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The Origin of Dihydroorotate Dehydrogenase Genes of Kinetoplastids, with Special Reference to Their Biological Significance and Adaptation to Anaerobic, Parasitic Conditions

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Abstract. *Trypanosoma cruzi* dihydroorotate dehydrogenase (DHOD), the fourth enzyme of the de novo pyrimidine biosynthetic pathway, is localized in the cytosol and utilizes fumarate as electron acceptor (fumarate reductase activity), while the enzyme from other various eukaryotes is mitochondrial membrane-linked. Here we report that DHOD-knockout *T. cruzi* did not express the enzyme protein and could not survive even in the presence of pyrimidine nucleosides, substrates for the potentially active salvage pathway, suggesting a vital role of fumarate reductase activity in the regulation of cellular redox balance. Cloning and phylogenetic analysis of euglenozoan *DHOD* genes showed that the euglenoid *Euglena gracilis* had a mitochondrial DHOD and that biflagellated bodonids, a sister group of trypanosomatids within kinetoplastids, harbor the cytosolic DHOD. Further, *Bodo saliens*, a bodonid, had an *ACT/DHOD* gene fusion encoding aspartate carbamoyltransferase (ACT), the second enzyme of the de novo pyrimidine pathway, and DHOD. This is the first report of this novel gene structure. These results are consistent with suggestions that an ancient common ancestor of Euglenozoa had a mitochondrial DHOD whose descendant exists in *E. gracilis* and that a common ancestor of kinetoplastids (bodonids and trypanosomatids) subsequently acquired a cytosolic DHOD by horizontal gene transfer. The cytosolic *DHOD* gene thus acquired may have contributed to adaptation to anaerobiosis in the kinetoplastid lineage and further contributed to the subsequent establishment of parasitism in a trypanosomatid ancestor. Different molecular strategies for anaerobic adaptation in pyrimidine biosynthesis, used by kinetoplastids and by euglenoids, are discussed. Evolutionary implications of the *ACT/DHOD* gene fusion are also discussed.

Key words: Dihydroorotate dehydrogenase — *Trypanosoma cruzi* — Bodonid — Kinetoplastid — Euglenozoa — Phylogenetic tree — Gene fusion — Horizontal gene transfer — Anaerobiosis

Introduction

Recent advances in molecular biology, genomics, and phylogenetics in the field of parasitology have provided insights into the evolutionary origin of protozoan parasites and into the intimate relationships

Sequence availability: The nucleotide sequence data reported here appear in the GenBank, EMBL, and DDBJ databases with the accession numbers AB120414, AB159227, and AB159228 for *Euglena gracilis* dihydroorotate dehydrogenase (DHOD), *Bodo saliens* aspartate carbamoyltransferase/dihydroorotate dehydrogenase (ACT/DHOD), and *B. caudatus* DHOD, respectively.

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between parasitic and related free-living protists (Arisue et al. 2002a–c; Baldauf et al. 2000; Bapteste et al. 2002; Gardner et al. 2002; Hashimoto et al. 1998; Katinka et al. 2001; Keeling 2001; Simpson et al. 2002; Sogin and Silberman 1998). However, the initial steps leading to a parasitic mode of life taken by a free-living ancestor remain mostly unknown. For example, *Plasmodium* spp. including human malaria parasites contain a plastid-like organelle, the apicoplast, suggesting that Apicomplexa arose from an alga and that apicoplast may have played a crucial role for the protozoan survival (Fichera and Roos 1997; Jomaa et al. 1999; Köhler et al. 1997; Wilson et al. 1996). Nevertheless, the contribution of this plastid (or ancestral apicoplast) in the initial steps of parasitism is still not clear.

Trypanosomatids are uniflagellated protists and cause important human diseases such as African sleeping sickness (*Trypanosoma brucei gambiense, T. brucei rhodesiense*), Chagas' disease (*Trypanosoma cruzi*), and leishmaniasis (*Leishmania* spp.). They live in a large variety of hosts including animals, plants, and protists. The trypanosomatids and biflagellated bodonids, a sister group of the former, are kinetoplast-bearing protists (kinetoplastids). The kinetoplastids belong to the phylum Euglenozoa, as well as euglenoids. While there is no free-living trypanosomatid reported so far, most bodonids are free-living organisms. Phylogenetic studies on small subunit ribosomal RNA (SSU rRNA) and heat shock protein 90 (HSP90) provided a notion that trypanosomatids and bodonids may have evolved from a common euglenozoan ancestor (Fernandes et al. 1993; Simpson et al. 2000, 2002). Such a closer relationship in these kinetoplastids suggests that a free-living bodonid(s) could be used as a good model for studying an initial stage of establishing parasitism, although the biochemical and molecular biological features of bodonids are little known.

Among the fundamental metabolic pathways, nucleotide biosynthesis is distinctive in trypanosomatids. They are unable to synthesize purines de novo, absolutely depending on the salvage pathway. In contrast, they possess both the de novo and the salvage pathways for pyrimidine synthesis. Generally, the de novo pyrimidine pathway consists of a six-enzyme cascade in the order carbamoyl-phosphate synthetase II (CPS II), comprising glutamine amidotransferase (GAT) and carbamoyl-phosphate synthetase (CPS) domains, aspartate carbamoyltransferase (ACT), dihydroorotase (DHO), dihydroorotate dehydrogenase (DHOD), orotate phosphoribosyltransferase (OPRT), and orotidine-5′-monophosphate decarboxylase (OMPDC). The characteristic protein structures and phylogenetic origins of trypanosomatid enzymes differ from those of other various eukaryotes (Nara et al. 2000). Further, we reported a pyrimidine-biosynthetic gene cluster that contains four separate and one fused genes, in the order, from the 5′-terminus, *CPS II, DHO, OMPDC/OPRT, ACT*, and *DHOD*, encoding all six enzymes of the pathway in *T. cruzi* (see Fig. 3A) (Gao et al. 1999). This gene cluster is also well conserved in another trypanosomatid, *Leishmania mexicana* (GenBank accession number AB029444), a pathogen of cutaneous leishmaniasis. To date, the *OMPDC/OPRT* gene fusion has only been observed in these trypanosomatids, while animals and plants are known to carry the conversely connected *OPRT/OMPDC*.

Phylogenetic studies of all the six enzymes from a great variety of organisms strongly suggest that trypanosomatids acquired *DHOD* and *OMPDC* genes by horizontal transfer (Nara et al. 2000). The origin of *OMPDC* was unclear, however. DHOD phylogeny clearly showed a monophyletic clade consisting of only four members, *T. cruzi*, *L. mexicana*, *Saccharomyces cerevisiae*, and *Lactococcus lactis A* (Nara et al. 2000). This type of DHOD is classified as the family 1A enzyme, which localizes in the cytosol and is capable of utilizing fumarate as electron acceptor, producing succinate. All other eukaryotes possess the family 2-type DHOD, which localizes in the mitochondrial inner membrane and uses ubiquinone as electron acceptor, delivering electrons to the respiratory chain (Andersen et al. 1994; Hines et al. 1986; Jensen and Björnberg 1998; Nagy et al. 1992; Nielsen et al. 1996). When *S. cerevisiae* lacking the family 1A *DHOD* gene was complemented with a family 2 *DHOD* gene from *Schizosaccharomyces pombe*, its viability was not recovered at low oxygen tensions, indicating that the family 1A DHOD is critical for pyrimidine synthesis and growth under anaerobiosis (Nagy et al. 1992). These data raise the questions, What is the biological significance of the family 1A DHOD in trypanosomatids? and Which organism is a close relative of the putative evolutionary ancestor that may have horizontally acquired the family 1A *DHOD* gene?

It should be pointed out that the family 1A DHOD functions under both aerobic and anaerobic conditions because of its independence of the mitochondrial respiratory chain. Acquisition of this enzyme (i.e., *DHOD* gene) should have been advantageous for organisms that contain an anaerobic mitochondrion or an aerobic but partially functioning mitochondrion. Notably, Euglenozoa includes species representing both types (Tielens et al. 2002; Tielens and Van Hellemond 1998). On one hand, the euglenoid *Euglena gracilis* respires normally under aerobic conditions but uses wax ester fermentation under anaerobic conditions when fatty acid formation serves as acceptor of electrons resulting from glycolysis (Inui et al. 1982). On the other hand, the trypanosomatid *T. brucei* bloodstream form possesses a poorly developed mitochondrion and its ATP

generation depends on breakdown of glucose, which is abundant in the blood (Misset et al. 1986; Opperdoes 1987; Visser et al. 1981). *T. cruzi* expresses all of the TCA cycle enzymes and respiratory chain throughout its life cycle but produces a considerable amount of succinate probably as an end product of glycolysis (Cannata and Cazzulo 1984; Cazzulo et al. 1985). Consistent with this potentially partial mitochondrial function, the protozoan DHOD serves as a major soluble fumarate reductase (Takashima et al. 2002).

