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Complementation of an *Escherichia coli adhE* mutant by the *Entamoeba histolytica EhADH2* gene provides a method for the identification of new antiamebic drugs

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ABSTRACT The pathogenic protozoan parasite *Entamoeba histolytica*, the cause of amebic dysentery and amebic liver abscess, is an obligate anaerobe, and derives energy from the fermentation of glucose to ethanol with pyruvate and acetyl coenzyme A as intermediates. We have isolated EhADH2, a key enzyme in this pathway, that is a NAD^+- and Fe^{2+}-dependent bifunctional enzyme with acetaldehyde dehydrogenase and alcohol dehydrogenase activities. EhADH2 is the only known eukaryotic member of a newly defined family of prokaryotic multifunctional enzymes, which includes the *Escherichia coli* AdhE enzyme, an enzyme required for anaerobic growth of *E. coli*. Because of the critical role of EhADH2 in the amebic fermentation pathway and the lack of known eukaryotic homologues of the EhADH2 enzyme, EhADH2 represents a potential target for antiamebic chemotherapy. However, screening of compounds for antiamebic activity is hampered by the cost of large scale growth of *Ent. histolytica*, and difficulties in quantitating drug efficacy *in vitro*. To approach this problem, we expressed the *EhADH2* gene in a mutant strain of *E. coli* carrying a deletion of the *adhE* gene. Expression of EhADH2 restored the ability of the mutant *E. coli* strain to grow under anaerobic conditions. By screening compounds for the ability to inhibit the anaerobic growth of the *E. coli*/EhADH2 strain, we have developed a rapid assay for identifying compounds with anti-EhADH2 activity. Using bacteria to bypass the need for parasite culture in the initial screening process for anti-parasitic agents could greatly simplify and reduce the cost of identifying new therapeutic agents effective against parasitic diseases.

The intestinal protozoan parasite *Entamoeba histolytica* causes amebic dysentery and amebic liver abscess, which are associated with significant morbidity and mortality worldwide. Amebiasis is currently treated with the drug metronidazole, which remains an effective agent in most cases. However, there can be significant side effects associated with metronidazole therapy including central nervous system complications and peripheral neuropathy (1, 2). In addition, the growing problem of metronidazole resistance among other protozoan parasites, such as *Trichomonas vaginalis* (3–5) and *Giardia lamblia* (6, 7), raises the specter of the development of metronidazole resistance in *Ent. histolytica*, and serves as an additional incentive for the development of new antiamebic agents. The search for new therapeutic agents for amebiasis has been hampered by the difficulties and cost of large-scale growth of *Ent. histolytica* and problems in developing quantitative measurements of drug efficacy *in vitro*.

Ent. histolytica is an anaerobic eukaryote that lacks mitochondria and ferments glucose to acetaldehyde and alcohol with pyruvate and acetyl coenzyme A (Ac-CoA) as intermediates (8, 9). Recently, we identified a potential target for antiamebic chemotherapy in the *Ent. histolytica* alcohol dehydrogenase/acetaldehyde dehydrogenase (EhADH2) molecule (10). EhADH2 is a bifunctional NAD^+-linked enzyme with both alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) activity, and is probably responsible for catalyzing two key steps in the *Ent. histolytica* fermentation pathway (9–11). The EhADH2 enzyme does not structurally resemble any known eukaryotic ADH or ALDH, but is homologous to the *Escherichia coli* AdhE enzyme and to similar enzymes found in some anaerobic or facultatively anaerobic bacteria (12–14). Here, we describe the expression of a functional EhADH2 protein in *E. coli* and the use of that recombinant protein to test the substrate specificity of the enzyme. More importantly, by using a plasmid containing the EhADH2 cDNA to complement an *E. coli* strain with a deletion of the *adhE* gene, we have produced a mutant *E. coli* that requires the *Ent. histolytica* enzyme for anaerobic growth. This *E. coli* strain can be used to screen compounds for their efficacy in inhibiting the *Ent. histolytica* EhADH2 enzyme. This approach represents a rapid and simple method for the identification of potential antiamebic drugs, and the method of using *E. coli* as a screening surrogate may be applicable to other parasites (e.g., *Cryptosporidium*) that are difficult or expensive to cultivate.

