Evidence for Lateral Transfer of Genes Encoding Ferredoxins, Nitroreductases, NADH Oxidase, and Alcohol Dehydrogenase 3 from Anaerobic Prokaryotes to *Giardia lamblia* and *Entamoeba histolytica*

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Giardia lamblia and *Entamoeba histolytica* are amitochondriate, microaerophilic protists which use fermentation enzymes like those of bacteria to survive anaerobic conditions within the intestinal lumen. Genes encoding fermentation enzymes and related electron transport peptides (e.g., ferredoxins) in giardia organisms and amebae are hypothesized to be derived from either an ancient anaerobic eukaryote (amitochondrate fossil hypothesis), a mitochondrial endosymbiont (hydrogen hypothesis), or anaerobic bacteria (lateral transfer hypothesis). The goals here were to complete the molecular characterization of giardial and amebic fermentation enzymes and to determine the origins of the genes encoding them, when possible. A putative giardia [2Fe-2S]ferredoxin which had a hypothetical organelle-targeting sequence at its N terminus showed similarity to mitochondrial ferredoxins and the hydrogenosomal ferredoxin of *Trichomonas vaginalis* (another luminal protist). However, phylogenetic trees were star shaped, with weak bootstrap support, so we were unable to confirm or rule out the endosymbiotic origin of the giardia [2Fe-2S]ferredoxin gene. Putative giardial and amebic 6-kDa ferredoxins, ferredoxin-nitroreductase fusion proteins, and oxygen-insensitive nitroreductases each tentatively supported the lateral transfer hypothesis. Although there were not enough sequences to perform meaningful phylogenetic analyses, the unique common occurrence of these peptides and enzymes in giardia organisms, amebae, and the few anaerobic prokaryotes suggests the possibility of lateral transfer. In contrast, there was more robust phylogenetic evidence for the lateral transfer of *G. lamblia* genes encoding an NADH oxidase from a gram-positive coccus and a microbial group 3 alcohol dehydrogenase from thermo-anaerobic prokaryotes. In further support of lateral transfer, the *G. lamblia* NADH oxidase and *adh3* genes appeared to have an evolutionary history distinct from those of *E. histolytica*.


Giardia lamblia, which is also known as *Giardia intestinalis* or *Giardia duodenalis*, is an amitochondriate, microaerophilic protist that causes intestinal malabsorption and diarrhea (1). *Entamoeba histolytica*, another amitochondriate, microaerophilic protist, causes amebic dysentery and liver abscess (40). Giardia organisms and amebae live under anaerobic conditions in the lumen of the intestines by means of fermentation enzymes which resemble those of some anaerobic prokaryotes and the microaerophilic protist *Trichomonas vaginalis* (a cause of vaginitis) (Fig. 1A) (7, 50, 58, 65). These *G. lamblia* and *E. histolytica* fermentation enzymes are absent from most eukaryotes, including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens*, for which whole-genome sequences are complete or nearly complete (2, 5, 48, 55). For example, pyruvate:ferredoxin oxidoreductase (PFOR), which is one of two keto-acid oxidoreductases in giardia organisms, decarboxylates pyruvate to acetyl coenzyme A (acetyl-CoA), with the production of CO_2 and reduced ferredoxin (35, 69, 72, 88, 89). Under anaerobic conditions, NADH is oxidized to NAD^+ when acetyl-CoA is reduced to

acetaldehyde and then ethanol by alcohol dehydrogenase E (ADHE) (76). Under microaerophilic conditions, NADH is oxidized by an NADH oxidase, while acetyl-CoA is converted to acetate by acetyl-CoA synthase (ACS), with the production of one ATP (8, 78). Although hydrogen gas has not been shown in cultures of axenic giardia organisms and amebae, each protist contains mRNA encoding an Fe hydrogenase, which may catalyze the transfer of electrons from ferredoxin to protons to form hydrogen (Fig. 1B) (10, 36; unpublished observations).

The fermentation pathway is the target of metronidazole, an important drug used to treat infections with giardia organisms, amebae, trichomonads, and anaerobic gram-negative bacteria (Fig. 1B) (28, 90). Metronidazole is a synthetic nitroimidazole which is activated when its nitro group is reduced by electrons from reduced ferredoxin (57). Metronidazole-resistant giardia organisms selected in vitro have decreased levels of PFOR and a 6-kDa ferredoxin (51, 87), while antisense inhibition of PFOR mRNAs causes transfected giardia organisms to become metronidazole resistant (15). Metronidazole-resistant amebae show increased expression of iron-containing superoxide dismutase and peroxiredoxin and decreased expression of ferredoxin 1 and flavin reductase (66, 93). The antigiardia drug furazolidone is a 5-nitrofuran which is also activated when its nitro group is reduced (28). Furazolidone-resistant giardia or-

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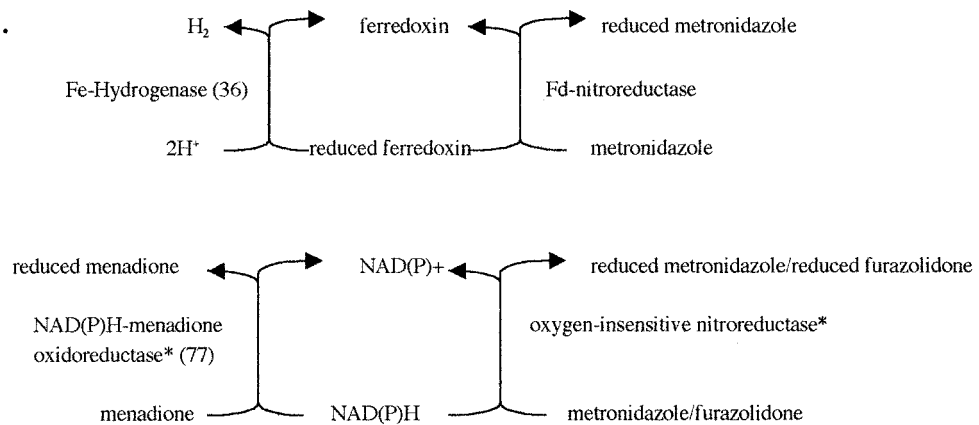
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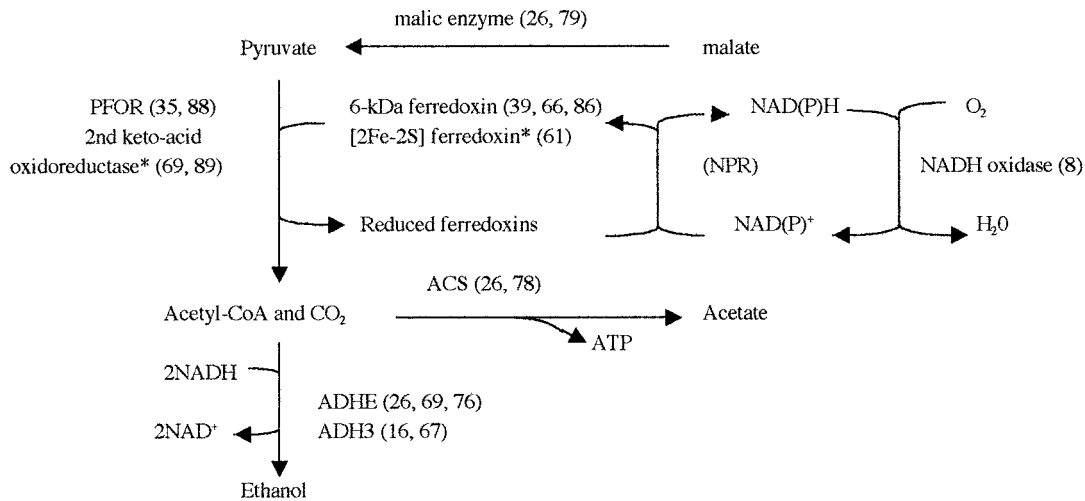
B.