In the present study, we demonstrate that the family 1A DHOD was essential for *T. cruzi* survival and we also characterize the counterpart enzymes in bodonids and euglenoids. To trace the evolutionary origin of the trypanosomatid family 1A DHOD, we performed phylogenetic analyses of these euglenozoan DHODs. Two bodonid species, *Bodo saliens* and *B. caudatus*, had the family 1A *DHOD* genes, and surprisingly, *B. saliens DHOD* occurred as an *ACT/DHOD* gene fusion; *ACT* encodes the second enzyme of the de novo pyrimidine pathway and this fused gene is reported here for the first time. A putative *E. gracilis* DHOD, derived from its cDNA sequence, contained a mitochondrial targeting sequence and was classified as a family 2 enzyme. The DHOD phylogeny, constructed from these and other data, showed that trypanosomatids and bodonids were monophyletic in the family 1A clade and that *E. gracilis* was placed in the eukaryotic family 2 clade. These results strongly suggest that the anaerobic de novo pyrimidine biosynthesis, probably elaborated by horizontal acquisition of the family 1A *DHOD* gene, may have been present in a common ancestor of free-living kinetoplastids and possibly have played an important role in the initial step(s) of establishing parasitism in an ancestor of the trypanosomatid lineage. Further, different molecular strategies for anaerobic adaptation in pyrimidine biosynthesis, taken by kinetoplastids and by euglenoids, are discussed. Evolutionary implications of the *ACT/DHOD* gene fusion are also discussed.

Materials and Methods

Organisms

The culture form (epimastigote) of *T. cruzi* Tulahuen strain was maintained in LIT medium as described (Nara et al. 1998). Briefly, epimastigotes were cultured at an initial cell density of 5×10^5/ml in 5 ml of LIT medium (No. 1029, ATCC medium formulations) in tightly capped 25-cm^2 culture flasks at 26°C and subcultured weekly. *Bodo saliens* (ATCC 50358) and *B. caudatus* (ATCC 50361) were purchased from the American Type Culture Collection. *B. saliens* was maintained in tightly capped 25-cm^2 culture flasks containing 5 ml of artificial seawater at 23°C with *Klebsiella pneumoniae* subsp. (ATCC 27889) as food. *B. caudatus* was cultured in tightly capped 25-cm^2 culture flasks containing 5 ml of the diluted LIT medium (0.05%) supplemented with the same bacterium. Both *B. saliens* and *B. caudatus* were inoculated at an initial cell density of 2×10^4/ml and subcultured at 2-week intervals. *K. pneumoniae* grown in Luria–Bertani (LB) medium were collected, washed with phosphate-buffered saline (PBS) at pH 7.2, and added to the protozoan cultures every 3 to 4 days. For the experiments, the bodonids were cultured in 150-cm^2 culture flasks containing 100 ml of the appropriate medium supplemented with *K. pneumoniae* and harvested at the late logarithmic phase of growth, having a cell density of 2×10^6/ml.

The cultures were centrifuged at 3000 rpm for 5 min at 4°C. The cell pellets were suspended in artificial seawater (for *B. saliens*) or in PBS, pH 7.2 (for *B. caudatus*), containing 1 mg/ml lysozyme and 40 μg/ml DNase I, incubated at room temperature for 30 min with gentle shaking, and centrifuged at 3000 rpm for 10 min at 4°C. This procedure was repeated seven times. The final preparations were verified microscopically to have a bacterial/protozoan cell ratio of less than 10%. Axenic *E. gracilis Klebs* (NIES-48 strain, Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan) was maintained in screw-capped glass tubes containing 0.1% sodium acetate at pH 7.0 and 0.4% yeast extract at room temperature under natural light conditions, with a passage every 3 weeks. For large-scale preparation, *E. gracilis* was inoculated at an initial cell density of 1.5×10^3/ml into 400 ml of medium in a 500-ml glass bottle. Cultures at the late logarithmic phase of growth, having a cell density of 1.5×10^5/ml, were harvested by centrifugation at 3000 rpm for 10 min at 4°C. The cells were washed twice with distilled water by resuspension and centrifugation.

Plasmids

Hygromycin- and tunicamycin-resistance genes (designated *HYG* and *TUN*, respectively) were prepared by *Hind*III/*Bam*HI digestion from the pTEX-derived plasmids, p72hyg72 and p72tun72, respectively (Cooper et al. 1993; Kelly et al. 1992; Nozaki and Cross 1994), kindly supplied by Dr. Tomoyoshi Nozaki, Department of Parasitology, National Institute of Infectious Diseases, Tokyo. These genes were individually subcloned into the *Hind*III/*Bam*HI site in pBluescript II KS(+). To amplify the 5′-upstream sequence of *T. cruzi DHOD* gene (at locus B in Fig. 1), a 3.1-kb DNA fragment which contains the *DHOD* gene was derived by *Hind*III/*Xho*I digestion from the protozoan genomic clone 28 (Nara et al. 2003) and was subcloned into the *Hind*III/*Sal*I site in pUC18. Using this recombinant plasmid as template, PCR was carried out in the presence of a universal M13 primer and a specific primer (5′-CGGGATCCAAGCTTCTTCTGCGTACGTACACC-3). The resulting PCR product of 1.2 kb was digested with *Hind*III and inserted into the *Hind*III site at the 5′-terminus of *HYG* or *TUN* in the above recombinant pBluescript II. To amplify the 3′-downstream sequence of *T. cruzi DHOD*, PCR was conducted using the above recombinant pUC18 as template, M13 reverse primer, and a specific primer (5′-CGGGATCCCGTGTCAAGA-CAATTGAGTGA-3′). After digestion of the PCR product with *Bam*HI, the resulting 0.9-kb DNA fragment was inserted into the *Bam*HI site at the 3′-terminus of *HYG* (or *TUN*) of the recombinant pBluescript II. Finally, the pBluescript II carrying a continuous sequence of the 5′-upstream sequence (1.2 kb), *HYG* or *TUN*, and the 3′-downstream sequence (0.9 kb) was established. We designated these continuous sequences as the *HYG* cassette and the *TUN* cassette (see Fig. 1A), respectively.

Disruption of the Trypanosoma cruzi DHOD *Gene*

The recombinant pBluescript II containing the *HYG* cassette was digested by *Sac*I/*Kpn*I and the cassette was purified. *T. cruzi* epimastigotes at the late logarithmic phase of growth were har-

