytica* that specifically react with sera from patients with amoebiasis. These include the cysteine-rich 170-kDa subunit of the galactose-inhibitable adherence lectin (7, 19) and the serine-rich 46- and 52-kDa (18) and cysteine-rich 29-kDa (21) antigens. These recombinant cell surface proteins have especially high-level reactivity with sera from patients with amoebic liver abscesses (15–17, 27).

The roles of cell surface proteins in the humoral immune response have been demonstrated (see above), but the immunological significance of nonstructural intracellular components remains unclear. Therefore, we attempted to clone and express the genes encoding more immunologically potent and specific major antigens, including the intracellular components, by immunoscreening the clones from a cDNA library generated with the total poly(A)$^+$ RNA from trophozoites and an expression vector.

Here, we describe the cloning of the genes derived from *E. histolytica* HM1:IMSS and the sequence analysis and serological evaluation of the gene products. The amino acid sequence predicted to be encoded by the open reading frame (ORF) of the clone showed homology to NADH-dependent butanol dehydrogenases of *Clostridium acetobutylicum* belonging to a new class of alcohol dehydrogenase (ADH) (23). The recombinant protein expressed from the clone in *Escherichia coli* had a molecular mass of 43.5 kDa, and it reacted in an enzyme-linked immunosorbent assay (ELISA) with 53 serum samples (88.3%) from 60 patients with amoebiasis.

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MATERIALS AND METHODS

Culture conditions for *E. histolytica*. *E. histolytica* HM1:IMSS was axenically cultured in Diamond's TYI-S-33 medium (4) in 50-ml plastic culture flasks

(Becton Dickinson, Franklin Lakes, N.J.). The cultures were harvested at 72 h in logarithmic growth phase after dispersion. After the culture flasks were chilled on ice, trophozoites were collected by centrifugation and washed in phosphate-buffered saline (PBS; pH 7.2).

Construction and screening of a cDNA library. Total RNA was isolated from about 1 g of the packed trophozoites by using guanidinium isothiocyanate (12). Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose column chromatography (12). The cDNA was synthesized and a cDNA library was constructed with cDNA synthesis and λgt11 cloning systems (Amersham, Buckinghamshire, United Kingdom) according to the manufacturer's protocols. The cDNA library was plated with soft agar on a Luria-Bertani plate and incubated for 3 h at 43°C. To make a replica of the library, a 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG)-impregnated Hybond C extra filter (Amersham) was placed on top of the soft agar and the plate was incubated at 37°C for 1 h to induce protein expression. A pool of sera from five patients with amoebiasis, which were intensely positive in an ELISA (Sigma Diagnostics, St. Louis, Mo.) and the indirect hemagglutination test (Japan Lyophilization Laboratory, Tokyo, Japan), was used as the primary antibody at a dilution of 1:500 for immunoscreening the clones from the cDNA library. Plaques were stained with peroxidase-conjugated rabbit anti-human immunoglobulin G (IgG; heavy plus light chains) (Jackson Immuno Research, West Grove, Pa.) at a dilution of 1:1,000 and its substrate, 3,3′-diaminobenzidine. Plaques showing positive reactions were selected and purified three times. PCR with the λgt11 primers (Takara, Otsu, Japan) was performed to estimate the size of the insert.

Southern blot hybridization. Southern blot hybridization was performed to confirm the origin of the cloned gene. Genomic DNA of *E. histolytica* was isolated from nuclei by the method of Tannich et al. (20). Approximately 10 μg of genomic DNA was digested with *Eco*RI, subjected to electrophoresis, and blotted onto a positively charged nylon membrane, Hybond-N+ (Amersham), according to published procedures (12). Labeling of cloned cDNA (clone 1223) and detection of the hybridized probe were performed with the ECL direct nucleic acid labeling and detection system (Amersham) according to the manufacturer's instructions. Genomic DNA of *E. coli* Y1090 was isolated by the same method (12), and 10 μg was digested with *Eco*RI and used as a negative control.

Sequencing of the cDNA. The cDNA insert cloned into λgt11 was subcloned into the plasmid vector pUC18 (Takara) and sequenced by dideoxy chain termination (13) by using an Auto Read sequencing kit and an A.L.F. DNA sequencer (Pharmacia Biosystems, Alameda, Calif.). The nucleotide and deduced amino acid sequences were compared with the data in GenBank, EMBL, and SWISS-PROT by using the genetic information processing software GENETYX version 9.0 (Software Development, Tokyo, Japan).