FIG. 1. Metabolic pathways. (A) Fermentation enzymes and other related peptides of *G. lamblia* and *E. histolytica*. Relevant references are shown in parentheses. Proteins identified only in giardia organisms are marked with an asterisk. (B) Other hypothetical electron acceptors for ferredoxin and NADH.

ganisms have also been selected in vitro, but the mechanism of resistance has not been characterized (87). It is not known whether giardia organisms have nitroreductases which enzymatically activate metronidazole and furazolidone, as described for *Helicobacter pylori* and *Escherichia coli*, respectively (30, 41, 46, 94).

There are three possible sources, which are not mutually exclusive, for *G. lamblia* and *E. histolytica* genes encoding fermentation enzymes, electron transport peptides, and assorted oxidoreductases. The amitochondriate fossil hypothesis suggests that the genes encoding these enzymes and peptides of *G. lamblia*, *E. histolytica*, and *T. vaginalis* were present in an ancient anaerobic eukaryotic ancestor which lacked the mitochondrial endosymbiont (7, 12, 65). These genes were then lost from the vast majority of eukaryotes after the acquisition of the mitochondrion. The amitochondriate fossil hypothesis was suggested by phylogenies of rRNA gene sequences which indicated that *G. lamblia* and *T. vaginalis* branched early from

the eukaryotic tree (80). Other amitochondriate protists (e.g., *E. histolytica* and the ciliate *Nyctotherus ovalis*), however, branched in an intermediate position or among the "crown" groups in rRNA phylogenies (24, 80, 91). Moreover, recent studies have suggested the luminal protists are secondarily amitochondriate, as the hydrogenosomes of *T. vaginalis* and *N. ovalis* are likely modified mitochondria (4, 6, 9, 71, 91) and *E. histolytica* contains an atrophic mitochondrion-derived organelle (crypton or mitosome) (13, 29, 52). The phylogeny of *G. lamblia* Hsp60, which lacks an N-terminal organelle-targeting sequence, suggests that its gene is mitochondrion derived (14, 68).

In contrast to the amitochondriate fossil hypothesis, the hydrogen hypothesis suggests that the *G. lamblia*, *E. histolytica*, and *T. vaginalis* fermentation genes may have been acquired with the mitochondrial endosymbiont, itself a facultative anaerobe (31, 53, 71). The hydrogen hypothesis, which provides an elegant biochemical explanation for the symbiosis

between the mitochondrial endosymbiont and a methanogenic archaebacterium, is supported by the presence within *T. vaginalis* hydrogenosomes of multiple fermentation enzymes similar to those of mitochondria (PFOR, [2Fe-2S]ferredoxin, Fe hydrogenase, malic enzyme, and succinyl-CoA synthetase) (10, 35–38, 42, 47, 58).

A third possibility is that the *G. lamblia* and *E. histolytica* genes encoding the fermentation enzymes may have been directly acquired from an anaerobic prokaryote or another microaerophilic eukaryote (lateral transfer hypothesis) (19, 20, 26, 59, 69). Phylogenetic analyses strongly suggest that *G. lamblia* genes encoding ADHE and a second keto-acid oxidoreductase, which resembles PFOR, were laterally transferred from anaerobic eubacteria, while the *G. lamblia* ACS gene was likely transferred from an archaeon (26, 69). *E. histolytica* fermentation genes were apparently transferred from a variety of prokaryotes, including a gram-positive eubacterium (ADHE), a thermoanaerobic, gram-positive eubacterium (alcohol dehydrogenase 1), and an archaeon (malic enzyme and ACS) (26, 45, 69, 95). While lateral transfers from prokaryotes to higher eukaryotes appear to be rare (2, 5, 48), recent whole-genome sequences of dozens of bacteria have shown that hundreds of genes encoding metabolic enzymes and virulence factors are frequently laterally transferred between eubacteria and between archaea and gram-positive and gram-negative eubacteria (20, 21, 49, 60, 62, 63).

With the goals of molecularly characterizing giardial and amebic fermentation enzymes and related peptides and of further exploring the origins of the genes that encode them, we identified here novel *G. lamblia* and *E. histolytica* genes encoding ferredoxins and ferredoxin-nitroreductase fusion proteins. We also identified genes encoding NADH oxidase and a hypothetical oxygen-insensitive nitroreductase (giardia organisms only), which may be involved in drug activation by the protists. We performed phylogenetic analyses on these giardial and amebic fermentation enzymes, as well as on a microbial group 3 alcohol dehydrogenase (ADH3) that had not been vigorously examined.

MATERIALS AND METHODS

Identification of *G. lamblia* and *E. histolytica* genes encoding fermentation enzymes. TBLASTN and an N-terminal sequence from a purified giardial 6-kDa ferredoxin (PIKLTAAKCSAAGACVDACPVSVFELP) (GenBank accession number S42359) were used to examine 50,000 shotgun sequences from the genomic DNA of the WB isolate of *G. lamblia* (3, 54, 86). We estimate that these single-pass reads cover 98% of the coding capacity of the *G. lamblia* genome. Three unique *G. lamblia* ferredoxin genes, as well as two genes that encoded hypothetical ferredoxin-reductase fusion proteins like those predicted from clostridial genomic sequences (11, 30, 62), were identified. The *G. lamblia* shotgun sequences were also searched with the *T. vaginalis* [2Fe-2S]ferredoxin, the *H. pylori* oxygen-insensitive nitroreductase, and the *E. coli* NADH oxidase; these searches yielded one gene encoding each protein (3, 8, 30, 41, 70). Similar methods were used to search 81,000 *E. histolytica* strain HM-1 shotgun sequences which likely cover 95% of the coding capacity of the amebic genome. We identified novel amebic genes encoding a second 2[4Fe-4S]ferredoxin, a nitroreductase, and an NADH oxidase (39). To confirm the identity of the *G. lamblia* and *E. histolytica* fermentation proteins, we searched the conserved domain database at GenBank by using RPS-BLAST, while homologous proteins in GenBank and in the database of unfinished microbial sequences were identified with BLASTP and TBLASTN, respectively (3). Organelle-targeting sequences were sought by using MITOP II (14).

Phylogenetic analyses. Amino acid sequences homologous to the *G. lamblia* and *E. histolytica* fermentation enzymes were aligned by using CLUSTAL W (85). Adjustments to the alignment were done manually, and regions of ambiguous alignment were removed by using the SEQLAB program (Genetics Computer Group, Madison, Wis.). Phylogenetic analyses were performed by using distance and parsimony methods with the computer program TREE-PUZZLE and the PHYLIP package (25, 81). Pairwise distances were computed by using TREE-PUZZLE in the Dayhoff model, with the inclusion of estimated amino acid frequencies, estimated proportions of invariant sites, and estimations of among-site variations for the remaining sites according to a gamma distribution (18). The optimal tree was inferred by using the Fitch-Margoliash algorithm with global rearrangements and 100 random-addition replicates (27). Bootstrap values were obtained by using 100 resampled data sets with the same model as that used for distance and by using the PHYLIP PROTPARS program for parsimony (25).

