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Giardia, *Entamoeba*, and *Trichomonas* Enzymes Activate Metronidazole (Nitroreductases) and Inactivate Metronidazole (Nitroimidazole Reductases)▽†

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Infections with *Giardia lamblia*, *Entamoeba histolytica*, and *Trichomonas vaginalis*, which cause diarrhea, dysentery, and vaginitis, respectively, are each treated with metronidazole. Here we show that *Giardia*, *Entamoeba*, and *Trichomonas* have oxygen-insensitive nitroreductase (*ntr*) genes which are homologous to those genes that have nonsense mutations in metronidazole-resistant *Helicobacter pylori* isolates. *Entamoeba* and *Trichomonas* also have *nim* genes which are homologous to those genes expressed in metronidazole-resistant *Bacteroides fragilis* isolates. Recombinant *Giardia*, *Entamoeba*, and *Trichomonas* nitroreductases used NADH rather than the NADPH used by *Helicobacter*, and two recombinant *Entamoeba* nitroreductases increased the metronidazole sensitivity of transformed *Escherichia coli* strains. Conversely, the recombinant nitroimidazole reductases (NIMs) of *Entamoeba* and *Trichmonas* conferred very strong metronidazole resistance to transformed bacteria. The Eh*ntr1* gene of the genome project HM-1:IMSS strain of *Entamoeba histolytica* had a nonsense mutation, and the same nonsense mutation was present in 3 of 22 clinical isolates of *Entamoeba*. While *ntr* and *nim* mRNAs were variably expressed by cultured *Entamoeba* and *Trichomonas* isolates, there was no relationship to metronidazole sensitivity. We conclude that microaerophilic protists have bacterium-like enzymes capable of activating metronidazole (nitroreductases) and inactivating metronidazole (NIMs). While *Entamoeba* and *Trichomonas* displayed some of the changes (nonsense mutations and gene overexpression) associated with metronidazole resistance in bacteria, these changes did not confer metronidazole resistance to the microaerophilic protists examined here.

Microaerophilic protists that are important human pathogens include *Giardia lamblia*, *Entamoeba histolytica*, and *Trichomonas vaginalis*, which cause diarrhea, dysentery, and vaginitis, respectively (1, 15, 34). Metronidazole is a nitroimidazole which was originally developed to treat *Trichomonas* infections but which has subsequently also become a mainstay for the treatment of infections caused by *Entamoeba*, *Giardia*, and anaerobic bacteria (17, 33, 38). Metronidazole damages the DNA in target cells when its nitro group is reduced by one electron to form a highly reactive and toxic radical anion (13). In bacteria, metronidazole is reduced and activated by enzymes called nitroreductases, which may be oxygen sensitive if they contain an N-terminal ferredoxin domain (2, 14, 24, 27).

Metronidazole resistance in *Helicobacter pylori*, which is an important cause of gastritis and gastric cancer, is frequently based upon nonsense mutations (premature stop codons) in oxygen-insensitive NADPH-nitroreductase (the *rdxA* gene product) and/or NADH-flavin oxidoreductase (the *frxA* gene product) (2, 14). Metronidazole resistance is widespread among *Helicobacter* strains in developing countries, such as India.

Strong metronidazole resistance in some strains of *Bacteroides* occurs by the overexpression of *nim* genes, which may be located on chromosomes or plasmids (36). A structural study of NimA from *Deinococcus* showed that metronidazole, in a reaction catalyzed by pyruvate, undergoes a two-electron reduction to form a nitroso group that is eventually reduced to an amine, which is not toxic (18).

Microaerophilic protists, which include *Giardia*, *Entamoeba*, and *Trichomonas*, are sensitive to metronidazole because they share metabolic properties with anaerobic bacteria (25, 29, 33, 38). Each of these protists, which live under anaerobic conditions in the lumen of the bowel or the vagina, is secondarily amitochondriate and lacks the enzymes for oxidative phosphorylation (5, 30, 37). Protist genes encoding fermentation enzymes appear to have been obtained by lateral gene transfer (LGT) from diverse bacteria (6, 8, 21, 22, 31). In *Entamoeba* and *Giardia*, these fermentation enzymes are present in the cytosol. In contrast, in *Trichomonas*, some of these enzymes are present in the hydrogenosome, a modified mitochondrion named for its production of hydrogen (4, 5, 26).

Metronidazole resistance is a major problem in clinical isolates of *Trichomonas* in the United States and elsewhere, while metronidazole-resistant *Entamoeba* and *Giardia* have for the most been prepared by selection in culture (9, 12, 17, 19, 20, 23, 32, 38, 39). Recently, a ferredoxin-nitroreductase fusion protein of *Giardia* (called GlNR1) which resembles oxygen-sensitive nitroreductases of bacteria and which appears to have been obtained by LGT was shown to have nitroreductase ac-

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tivity when it was expressed as a recombinant protein in bacteria (24, 27).