Expression and purification of the recombinant protein. The cDNA was subcloned into the *Eco*RI site of the expression vector pRSET B (Invitrogen, San Diego, Calif.). This expression vector has a T7 promoter for expression and a metal binding domain consisting of six histidine residues for the affinity-chromatographic purification of the recombinant protein. *E. coli* XL1-Blue was transformed by electroporation with the Gene Pulser system (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's protocol. The transformants were incubated in 200 ml of SOB medium (12) at 37°C overnight. The recombinant protein was expressed by infection with M13/T7 phage (10^{12} PFU) at a multiplicity of infection of 5 in the presence of 0.5 mM IPTG. After 4 h in the presence of T7 polymerase induced by infection with the phage, the cells were harvested by centrifugation at 3,000 × *g* for 15 min. The pellet was resuspended in 20 ml of binding buffer (20 mM phosphate, 500 mM NaCl, pH 7.8) and cooled for 15 min on ice. The cells were lysed by three cycles of sonication for 10 s at a medium setting with the tube being held on ice and by four freeze-thaw cycles. The lysate was centrifuged at 43,000 × *g* for 20 min at 4°C to remove cell debris. The presence of the expressed protein of interest was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of an aliquot of the supernatant.

The recombinant protein was purified by using nickel nitrilotriacetic acid resin (Qiagen, Chatsworth, Calif.), which has a high affinity for the six histidine residues derived from the expression vector pRSET B. The resin (1 ml) was resuspended in 1 ml of the binding buffer (pH 7.8) after two washes with the same buffer. The lysate was incubated with the resin mixture at room temperature for 15 min with gentle shaking, and the resin was poured into a column and set to Econo System (Bio-Rad Laboratories). After being washed with buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 6.0), the bound protein was eluted with the same buffer containing 500 mM imidazole. The eluate was dialyzed against PBS five times at 4°C. The protein concentration was determined as described by Bradford (2). The purity and specificity of the protein were determined by SDS-PAGE and Western immunoblotting.

Evaluation of the antigenicity of the recombinant protein by ELISA using patients' sera. Sixty serum samples from patients diagnosed as having amoebiasis by stool and/or serological examination were used to antigenically evaluate the recombinant protein. Forty were from patients with amoebic colitis resulting in bloody dysentery or with liver abscess in hospitals in Osaka Prefecture, Japan (group I). Twenty serum samples were from asymptomatic cyst carriers, asymptomatic seropositive patients, or patients with mild diarrhea in a mental hospital in Osaka Prefecture (group II). The grouping of patients according to clinical status is shown in Table 1. For the negative control, 54 serum samples were collected from healthy Japanese donors from 1 to 40 years old without anamnesis of amoebiasis residing in Osaka Prefecture.

TABLE 1. Antigenic evaluation of the recombinant protein by ELISA using sera from amoebiasis patients and healthy controls

Group by clinical status	No. of subjects	Result of ELISA	
		No. positive (%)	Mean OD
Patient group I			
Patients with amoebic colitis	26	21 (80.8)	0.39
Patients with liver abscess	14	12 (85.7)	0.39
Subtotal	40	33 (82.5)	0.39
Patient group II			
Patients with mild diarrhea	4	4 (100)	0.43
Asymptomatic cyst carriers	5	5 (100)	0.64
Asymptomatic seropositives	11	11 (100)	0.41
Subtotal	20	20 (100)	0.47
Patient group total	60	53 (88.3)	0.42
Healthy control group	54	1 (1.9)	0.03

Each well of 96-well microtiter plates (Sumitomo, Tokyo, Japan) was coated with 100 μl of 0.1 M carbonate buffer (pH 9.8) containing 100 ng of the recombinant protein. After incubation of the plate at 4°C overnight, 5% skim milk was added to each well and the plates were incubated at 37°C overnight. All serum samples were diluted 1:1,000 (for detection of IgG antibody) or 1:200 (for detection of IgM antibody) in PBS containing 0.1% Tween 20 and 1% skim milk and were tested in triplicate. The optimal amount of recombinant protein and the optimal serum dilution were determined by checkerboard titration in advance. For ELISA, the wells were incubated with 100 μl of diluted serum for 1 h at 37°C; this was followed by a 1-h incubation at 37°C with 100 μl of peroxidase-conjugated rabbit anti-human IgG (heavy plus light chains; Jackson Immuno Research) at 1:10,000 or peroxidase-conjugated goat anti-human IgM (5FCμ; Organon Teknika, West Chester, Pa.) at 1:2,000. For the color reaction, 50 μl of 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added and the A_{655} was measured with an automatic microplate reader (Bio-Rad Laboratories) after the reaction proceeded for 30 min. Between the sequential reaction steps, the wells were washed more than three times with PBS–0.1% Tween 20. An OD value greater than 0.1 (mean OD value plus double the standard deviation in healthy controls) was regarded as a positive result (16).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCIB nucleotide sequence databases with the accession number D49910.