Nucleotide sequence accession numbers. The sequences of the *G. lamblia* and *E. histolytica* genes encoding NADH oxidase have been deposited in GenBank with the accession numbers AF454832 and AF454833, respectively. The other *G. lamblia* fermentation proteins were encoded by the following shotgun clones (GenBank accession numbers): Fd1 (AC058736), Fd2 (AC047574), Fd3 (AC064773), Fd-NR1 (AC028243), and Fd-NR2 (AC059846). The GenBank accession number for oxygen-insensitive nitroreductase is AC075294. The GenBank accession numbers for the *E. histolytica* shotgun clones Fd2 and Fd-NR1 are AZ529242 and AZ535545 (incomplete open reading frame), respectively. In most instances, there were numerous overlapping shotgun sequences, which were used to check the predicted open reading frames.

RESULTS

A predicted *G. lamblia* [2Fe-2S]ferredoxin is similar to hydrogenosomal and mitochondrial ferredoxins, but the phylogenetic signal has been lost. Ferredoxins with [2Fe-2S] iron-sulfur centers are present in organelles of eukaryotes (mitochondria, chloroplasts, and hydrogenosomes), as well as the cytosols of numerous facultative gram-negative eubacteria (41, 49, 74, 92). The *G. lamblia* gene encoding a putative [2Fe-2S] ferredoxin contained a 35-bp intron, which is the first intron identified to date in giardia organisms (61). In contrast, no [2Fe-2S]ferredoxin gene was identified in *E. histolytica* (3). The predicted *G. lamblia* [2Fe-2S]ferredoxin, which was 133 amino acids long, contained four Cys residues and numerous other amino acids that are conserved in 12-kDa ferredoxins (pFam00111 in the conserved-domain GenBank database) (Fig. 2A) (3, 41, 74). Because the phylogenetic trees of 15 eukaryotic and 17 eubacterial [2Fe-2S]ferredoxins were star shaped and contained few nodes with significant bootstrap support (data not shown), it was not possible to determine whether the *G. lamblia* [2Fe-2S]ferredoxin gene was derived from the mitochondrial endosymbiont (hydrogen hypothesis) or another eubacterium (lateral transfer hypothesis) (26, 53, 69, 71). Interestingly, the predicted *G. lamblia* [2Fe-2S]ferredoxin contained a possible 18-amino-acid organelle-targeting sequence at its N terminus (probability, 0.96) (Fig. 2A) (14). This N-terminal peptide (MSLLSSIRRFITFRVVQQ), which was missing an Arg at −2, was rich in Ser and Leu, as described for proteins targeted to hydrogenosomes of *T. vaginalis* (6, 9, 10, 37, 38, 47).

Identification of eight *G. lamblia* and five *E. histolytica* genes encoding putative peptides containing 2[4Fe-4S] or [4Fe-4S] [3Fe-4S] iron-sulfur centers. Ferredoxins with 2[4Fe-4S] or [4Fe-4S] [3Fe-4S] iron-sulfur centers are present as 6-kDa peptides and as part of longer oxidoreductases (e.g., Fe hydrogenases and PFOR) in luminal protists, archaea, anaerobic gram-positive eubacteria, and some gram-negative eubacteria, which are facultative anaerobes (3, 7, 10, 11, 17, 35, 36, 38, 39, 62, 64, 66, 69, 86, 88, 89). In contrast, these ferredoxins and proteins containing domains like those in ferredoxins are ab-

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Gl                                                                  MSLLSSIRRFITFRVVQQGVEHTVSGAVGQSLLDAIKA.AH
Tv                                                                  MLSQVCRFGTITAVKGGVKKQLKFEDDQTLFTVLTEAGL
Tb                 MLSATLGTLGTSCGRGFLQRQGLLTTFMGRFCHSGGGGNNQNNDSSNSGNDGSSTPGAIRVNVTTAEGEKITFSAPSGLTLMEALRDVAR
Sc                 MLKIVTRAGHTARISNIAAHLLRTSPSLLTRTTTTTRFLPFSTSSFLNHGHLKKPKPGEELKITFILKDGSQKTYEVCEGETILDIAQG.HN
Hs                 MAAAGGARLLRAASAVLGGPAGRWLHHAGSRAGSSGLLRNRGPGGSAEASRSLSVSARARSSSEDKITVHFINRDGETLTTKGKVGDSLLDVVVE.NN
Rp                                                                  MLRKIKVTFIINDEEERTVEAPIGLSILEIAHS.ND
As                                                                  .ATYKVTLINEEEGINAILEVADDQTILDAGEE.AG
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Ferredoxin domain

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Gl    IPIQD..ACEGHLGCGTCGVYLDKKTYKRIPRATKEEAVLLDQVPNPKPTSRLSCAVKLSSMLEGATVRIPSFNKNVLSESDILASEEKKRHGQH*
Tv    MSADD..TCQGNKACGKC...ICKHVSGKVAAAEDDEKEFLEDQP...ANARLACAITLSGENDGAVFEL*
Tb    VDIEA..ACDGTCACSTCHVILREEDFGKLTAASEDEMDMLDLAPXVTPTSRLACQVKLSKELDGITLQMPSETTNEMR*
Sc    LDMEG..ACGGSCACSTCHVIVDPDYYDALPEPEDDENDMLDLAYGLTETSRLGCQIKMSKDIDGIRVALPQMTRNVNNNDFS*
Hs    LDIDGFGACEGTLACSTCHLIFEDHIYEKLDAITDEENDMLDLAYGLTDRSRLGCQICLTKSMDNMTVRVPETVADARQSIDVGKTS*
Rp    LDLEG..ACEGSLACATCHVMLEEEFYNKLKKPTEAEEDMLDLAFGLTDTSRLGCQIILTEELDGIKVRLPSATRNIKL*
As    LDLPS..SCRAG.SCSTC...AGKLVSGAAPN.QDDQ.AFLDDDQ.LAAGWVMTCVAYPTGDCTIMTHQESEVL*
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B