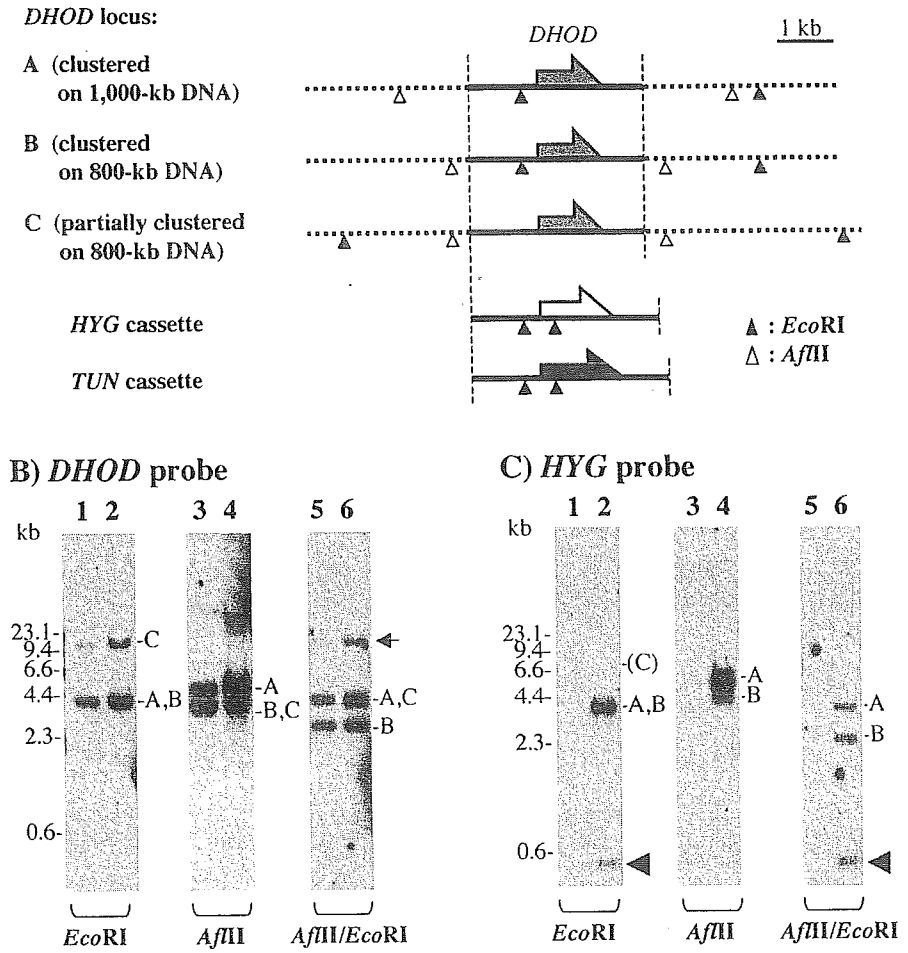


Fig. 1. Southern blot analyses of *DHOD* loci in the wild-type *Trypanosoma cruzi* (Tulahuen strain) and in its *Δdhod::HYG* transformant. A Schematic representation of *DHOD* loci in the wild-type *T. cruzi*, drawn on the basis of Southern hybridization patterns in B. Locus A occurs in the pyrimidine-biosynthetic gene cluster on a 1000-kb chromosomal DNA, locus B in the pyrimidine-biosynthetic gene cluster on an 800-kb chromosomal DNA (see Fig. 3A), and locus C in the partial pyrimidine-biosynthetic gene cluster on the same or same-sized 800-kb chromosomal DNA. Hygromycin- and tunicamycin-resistance gene cassettes (*HYG* and *TUN* cassettes, respectively) contained the 5′-upstream (1.2 kb) and the 3′-downstream (0.9 kb) sequences of the locus B (see Materials and Methods). **B** Southern hybridization patterns with a *DHOD*-specific probe for the wild-type *T. cruzi* (lanes 1, 3, and 5) and its *Δdhod::HYG* transformant (lanes 2, 4, and 6). The protozoan DNA (1 μg/lane) was digested with *Eco*RI (lanes 1 and 2), *Afl*II (lanes 3 and 4), or *Eco*RI plus *Afl*II (lanes 5 and 6), electrophoretically separated, and hybridized with the *DHOD*-specific probe. The band derived from each *DHOD* locus is indicated as A, B, or C. Weak band shown by an arrow indicates an incomplete digestion with *Afl*III plus *Eco*RI, with a size corresponding to the band of *Eco*RI digestion (lanes 1 and 2). **C** Southern hybridization patterns with an *HYG*-specific probe for the wild-type *T. cruzi* (lanes 1, 3, and 5) and *Δdhod::HYG* transformant (lanes 2, 4, and 6). The protozoan DNA (1 μg/lane) was digested and electrophoresed as in B and hybridized with the *HYG*-specific probe. The bands at 550 bp (shown by arrowheads) are derived from the *HYG* cassette by internal digestion with *Eco*RI.

vested, washed with PBS three times, and suspended in 7 m*M*. sodium phosphate (pH 7.5) containing 272 m*M* sucrose at a cell density of 2×10^7 cells/ml. This cell suspension (400 μl) and the *HYG* cassette (25 μg/50 μl TE) were placed in a disposable cuvette (0.2-cm electrode gap; Bio-Rad Laboratories) and kept on ice for 15 min prior to electroporation. Using Gene Pulser apparatus (Bio-Rad Laboratories), electroporation was performed at 300 V and 500 μF with three pulses. The time constant ranged from 35 to 80 ms for each pulse. The cells were immediately mixed with 1 ml of ice-cold LIT medium by thorough pipetting, transferred into a 25-cm^2 flask containing 3.5 ml of LIT medium, and incubated at 27°C for 2 days. The transformant (designated the *HYG* transformant) was then cultured in LIT medium supplemented with 100 μg/ml of hygromycin (Wako Pure Chemical Industries Co., Tokyo). Then the *TUN* cassette was prepared from the recombinant pBluescript II by digestion with *Sac*I/*Sal*II and introduced in the same way into the *HYG* transformant. The *HYG and TUN* double transformant was cultured in LIT medium for 2 days. The culture was then supplemented with 100 μg/ml hygromycin and 1 μg/ml tunicamycin.

Subcellular Fractionation and DHOD Assay

Freshly harvested *B. saliens* cells (1.4×10^9 cells) were suspended in 1 ml distilled water containing a protease inhibitor cocktail (Complete Mini, Roche Diagnostics), disrupted by several passages through a No. 21-gauge needle, and kept on ice for 20 min. Microscopic observation revealed no viable cells in the homoge-

nate. The homogenate was centrifuged at 1500*g* for 10 min at 4°C to remove bacterial cells and cell debris. The supernatant was centrifuged at 27,000*g* for 10 min at 4°C and the pellet was suspended in 100 µl of distilled water and designated the *B. saliens* low-speed pellet. The supernatant was further centrifuged at 150,000*g* for 60 min at 4°C. The resulting supernatant and pellet suspended in 100 µl of distilled water were defined as the *B. saliens* cytosol and high-speed pellet, respectively. For the assay of *K. pneumoniae* DHOD, the bacteria were cultured in LB medium at 37°C for 15 h, collected by centrifugation at 1500*g* for 10 min at 4°C, and washed with PBS for three times. The bacterial cell samples were suspended in PBS, transferred to a Cell Disruption Bomb (Model 4639; Parr Instrumental Co.), and disrupted at a pressure of 1500 psi. The lysate was centrifuged at 1500*g* for 5 min at 4°C to remove cell debris and then at 150,000*g* for 60 min at 4°C. The supernatant and pellet were designated the *K. pneumoniae* cytosol and menbrane fractions, respectively. DHOD activity was measured spectrophotometrically by orotate production. Briefly, 100 µl of the reaction mixture containing 1 m*M* dihydroorotate, 1 m*M* fumarate or 20 µ*M* ubiquinone-1 (Q1), and 100 m*M* potassium phosphate, pH 7.0, was preincubated at 37°C for 5 min and then mixed with 40 µg of the cell lysates, followed by further incubation for 1 min. The enzyme activity was monitored spectrophotometrically at 300 nm for fumarate using an absorption coefficient (ε) of 3.13 mM^{-1} cm^{-1} and at 287 nm for Q1 using an ε of 7.15 mM^{-1} cm^{-1} (Takashima et al. 2002).

Identification of DHOD cDNA in Euglenozoa

PCR primers were designed to amplify cDNA fragments corresponding to conserved region of DHODs, belonging to family 1A and family 2. For the enzyme of family 1A, the forward primers, CF1 (5′-GGATTCAAYCCITTYATGAAYGC-3′, according to the IUB format) and CF2 (5′-GGATTCATGGGIYTICCIAA-3′), and the reverse primers, CR1 (5′-AAGCTTTTICCIGGIACRTTIGG-3′) and CR2 (5′-AAGCTTACRTTIGCIAGIGCIGT-3′), were synthesized. For the enzyme of family 2, the forward primers, MF1 (5′-GAATTCGCIGCIGGITTYGAYAA-3′) and MF2 (5′-GAATTCATYAAYMGITAYGGITT-3′), and the reverse primers, MR1 (5′-GTCGACARICCIGGIGTRTTIGG-3′), and MR2 (5′-GTCGACACIARISWIGCICCIGC-3′), were used. Total RNA was extracted from *B. saliens*, *B. caudatus*, or *E. gracilis* with TRIZOL reagent (Invitrogen) and cDNA was synthesized in the presence of oligo(dT) primer. cDNA-PCR was performed using the outer primer set CF1/CR2 or MF1/MR2, followed by the second PCR (nested PCR) using the inner primer set CF2/CR1 or MF2/MR1. We obtained the expected PCR products from bodonids and *E. gracilis* only when CF2/CR1 and MF2/MR1 were used, respectively. The PCR products were cloned into a PCR vector (TOPO TA cloning; Invitrogen) and sequenced using an automated DNA sequencer.