MATERIALS AND METHODS

***E. coli* and *Ent. histolytica* Strains and Culture Conditions.** The *E. coli* strains DH5α, BL21(DE3), and SHH31 (Δ*adh zch*::Tn10 *fadR met tyrT*) (15) were used for transformation and the expression of recombinant EhADH2. Aerobic cultures were grown in Luria–Bertani broth medium with agitation at 37°C. For anaerobic growth, bacteria were incubated in anaerobic jars, BBL GasPak System under an H_2-CO_2 atmosphere generated by BBL GasPak Anaerobic System Envelopes (Becton Dickinson). Anaerobic indicator strips were used to ensure anaerobic conditions. M9 minimal medium used for anaerobic growth was supplemented with glucose at 0.25%, 1 mM thiamine, 0.1 mM $CaCl_2$, 1.2 mM $MgSO_4$, and the following trace minerals: 50 μM Fe, 5 μM Se, 5 μM Mo, 5 μM Mn(16). Anaerobic liquid cultures were grown without agitation in tubes inside the anaerobic jars at 37°C. Solid media contained 1.5% Bacto Agar (Difco). Trophozoites of *Ent. histolytica* HM1:IMSS were cultured axenically in BYI-S-33 medium as described (17).

Construction of the EhADH2 Expression Vectors. Two expression vectors were used for prokaryotic expression of EhADH2: (*i*) the T7 promoter-based vector pET3a (Novagen) (18) and (*ii*) the recA promoter-based vector pMON2670 (19).

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Abbreviations: ADH, alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase; GST, glutathione *S*-transferase; Ac-CoA, acetyl coenzyme A.

The sequences flanking the EhADH2 coding region were modified by the incorporation of a *Bam*HI site next to the termination codon TAA at the 3′ end of EhADH2 and a *Nco*I site at the initiating ATG codon using PCR with the EhADH2 cDNA as the template (10). The EhADH2 sequence was then ligated in frame into *Nco*I and *Bam*HI digested pET3a as two fragments, *Nco*I/*Pst*I and *Pst*I/*Bam*HI to construct the expression vector pET/EhADH2. To construct pMON/EhADH2, the coding sequences were ligated in frame into *Nco*I/*Sac*I-digested pMON2670 as two fragments, *Nco*I/*Pst*I and *Pst*I/*Sac*I.

Expression of Recombinant EhADH2 in *E. coli* SHH31 (Δ*adhE*). EhADH2 was first expressed in *E. coli* SHH31 using the pMON/EhADH2 vector. Subsequently, the SHH31 strain was lysogenized by λDE3 using a lysogenization kit (Novagen) according to the manufacturers protocol. EhADH2 was then expressed in SHH31(DE3) using the pET/EhADH2 vector. SDS/PAGE analysis of bacterial lysates for expression of recombinant EhADH2 was performed as described (20). Western blot analysis was performed using a 1:500 dilution of rabbit antiserum raised to a recombinant 6His-EhADH2 fusion protein using previously described methods (20). Complementation of the Δ*adhE* mutation by EhADH2 was tested by measuring the anaerobic growth of *E. coli* SHH31 transformed with pMON/EhADH2 on minimal glucose media compared with that of *E. coli* SHH31 transformed with pMON2670.

Assay of ADH and ALDH Activity of Bacterial Lysates and Purified Recombinant EhADH2. ADH activity of the supernatant fraction from bacterial lysates or of the purified recombinant enzyme was assayed spectrophotometrically by measuring the decrease in absorbance at 340 nm following the oxidation of NADH to NAD (21). The cuvette contained 6 mM DTT, 5 mM $MgSO_4$, 0.1 mM $Fe(NH_4)_2(SO_4)_2$, 0.4 mM NADH, 10 mM acetaldehyde, and 0.1 M Mops-KOH buffer (pH 7.5) to give a final volume of 1.0 ml. ALDH activity was assayed using the same method, with the substitution of 0.1 mM Ac-CoA for acetaldehyde in the reaction buffer. A unit of enzyme activity is defined as the micromoles of product formed per min of incubation at room temperature.