The goal of the present study was to determine how well two bacterial models for metronidazole resistance (nonsense mutations in *Helicobacter* genes encoding oxygen-insensitive nitroreductases that activate metronidazole and the overexpression of *Bacteroides nim* genes encoding enzymes that inactivate metronidazole) apply to microaerophilic protists. The specific questions asked in the present study included the following. Do *Entamoeba*, *Trichomonas*, and *Giardia* have homologs of bacterial nitroreductases and nitroimidazole reductases (NIMs)? Do recombinant protist nitroreductases and NIMs have the expected enzyme activities? Are there nonsense mutations in these protist genes like those that have been described for the *ntr* genes of metronidazole-resistant *Helicobacter* strains? Are any of the *nim* genes overexpressed in metronidazole-resistant protists, as has been described for the *nim* genes of metronidazole-resistant *Bacteroides* strains? How do nonsense mutations and/or the overexpression of the *ntr* and the *nim* genes relate to the metronidazole sensitivities of axenized *Trichomonas* and *Entamoeba*?

MATERIALS AND METHODS

Parasites examined. Genome project strains of *Giardia lamblia* (strain WB), *Entamoeba histolytica* (strain HM-1:IMSS), and *Trichomonas* (strain G3) were all grown in axenic culture by standard conditions. For the testing of metronidazole sensitivity and for the determination of *ntr* and *nim* gene expression, we grew two other model strains of *Entamoeba* (strains 200:NIH and Rahman) and *Trichomonas* (strains B2RC7 and S1). To test for polymorphisms in the *ntr* and *nim* genes, we obtained the DNA of 18 *Entamoeba* clinical isolates from Egbert Tannich of the Bernard Nochte Institute for Tropical Medicine in Hamburg, Germany. We obtained the DNA of four *Entamoeba* clinical isolates from William Petri of the University of Virginia. We obtained the DNA of six *Trichomonas* clinical isolates from Evan Secor of the Centers for Disease Control and Prevention.

Bioinformatic methods. Searches of the NCBI GiardiaDB database or databases managed by The J. Craig Venter Institute for sequences that matched the predicted proteins of *Giardia*, *Entamoeba*, and *Trichomonas*, which were derived by whole-genome sequencing, were done with the BLASTP program and the *Giardia* ferredoxin-nitroreductase and *Bacteroides fragilis* NimA (3, 6, 21, 22, 27).

Recombinant expression of protist nitroreductases and NIMs. Two *Entamoeba histolytica* nitroreductase genes (Eh*ntr1* and Eh*ntr2*) were amplified from the genomic DNA of strains 200:NIH and HM-1:IMSS, respectively (see Table S1 in the supplemental material for all primers used). A third *Entamoeba histolytica* nitroreductase gene (Eh*ntr3*) was amplified from the cDNA of HM-1:IMSS with gene-specific primers, as the gene contains an intron. Nitroimidazole resistance genes (*nim*) from *Entamoeba histolytica* (Eh*nim*) and *Trichomonas vaginalis* (Tv*nim*) were amplified with gene-specific primers from genomic DNAs. The BamHI and XhoI restriction sites were introduced into the sense and antisense primers, respectively. The amplicons were cloned into the expression vector pQE30 (Qiagen), which produces a recombinant protein with an N-terminal His tag. The protein was expressed in *Escherichia coli* strain M15 (*lacI* Kan^r on pREP4 F$^-$ *recA*$^+$ *uvr*$^+$ *lon*$^+$ *lac*) with pREP4. Isopropyl-β-D-thiogalactopyranoside (IPTG)-induced soluble recombinant protein was purified on a nickel-nitrilotriacetic acid affinity column and was eluted with 200 mM imidazole. To recover the proteins in the pellet (EhNTR3), the inclusion body was dissolved in 8 M urea and the mixture was briefly centrifuged, and then the supernatant was loaded onto the column. Slow renaturation of the protein was done with a gradient of urea (6 M, 4 M, 2 M, and 1 M), and finally, the protein was eluted with 200 mM imidazole.

The single nitroreductase gene from *Giardia* (Gl*ntr1*) and 1 of 11 nitroreductase genes of *Trichomonas* (Tv*ntr8*) were amplified from genomic DNAs with specific primers. Amplified *Giardia* and *Trichomonas* nitroreductase genes were cloned into the pGEX6P vector (Amersham Biosciences) at the EcoRI-XhoI and BamHI-XhoI sites, respectively (35). *E. coli* strain BL21 was used for the overexpression of recombinant proteins, which were allowed to bind to glutathione-conjugated beads overnight. The bound protein was eluted with 15 mM reduced glutathione and was dialyzed against phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride to remove the glutathione. The purified protein was immediately used for the enzyme assays or was stored at −20°C in 10% glycerol.