Cloning of a DHOD *Gene from* Bodo saliens

The protozoan total DNA was partially digested with *Sau*3AI, ligated with *Bam*HI-restricted λ DASH II arms, and packaged using the Giga Pack III Gold Packaging Extract (Stratagene). The genomic DNA library thus constructed had a plaque forming activity of 1.7×10^5 pfu/ml, with an average insert length of 20 kb. Plaque hybridization was carried out using a DNA probe (a partial cDNA fragment of the *B. saliens DHOD*) labeled with DIG (PCR DIG probe synthesis kit; Roche Diagnostics). A single positive clone containing a 20-kb DNA insert was obtained by screening 5×10^5 plaques. The insert DNA was restricted with appropriate enzymes and subcloned into pBluescript II, and an open reading frame of the *DHOD* gene was completely sequenced.

Cloning of cDNA Encoding DHOD from Euglena gracilis *and* B. caudatus

Based on the nucleotide sequence of the PCR product derived from *E. gracilis* cDNA using the primer set MF2/MR1, we attempted to clone a full-length cDNA by 5′- and 3′-RACE. For 5′-RACE, the forward primer (5′-ACACTTTCTGAGTGTCTATT-3′) was designed to match the spliced leader (SL) sequence of *E. gracilis*. cDNA specific for the *E. gracilis DHOD* was synthesized by reverse transcription using MR1, and against this cDNA preparation, PCR was carried out using the forward and reverse primer (5′-CTCCTGGAGCTTGTTCTTGC-3′). The resulting PCR product was subcloned into TOPO TA cloning vector and sequenced. 3′-RACE for the *E. gracilis DHOD* was performed as described (Watanabe and Gray 2000) with a minor modification. cDNA was produced by reverse transcription using a oligo(dT) primer having the anchor sequence (5′-AATAAAGCGGCCGCGGATCCAA-TTTTTTTTTTTTTTTT-3′). PCR was carried out using the forward (5′-ACCTCGAGATGGAGGTGTG-3′) and reverse (5′-AATAAAGCGGCCGCGGATCCAA-3′) primers and the derived DNA fragment was subcloned and sequenced. To examine accuracy of the cDNA sequence, the full-length cDNA of *DHOD* was amplified using the forward (5′-ATGTCTGTTTTTCGAAACCA-3′) and reverse (5′-TCACGAGGATGGGGTGGGAC-3′) primers, subcloned, and sequenced.

The *B. caudatus* DHOD cDNA was cloned using RACE system. Unfortunately, 5′-RACE did not yield any bands. The 3′-RACE was conducted using the *B. caudatus* DHOD-specific primer (5′-GGAGCTGAACCTTTCGTGTC-3′) and the anchor-specific primer and the anchored cDNA as mentioned above and yielded the expected band of about 700 bp. The cDNA was subcloned into a TA vector and sequenced.

Phylogenetic Analysis

All sequence data, apart from the *B. saliens* ACT sequence and the DHOD sequences from *B. saliens* and *E. gracilis* that were obtained in this work, were collected from public databases, including genome sequencing project databases. Multiple alignments for 43 ACT sequences and for 40 DHOD and 4 related sequences (dihydropyrimidine dehydrogenase [DPYD] and putative oxidoreductase [YEIA]) were obtained with the SAM version 2.0 program (Hughey and Krogh 1996). The alignments were corrected manually. Unambiguously aligned 168 and 119 positions, respectively, from ACT and DHOD were selected and used for phylogenetic analyses. Data files for the original alignments and selected sites are available from the authors on request.

The maximum likelihood (ML), distance matrix (DM), and maximum parsimony (MP) methods for protein phylogeny were applied to the data sets using the CODEML program in PAML3.1 (Yang 1997) and PROML, PROTDIST, NEIGHBOR, PROTPARS, SEQBOOT, and CONSENSE programs in PHYLIP3.6a (Felsenstein 2002). In the ML analysis, an initial tree search was done by applying PROML with the JTT-F model for amino acid substitution process, assuming homogeneous rates across sites. Based on me best tree, a Γ shape parameter (α) of the discrete Γ-distribution with eight categories that approximates site rates was estimated by PAML. By using the α value, a further tree search with the JTT-F + Γ model with eight site-rate categories was done by PROML with a global rearrangement option, producing the final best tree. In the DM analysis, ML estimates for pairwise distances among sequences analyzed were calculated using PROTDIST, based on the Dayhoff PAM model with rate variation among sites allowed. Then the neighbor joining (NJ) tree was reconstructed from the distances using NEIGHBOR. In the MP analysis, the MP tree was searched by PROTPARS. Bootstrap

analysis for each of the three methods was performed in the same way by applying PROML, PROTDIST + NEIGHBOR, or PROTPARS to the resampled data sets produced by SEQBOOT. One hundred and 1000 resamplings were performed for the ML and the DM and MP analyses, respectively.

Results

Construction of DHOD-*Knockout* Trypanosoma cruzi

In the *T. cruzi* Tulahuen strain, the *DHOD* gene is located at the 3′-terminus of the pyrimidine-biosynthetic gene cluster on a 1000-kb and an 800-kb chromosomal DNA (Gao et al. 1999) (see Fig. 3A). In addition, a partial gene cluster containing *ACT* and *DHOD* was reported to be on an 800-kb DNA (Nara et al. 2003) (Fig. 1A). Thus, the *T. cruzi* Tulahuen strain possesses the three distinct *DHOD* loci in its nucleus, designated loci A, B, and C. To investigate the physiological role of the *T. cruzi* DHOD localized in the cytosol (Nara et al. 2000; Takashima et al. 2002), we developed a *DHOD*-knockout *T. cruzi* using the *HYG* cassette (Fig. 1). This transformant grew normally in LIT medium in the presence of hygromycin. Southern blotting using an *HYG*-specific probe showed no band corresponding to the *DHOD* locus C (lane 2 in Fig. 1C), indicating that the *HYG* cassette was not introduced into this locus, while it showed the bands corresponding to loci A and B. (Fig. 1C). These data imply that homologous recombination with the *HYG* cassette may have occurred at locus A in some protozoan cells, at locus B in other cells, and at loci A and B in some cells, of the whole *T. cruzi* cell population analyzed. There was no unexpected band due to episomal copies or due to inappropriate insertions of the *HYG* cassette (Fig. 1B and C), suggesting that the homologous recombination took place specifically at the *DHOD* loci A and B. We also established the *Δdhod::TUN* transformant by transfecting the *TUN* cassette and similar results were obtained (data not shown).

The *Δdhod::HYG* transformant was then transformed with the *TUN* cassette in order to disrupt the remaining *DHOD* loci (Fig. 2). The resulting transformant was viable at the beginning of cultivation in the presence of hygromycin and tunicamycin but failed to survive 2 weeks later. The experiment was repeated, with no resultant double-transformant established. Addition of pyrimidine nucleosides, uridine, cytidine, and thymidine (200 μ*M* each) to the medium did not enable the double-transformed parasites to grow (data not shown). Consistent with this evidence, the *HYG* and *TUN* double transformant, when harvested after 7 to 10 days in culture, expressed no detectable levels of DHOD protein

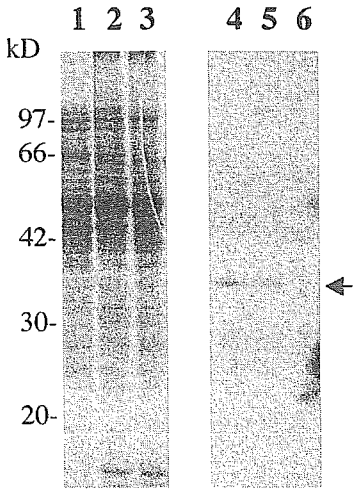


Fig. 2. Detection of dihydroorotate dehydrogenase (DHOD) from culture forms (epimastigotes) of *dhod* transformants of *Trypanosoma cruzi*. SDS-PAGE patterns of epimastigote lysates (lanes 1, 2, and 3) and the corresponding western blotting patterns (lanes 4, 5, and 6) with anti-*T. cruzi* DHOD antibody. Lanes 1 and 4, wild type; lanes 2 and 5, *HYG* transformant; lanes 3 and 6, *HYG* plus *TUN* transformant. *T. cruzi* DHOD is calculated to be 34 kD and indicated by the arrow. Samples containing 30 μg proteins were loaded onto individual lanes.

(Fig. 2). In contrast, the single (*HYG*) transformant expressed the DHOD protein at a level roughly comparable to that found in the wild-type *T. cruzi* epimastigotes. These results indicate that the loss of viability in the double transformant is due to the completely suppressed level of DHOD protein.