RESULTS

Cloning and sequencing of the cDNA. A cDNA library of 3 × 10^6 PFU was immunoscreened by using patients' sera with high titers of antibody to *E. histolytica*, and 55 suspected plaques were selected. Consequently, 35 positive clones were isolated after three plaque purification steps. The sizes of the inserts estimated by PCR with the λgt11 primer were from 500 to 1,300 bp. The largest clone was sequenced, showing that the insert consisted of 1,223 nucleotides. (This clone was tentatively designated clone 1223.) The putative ORF of the insert contained 1,188 nucleotides from the 3rd nucleotide at the 5′ upstream region to the 1,190th nucleotide at the 3′ downstream region. The A+T content of the ORF was 66.4%, and it was AT rich. A search of the GenBank and EMBL data banks revealed that the nucleotide sequence of the insert had 57% homology to that of the NADP$^+$-dependent ADH gene (EhADH1) (6), 42% homology to that of the NAD$^+$-dependent acetaldehyde-alcohol dehydrogenase gene (EhADH2) (3, 25), and 48% homology to that of an NADP$^+$-dependent aldehyde dehydrogenase gene (EhALDH1) (26) of *E. histolytica*.

Analysis of amino acid sequence encoded by clone 1223. The amino acid sequence predicted to be encoded by the ORF contained 396 residues. The molecular mass of the protein from the clone was calculated by software to be 43.5 kDa. The

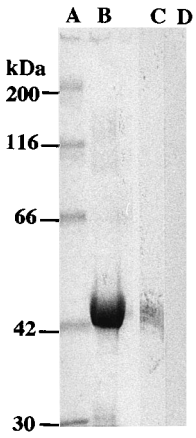

FIG. 1. SDS-PAGE and Western immunoblot analysis of the purified recombinant protein. Reduced recombinant protein (5 μl) was loaded onto a 10% linear polyacrylamide slab gel and resolved by electrophoresis at a constant current of 50 mA. After electrophoretic Western blotting of the recombinant protein onto a polyvinylidene difluoride membrane, the slab gel was stained with Coomassie brilliant blue (lane B). Immobilized recombinant protein on the polyvinylidene difluoride membrane was detected by using sera from a patient with amoebiasis (lane C) and a healthy control (lane D) diluted 1:500 followed by 1:1,000-diluted horseradish peroxidase-conjugated anti-human IgG antiserum. The mobilities and molecular masses of marker proteins are shown in lane A.

amino acid sequence showed 32.5 and 32.3% homology to those of the NADH-dependent butanol dehydrogenases I (bdh A) and II (bdh B) of *C. acetobutylicum*, respectively (23). In addition, 29 of the 34 consensus positions which characterize bdh A and bdh B as belonging to the new class of ADH were also well conserved in clone 1223 (data not shown).

The amino acid sequences encoded by EhADH1, EhADH2, and EhALDH1 have positional identities of 28, 24, and 24% to that of clone 1223, respectively (3, 6, 25, 26). With the exception of the sequence GGGS present in the EhADH2 protein, none of the conserved positions in these four proteins corresponded with each other (3, 25).

Southern blot analysis. Genomic DNA from *E. histolytica* was probed with labeled clone 1223, and positive fragments were found. No fragments were detected in the negative control (data not shown).

Expression and purification of the recombinant protein. A single colony of *E. coli* transformed with the recombinant pRSETB expression vector was cultured in 200 ml of SOB medium (12) overnight. The 200-ml culture was sequentially processed to purify the recombinant protein as described in Materials and Methods, and finally 2 ml of eluate was obtained. The protein concentration of the eluate was determined to be 696 μg/ml and the net weight of the protein was 1,392 μg, which corresponds to the amount of antigen required to test about 4,000 serum samples by ELISA. The molecular mass of the purified protein was 43.5 kDa on the basis of its mobility in SDS-PAGE, and this value was in agreement with the molecular size predicted from the ORF of clone 1223. The immunological specificity of the protein was demonstrated by the electrophoretic profile showing a single band in Western immunoblots with the serum of a patient with amoebiasis (Fig. 1).