Nitroreductase and NIM enzyme assays. The nitroreductase assay was performed under the conditions described previously (14). The reaction mixture contained Tris-acetate (100 mM Tris-HCl, 50 mM acetate buffer, pH 7.0), 50 μM metronidazole, 0.3 mM NADPH or NADH, and enzyme in a 1-ml reaction volume. The assay was carried out at 25°C in a UV-visible spectrophotometer (Lambda 25; Perkin-Elmer) in quartz cuvettes with a 1-cm path length. The results of the assay were determined by measurement of the oxidation of NADPH or NADH at 340 nm (E = 6.22 mM^{-1} cm^{-1}) or by determination of the reduction of metronidazole at 320 nm (E = 9.2 mM^{-1} cm^{-1}). The NADH concentration was varied while the concentration of metronidazole (50 μM) was kept constant. Alternatively, the metronidazole concentration was varied while the NADH concentration (0.3 mM) was kept constant. The initial velocity of the purified enzyme was measured by determining the amount of change in the substrate concentration at 10-s intervals. The Michaelis-Menten constant (K_m) and the maximal velocity (V_{max}) were determined by using a Lineweaver-Burk double-reciprocal plot. All the experiments were performed in duplicate or triplicate with a minimum of four substrate concentrations. Enzymatic specific activities are reported as micromoles per minute per milligram of protein.

The assay methods used for the NIMs, which reduce metronidazole in the presence of NADH or NADPH, were similar to those used for the nitroreductases. *E. coli* cells harboring the empty pQE30 vector exhibited no significant amounts of enzyme activity.

Tests of metronidazole sensitivity of *E. coli* expressing recombinant nitroreductases and NIMs. The sensitivity of transformed *E. coli* cells expressing protist NIMs to metronidazole was determined by a modified broth dilution procedure. The Eh*nim* and Tv*nim* genes were cloned into the pQE30 vector and expressed in *E. coli* strain JM109, which is relatively sensitive to metronidazole. IPTG-treated *E. coli* cells were incubated at 37°C for 16 h in the presence of metronidazole at concentrations ranging from 0 μg/ml to 2 mg/ml. The bacterial growth was measured in a UV-visible spectrophotometer (DU 500; Beckman) at 600 nm. The experiment was performed in duplicate and was repeated at least three times.

Similar methods were used to determine whether the expression of *Entamoeba* NTR1 or NTR2 increases the sensitivity of transformed *E. coli* cells to metronidazole. The Eh*ntr1* and Eh*ntr2* genes were cloned into the pQE30 vector and were expressed in *E. coli* strain JM109. Bacterial growth in the presence of metronidazole was measured in liquid culture.

Identification of introns and nonsense mutations in *ntr* and *nim* genes of *Entamoeba* and *Trichomonas*. Genome project strains of *Entamoeba* (strain HM-1:IMSS) and *Trichomonas* (strain G3) have predicted introns and/or in-frame nonsense mutations in some of the *ntr* and *nim* genes (6, 21). We confirmed that these strains had the introns and stop codons, as well as those identified in other isolates, in three different ways. First, to verify the presence of introns, we performed PCR and reverse transcription-PCR (RT-PCR) with genomic DNA and total RNA, respectively, and we compared the sizes of the products by agarose gel electrophoresis. Second, the amplified products from PCR and RT-PCR were cloned and sequenced. Third, Western blots were performed with total protein, which was isolated from exponentially growing *Entamoeba* (strains HM-1:IMSS, 200:NIH, and Rahman) and separated in a 4 to 20% precast sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad). The proteins were transferred onto polyvinylidene difluoride membranes, and affinity-purified rabbit antibodies to recombinant EhNTR1 were used for hybridization. Bound antibody was detected with a chemiluminescence kit (Pierce).

Tests of metronidazole sensitivity of cultured *Entamoeba* and *Trichomonas*. Trophozoites (10,000 per ml) were grown in TYI-S-33 (tryptone-yeast extract) (*Entamoeba*) or TYM (tryptone-yeast extract-maltose) (*Trichomonas*) medium, each of which was supplemented with 10% heat-inactivated fetal bovine serum, at 37°C in the presence of various concentrations of metronidazole dissolved in 100% dimethyl sulfoxide. After 48 h, the number of viable parasites was counted with a hemocytometer and by the use of trypan blue to identify dead organisms. All experiments were run twice in triplicate with protists treated with dimethyl sulfoxide only (controls).

Real-time PCR for measurement of *ntr* and *nim* gene expression by cultured *Entamoeba* and *Trichomonas*. Total RNA was isolated from mid-log-phase *Entamoeba* and *Trichomonas* with the Trizol reagent (Invitrogen), and the RNA was treated with a DNA-free reagent (Ambion), according to the manufacturer's instructions. The RNA was reverse transcribed with a RETROscript kit (Ambion) by using oligo(dT)$_{18}$. Real-time PCR was carried out by the SYBR green method in a 96-well plate format with a Stratagene MX4000 cycler. A typical reaction mixture contained 12.5 μl of twice-concentrated Brilliant SYBR green QPCR master mix (Stratagene), primers (100 nM), and template cDNA in a total volume of 25 μl. The thermal profile for amplification was 95°C for 10 min,