Cloning and Identification of Novel ACT/DHOD *Gene Fusion from* Bodo saliens

The trypanosomatid DHOD belongs to the family 1A type exceptionally in eukaryotes (Nara et al. 2000). We therefore attempted to estimate the origin of the horizontal acquisition of the family 1A *DHOD* in Euglenozoa including euglenoids and kinetoplastids (bodonids and trypanosomatids). For this purpose, nested PCR was conducted to amplify the conserved regions of *DHOD* in the euglenoid *E. gracilis* and the bodonids *B. caudatus* and *B. saliens* (see Materials and Methods for details) and resulted in amplification of the family 2-specific cDNA fragment in *E. gracilis* and the family 1A-specific cDNA in the bodonids. Using the *B. saliens*-specific cDNA fragment labeled with DIG, a genomic library of this species was screened and a single positive clone was obtained. Surprisingly, the open reading frame of the presumed *DHOD* gene was extremely long (1944 bp) (GenBank accession number AB159227) and its 5′-half encoded a putative ACT, the second enzyme of the de novo pyrimidine biosynthetic pathway (Fig. 3A). The open reading frame encoded, from the 5′-terminus, the ACT domain (933 bp), a possible

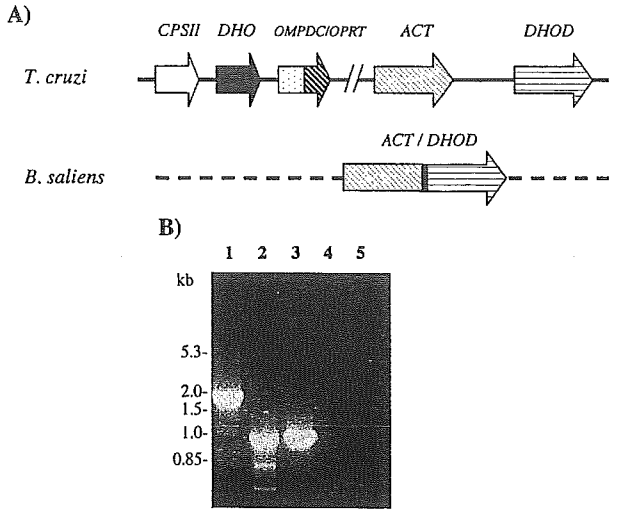


Fig. 3. The *ACT/DHOD* gene fusion in *Bodo saliens*. **A** Schematic comparison of the pyrimidine-biosynthetic genes in *Trypanosoma cruzi* and the *ACT/DHOD* gene fusion in *Bodo saliens*. *T. cruzi* has the six genes tandemly arrayed, from the 5′-terminus, *CPS II, DHO, OMPDC/OPRT, ACT*, and *DHOD* (Gao et al. 1999). *B. saliens* possesses an *ACT/DHOD* gene fusion. **B** Detection of transcripts of the *ACT/DHOD* gene fusion. Poly(A) RNA from *B. saliens* was reverse-transcribed in the presence (lanes 1, 2, and 3) or absence (lane 4) of oligo(dT) primer. PCR was then performed using the primer sets specific for the full lengths of *ACT/DHOD* (lanes 1 and 5), *ACT* (lane 2), and *DHOD* (lane 3). No band was detected without reverse transcription (lane 4). Total RNA from *K. pneumoniae* (cocultured as bait for *B. saliens*) was also reverse-transcribed using random hexamers, resulting in no positive PCR product (lane 5).

linker domain (66 bp), and the DHOD domain (945 bp). Reverse transcriptase-based PCR yielded the expected sizes of the PCR products, which corresponded to *ACT/DHOD* (including the linker portion), *ACT*, and *DHOD* (Fig. 3B). These results indicated that the *ACT/DHOD* gene fusion was not a pseudogene and was apparently transcribed.

We measured the *B. saliens* DHOD activity with fumarate or ubiquinone-1 as electron acceptor (Table 1) and showed that the cytosolic enzyme much preferred fumarate to ubiqunone-1 and that no measurable activity was associated with low- and high-speed pellet fractions. These results are diagnostic of a family 1A-type enzyme (Takashima et al. 2002). On the other hand, the *K. pneumoniae* DHOD activity was ubiquinone-dependent and associated with the membrane fraction (Table 1), consistent with the properties of a family 2 enzyme. These observations indicate that the protozoan enzyme preparations were not contaminated with the bacterial enzyme.

The deduced amino acid sequence of the *B. saliens* DHOD domain shared a higher identity with those of the enzymes in family 1A (*T. cruzi*, 59%; *L. mexicana*, 57%; *S. cerevisiae* and *Lactococcus lactis A*, 53%) than those of the enzymes in family 2 (human, 28%; rat, 29%). Figure 4 shows an alignment of the two

Table 1. Subcellular fractionation of DHOD activity in *B. saliens* and in *K. pneumoniae*

Species	Fraction	DHOD activity ($nmol^{-1}$ min^{-1} mg^{-1})[a]	
		Fumarate	Ubiquinone-1
B. saliens	Cytosol	31.7	6.87
	Low-speed pellet	N.D.[b]	N.D.
	High-speed pellet	N.D.	N.D.
K. pneumoniae	Cytosol	N.D.	N.D.
	Membrane	N.D.	29.3

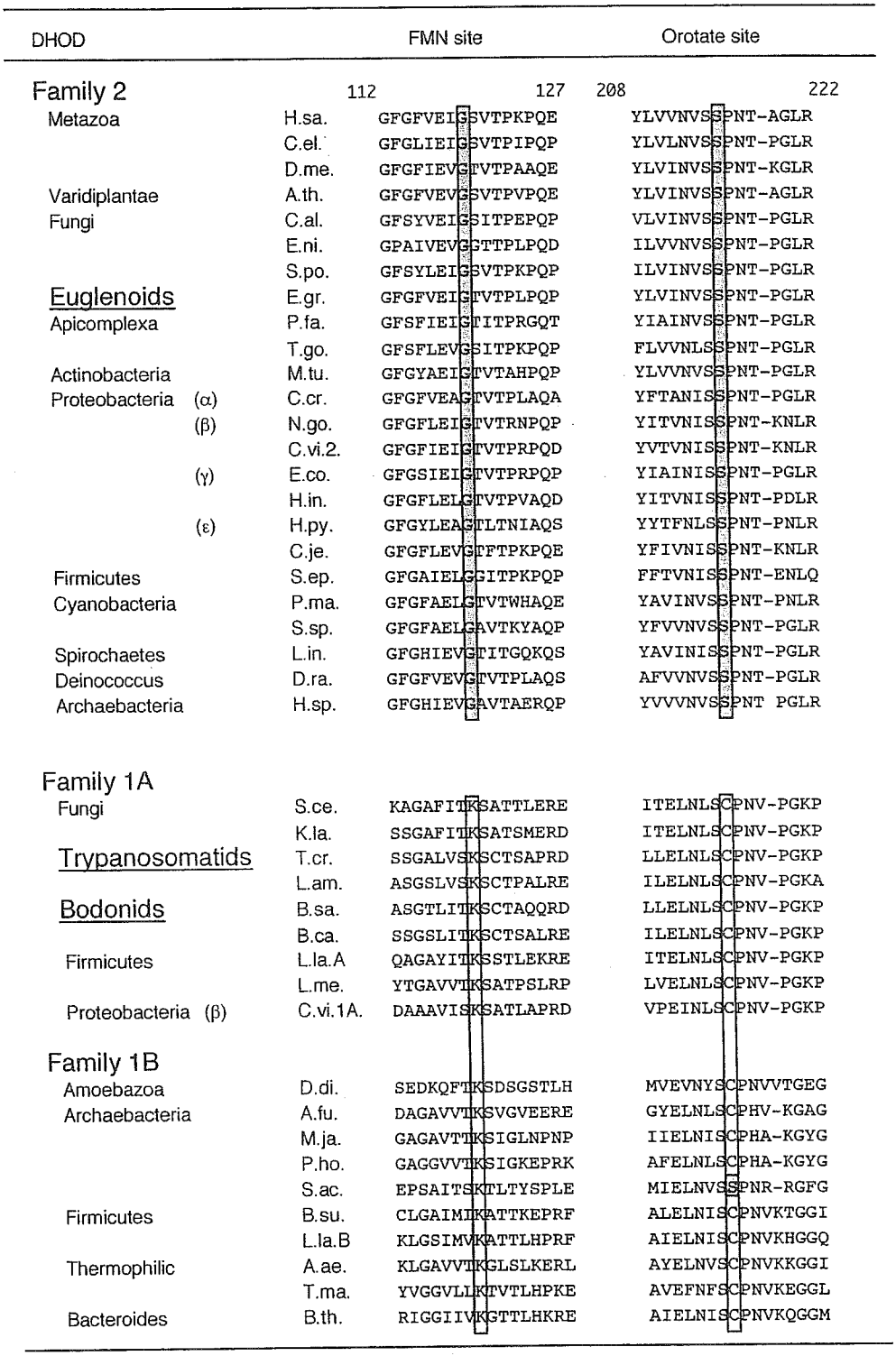
[a]DHOD activities were measured in the presence of fumarate and ubiquinone-1 as electron acceptors, respectively.
[b]Not detected.