To study the substrate specificity and kinetics of the purified recombinant EhADH2 molecule, the spectrophotometric assay of ADH activity was again used, but this time the assay was performed in the reverse direction, with 5 μg of the purified enzyme in the presence of 50 mM glycine/NaOH buffer (pH 9.5) containing 6 mM dTT, 5 mM $MgSO_4$, 0.1 mM $Fe(NH_4)_2(SO_4)_2$, 1 mM NAD^+, and varying concentrations of the substrate alcohol to be tested (11). The K_m and K_{cat} values expressed were determined using nonlinear regression to fit the values for initial velocity and substrate concentration to the Michaelis-Menten equation.

Purification of Recombinant EhADH2. A 1-liter culture of *E. coli* SHH31(DE3) carrying pET/EhADH2 was grown overnight under aerobic conditions. The bacteria were collected by low speed centrifugation, resuspended in 20 mM Mops-KOH buffer (pH 7.5), disrupted by sonication, and sedimented by centrifugation at 150,000 × *g* for 1 hr at 4°C. The supernatant was brought to 35% saturation with solid ammonium sulfate and stirred for 1 hr at 4°C. The suspension was centrifuged at 15,000 × *g* for 20 min at 4°C. The supernatant was dialyzed extensively against 20 mM Mops-KOH (pH 7.5), and chromatographed over a 1.6 cm × 90 cm Sepharose CL-6B (Sigma) gel filtration column equilibrated with 20 mM Mops-KOH buffer. Using a flow rate of 0.4 ml/min, fractions were collected and screened for NAD^+ dependent ADH activity.

EhADH2 Inhibition Assay. *E. coli* SHH31 transformed with pMON/EhADH2 were inoculated into M9 minimal liquid medium and grown under anaerobic or aerobic conditions in the presence or absence of pyrazole (Sigma) at concentrations of 5 to 20 mM. Growth was monitored by determining the OD at 600 nm at 24 and 48 hr postinoculation. To study inhibition of *Ent. histolytica* growth, standard culture tubes containing an initial inoculation of 4×10^3 per tube *Ent. histolytica* HM1:IMSS trophozoites were incubated for 4 days in the presence or absence of pyrazole at a concentrations of 5 to 40 mM. Viable trophozoites were counted using a hemocytometer at days 2 and 4, and the number of trophozoites per ml was recorded.

RESULTS

Expression of Functional EhADH2 in *E. coli*. Nucleotides 3–2,620, representing the entire coding region of the EhADH2 cDNA clone, were first expressed as glutathione *S*-transferase (GST) and 6His-EhADH2 fusion proteins, using the pGEX-KG (22) and pQE (Qiagen, Chatsworth, CA) vectors, respectively. However, neither recombinant fusion protein possessed detectable ADH or ALDH activity (data not shown). The 6His-EhADH2 recombinant protein was purified and used to generate a specific anti-EhADH2 antiserum. The EhADH2 protein was expressed without a fusion partner using the pET/EhADH2 construct as described. As shown in Fig. 1*A*, *E. coli* BL21(DE3) containing the pET/EhADH2 plasmid produced a protein at 96 kDa (the predicted size of the EhADH2 protein) (lane 3), whereas *E. coli* BL21 (DE3) transformed with the pET3a vector alone did not show a species at 96 kDa (lane 1). To confirm that the species at 96 kDa was EhADH2, Western blot analysis of the SDS/PAGE separated bacterial lysates with antiserum to the 6His-EhADH2 recombinant protein was performed. Anti-EhADH2 antiserum bound to the species at 96 kDa in lysates from BL21(DE3) expressing pET/EhADH2 (Fig. 1*B*, lane 3), but not in control lysates of BL21 (DE3) transformed with the pET vector alone (Fig. 1*B*, lane 1).

The ADH and ALDH activity of the recombinant EhADH2 protein was first assessed by measuring the enzymatic activity of lysates obtained from aerobically grown *E. coli* expressing the pET/EhADH2 plasmid and from *E. coli* BL21 (DE3) containing the pET vector alone. As shown in Table 1, lysates from pET/EhADH2-transformed bacteria expressing the 96-kDa EhADH2 enzyme had high levels of ADH and ALDH activity when compared with lysates from control *E. coli* BL21(DE3) containing the pET vector alone.