TABLE 1. Kinetic properties of recombinant nitroreductases of *Entamoeba*, *Giardia*, and *Trichomonas*

Protein	Substrate	K_m (nM)	V_{max} (μmol min^{-1})	V_{max}/K_m
EhNTR1	NADH	57	0.068	1.19
	Metronidazole	67	0.027	0.40
EhNTR2	NADH	62	0.084	1.35
	Metronidazole	81	0.031	0.38
EhNTR3	NADH	66	0.033	0.50
	Metronidazole	83	0.014	0.17
GlNTR	NADH	24	1.698	70.75
	NADPH	19	2.222	116.9
	Metronidazole	206	0.347	1.68
TvNTR8	NADH	16	1.661	106.5
	Metronidazole	78	0.329	4.21

followed by 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s. The primers used for real-time PCR were designed (Table 1) by using OligoPerfect Designer software (Invitrogen). Gel electrophoresis was carried out with representative samples to confirm the product size. The relative quantities of the mRNA species were determined with MX4000 software (version 4.20; Stratagene) by using the *Entamoeba* or *Trichomonas* actin gene as a calibrator.

Nucleotide sequence accession number. The nucleotide sequence of the Eh*ntr1* gene of the HK9 strain of *Entamoeba histolytica* has been submitted to the GenBank database and can be found under accession number ABE99820.

RESULTS AND DISCUSSION

***Giardia*, *Entamoeba*, and *Trichomonas* genomes predict different sets of enzymes which might activate (nitroreductases) or inactivate (NIMs) metronidazole.** Genes encoding putative oxygen-insensitive nitroreductases, which lack an N-terminal ferredoxin domain, were present in a single copy in *Giardia* (Gl*ntr1*), 3 copies in *Entamoeba* (Eh*ntr1* to Eh*ntr3*), and 11 copies in *Trichomonas* (Tv*ntr1* to Tv*ntr11*) (see Table S1 in the supplemental material).

Bacteroides fragilis NimA, which confers metronidazole resistance, was used to identify a single putative *nim* gene from *Entamoeba* (Eh*nim1*) and three putative *nim* genes from *Trichomonas* (Tv*nim1* to Tv*nim3*) (see Table S1 in the supplemental material). *Nim* genes appeared to be absent from *Giardia*.

Putative oxygen-sensitive nitroreductase (*fdntr*) genes, which have a ferredoxin domain at the N termini of the predicted enzymes, were present in two copies in *Giardia* and one copy in *Entamoeba* (24, 27). In contrast, *fdntr* genes appeared to be absent from the genome of *Trichomonas*. These results show that each microaerophilic protist has different combinations of nitroreductases and NIMs.

Phylogenetic reconstructions of protist and bacterial *ntr* and *nim* genes strongly suggested that *Entamoeba* and *Giardia* received their *ntr* genes from two different bacteria, while *Entamoeba* and *Trichomonas* received their *nim* genes from two different bacteria (see Fig. S1 and S2 in the supplemental material).

Recombinant nitroreductases of *Giardia*, *Entamoeba*, and *Trichomonas* reduce metronidazole. All the nitroreductases (i.e., *Giardia lamblia* NTR1 [GlNTR1]; *Entamoeba histolytica* NTR1, NTR2, and NTR3 [EhNTR1, EhNTR2, and EhNTR3, respectively]; and *Trichomonas vaginalis* NTR8 [TvNTR8]) which were expressed as recombinant enzymes in the cytosol of *E. coli* reduced metronidazole (Table 1). While the *Entamoeba* and *Trichomonas* nitroreductases used NADH as the electron donor, the *Giardia* nitroreductase used either NADH or NADPH. The K_ms for NADH and metronidazole were similar for the nitroreductases examined, but the activities of the recombinant *Giardia* and *Trichomonas* nitroreductases were much greater than those of the *Entamoeba* nitroreductases.

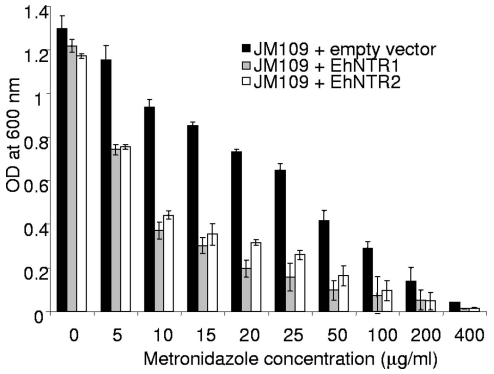

FIG. 1. Recombinant expression of *Entamoeba* nitroreductases increased the sensitivity of transformed bacteria to metronidazole. *E. coli* JM109 was transformed with either the Eh*ntr1* gene, the Eh*ntr2* gene, or an empty PQE30 vector. The expression of each recombinant *Entamoeba* nitroreductase was induced with IPTG, and the bacteria were grown in the presence of serial dilutions of metronidazole for 16 h, at which time the optical density (OD) at 600 nm was measured.