important regions and their indispensable residues (boxed) for binding with flavin (cofactor) and orotate (product) (Nara et al. 2000). In *B. caudatus*, we were able to clone the 3′-portion of the DHOD cDNA, which includes the flavin and orotate sites. The amino acid sequences of both the *B. saliens* and the *B. caudatus* DHOD were well aligned with the enzymes in family 1A, especially with those from trypanosomatids, and the indispensable residues were completely conserved among them (Fig. 4). These results indicate that the *DHOD* genes in bodonids and in trypanosomatids share a common evolutionary origin.

Discovery of the *ACT/DHOD* gene fusion in *B. saliens* enabled us to analyze not only DHOD but also ACT in kinetoplastids. The amino acid residues constituting the active site of ACT were identified by crystallography of *Escherichia coli* ACT (Lipscomb 1994). All critical amino acid residues are conserved in the predicted *B. saliens* ACT domain and are identical among *T. cruzi*, *L. mexicana*, and *B. saliens* (data not shown).

cDNA Cloning for DHOD from Euglena gracilis

We examined whether the sister group of kinetoplastids, e.g., euglenoids, has the family 1A *DHOD* gene or the *ACT/DHOD* gene fusion. Using *E. gracilis*, which possesses introns in its genomic DNA and a mini-exon at 5′-termini in mRNA molecules, we determined the cDNA sequence encoding DHOD by 5′- and 3′-RACE. The *ACT/DHOD* gene fusion was not detected in this euglenoid. An open reading frame of this cDNA encodes 435 amino acid residues (GenBank accession number AB120414). Interestingly, the predicted amino acid sequence shared a higher identity with those of the family 2 DHODs (human, 50%; rat, 48%; *Arabidopsis thaliana*, 47%) and less identity with those of the family 1A counterparts (*T. cruzi*, 22%; *L. mexicana*, 24%; *B. saliens*, 26%; *S. cerevisiae* 20%). The MitoProt program



(http://ihg.gsf.de/ihg/mitoprot.html) showed the existence of a mitochondrial targeting signal at its N-terminus with a probability of 0.9989, characteristic in family 2 DHOD. The flavin and orotate binding sites of the *E. gracilis* DHOD were perfectly aligned with other various DHODs in family 2 (Fig. 4). We could not obtain any additional cDNA fragments encoding the family 1A or family 1B DHOD in this experiment. These data indicate that, in contrast to the bodonids, *E. gracilis* carries the family 2 *DHOD* gene.

DHOD Phylogeny

The overall structures of the DHOD trees inferred by three methods, ML, DM, and MP, were almost the

same as a previously published one (Nara et al. 2000) in which the phylogenetic relationships reflect the cellular localization and catalytic properties of DHOD but are discordant with accepted taxonomic relationships (Fig. 5). When DPYD and YEIA sequences were used as outgroups, family 2 and family 1A each formed a monophyletic clade with clear BP support for all methods used, but no support was obtained for the monophyly of family 1B. As expected from its amino acid sequence, *B. saliens* was positioned in the kinetoplastid clade with moderate BP support. The close affinity of *B. saliens* with trypanosomatids (*Trypanosoma* and *Leishmania*) is consistent with the trees obtained for SSUrDNA (Fernandes et al. 1993) and HSP90 (Simpson et al. 2002). In contrast, the *E. gracilis* enzyme clustered in the eukaryotic clade within the family 2 subtree.

Although there was no clear BP support for their monophyletic origin, DHOD sequences from diverse eukaryotic lineages including Metazoa, Fungi, Viridiplantae, euglenoids, and Apicomplexa are all clustered within the family 2 eukaryotic clade, suggesting that eukaryotes originally had the family 2 enzyme. If this was the case, then Euglenozoa might have had the family 2 DHOD and a common ancestor of kinetoplastids had acquired the family 1A gene by a later horizontal transfer.

ACT Phylogeny

Three phylogenetic methods, ML, DM, and MP, consistently demonstrated with high BP support that ACT sequences are clearly divided into two groups A and B (Fig. 6). Group A comprises all eukaryotes and several prokaryotic lineages including proteobacteria and archaebacteria, while group B comprises eubacterial lineages including firmicutes and proteobacteria. The separation of the two groups was clearly supported also by the alignment with some insertion/deletions and some conserved amino acid residues shared only by one of the group (data not shown). Within group A the ML and DM trees reconstructed the monophyly of eukaryotes with only low support. The MP tree did not reconstruct the monophyly of eukaryotes, with *Leptospira* located as a sister group to the common ancestor of Viridiplantae and *Chlorella* virus (data not shown). The kinetoplastids clearly form a monophyletic clade within the eukaryotic subtree with high BP values (97, 94, and 88% for ML, DM, and MP) including the ACT domain encoded by the *B. saliens ACT/DHOD* gene. The position of *B. saliens* within the kinetoplastid subtree was consistent with the DHOD tree and also with the SSUrRNA and HSP90 phylogenies. The kinetoplastid subtree indicates that the ACT and DHOD genes might have fused in *B. saliens* or in the bodonid lineage. Cloning and further analysis of ACT and DHOD genes in other bodonid species are necessary to place the root of the *ACT/DHOD* gene fusion.

In Metazoa, Fungi, and *Dictyostelium*, the first three enzymes in the de novo pyrimidine biosynthetic pathway are fused into a multienzyme protein, CAD, in the order CPSII, DHO, and ACT. In Fungi, the DHO domain is inactive and the DHO activity is carried by an additional enzyme. Since kinetoplastids are distantly related to Metazoa, Fungi, and *Dictyostelium* (Baldauf et al. 2000; Bapteste et al. 2002), our findings may suggest that the kinetoplastid ACT is an ancient form of the enzyme that gave rise to a part of the multienzyme (CAD). However, we cannot rule out the possibility that the fused gene of the multienzyme was already present in the common ancestor of all eukaryotes and was secondarily separated into CPSII, DHO, and ACT on the branch leading to the common ancestor of Euglenozoa and other protist lineages.

Fig. 4. Comparison of characteristic regions of amino acid sequences of dihydroorotate dehydrogenase (DHOD) in nature. Indispensable residues (shaded and open boxes) for binding with FMN and orotate are different between membrane-bound (shaded boxes) and cytosolic (open boxes) DHODs, i.e., the enzymes in family 2 and in families 1A and 1B, respectively. Abbreviations of species: H.sa., *Homo sapiens*; C.el., *Caenorhabditis elegans*; D.me., *Drosophila melanogaster*; A.th., *Arabidopsis thaliana*; C.al., *Candida albicans*; E.ni., *Emericella nidulans*; S.po., *Schizosaccharomyces pombe*; E.gr., *Euglena gracilis*; P.fa., *Plasmodium falciparum*; T.go., *Toxoplasma gondii*; M.tu., *Mycobacterium tuberculosis*; C.cr., *Caulobacter crescentus*; N.go., *Neisseria gonorrhoeae*; C.vi.2, *Chromobacterium violaceum*; E.co., *Escherichia coli*; H.in., *Haemophilus influenzae*; H.py., *Helicobacter pylori*; C.je., *Campylobacter jejuni*; S.ep., *Staphylococcus epidermidis*; P.ma., *Prochlorococcus marinus*; S.sp., *Synechocystis* sp.; L.in., *Leptospira interrogans*; D.ra., *Deinococcus radiodurans*; H.sp., *Halobacterium* sp.; S.ce., *Saccharomyces cerevisiae*; K.la., *Kluyveromyces lactis*; T.cr., *Trypanosoma cruzi*; L.am., *Leishmania amazonensis*; B.sa., *Bodo saliens*; B.ca., *Bodo caudatus*; L.la.A., *Lactococcus lactis A*; L.me., *Leuconostoc mesenteroides*; C.vi.1A, *Chromobacterium violaceum*; D.di., *Dictyostelium discoideum*; A.fu., *Archaeoglobus fulgidus*; M.ja., *Methanococcus jannaschii*; P.ho., *Pyrococcus horikoshii*; S.ac., *Sulfolobus acidocaldarius*; B.su., *Bacillus subtilis*; L.la.B., *Lactococcus lactis B*; A.ae., *Aquifex aeolicus*; T.ma., *Thermotoga maritima*; B.th., *Bacteroides thetaiotaomicron*. Accession numbers for these sequences are shown in Fig. 5, except for *C. violaceum* (Nos. NP_903221 and NP_901482 for C.vi.1A and C.vi.2, respectively).