***EhADH2* can Complement (Δ*adhE*) in *E. coli*.** To determine whether the EhADH2 gene product would complement the *E. coli adhE* gene, we expressed EhADH2 in *E. coli* SHH31 (Δ*adhE*) (15). This strain produces no AdhE enzyme and is unable to grow in M9/glucose minimal media under anaerobic conditions (15). As shown in Fig. 2, *E. coli* SHH31 transformed with pMON/EhADH2 was able to grow on M9 minimal medium agar under both aerobic and anaerobic conditions, whereas *E. coli* SHH31 transformed with pMON alone could only grow under aerobic conditions. Thus, the product of the amebic EhADH2 gene can complement the *E. coli* (Δ*adhE*) mutation.

We confirmed that SHH31 was producing the EhADH2 protein by examining bacterial lysates from both SHH31 transformed with pMON/EhADH2 and lysogenized SHH31(DE3) expressing the pET/EhADH2 vector. As shown in Fig. 1*A*, expression of EhADH2 was detected in SHH31/pMON/EhADH2 (lane 7) and SHH31(DE3)/pET/EhADH2 vector (lane 5). The identity of the 96 kDa species as EhADH2 was confirmed by Western blot analysis using anti-EhADH2 antiserum (Fig. 1*B*, lanes 5 and 7). Lysates obtained both from SHH31(DE3) transformed with pET/EhADH2 and from SHH31 transformed with pMON/EhADH2 contained detectable ADH and ALDH activity (Table 1), whereas lysates from the parent strains showed no detectable ADH or ALDH activity.

Purification and Determination of the Substrate Specificity of Recombinant EhADH2. By expressing EhADH2 in *E. coli* SHH31, we had a source of recombinant EhADH2 without any possible contamination by the bacterial AdhE enzyme. Because greater ADH and ALDH activity was detected in lysates

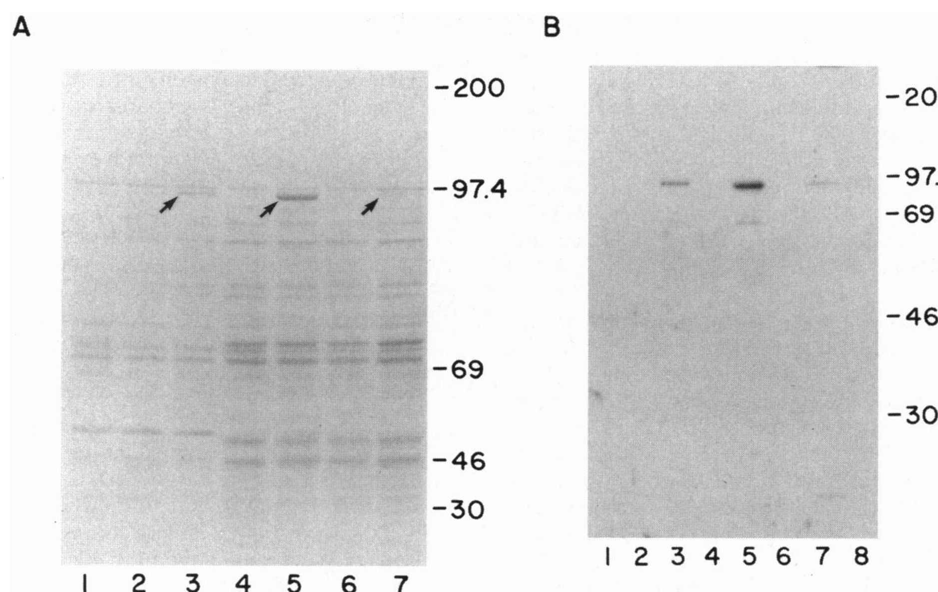

FIG. 1. Expression of EhADH2 by *E. coli*. (*A*) Coomassie blue staining of SDS/PAGE separated lysates from: lane 1, BL21(DE3); lane 2, BL21(DE3)/pET-3a; lane 3, BL21(DE3)/pET/EhADH2; lane 4, SHH31; lane 5, SHH31(DE3)/pET/EhADH2; lane 6, SHH31/pMON2670; lane 7, SHH31/pMON/EhADH2. A band at 96 kDa (arrows) is seen in lysates from strains expressing EhADH2 (lanes 3, 5, and 7) and not from control strains. (*B*) Immunoblotting of lysates with anti-EhADH2 serum. Lane assignments are identical as in *A*; lane 8 is lysates from *Ent. histolytica* HM1:IMSS. A species at 96 kDa is detected in *E. coli* lysates expressing EhADH2 (lanes 3, 5, and 7) and in *Ent. histolytica* (lane 8). Molecular mass standards (in kDa) are indicated at the right of each panel.