Two *Entamoeba* nitroreductases increased the metronidazole sensitivity of transformed strain JM109 of *E. coli* by greater than threefold (Fig. 1). JM109 transformed with an empty vector had a 50% effective concentration (EC_{50}) slightly greater than 25 μg/ml metronidazole, while JM109 transformed with EhNTR1 and JM109 transformed with EhNTR2 had EC_{50}s of 7 to 8 μg/ml metronidazole.

***Entamoeba* and *Trichomonas* NIMs confer metronidazole resistance to *E. coli*.** *E. coli* JM109 cells transformed with either

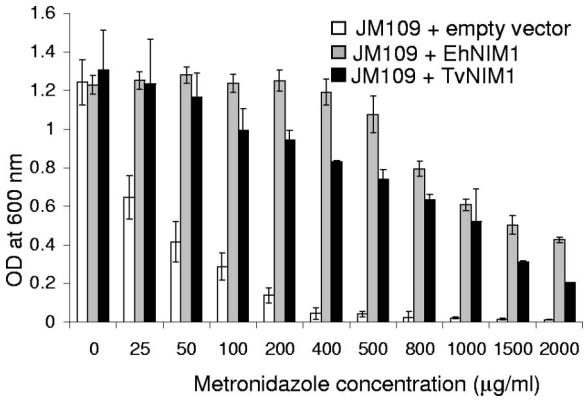

FIG. 2. Recombinant expression of *Entamoeba* and *Trichomonas* NIMs conferred metronidazole resistance to transformed bacteria. *E. coli* JM109 was transformed with either the Eh*nim1* gene, the Tv*nim1* gene, or an empty PQE30 vector. The expression of either the recombinant *Entamoeba* or the *Trichomonas* NIMs was induced with IPTG, and the bacteria were grown in the presence of serial dilutions of metronidazole for 16 h, at which time the optical density (OD) at 600 nm was measured.

TABLE 2. Kinetic properties of a recombinant EhNIM1 of *Entamoeba*

Substrate	K_m (nM)	V_{max} (μmol min^{-1})	V_{max}/K_m
NADH	57	0.042	0.736
NADPH	96	0.033	0.34
Metronidazole	8	0.057	6.25

the *Entamoeba* or the *Trichomonas nim* gene were 30 and 20 times more resistant to metronidazole, respectively, than *E. coli* cells transformed with an empty vector (Fig. 2). JM109 transformed with an empty vector had an EC_{50} of ~25 μg/ml metronidazole, while JM109 transformed with EhNIM1 and JM109 transformed with TvNIM1 had EC_{50}s of 750 and 500 μg/ml metronidazole, respectively. These results show that EhNIM1 and TvNIM1 confer strong metronidazole resistance to transformed *E. coli* cells.

The kinetics of the reduction of metronidazole by *Entamoeba* NIM1 showed that either NADPH or NADH may be an electron donor (Table 2).

An *Entamoeba* nitroreductase gene (Eh*ntr1*) has a nonsense mutation in the genome project strain and some clinical isolates. One of the goals of the present study was to determine whether there are any nonsense mutations in protist *ntr* genes, as have been shown for the *ntr* genes of metronidazole-resistant *Helicobacter* (2, 14). No nonsense mutations or introns were identified in the 11 *ntr* genes of genome project strain G3 of *Trichomonas* or the single *ntr* gene of genome project strain WB of *Giardia*. In contrast, the Eh*ntr1* gene of genome project strain HM-1:IMSS of *Entamoeba*, which was previously predicted to have a zero-frame 81-bp intron, appeared to have a nonsense mutation for the following reasons (Fig. 3).

First, the Eh*ntr1* genes from two other axenized strains of *Entamoeba* (strains 200:NIH and Rahman) had a single base change compared with the Eh*ntr1* sequence of HM-1:IMSS, so that their Eh*ntr1* genes had an open reading frame that was not interrupted by an intron (Fig. 3A). The wild-type, intron-less Eh*ntr1* gene was also present in the PCR products of 19 of 22 clinical isolates of *Entamoeba*, while 3 clinical isolates had the exact same nonsense mutation in Eh*ntr1* as genome project strain HM-1:IMSS (see Fig. S3 in the supplemental material).