Ferredoxin domains

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Gl1           MPIKLTASKCSA..AGACVDACPVSVFELPQGAKVVLTPNIDACVECGACVSACPEGALEL*
Gl2           MPILLHEEKCDA..CLTCVGTCPVGIISEEKGALKVT..HVEDCTECGDCVVSCHTTALEVDV*
Gl3           MQRIILDVDACTL..CGQCVQACPLSILVIQNNKISLR..DPHKCFQCLQCMQICPEDCFSFPPVYEIDTDTQNKS*
Eh1           MGKITIVNIDDCVA..CGACSGTCPQSVLEVNDHVEIKN...PDDCIGCGACVDACPQGVLKN*
Eh2    MSKVFTSKTVKITINQDDCIG..CEACVQNCPVQVYEMIDGKSNAT..NVDSCCKTLSCVSVCPKVSAITVDDI*
Dv            MGWTVTVDTDKCTG..DGECVDVCPVEVYELQDGKAVPV..NEEECLGCESCVEVCEAGAITVEEN*
Cp            MAYKIADSCVS..CGACASECPVNAISQGDSIFVID...ADTCIDCGNCANVCPVGAPVQE*
Af            MIAVLGCQG..CGTCSRVCPTDAIIREGGKVVRI..DQSKCKECYECVKACPYGALIVMD*
Mj            MVIMAVTIDYSLCKGAECAECVNNCPMEVFEIEGDKVVVA..RPDDCTYCGVCEDVCPTGAVKVEPE*
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FIG. 2. Putative ferredoxins of *G. lamblia* and *E. histolytica*. (A) Alignment of a hypothetical [2Fe-2S]ferredoxin of *G. lamblia* (*Gl*) (GenBank accession number AF393829) in single-letter code with those of other eukaryotes (*T. vaginalis* [*Tv*] [102172], *Trypanosoma brucei* [*Tb*] [incomplete microbial genome database], *S. cerevisiae* [*Sc*] [6325004], and *H. sapiens* [*Hs*] [4758352]) and eubacteria (*Rickettsia prowazekii* [*Rp*] [15604072] and *Aphanothece sacrum* [*As*] [119940]). Cys residues, which form ligands with the iron-sulfur site, and other residues identical in these ferredoxins are shaded in dark gray. Residues with conservative amino acid substitutions are shaded in light gray. N-terminal organelle-targeting sequences, which are proven (*T. vaginalis*, *S. cerevisiae*, and *H. sapiens*) or hypothetical (*G. lamblia*), are underlined (10, 14). An asterisk indicates the end of each protein. Gaps are indicated by dots. (B) Alignment of three hypothetical 6-kDa ferredoxins of *G. lamblia* (*Gl*1 to *Gl*3) with selected ferredoxins of *E. histolytica* (*Eh*1 and *Eh*2) (GenBank accession number 158942), eubacteria (*Desulfovibrio vulgaris* [*Dv*] [944972] and *Clostridium perfringens* [*Cp*] [119990]), and archaea (*Archaeglobus fulgidus* [*Af*] [11499725] and *Methanococcus jannaschii* [*Mj*] [15668371]). Residues are shaded as described for panel A.

sent from most eukaryotes (2, 5, 48, 55). When the *G. lamblia* random sequences were searched with an N-terminal peptide sequence from a partially purified *G. lamblia* ferredoxin by using TBLASTN, three genes encoding ~6-kDa ferredoxins (Gl Fd1 to Gl Fd3) were discovered (Fig. 2B) (3, 86). The partial *G. lamblia* ferredoxin sequence also revealed two *G. lamblia* genes encoding hypothetical ferredoxin-nitroreductase fusion proteins (Fd-NR1 and Fd-NR2), as well as previously identified genes encoding PFOR, the second keto-acid oxidoreductase, and Fe hydrogenase (see below) (Fig. 1 and 3A) (3, 30, 35, 36, 44, 62, 69, 88, 89; J. Nixon, J. Field, A. McArthur, M. Sogin, and J. Samuelson, unpublished observations). When we searched *E. histolytica* shotgun genomic sequences with the published amebic 2[4Fe-4S]ferredoxin (Eh Fd1) sequence by using TBLASTN, we discovered genes encoding a second 6-kDa ferredoxin (Eh Fd2) and ferredoxin-nitroreductase, as well as previously recognized genes encoding PFOR and Fe hydrogenase (Fig. 2B and 3A) (3, 36, 39, 64, 66, 69). Like the *G. lamblia* and *E. histolytica* PFORs and Fe hydrogenases, none of the putative 6-kDa ferredoxins or ferredoxin-nitroreductases contained an organelle-targeting sequence (14, 35, 69; Nixon et al., unpublished observations). These fermentation proteins are likely cytosolic, as has been shown for amebic Fe

hydrogenase and alcohol dehydrogenase 1 (29, 45, 52) and giardial fermentation enzymes (22, 50).

Putative *G. lamblia* and *E. histolytica* 6-kDa ferredoxins, ferredoxin-nitroreductase, and oxygen-insensitive nitroreductase provide tentative evidence for lateral gene transfer from prokaryotes. One predicted *G. lamblia* 6-kDa ferredoxin (Fd1), which was 59 amino acids long, matched the N-terminal peptide sequence of the purified ferredoxin (86). Two other predicted *G. lamblia* 2[4Fe-4S]ferredoxins, Fd2 and Fd3, which were 59 and 72 amino acids long, respectively, were new (Fig. 2B). *G. lamblia* Fd3 contained two conserved sequences (CxxCxxCxxxCP) which form ligands with 2[4Fe-4S] iron-sulfur centers (pFam00037) (11, 17). In contrast, an Ala is substituted for the second Cys in the N-terminal iron-sulfur center of *G. lamblia* Fd1, a His is substituted for Pro in the C-terminal iron-sulfur center of *G. lamblia* Fd2, and a Thr is substituted for Cys in the C-terminal iron-sulfur center of *E. histolytica* Fd2. These ferredoxins likely contain one [4Fe-4S] iron-sulfur center and one [3Fe-4S] iron-sulfur center, as previously suggested for Fd1 (11, 86). Besides the Cys residues, there was limited amino acid conservation in other positions of the protist 6-kDa ferredoxins. Phylogenetic trees, which included 5 protist ferredoxins, 15 eubacterial ferredoxins, and 23 archaeal

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Ferredoxin domain

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Gl1  MSRFPEVDEDLCIGCNVCVQGCPTQCIEVNAET..RKLVFRAKETCISCGHCASVCRTAAISMFAILPDTV...EEEIMAD.NPVVRSIKMARQHR
Gl2  MVEGYPVFLTDTCTGCNMCVEVCPKAVLKVDPET..KVVAYANRDNCIFCGHCGAICPTESISMFGIT...PESEENAIKTD.PPAVRAVKMARTCR
Eh   MANLTINTDVCIGCGMCTKECPCRVLKVEGGK..ALVDEKQSPNCMSCGHCQSICKAGAISLFEQK.......CEPLPDEHTSVIGAIKTRRSVR
Cd   MNMKYQHIIEVDKELCIGCGLCKNDCPVNNIIIENKK.....SVIKKQDCLMCGHCAAICPTKAITLTGFD.EPPIELTNKPKLDSDELLMAIKSRRSIR
Ca   MNLITVNHDKCVKCGICVNECPEQIIKMKENS..P..EDVCPQKCIACGHCVAVCPKEAIDNVKTPLINQKSSKKFSKLTPAEAENFLRSRRSIR
Ct   MIDFNVDSARCTRCGQCVADCPSRIIVMATGEY.PSIAPGKEFSCLRCEHCLAVCPEAAISILGFSPDGSTPLKGNLPET.DQLETLIAGRRSVR
Dv   MAFFEVDAQRCKRDDICIAECPIHIIISDRDSGVPKMRDGAESVCIRCGHCVAVCPHGAVRIEDMPIEAFTPVDKSLAIDARQAEQFLRGRRSIR
Gs   MPELIVTDAACTRCGACVSVCPLRIVELPEGNLPPRFTEEGAGRCIICGHCEAVCPTAALAVDDPRLDPAVYSPPTAPFAPAELDAYLRMRRSIR
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Beginning Nitroreductase domain