Evaluation of antigenicity of the recombinant protein by ELISA with patients' sera. To evaluate the antigenicity of the recombinant protein, we performed an ELISA using the recombinant protein as the antigen and sera from two clinical groups of patients and a healthy control group. For group I, 33

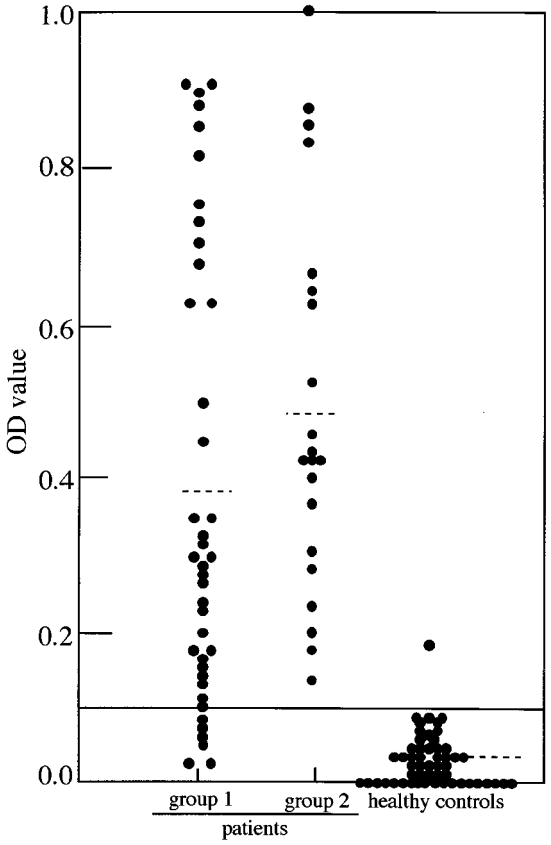

FIG. 2. Antigenic evaluation of the recombinant protein by ELISA using sera from patients with amoebiasis and healthy controls (by individual). Individual OD values for patient and healthy control groups were plotted. The solid line represents the cutoff value for positivity, and the dotted lines indicate the mean ODs. Grouping of the patients according to clinical status is shown in Table 1.

(82.5%) of 40 serum samples were antibody positive, with a mean OD value of 0.39. There were no significant differences between patients with amoebic colitis and those with liver abscesses in the prevalence of the antibody and the intensity of the reaction. All 20 serum specimens (100%) of group II were positive for IgG antibody, with a mean OD value of 0.47 and a maximal reactivity of 0.64 in asymptomatic cyst carriers. In all, 53 serum samples (88.3%) obtained from 60 patients were positive for IgG antibodies against the recombinant protein. In contrast, of 54 serum specimens from healthy controls, 53 were negative and 1 was positive, with the latter having a borderline OD value (Table 1; Fig. 2). However, this sample was negative in both tests with commercial serodiagnostic kits (an ELISA kit from Sigma Diagnostics and an indirect hemagglutination kit from Japan Lyophilization Laboratory). The rate of correspondence between the results of our ELISA using the recombinant protein and those of other diagnostic methods was 93.0% (Table 1). Thus, the recombinant protein had potent and specific antigenicity for amoebiasis. No serum had IgM antibody to the recombinant protein (data not shown).

DISCUSSION

In this study, we cloned a gene from *E. histolytica* and expressed the recombinant protein to improve serodiagnosis of amoebiasis. In general, the recombinant protein used as an antigen has several advantages compared with crude or puri-

TABLE 2. Serodiagnosis with the different recombinant proteins as antigens

Recombinant protein	No. of positives (%) in clinical status group[a]			Reference
	Symptomatic (invasive)		Asymptomatic	
	Amoebic liver abscess	Amoebic colitis		
170 kDa (1–1,202 amino acids)	52/57 (91)	2/3 (67)	ND	27
170 kDa (758–1,134 amino acids)	108/113 (95)	ND	3/4 (75)	15
SREHP	49/61 (80)	4/4 (100)	ND	17
29 kDa	13/17 (76)	9/113 (7.9)	6/54 (11)[b]	16
Clone 1223	12/14 (85.7)	21/26 (80.8)	20/20 (100)	This study

[a] Number of positives per total number of serum specimens. ND, not determined.
[b] Includes *E. dispar* cases.

fied antigen prepared from the trophozoites. The recombinant protein is chemically pure and antigenically specific and can be readily mass-produced. On the other hand, it is troublesome and time-consuming to maintain trophozoite cultures under the best conditions for providing a large amount of cell material to produce an excellent antigen. The gene which we succeeded in cloning (clone 1223) had all the above-mentioned advantages and enabled us to make accurate serodiagnoses of amoebiasis.