Discussion

All species of trypanosomatids are parasitic and phylogenetically form a monophyletic clade, while bodonids, a sister group of trypanosomatids within the kinetoplastids, are free-living organisms but contain a few parasitic species (Fernandes et al. 1993; Simpson et al. 2000, 2002). The paraphyly of bodonids and the later branching of trypanosomatids suggest that the trypanosomatids might have evolved

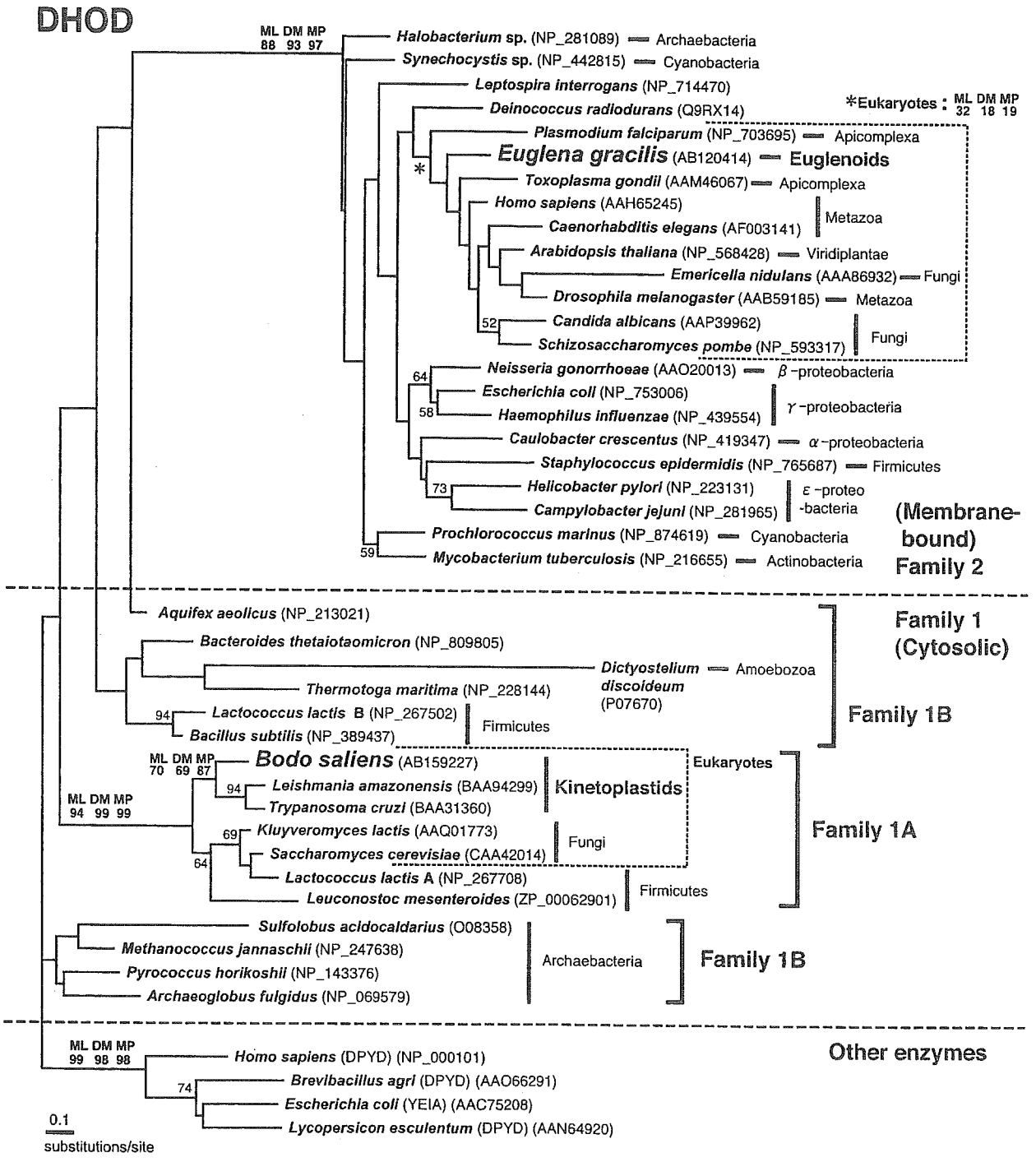


Fig. 5. The maximum likelihood (ML) tree of the dihydroorotate dehydrogenase (DHOD) and related sequences inferred by the JTT-F + Γ model with across-site rate heterogeneity taken into consideration. The α-value of the Γ shape parameter used in the analysis is 0.9638. Bootstrap proportions (BPs) by the ML method are attached to the internal branches. Unmarked branches have < 50% BP. For the four nodes of interest, BP values by the distance matrix (DM) and maximum parsimony (MP) methods are also shown. The length of each branch is proportional to the estimated number of substitutions. Unambiguously aligned 119 amino acid sites were used for the analysis. These correspond to residues 343–359, 372–377, 385–392, 399–409, 435–438, 441–450, 460–468, 497–503, 526–532, 554–565, and 581–608 of the *B. saliens* sequence.

from a bodonid species (or its ancestor). We hypothesized that the gene(s) acquired secondarily in a common ancestor of kinetoplastids had played a role not only in the primary adaptation of bodonids but also in the subsequent parasitic adaptation of trypanosomatids. To test this hypothesis, we focused on the kinetoplastid DHOD for the following reasons. First, the family 1A DHOD is essential for a facultatively anaerobic yeast to grow under anaerobic conditions (Nagy et al. 1992). Second, a phylogenetic study suggested that the kinetoplastids may have acquired horizontally the family 1A *DHOD* gene