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Gl1  FFRSTSISPQEVLRILNIAKYSGANAYNSAFHFTAVDLK..RNNVDAVIEVFVTPLSKTSKYAWLP....KVRAHG...GDPIFHGAPWLILISTGHNE
Gl2  KYHPEPLPKEDLMQALSIAKYSASSSNIRPLHFTVLS.....RSLMDTLSLSIAKEVSKNPRYSKVA....ALVEKG...IDVVFRGAPHMILMSAPADK
Eh   KWKG.AIPNEELKEMINLIKFAPSACNYRPIKFMVINRP...KLTEVLTHLASRAANLETTPAIFR....PVCAYQ.AKVDIIGRD-
Cd   KFKDKEVSSEIIKQIIEAGRYTPSAKNSQDVSYIVLDNKK..SIYENEAVKFFRKIKPIANIAIKYS...KEVEIDD....NFFFKHAPIAIMIITKDKI
Ca   SYKKTSVPREKLMELVNIAHFAPTGSNLQSVSYIIIDD....RNILNMAIELTAKELERDELLSKRY..AAYIESYLSKGIDSILRGAPCLVLATADVDF
Ct   RYCPENLSPELIDKLITVASCAPTGVNARKVRFTVLDDREKTAHFRDEVMERLVRLLEEEALPESKAYYARFVKVWQKHKIDLVFRDAPHLLIATAPKSL
Dv   TFRDTPVPHDQLAQIMETVRWAPSASHKQPVHWVMVETPSAVREMAELVVDWMTEVRKENPELARRFNVAGLIAGWR.KGQDLILRGAPHLAVAWTEPAA
Gs   HFRPEPVPRETIAGMLDVVRYAPTGSNSQRVKWLIIHDTAEVRRLTGLVVDWLRSQQEGDTHLSRHFNIAAMIRSWR.KGNDPICRNAPHLAIAYTDRRH
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End Nitroreductase domain