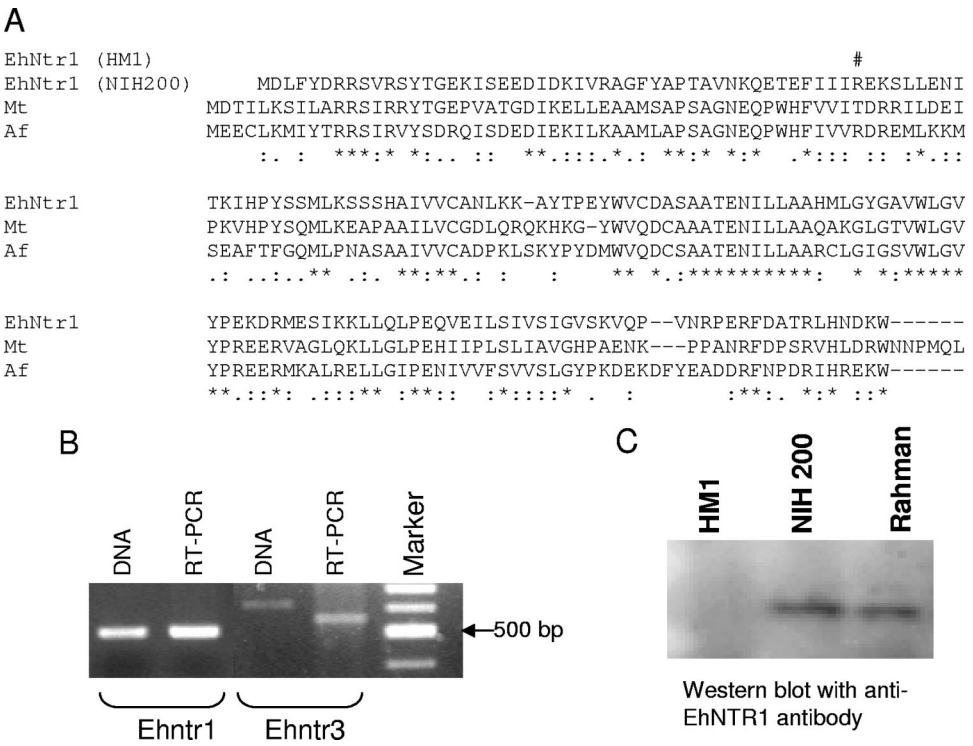

FIG. 3. A nonsense mutation (in-frame stop codon) was present in the Eh*ntr1* gene of genome project strain HM-1:IMSS of *Entamoeba* and three clinical isolates. (A) The nitroreductases of *Entamoeba* strains HM-1:IMSS and 200:NIH, as well as those *Methanothermobacter thermautotrophicus* (Mt) and *Archaeoglobus fulgidus* (Af), were aligned by using the single-letter code. In this alignment, identical amino acids are marked with an asterisk, while similar amino acids are marked with a colon or a period. Within a putative zero-frame 81-bp intron of EhNTR1 of genome project strain HM-1:IMSS, there was a stop codon (#) where there was an Arg (R) in the wild-type EhNTR1 of 200:NIH. The stop codon, which was also present in the Eh*ntr1* genes of 3 of 22 clinical isolates examined (isolates BM1, CM2, and H22), was present in a region that is conserved in bacterial nitroreductases. (B) PCR and RT-PCR with Eh*ntr1* gene primers from HM-1:IMSS DNA and RNA, respectively, produced products of the same size, arguing against the presence of an in-frame intron in the Eh*ntr1* gene. In contrast, the product obtained by RT-PCR with Eh*ntr3* gene primers from HM-1:IMSS RNA was smaller than the product obtained by PCR from DNA, consistent with the presence of an intron in the Eh*ntr3* gene of *Entamoeba*. The sequence of the Eh*ntr3* RT-PCR product confirmed the presence of the intron at the position predicted by the genome project (data not shown). (C) Western blotting with a rabbit polyclonal antibody to a recombinant EhNTR1 protein showed that strains 200:NIH and Rahman, which had the wild-type Eh*ntr1* gene, both expressed the NTR1 protein. In contrast, strain HM-1:IMSS, which had a nonsense mutation in the Eh*ntr1* gene, did not express the NTR1 protein.

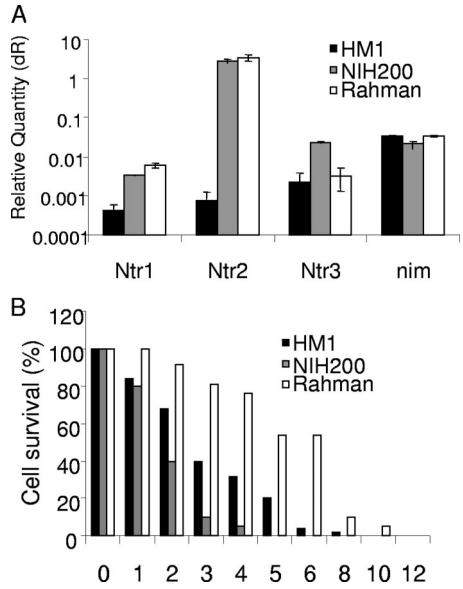

FIG. 4. There was no correlation between the expression of nitroreductase and NIM mRNAs and the sensitivities of three axenized strains of *Entamoeba* to metronidazole. (A) Results of real-time PCR with actin as a calibrator plotted on a log scale showing that the level of expression of nitroreductase mRNAs is markedly decreased in strain HM-1:IMSS compared with the levels of expression in strains 200:NIH and Rahman, while the levels of NIM expression are similar. These results predict that strain HM-1:IMSS would be the least sensitive to metronidazole, while the other two strains would have similar sensitivities to metronidazole. dR, baseline subtracted fluorescent reading. (B) In contrast, strain Rahman was the least sensitive to metronidazole, strain HM-1:IMSS was intermediate in its sensitivity to metronidazole, and strain 200:NIH was the most sensitive to metronidazole.