from SHH31(DE3)/pET/EhADH2 (Table 1), we used this system for purification of recombinant EhADH2. Purification of recombinant EhADH2 from lysates of pET/EhADH2 transformed *E. coli* SHH31(DE3) was accomplished using ammonium sulfate precipitation and gel filtration on Sepharose CL-6B (Fig. 3). Purity was assessed using Coomassie staining of SDS/PAGE separated fractions (Fig. 3) and measuring ADH activity. The purified recombinant EhADH2 retained both ADH and ALDH activity (Table 2). Based on gel filtration, the molecular mass for the recombinant EhADH2 enzyme was greater than 200 kDa; a similar pattern was seen with the purification of the native *Ent. histolytica* enzyme (11), and suggests the recombinant enzyme forms multimers similar to those seen with the *E. coli* AdhE protein and native EhADH2 (11).

We used the purified recombinant enzyme to study the substrate specificity of EhADH2. We found that only the primary alcohols ethanol, 1-propanol, and butanol were substrates for the enzyme (Table 2). No reactivity with isopropanol or sec-butanol was detected, and neither retinol nor methanol were substrates for the enzyme (data not shown). These results are similar to those seen with the *E. coli* AdhE enzyme that uses ethanol, 1-propanol, and 1-butanol as a substrate, but does not use methanol or secondary or branched-chain alcohols (unpublished observations). The K_m value obtained for the recombinant EhADH2 enzyme for ethanol (85 mM) is essentially identical to that reported for the native EhADH2 enzyme (80 mM) (11), as were the K_m values for NAD^+ and NADH, whereas the K_m value for acetaldehyde was similar to that reported for native enzyme (0.15 mM) (11). The K_m for the *E. coli* AdhE enzyme for ethanol is 30 mM (unpublished observations). Measurements of ALDH activity confirmed the identity in substrate specificity between the recombinant and native EhADH2 enzymes, as K_m values for Ac-CoA and NADH were essentially identical between the recombinant and native enzymes (11).

Table 1. Comparison of the NAD^+-dependent ADH and ALDH activities in the crude lysates of *E. coli* expressing EhADH2 and control strains

E. coli strains	ADH	ALDH
BL21(DE3) with pET/EhADH2	524	90
SHH31(DE3) with pET/EhADH2	417	72
SHH31 with pMON/EhAHD2	82	14
BL21(DE3)	ND	ND
SHH31	ND	ND

Values are represented as milliunit per milligram. A unit of enzyme activity is defined as a micromole of product formed per minute of incubation. ND, not detectable.

Screening for Compounds with anti-EhADH2 Activity Using *E. coli* SHH31 Transformed with pMON/EhADH2. The successful complementation of the Δ*adhE E. coli* strain SHH31 by EhADH2 and the demonstration that the recombinant EhADH2 enzymes substrate specificity appears identical to the native EhADH2 enzyme provided a potential system for the rapid screening of compounds to identify those capable of inhibiting EhADH2. In this protocol, compounds could first be administered to *E. coli* SHH31 expressing EhADH2, and the effect of the compound on both aerobic and anaerobic growth of the bacteria measured. Compounds that specifically inhibit EhADH2 should inhibit anaerobic growth of SHH31/pMON/EhADH2, but should not significantly alter aerobic growth of this strain. Compounds with inhibitory activity on anaerobic bacterial growth could then be screened for their effects on amebic growth and for their ability to inhibit the recombinant EhADH2 enzyme. To determine whether such a screening system was feasible, we performed a pilot study using the compound pyrazole, which is known to be a potent inhibitor of NAD^+-dependent alcohol dehydrogenases (23). As shown in Fig. 4, pyrazole in a dose-dependent manner significantly inhibited the anaerobic growth of SHH31/pMON/EhADH2, but had a much reduced effect on SHH31/pMON growing under aerobic conditions. We next examined whether pyrazole could inhibit the growth of *Ent. histolytica* trophozoites. As shown in Fig. 5, at a concentration of 20 to 40 mM, pyrazole significantly inhibited amebic growth. Finally, the K_i of pyrazole for the recombinant EhADH2 molecule was measured and found to be 7.24 mM.