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Gl1  DDPKDDGPGLDAFTQAHMLYRDLQLHSEIRGYGTHLCRILQPHLNDVQSLSDSCPNIFDVPQSHTVLLAFVLGIGAMKYHRPLLLPDLVEGIDVTFVK*
Gl2  ....AAVAMADASIAGRDIQLNAESMGLGMFWCGFLLAAVASSQ....ELHDICGVPEGHKILMAMGIGRPKVKFARPALRRNLEEGVDITFK*
Cd   SG.............SLAASNMALMAESYGLGVLFSGYFSDVANNSP....KLKKLLGLKRSNHVLTTLVIGYPDVKYRRTAQK....EVATVRYL*
Ca   PRGREN.........SIFSLAYMELYAPTLGLGSCWAGVFEKIALKDNS...PMLKLFNVPKGKKITGAVMVGYPKYNYPRLVDR....NPLEVNFYQSK*
Ct   STPRQD.........CVIALTTFELYAQACGIGTLWNGIATWAIEEMLP....EMRQRLGIPDDHAFGYAMLFGKPAVRYARTVQH....HPPEIYRVP*
Dv   TWPEVD.........CAVALTYLELAAHAHGVGCCWAGVFYTYAANAYG....PLREALGLPQDARVCGGQMMGYARFRYRRLPWR....KPLAIDWK*
Gs   PTVAVD.........AVIAATHLDILAPAHGVGTCWAGFFQMAAEAWE....PLQQAIGLPEGHHAGYTLLLGYPAVTYRRPPRR....NRLDVVWR*
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Gl  　　　　　　　　　　　　　　　　　　　　MPPICDAILRRRAIKQ.YTKESVSIEAIEYLRKVAVAIPTGHNTRHTEFAFVTNPR
Ca  　　　　　　　　　　　　　　　　　　　　MIDLLKTRRSIRK.YKNKEIEKEKVDTLLKAALLAPTSMGKRSWEFIAVTNKN
Tm  　　　　　　　　　　　　　　　　　　　MSIIYRRRSIRK.YQRKDVPDELVKEIIRAAMHAPSACNQQPWHFVVIRNEE
Cs  MDKKIDTKRSWPKGYEPKEETEDDLSTRLGLLETILKAKPEKKASTDIFKVMATRRSTRK.FSKNPVEKWKIDKILAAADTAPTAGNFQGFEIFYVNDKE
Bh  　　　　　　　　　　　　　　　　　　　MYIQSHLANVIKERRSIKNGYLDQEVPLELVEQLLEVAVWAPTHGLREPWRFIFVSSEQ
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Gl  ...IIKTISQAKGEKA....EYMQHAKLLIVVMGIQN.........DGVTSIATDMSIAAAMIQ...LACSDFGLACSWEQFYGRKNVYGEDSERIVLDV
Ca  ...LISELSTARKMGS....QFLKGAPLVIVVVENP.........EATDAYIEDGAIASTLIQ...VTAHSMGLGSCWCHVRNRDRIDYDTTE.AFIKT
Tm  ...TKKKIAEIHPH.A....QMSAEAPVVILVCGDPT.......LEKCKGYWIQDCSAAVENLL...LRATELGLGAVWCGVYPRE..ER..VK.AFREL
Cs  ...TKEELVKASNN......QPYVNTPLVLIFCMNPSRVKLDFSPKVIMKFSIQDATLAAAYSQ...LAASALGLSSIWIGMFDEE.........KVKKI
Bh  KETFVAQLVQTFPKEMQENRRNYFSQPAAFLIVVMQE........DPRQKQWEEDFGAVSSLIQNFQLLAWEQKLGVVWKTNPHIY.......DPKVRDL
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```
Gl  LRLLNSPNRRVLCALAIGYPAVQMP..PADMHENGRIYMIE*
Ca  LLNI.PENLKVECMLGIGYPDEERKAYTDEDLALNKLHYDKYSK*
Tm  LG.I.PEHVVPFALVPVGYPAEHP..VQVDRFKPERIHYEKW*
Cs  IG...TE.LRPTSILCIGYPIKKRP.AKLRRQLKELIHVVNKKD*
Bh  VG..VAPGEKIVGFLHLGYFDEVPKAMPRTSVKEKLTVYDGK*
```

FIG. 3. Putative nitroreductases of *G. lamblia* and *E. histolytica*. (A) Alignment of two hypothetical ferredoxin-nitroreductase fusion enzymes of *G. lamblia* (*Gl*1 and *Gl*2) with those of *E. histolytica* (*Eh*), *Clostridium difficile* (*Cd*) (incomplete microbial genome database), *Clostridium acetobutylicum* (*Ca*) (GenBank accession number 15026573), *Chlorobium tepidum* (*Ct*) (incomplete microbial genome database), *D. vulgaris* (*Dv*) (incomplete microbial genome database), and *Geobacter sulfurreducens* (*Gs*) (incomplete microbial genome database). Residues are shaded as described in the legend to Fig. 2A. A dash indicates the end of the available sequence for the *E. histolytica* ferredoxin-nitroreductase, which was truncated. Gaps are indicated by dots. (B) Alignment of putative oxygen-insensitive nitroreductases of *G. lamblia* (*Gl*) with those of *Bacillus halodurans* (*Bh*) (GenBank accession number 15612866), a *Crenarchaeote* sp. (*Cs*) (14548151), *C. acetobutylicum* (*Ca*) (15896791), and *T. maritima* (*Tm*) (15643149).

2[4Fe-4S]ferredoxins, were star shaped and contained no nodes with significant bootstrap support (data not shown) (25, 27).

Ferredoxin-nitroreductase fusion proteins, which have an N-terminal 2[4Fe-4S]ferredoxin domain and a C-terminal nitroreductase domain, have been predicted from the whole genome sequence of *Clostridium acetobutylicum* (11, 44, 62). The *G. lamblia* sequence contained two genes that encoded putative ferredoxin-nitroreductases (Fd-NR1 and Fd-NR2), which were 278 and 265 amino acids long, respectively, while an *E. histolytica* shotgun sequence encoded a partial ferredoxin-nitroreductase, which was missing its C terminus (Fig. 3A).

The protist ferredoxin-nitroreductases each had a conserved 2[4Fe-4S] iron-sulfur center at the N terminus, which was followed by a C-terminal nitroreductase domain (pFam00881) (3, 11, 17, 44). Phylogenetic trees, which included three protist and seven eubacterial ferredoxin-nitroreductases, were star shaped and contained few nodes with significant bootstrap support (data not shown) (25, 27).

Oxygen-insensitive nitroreductases, which lack a ferredoxin domain, are present in archaea and eubacteria (30, 41, 44, 94). *H. pylori* and *E. coli* nitroreductases reduce and activate nitro groups of metronidazole and furazolidone, respectively, so that *H. pylori* and *E. coli* mutants lacking nitroreductase activity are

resistant to each drug (30, 44, 94). When the *G. lamblia* and *E. histolytica* shotgun sequences were searched with TBLASTN and an *H. pylori* nitroreductase sequence, a *G. lamblia* gene encoding a putative nitroreductase was predicted, while no nitroreductase was identified in *E. histolytica* (3, 30). The *G. lamblia* nitroreductase was 176 amino acids long and contained a conserved nitroreductase domain (pFam00881), which was like that present in the *G. lamblia* ferredoxin-nitroreductase described above (Fig. 3B) (42). In BLASTP searches, only three eubacterial sequences and one archaeal sequence showed significant homology to the giardial nitroreductase, and the greatest similarity was found with a *C. acetobutylicum* sequence (3, 62).

In summary, putative giardial and amebic 6-kDa ferredoxins, ferredoxin-nitroreductases, and oxygen-insensitive nitroreductases resemble each other and those of some anaerobic eubacteria and archaea. Because there were not enough sequences to perform meaningful phylogenetic analyses, only their common occurrence in giardia organisms, amebae, and a few anaerobic prokaryotes suggests the possibility of their lateral transfer (19–21, 26, 69). Because the 6-kDa ferredoxin and the ferredoxin-nitroreductase are absent from proteobacteria, it is likely that the giardial and amebic genes are not derived from the mitochondrial endosymbiont (hydrogen hypothesis) (31, 53, 71).