Recently, several recombinant cell surface antigens of *E. histolytica* which are useful in serodiagnosis have been reported. When used in ELISA or Western blotting, the recombinant 170-kDa surface protein reacted with more than 80% of sera from patients with amoebic liver abscess (15, 27). Similar findings have been obtained with the recombinant membrane serine-rich 46- to 52-kDa (SREHP) antigens (17) and cysteine-rich 29-kDa antigen (16). On the other hand, serodiagnosis of four asymptomatic cyst carriers by ELISA using the 170-kDa recombinant antigen prepared by Soong et al. (15) gave a positivity of 75%, which was lower than that of patients with amoebic liver abscess (95%). A similar relationship in correlative rates in these two clinical states was obtained by ELISA using the 29-kDa recombinant antigen (16), although most of the asymptomatic cyst carriers tested in that study were infected with *Entamoeba dispar*. In contrast, the recombinant protein derived from clone 1223 in this study was more reactive with sera from asymptomatic cyst carriers (100%) than with sera from patients with invasive amoebiasis (82.5%), suggesting that our recombinant protein is more useful for detecting chronic amoebiasis, especially in asymptomatic cyst carriers (summarized in Table 2).

The sera from patients with either amoebic liver abscess or amoebic colitis showed a mean OD value of 0.2 or 0.02, respectively, in ELISA at a serum dilution of 1:500 using Soong's 29-kDa antigen (15, 16), while in patients with amoebic liver abscess the OD was 0.7 using the 170-kDa antigen (OD values were estimated from figures in references 15 and 16) at the same serum dilution. On the other hand, in patients with either amoebic liver abscess or amoebic colitis, the OD was 0.39 using our antigen at a serum dilution of 1:1,000. Consequently, the ELISA using our antigen was markedly more sensitive than that using the 29-kDa antigen and had almost the same sensitivity as that using the 170-kDa antigen.

Abd-Alla et al. (1) tested Egyptian amoebiasis patients by ELISA using the purified cell surface 170-kDa antigen. The positivity rates obtained with their antigen drastically fluctuated depending on the healthy-control value. When unreasonably matched healthy controls residing in an area of nonendemicity (North America) were used in a study of patients residing in an area of endemicity (Egypt), a high seropositivity rate was attained with an unfavorable positivity of 64% even in healthy Egyptians. On the other hand, when matched healthy controls residing in the same area of endemicity (Egypt) as patients were used, the correlative rate using the 170-kDa antigen decreased markedly in patients with amoebic colitis and asymptomatic cyst carriers, allowing the differentiation between them and patients with amoebic liver abscess, whereas a high seropositivity rate specific to asymptomatic amoebic cyst carriers was obtained with our antigen without affecting the sensitivity, even when the patients were matched with healthy controls residing in the same area of endemicity (Osaka Prefecture, Japan).

By means of a computer-assisted homology search using genetic information and processing software, the predicted amino acid sequence from clone 1223 showed a homology of around 30% to those of bdh A and bdh B of *C. acetobutylicum*. These enzymes belong to a new class of ADH whose members are distinct from long-chain zinc-containing or short-chain zinc-lacking types of dehydrogenases; their 34 strictly conserved positions characterize them as being in a new enzyme class with this consensus primary structure (23). In addition to the overall amino acid sequence homology of about 30%, 29 of the 34 consensus positions were well conserved in the sequence encoded by clone 1223. Deviation from the consensus positional conservation to this extent is often observed among other enzymes belonging to this class (23). In contrast, there was no positional conservation in the other three types of alcohol and aldehyde dehydrogenases of *E. histolytica* except for the sequence GGGS in the EhADH2 protein (3, 6, 25, 26). These lines of evidence suggest that clone 1223 encodes a putative ADH belonging to a new class of ADH and that it is different from the dehydrogenases already identified for *E. histolytica*, although this speculation remains to be proved by further biochemical studies of the putative enzyme. The enzymatic activity of our recombinant protein is under study.

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