DISCUSSION

The EhADH2 molecule is a bifunctional NAD^+/Fe^{2+}-dependent enzyme with both ADH and ALDH activities (10,

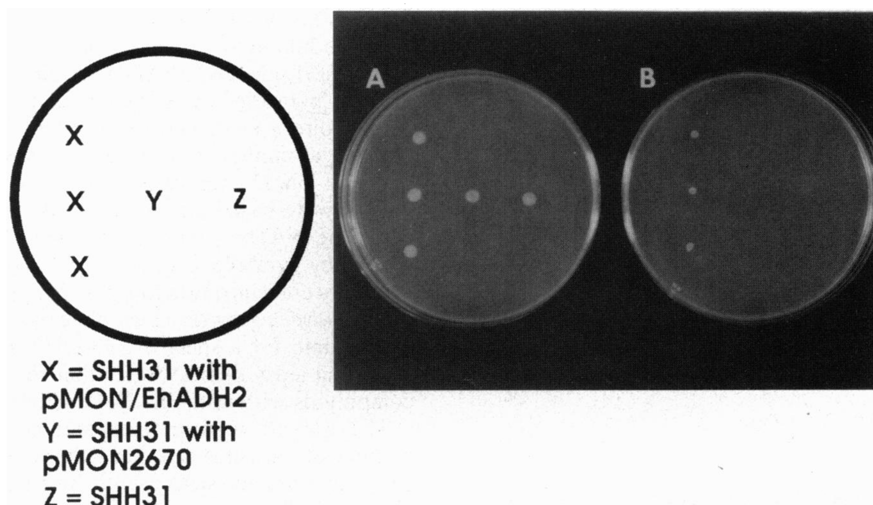

FIG. 2. Complementation of *E. coli* Δ*adhE* strain SHH31 by expression of EhADH2. (*A*) Under aerobic conditions, all *E. coli* strains grow. (*B*) Under anaerobic conditions, SHH31 expressing EhADH2 (SHH31/pMON/EhADH2) can grow (colonies indicated by "X"), but SHH31 transformed with the pMON 2670 vector alone (colonies marked by "Y") or untransformed SHH31 (colonies marked "Z") show no growth.

11). It appears to be a critical enzyme in the amebic glucose to ethanol pathway, catalyzing two reactions in fermentation: (*i*) the conversion of Ac-CoA to acetaldehyde (rather than pyruvate to acetaldehyde as is seen in most organisms) and (*ii*) the conversion of acetaldehyde to ethanol (8, 9). The EhADH2 molecule is homologous to certain enzymes present in facultatively or obligate anaerobic bacteria (12–14). The best studied of these enzymes is the *E. coli* AdhE molecule, an NAD^+-dependent enzyme that also uses Fe^{2+} as a cofactor, and possesses ADH, ALDH, and pyruvate-formate-lyase deactivase activities (12, 21). The AdhE enzyme is required for anaerobic growth of *E. coli* and expression of this gene is induced by anaerobic conditions (24). In addition to its critical role in the amebic fermentation pathway, the EhADH2 molecule may serve other functions in *Ent. histolytica* as well. The EhADH2 protein was originally isolated because of its ability to bind extracellular matrix proteins such as laminin and fibronectin (10), and it has been recently shown that the EhADH2 molecule, or an isoform, is shed or secreted by amebic trophozoites (25).