***G. lamblia* genes encoding putative NADH oxidase and ADH3 provide more robust support for lateral gene transfer.** Under microaerophilic conditions, giardia organisms oxidize NADH and reduce two protons to form NAD^+ and water (8). NADH oxidases belong to a superfamily of pyridine nucleotide disulfide oxidoreductases (pFam00070) which also includes NADH peroxidase, glutamate synthase, and the dihydrolipo-amide dehydrogenase component of pyruvate dehydrogenase E3, as well as reductases of glutathione, thioredoxin, trypanathione, nitrite, and mercuric ion (3, 32, 70, 84). When the *G. lamblia* and *E. histolytica* shotgun sequences were searched with an *E. coli* NADH oxidase by using TBLASTN, one gene was identified for each protist. The predicted *G. lamblia* NADH oxidase, which included the N-terminal peptide sequence from the purified protein, was 458 amino acids long, and the predicted *E. histolytica* NADH oxidase was 447 amino acids long (8). Distance-based phylogenetic analyses strongly suggested that the *G. lamblia* NADH oxidase gene shared common ancestry with genes of gram-positive anaerobes, including *Lactococcus* and *Enterococcus* spp. (Fig. 4). In contrast, the *E. histolytica* NADH oxidase gene appeared to share common ancestry with genes of *Thermotoga maritima* and *Microscilla* spp.

While giardial and amebic ADHE enzymes convert acetyl-CoA to ethanol, the functions of protist ADH3 are not known (16, 67, 76, 95). Phylogenetic trees were drawn with 4 eukaryotic, 4 gram-positive eubacterial, 11 gram-negative eubacterial, and 3 archaeal ADH3 enzymes (Fig. 5). These trees strongly suggested that the putative *G. lamblia* ADH3 gene shared common ancestry with those of a thermoanaerobic gram-positive eubacterium and an archaeon, while the putative *E. histolytica* ADH3 gene shared common ancestry with those of gram-negative eubacteria. In addition, a yeast gene encoding ADH3 shared common ancestry with those of a distinct set of gram-negative eubacteria.

These results suggest that the protist NADH oxidase and

ADH3 genes were laterally transferred from different anaerobic prokaryotes, as previously shown for the *G. lamblia* and *E. histolytica* genes encoding malic enzyme and ADHE (26, 69). However, we cannot rule out the possibilities that (i) two paralogues of each NADH oxidase and ADH3 gene existed within the prokaryotic donor; (ii) both paralogues of each gene were transferred to giardia organisms and amebae; and (iii) giardia organisms lost one paralogue, while amebae lost the other paralogue. Similarly, we cannot eliminate the possibilities that (i) each of these paralogues existed in a common eukaryotic ancestor; (ii) one paralogue was lost from giardia organisms, and the other was lost from amebae; and (iii) both paralogues of each gene were lost from the vast majority of eukaryotes (amitochondriate fossil hypothesis) (7, 12, 65).

DISCUSSION

Molecular characterization of giardial and amebic fermentation proteins and identification of possible protist nitroreductases which may be involved in the activation of metronidazole and furazolidone. *G. lamblia* and *E. histolytica* genes encoding 6-kDa ferredoxins and the NADH oxidase were predicted by biochemical studies (8, 66, 86). In contrast, we were unable to identify a giardial gene encoding a ferredoxin: NAD(P)H oxidoreductase, which was also predicted (7). As greater than 98% of the giardial genome has been sampled by shotgun sequencing, it is likely that most of the fermentation enzymes of giardia organisms have now been molecularly characterized (Fig. 1) (54). Giardial genes encoding [2Fe-2S]ferredoxin, the ferredoxin-nitroreductases, and the oxygen-insensitive nitroreductases and the amebic ferredoxin-nitroreductase gene were not previously predicted. Nitroreductases and ferredoxins are potentially important for an understanding of how giardia organisms and amebae are susceptible to metronidazole and furazolidone and how these protists might become resistant to these drugs (28, 90). For example, it is likely that the [2Fe-2S]ferredoxin, which is expressed at levels similar to those of the 2[4Fe-4S]ferredoxin (unpublished data), is also a source of electrons to reduce nitro groups of antigiardial drugs (28, 51, 57, 90). It is possible that the transfer of electrons from ferredoxin to metronidazole and furazolidone is catalyzed by oxygen-insensitive nitroreductases in giardia organisms, as has been described for *H. pylori* and *E. coli*, respectively (30, 46, 90, 94). It remains to be determined whether the ferredoxin-nitroreductase, which may be an oxygen-sensitive nitroreductase because of the iron-sulfur group, is also involved in the reduction of nitro groups and the activation of drugs against giardia organisms, amebae, and clostridia.

Weak support for the hydrogen hypothesis. A prediction of the hydrogen hypothesis is that some of the genes encoding protist fermentation enzymes resembling those of bacteria will be endosymbiont derived (53, 71, 72). In support of this idea, hydrogenosomes of *T. vaginalis* contain multiple fermentation enzymes (PFOR, [2Fe-2S] ferredoxin, Fe hydrogenase, malic enzyme, and succinyl-CoA synthetase), which are similar to those of mitochondria and have organelle-targeting sequences at their N termini (6, 10, 35–38, 42, 47). With the possible exception of IscS, phylogenetic analyses have not been able to show that it was the mitochondrial endosymbiont rather than some other gram-negative eubacterium which donated these
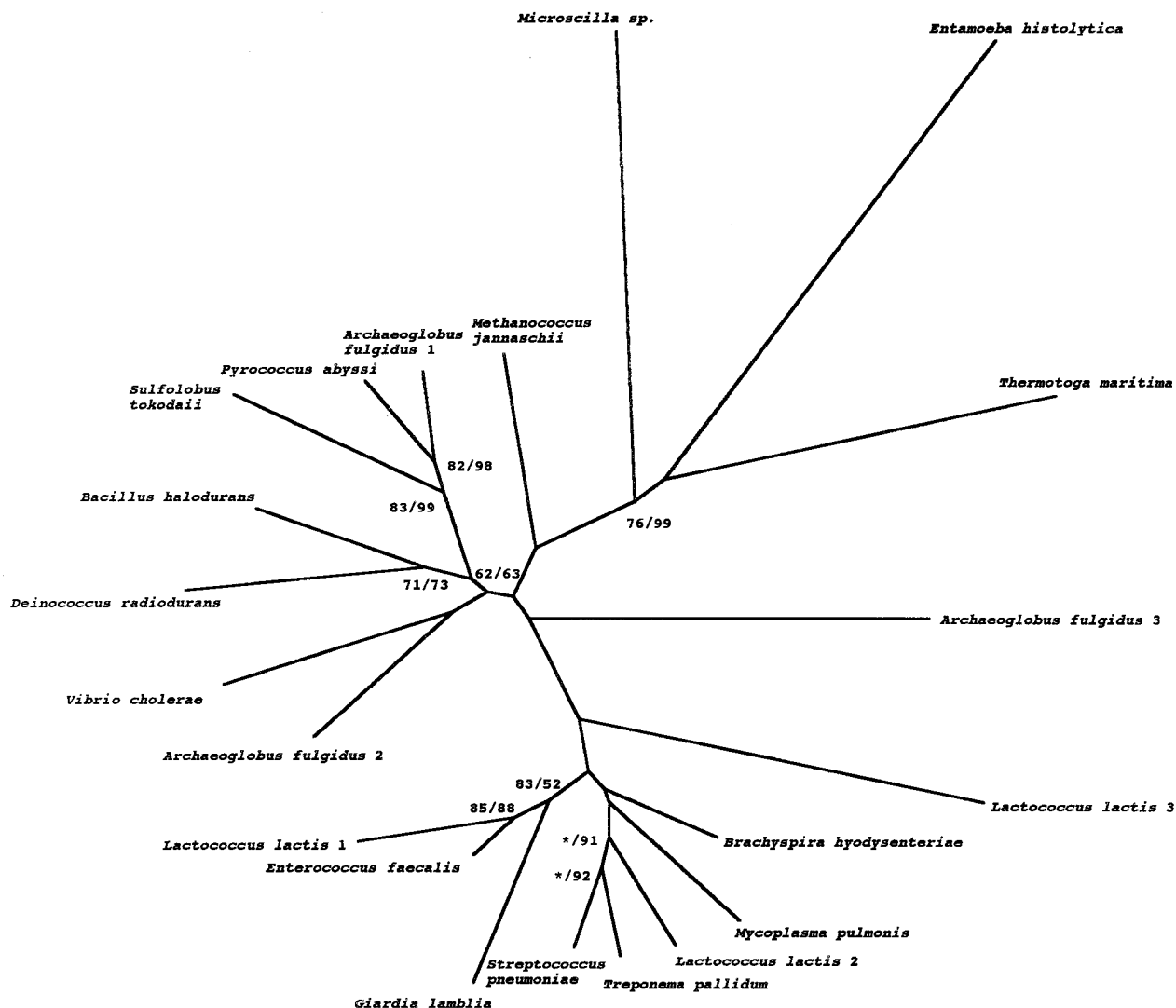