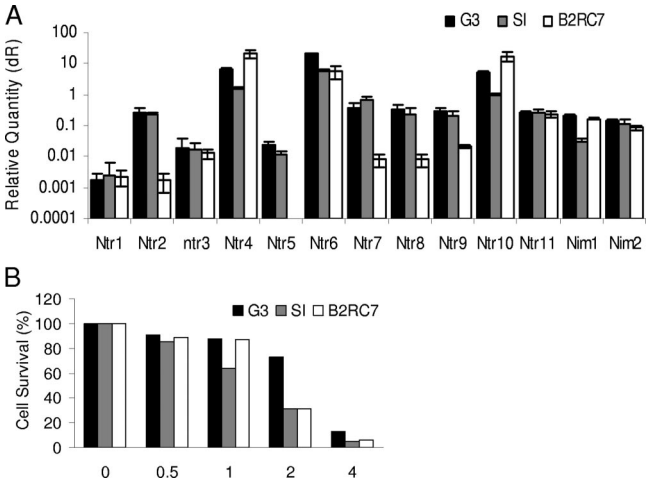

FIG. 5. There was no correlation between the expression of nitroreductase and NIM mRNAs and the sensitivities of three model strains of *Trichomonas* to metronidazole. (A) The levels of expression of nitroreductases, as measured by real-time PCR by using actin as a calibrator, by 11 *Trichomonas* varied by multiple log units. In contrast, they showed similar levels of expression of NIMs. These results were so complex that it was not possible to predict which of the three model strains of *Trichomonas* would be the most sensitive or the most resistant to metronidazole. dR, baseline subtracted fluorescent reading. (B) Indeed, none of these axenized strains of *Trichomonas* were resistant to metronidazole.

An intron-less *ntr1* gene was also predicted from the whole-genome sequence of *Entamoeba dispar*.

Second, the predicted intron in Eh*ntr1* of the genome project strain caused a deletion of residues which are conserved in bacterial nitroreductases (Fig. 3A). Third, RT-PCRs with Eh*ntr1* gene-specific primers from strains HM-1:IMSS, 200:NIH, and Rahman RNAs failed to produce a spliced product (Fig. 3B). In contrast, RT-PCR of the Eh*ntr3* gene showed removal of its predicted intron (Fig. 3B). Fourth, antibodies to recombinant EhNTR1 bound to a 20-kDa protein band in Western blots of proteins from *Entamoeba* strains 200:NIH and Rahman, which had the wild-type Eh*ntr1* gene (Fig. 3C). In contrast, these anti-EhNTR1 antibodies failed to bind to proteins from HM-1:IMSS, which had the nonsense mutation in its Eh*ntr1*gene (Fig. 3C).

To our knowledge, this is the first report of the identification of a polymorphic nonsense mutation in an *Entamoeba* gene, although pseudogenes for *Entamoeba* P glycoproteins and *Entamoeba dispar* cysteine proteinase 5 have been observed (11, 41). We were able to identify just eight other genes with nonsense mutations in genome project strain HM-1:IMSS of *Entamoeba* (our unpublished data).

Trichomonas nim genes included a nonsense mutation in Tv*nim1* in strain S1 and a truncated Tv*nim3* gene in all three strains examined (see Fig. S4 in the supplemental material). We were able to identify nonsense mutations in ~5% of the predicted *Trichomonas* genes (our unpublished data).

Nitroreductase and NIM mRNAs are variably expressed by cultured *Entamoeba* and *Trichomonas*, but there is no relationship to metronidazole sensitivity. The goal of the evaluation of nitroreductase and NIM mRNA expression was to determine whether there is any relationship between either *ntr* or *nim* gene expression by *Entamoeba* and *Trichomonas* or the presence of nonsense mutations in their *ntr* or *nim* genes and the sensitivity of these microaerophilic protists to metronidazole.

Entamoeba genome project strain HM-1:IMSS strain had an Eh*ntr1* gene with a nonsense mutation, and the Eh*ntr2* gene of HM-1:IMSS was expressed at a level 3 orders of magnitude less than the level of Eh*ntr2* expression by strains 200:NIH and Rahman. In addition, the level of expression of the Eh*ntr3* gene of HM-1:IMSS was 1 order of magnitude less than that of the Eh*ntr3* gene of 200:NIH, while all three *Entamoeba* strains examined had similar levels of expression of the Eh*nim1* gene (Fig. 4A; note that the plots of gene expression are on a log scale). These results predicted that strain HM-1:IMSS amebae would be much less sensitive to metronidazole than the other *Entamoeba* strains because HM-1:IMSS has fewer nitroreductases that activate metronidazole. While strain HM-1:IMSS was slightly less sensitive to metronidazole than strain 200:NIH, strain Rahman was the least sensitive to metronidazole (Fig. 4B). These results suggest that there is no relationship between metronidazole sensitivity and Eh*ntr* gene expression and/or the presence of nonsense mutations in the Eh*ntr* genes of the three axenized *Entamoeba* strains examined here.