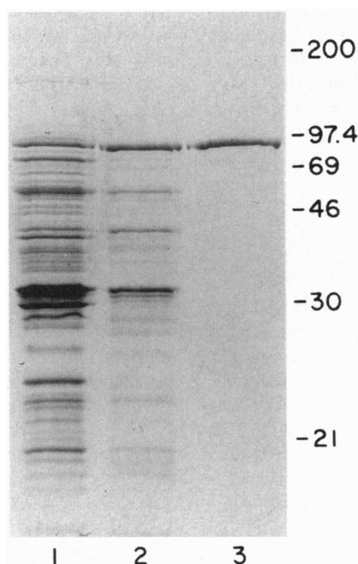

FIG. 3. Purification of recombinant EhADH2. Coomassie blue staining of SDS/PAGE separated samples of: lane 1, lysate of SHH31(DE3)/pET/EhADH2; lane 2, 35% ammonium sulfate precipitate fraction of lysates from lane 1; lane 3, fraction containing EhADH2 obtained from the gel filtration of ammonium sulfate precipitated lysates (lane 2) on a column of Sepharose CL-6B.

Here we have reported the successful expression of a functional EhADH2 molecule in *E. coli*. Our initial approach was to express EhADH2 as either a GST- or 6His-fusion protein. However, in both cases while a fusion protein was successfully expressed, it had no enzymatic activity. This contrasts with the findings for the $NADP^+$-dependent ADH of *Ent. histolytica* (EhADH1), which retained ADH activity as a GST-fusion protein (26). This difference may reflect a requirement for multimer formation for EhADH2 activity that could not be achieved by fusion proteins. Both the native EhADH2 enzyme and the homologous *E. coli* AdhE enzyme form multimers that array into helical structures of up to 100 nm (when viewed by electron microscopy) called spirosomes (11, 21). The functional recombinant EhADH2 enzyme we produced in *E. coli* had a molecular mass of greater than 200 kDa by gel filtration, consistent with multimer formation. We were able to use the purified recombinant enzyme to look at the substrate specificity of EhADH2. The K_m values for ethanol, NAD^+, NADH, acetaldehyde, and Ac-CoA were comparable with those obtained for the native amebic enzyme (11). We were able to demonstrate that in addition to ethanol, the primary alcohols butanol and propanol are substrates for EhADH2, but methanol, retinol, isopropanol, and sec-butanol are not. The substrate specificity of the ADH portion of EhADH2 clearly differs from the $NADP^+$-dependent EhADH1 that preferentially uses branched-chain alcohols (26).

Table 2. Enzyme activities and K_m values of the purified recombinant EhADH2

Reactions	K_{cat}, mol substrate per mol enzyme per min	K_m, mM
Acetaldehyde + NADH	854	
Acetaldehyde		0.23
NADH		0.28
Ac-CoA + NADH	154	
Ac-CoA		0.04
NADH		0.17
Ethanol + NAD^+	461	
Ethanol		85
NAD^+		0.55
1-Propanol + NAD^+	326	
1-Propanol		40
NAD^+		0.25

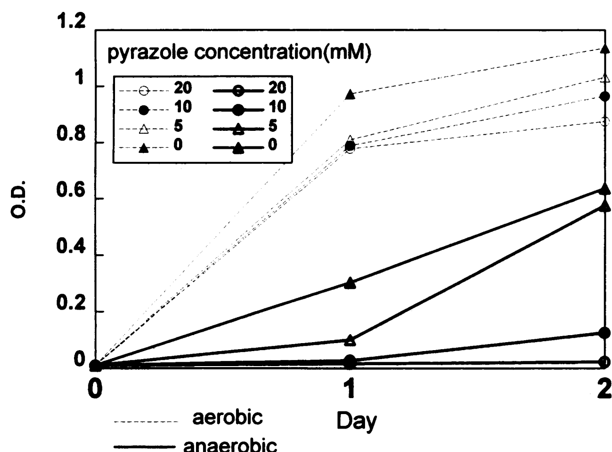

FIG. 4. Inhibition of the anaerobic growth of Δ*adhE* mutant *E. coli* complemented with EhAHD2 by pyrazole. SHH31/pMON/EhADH2 was inoculated on the M9 minimal liquid media containing pyrazole at the indicated concentrations, and incubated aerobically or anaerobically for 2 days. O.D. at 600 nm were read at 1 and 2 days postinoculation to assess the growth of the bacteria.