FIG. 4. Phylogenetic tree drawn by distance methods for NADH oxidases of *G. lamblia*, *E. histolytica*, archaea (*A. fulgidus* 1, 2, and 3 [GenBank accession numbers 11498007, 11498012, and 11498556, respectively]; *M. jannaschii* [15668830]; a *Microscilla* sp. [14518332]; *Pyrococcus abyssi* [14521611]; and *Sulfolobus tokodaii* [15922579]), and eubacteria (*B. halodurans* [15616338]; *Brachyspira hyodysenteriae* [642030]; *Deinococcus radiodurans* [15806015]; *Enterococcus faecalis* [547994]; *Lactococcus lactis* 1, 2, and 3 [15672373, 15674108, and 15672768, respectively]; *Mycoplasma pulmonis* [15828494]; *Streptococcus pneumoniae* [4416519]; *T. maritima* [15643161]; *Treponema pallidum* [15639906]; and *Vibrio cholerae* [15601402]). Bootstrap values at nodes were from distance/parsimony methods. An asterisk indicates where a bootstrap value was below 50%. Nodes were left blank when both distance and parsimony analyses failed to identify the same bifurcating branch in over 50% of the bootstrap replicates.

trichomonad fermentation genes (26, 35–38, 72, 83). Similarly, with the exception of IscS, it has not been possible to show that *G. lamblia* and *E. histolytica* genes encoding fermentation enzymes were derived from the mitochondrial endosymbiont rather than some other eubacterium (26, 35–38, 72, 83). In support of the endosymbiont hypothesis, a putative organelle-targeting sequence is present at the N terminus of the giardial [2Fe-2S]ferredoxin, while a peptide at the N terminus of the *G. lamblia* adenylate kinase also may have once been an organelle-targeting sequence (14, 73). Arguing against the hydrogen hypothesis are the facts that some of the fermentation enzymes of giardia organisms and amebae (e.g., 6-kDa ferredoxins, ferredoxin-nitroreductases, and Fe hydrogenases) are present in other luminal protists, gram-positive anaerobes, and

archaea but are absent from proteobacteria, which resemble the mitochondrial endosymbiont (3, 7, 60, 62, 65, 69).

Weak and strong evidence for lateral transfer of genes encoding fermentation enzymes from anaerobic prokaryotes to giardia organisms. Because of the rarity of similar sequences in GenBank and the database of unfinished microbial sequences, it was not possible to perform phylogenetic analyses that might demonstrate that *G. lamblia* genes encoding ferredoxin-nitroreductases and oxygen-insensitive nitroreductases were laterally transferred from anaerobic bacteria (Fig. 1) (26, 69). In addition, the *G. lamblia* and *E. histolytica* 6-kDa ferredoxins were short and lost any phylogenetic signal matching them with a specific bacterium. Although it is likely that the giardial and amebic genes encoding these fermentation en-
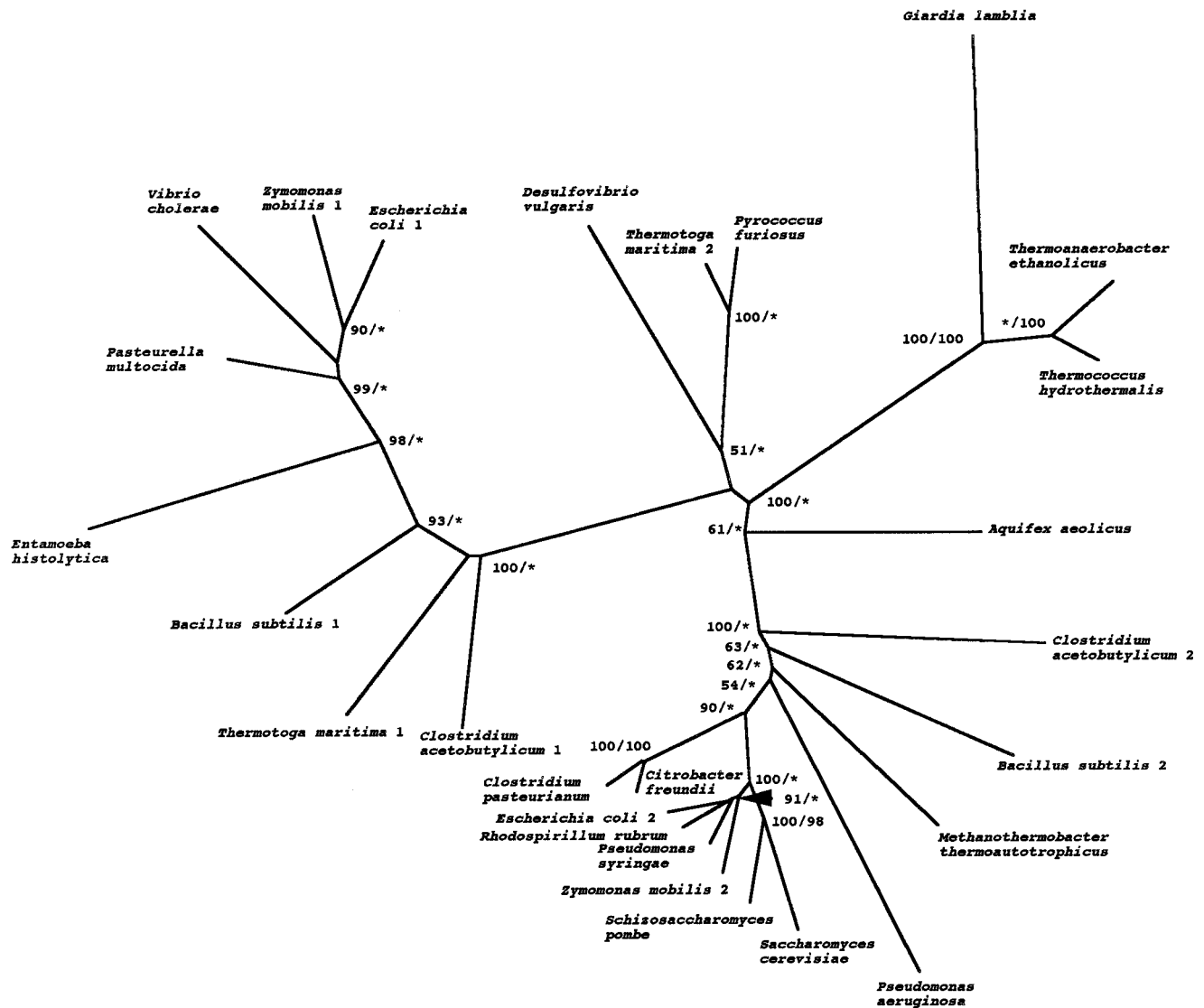
FIG. 5. Phylogenetic tree drawn by distance methods for ADH3 enzymes from *G. lamblia* (GenBank accession number 7288867), *E. histolytica* (1502307), other eukaryotes (*S. cerevisiae* [3337] and *Schizosaccharomyces pombe* [1168356]), archaea (*Pyrococcus furiosus* [3288811], *Methanothermobacter thermoautotrophicus* [7431292], and *Thermococcus hydrothermalis* [2765364]), and eubacteria (*Aquifex aeolicus* [7431295], *Bacillus subtilis* 1 and 2 [7431302 and 3023262, respectively], *Citrobacter freundii* [1169299], *C. acetobutylicum* 1 and 2 [144714 and 15896543, respectively], *Clostridium pasteurianum* [2393887], *D. vulgaris* [incomplete microbial genome database], *E. coli* 1 and 2 [9911058 and 3025295, respectively], *Pasteurella multocida* [15602014], *Pseudomonas aeruginosa* [12698394], *Pseudomonas syringae* [incomplete microbial genome database], *Rhodospirillum rubrum* [4519177], *Thermoanaerobacter ethanolicus* [9863860], *T. maritima* 1 and 2 [7431300 and 7431301, respectively], *V. cholerae* [15601458], and *Zymomonas mobilis* 1 and 2 [113381 and 4378173, respectively]). Bootstrap values are marked as described in the legend to Fig. 4.

zymes were laterally transferred, the evidence is weak, and we cannot rule out the possibility that these genes were present in an anaerobic eukaryotic ancestor but were subsequently lost from the vast majority of eukaryotes (amitochondriate fossil hypothesis) (7, 12, 65). In contrast, phylogenetic analyses suggest that numerous giardial genes encoding fermentation enzymes (NADH oxidase, ADH3, ADHE, malic enzyme, and second keto-acid oxidoreductase) have prokaryotic sources different from those of amebae 26, 62, 79; this study). *G. intestinalis* genes encoding aldolase, arginine deiminase, carbamate kinase, glucokinase, glucosephosphate isomerase, NAD(P)H: menadione oxidoreductase, and phosphoenolpyruvate carboxykinase also appear to have been laterally transferred from

prokaryotes (33, 34, 43, 56, 77, 82). These observations suggest that despite numerous lateral transfers of genes from one prokaryote to another (20, 21, 49, 60, 63), one can still discern distinct lateral transfer events which brought prokaryotic genes to giardia organisms. These lateral transfers likely occurred a long time ago, as the predicted peptide sequences are relatively divergent and there is no biased codon usage, as is the case for recent lateral transfers between eubacteria (20, 21, 26, 49, 60, 63, 69). Although the mechanisms responsible for the acquisition of bacterial genes by giardia organisms and amebae are not known, these protists may be transformed by bacterial plasmids which lack a specific origin of replication (23, 52).

While lateral gene transfer appears to be important for the

adaptation of giardia organisms and amebae, lateral gene transfer among higher eukaryotes appears to have been rare (2, 5, 48, 75). It seems most appropriate then to think of giardia organisms, amebae, and other microaerophilic protists as exceptional eukaryotes, not only in their anaerobic life style but also in the mechanisms by which they may have acquired the genes that encode their fermentation enzymes and related peptides.

ACKNOWLEDGMENTS

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