The levels of expression of the 11 Tv*ntr* genes varied by 4 orders of magnitude among the three *Trichomonas* strains examined here (Fig. 5A; note that the plots of gene expression

are on a log scale). Genome project strain G3 and the S1 strain of *Trichomonas* had similar levels of Tv*ntr* and Tv*nim* gene expression, suggesting that they would have similar sensitivities to metronidazole. In contrast, strain B2RC7 expressed some Tv*ntr* genes at higher levels and some Tv*ntr* genes at lower levels than those by strains G3 and the S1, so it was difficult to predict the metronidazole sensitivity of B2RC7 compared with the sensitivities of the other strains. As it turns out, all three *Trichomonas* strains were relatively sensitive to metronidazole (Fig. 5B). These results suggest that there is no relationship between metronidazole sensitivity and Tv*ntr* gene expression and/or the presence of nonsense mutations in the Tv*ntr* genes of the three axenized *Trichomonas* strains examined here.

Major conclusions and unanswered questions. The results presented here show that microaerophilic protists have different combinations of enzymes which activate metronidazole (nitroreductases and ferredoxin-nitroreductase fusions) or inactivate metronidazole (NIMs) in bacteria (2, 14, 18, 24, 27, 36). While it is likely that these *ntr* and *nim* genes, which are absent from the vast majority of eukaryotes, were obtained from anaerobic bacteria by LGT, the phylogenetic analyses were not conclusive for some of the protist genes (see Fig. S1 and S2 in the supplemental material) (8, 21). In addition, there was no evidence that LGT directly contributes to metronidazole resistance in these protists.

The most important results were that all of the *Giardia*, *Entamoeba*, and *Trichomonas* enzymes examined here activate metronidazole (nitroreductases) or inactivate metronidazole (NIMs) when they are expressed in *E. coli*. These results, as well as the demonstration of nitroimidazole activation by a *Giardia* ferredoxin-nitroreductase fusion enzyme (24), strongly suggest that these enzymes may contribute to metronidazole activation or inactivation in these microaerophilic protists, as has been demonstrated in bacteria (2, 14, 18, 36). A recombinant *Trichomonas* ferredoxin:NADH was also shown to reduce metronidazole, although the kinetics were not determined (16). Similarly, a recent report showed that the knockout of a *Trypanosoma* nitroreductase confers cross-resistance to nifurtimox and benznidazole (40).

The nitroreductases of *Giardia*, *Entamoeba*, *and Trichomonas*, as well as the NIM of *Entamoeba*, which lacked targeting sequences, are likely present in the cytosol. In contrast, *Trichomonas* NIMs, which contained organelle-targeting sequences, are likely present in the hydrogenosome (4). The results for *Trichomonas*, which suggest that metronidazole might be activated by nitroreductases in the cytosol and inactivated by NIMs in the hydrogenosome, are somewhat surprising, because previous reports suggested that metronidazole is activated in the hydrogenosome (7, 16, 17, 28). It is possible, then, that there are multiple locations for metronidazole activation in *Trichomonas* and that there are competing reactions with metronidazole in the hydrogenosome.

As is the case in bacteria (2, 14, 18, 36), there were nonsense mutations in protist nitroreductase and *nim* genes (Eh*ntr1* in strain HM-1:IMSS, Tv*nim1* in strain S1, and Tv*nim3* in all *Trichomonas* strains examined) and marked differences in the mRNA expression of numerous *ntr* and *nim* genes in these microaerophilic protists. However, in contrast to *Helicobacter* and *Bacteroides*, the presence of nonsense mutations in *ntr* genes and the overexpression of *nim* mRNAs did not accurately predict the metronidazole sensitivity of the axenized *Entamoeba* and *Trichomonas*.

While the present studies have focused on the nitroreductases and NIMs of *Giardia*, *Entamoeba*, and *Trichomonas*, previous observations show that the metronidazole sensitivities of these microaerophilic protists may also be determined by (i) the availability of reduced ferredoxin or NAD(P)H to activate or inactivate metronidazole (7, 10, 16, 17, 20, 28) or (ii) the ability of these organisms to quench the reactive species generated by metronidazole activation (19, 23, 32, 39). In *Trichomonas*, low-level, microaerophilic resistance to metronidazole is associated with impaired oxygen scavenging, which results in the reoxidation of metronidazole or the removal of electrons by oxygen (17). In contrast, high-level, anaerobic resistance to metronidazole is associated with marked changes in hydrogenosomal enzymes that metabolize pyruvate (PFOR and hydrogenase) or metabolize malate (malic enzyme and NADH:ferredoxin oxidoreductase) (the so-called alternative pathway) (7, 16, 28).

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