No structural homologue to the full-length EhADH2 has yet been found among eukaryotic ADH or ALDH enzymes, although there are eukaryotic ALDH enzymes with some homology to the N-terminal (ALDH) domains of EhADH2 and related prokaryotic molecules (13). The unique structure of the EhADH2 molecule among eukaryotic ADH molecules and its critical role in the amebic fermentation pathway would appear to make it an ideal target for antiamebic chemotherapy. However, the cost of growing *Ent. histolytica* in culture, and the cumbersome methods for measuring growth inhibition (counting viable trophozoites), make large-scale screening of compounds for antiamebic activity difficult. As an approach to this problem, we developed a screening system for compounds with anti-EhADH2 activity that uses inhibition of anaerobic bacterial growth (easily quantitated by measuring the O.D. of liquid bacterial cultures) to identify effective compounds. We produced an *E. coli* strain that requires EhADH2 activity to grow under anaerobic conditions by using the EhADH2 gene to complement a mutant strain of *E. coli* containing an engineered deletion in the *adhE* gene. Compounds capable of inhibiting anaerobic, but not aerobic, growth of this strain are potential specific inhibitors of EhADH2 activity. The feasibility of this approach was tested using the compound pyrazole, which is known to inhibit NAD+-dependent ADH enzymes. Pyrazole inhibited anaerobic but not aerobic growth of the *E. coli* SHH31/pMON/EhADH2 strain, and consistent with this finding, pyrazole was shown to inhibit both *Ent. histolytica* trophozoite growth and the purified recombinant EhADH2 enzyme at similar concentrations, suggesting the effects of pyrazole on *E. coli* anaerobic growth and *Ent. histolytica* growth were based on inhibition of EhADH2. In this regard, while the $NADP^+$-dependent EhADH1 molecule is also inhibited by pyrazole (26), the K_i for pyrazole and EhADH1 is 1.4 μM, a concentration range where pyrazole had no effect on *Ent. histolytica* growth. Thus, while pyrazole does not represent a candidate for a specific EhADH2 inhibitor, its use in this screening assay demonstrates that this approach can identify compounds with anti-EhADH2 activity.

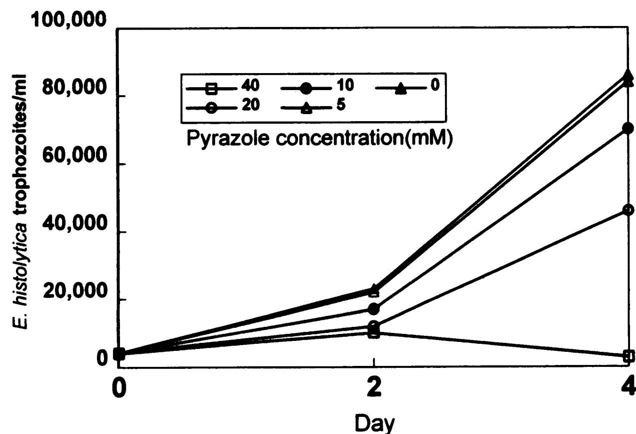

FIG. 5. Pyrazole inhibits *Ent. histolytica* growth. Culture tubes containing *Ent. histolytica* HM1:IMSS trophozoites with an inoculation dose of 4×10^3 per tube were incubated for 4 days with pyrazole in concentrations ranging from 5 to 40 mM. The number of viable amebic trophozoites at 2 and 4 days postinoculation is indicated.

The growth requirements and complex life cycles of a number of parasites can make the identification of new antiparasitic drugs and susceptibility testing of existing compounds difficult and costly endeavors. In addition, genetic systems that allow targeted mutations are poorly developed or nonexistent for a number of protozoan and helminthic parasites. Here, we have taken advantage of the presence of homologous genes in *E. coli* and the parasite *Ent. histolytica* that encode an enzyme required for a selectable function (the ability to grow anaerobically), the ability to generate bacteria with mutations of that gene, and the ability to complement that mutation with the parasitic gene, to devise a method for rapidly identifying specific inhibitors of the parasitic enzyme. While it remains to be determined how widely applicable this strategy will be, using bacteria to bypass the need for parasite culture in the initial screening process for antiparasitic agents could greatly simplify and reduce the cost of identifying new therapeutic agents effective against parasitic diseases.

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