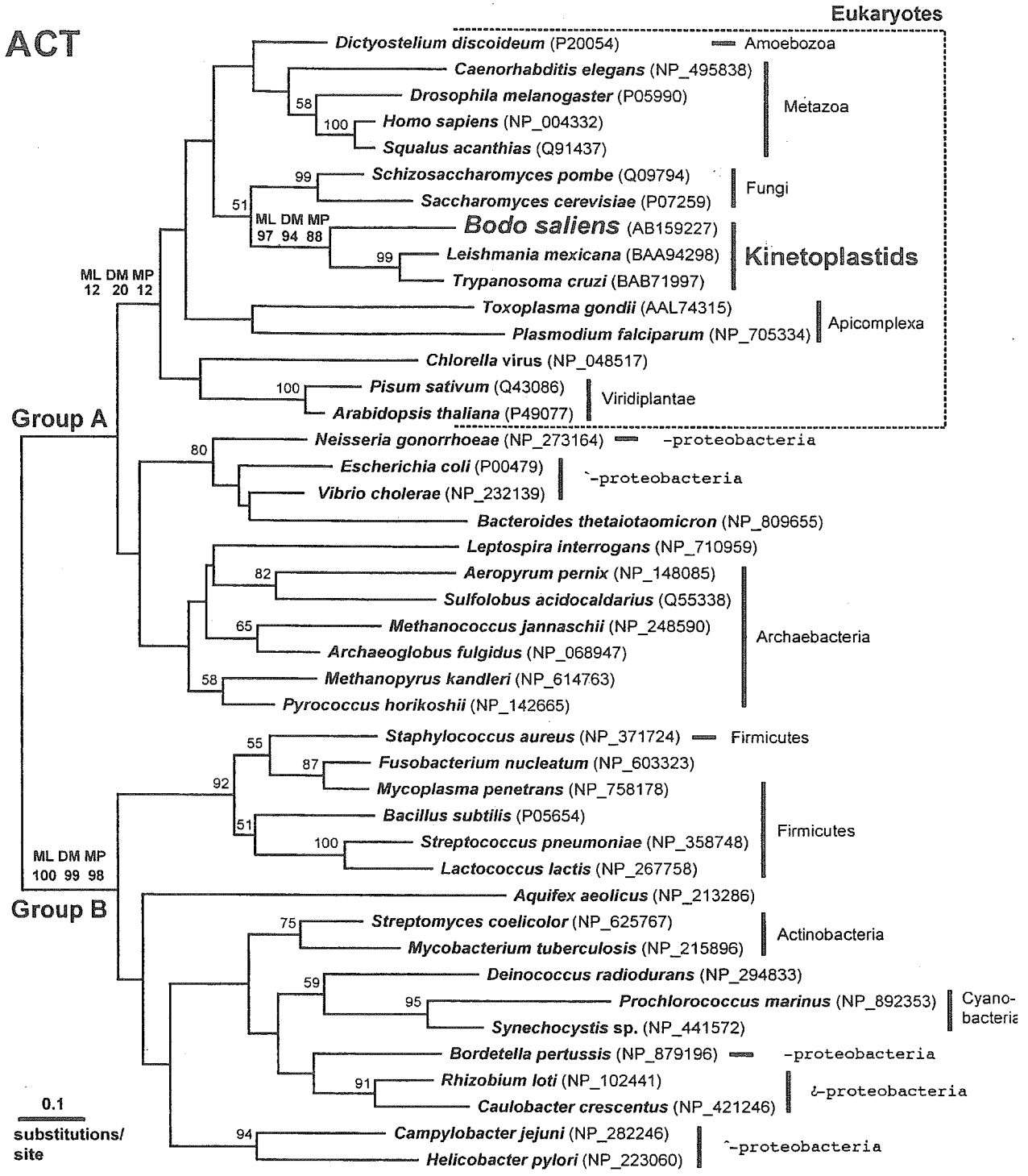


Fig. 6. The maximum likelihood (ML) tree of the aspartate carbamoyltransferase (ACT) sequences inferred by the JTT-F + Γ model with across-site rate heterogeneity taken into consideration. The α-value of the Γ shape parameter used in the analysis is 0.6681. Bootstrap proportions (BPs) by the ML method are attached to the internal branches. Unmarked branches have < 50% BP. For the three nodes of interest, BP values by the distance matrix (DM) and maximum parsimony (MP) methods are also shown. The length of each branch is proportional to the estimated number of substitutions. Unambiguously aligned 168 amino acid sites were used for the analysis. These correspond to residues 13–31, 51–79, 84–88, 90–104, 106–112, 129–152, 160–181, 191–194, 224–230, 232–241, 269–275, 279–284, and 295–307 of the *B. saliens* sequence.

(Nara et al. 2000). Third, the family 1A DHOD indeed functions in *T. cruzi* as a soluble enzyme with fumarate reductase activity, utilizing fumarate as electron acceptor (Takashima et al. 2002). Lastly, bodonids are distributed in the diverse and extreme place including deep-sea hydrothermal vents (Atkins et al. 2000), suggesting that they may survive in anaerobic environments.

Biological Significance of the Family 1A DHOD in Trypanosoma cruzi

The present study demonstrated that DHOD is essential for survival and growth of *T. cruzi*, as shown by the *HYG* and *TUN* double transformant of this organism. In this case, at least two of the three *DHOD* loci were disrupted, thus suppressing DHOD protein expression in *T. cruzi* Tulahuen strain. Pyrimidine nucleosides, substrates for the potentially active salvage pathway, could not complement the suppressed DHOD expression. Fox and Bzik (2002) previously demonstrated that de novo pyrimidine biosynthesis is essential for in vivo growth of an apicomplexan parasite, *Toxoplasma gondii*. These findings correlate well with the fact that *T. gondii* lacks many potential salvage enzyme activities and is able to incorporate only uracil as a pyrimidine precursor. In contrast, Gutteridge and Gaborak (1979) demonstrated that all three developmental forms of *T. cruzi* were capable of taking up exogenous pyrimidine bases and nucleosides; the cultured epimastigotes, in particular, could rely on exogenously added pyrimidines for the growth. This striking contrast in the salvage ability between the two species thus highlights the essential role of the "bifunctional" DHOD in *T. cruzi*. It is likely that the incorporated pyrimidines could bypass the suppressed pyrimidine-biosynthetic DHOD activity but could not bypass the suppressed fumarate reductase activity, another important function of the family 1A DHOD, strongly suggesting that the fumarate reductase activity was needed to maintain the cellular redox balance for their survival.

Evolutionary Origin of the Family 1A DHOD in Kinetoplastids

Phylogenetic analysis showed a monophyly of the family 1A DHOD, including *B. saliens* and trypanosomatid species, while the euglenoid *E. gracilis* is grouped within the family 2 clade (Fig. 5). Although we cannot rule out the possibility that euglenoids possessed and have lost the family 1A DHOD, these results may imply that the family 1A DHOD has been acquired by lateral gene transfer. In addition to the family 1A DHOD sequences shown in Fig. 5, we have recently found a β-proteobacterial (*Chromobacterium violaceum*) sequence (Fig. 4) and five Saccharomycetaceae sequences (data not shown). Interestingly, *C. violaceum* possesses both family 1A and family 2 DHOD (Fig. 4). Since the proteobacterial DHOD is a family 2 type already examined, the most likely explanation is that the family 1A DHOD has been laterally transferred to *C. violaceum*. Although taxon sampling is still limited, distribution of the family 1A DHOD in phylogenetically distant groups of both prokaryotes and eukaryotes is obvious and attributable to horizontal gene transfer across the kingdoms.

At present, it is difficult to locate the root of the family 1A subtree and to verify lateral gene transfer from prokaryotes to eukaryotes, and vice versa. Weak association of family 1A with the archaeal family 1B may render it possible that the eukaryotic family 1A is a descendant of family 1B. However, the facts that Fungi and kinetoplastids are distantly related groups and that there are no known examples of coexistence of the family 1A and 1B DHOD in eukaryotes strongly suggest a lateral gene transfer rather than a vertical inheritance of the ortholog. The evidence that the presence of the family 1A DHOD in Fungi is very limited and restricted to the subgroup Saccharomycetaceae also supports this hypothesis. Thus, the most likely scenario is that the family 1A DHOD evolved from the family 1B ortholog (probably in anaerobic prokaryotes) and then was laterally transferred independently to the ancestors of kinetoplastids and Saccharomycetaceae. Efforts to collect more taxons from both prokaryotes and eukaryotes would allow us to establish the root of the family 1A DHOD subtree.

Evolutionary Implication of DHOD in Anaerobiosis in Euglenozoa

Since the family 1A DHOD is essential for de novo pyrimidine synthesis in anaerobically growing yeast (Nagy et al. 1992), the discovery of the family 1A *DHOD* gene in *B. saliens* supports the idea that a possible adaptation to anaerobic environmental conditions may have happened in a common ancestor of kinetoplastids. Bloodstream forms of *T. brucei* lack the classic TCA cycle but express the mitochondrial alternative oxidase for the regeneration of NADH derived from glycolysis (Chaudhuri and Hill 1996; Opperdoes et al. 1977). Glycerol-3-phosphate dehydrogenase, as well as an alternative oxidase, oxidizes NADH, resulting in the production of glycerol as well as pyruvate (Clayton and Michels 1996; Tielens and Van Hellemond 1998). Promastigotes of *Phytomonas* produce anaerobically ethanol as well as glycerol via fermentation (Chaumont et al. 1994). In contrast, *T. cruzi* and *Leishmania* spp. are dependent on respiration but produce a variety of end products, such as acetate, pyruvate, and succinate (Clayton and Michels 1996; Tielens and Van Hellemond 1998). Since the family 2 DHOD uses preferentially ubiquinone as electron acceptor, acquisition of the ubiquinone-independent, family 1A DHOD may have enabled an ancestral kinetoplastid to adapt to anaerobiosis and possibly enabled a trypanosomatid (or its ancestor) to establish the initial step(s) of

parasitism. It is noteworthy that our preliminary experiment on *T. cruzi* growth showed that the cultured epimastigotes were able to proliferate rapidly at low oxygen tensions of less than 0.1% O_2 and at 5% CO_2 (Annoura and Nara, unpublished observation).

The *E. gracilis* DHOD is a family 2-type enzyme (Figs. 4 and 5) and positioned within the monophyletic clade of eukaryotes. This is inconsistent with the fact that *E. gracilis* ferments and proliferates under anaerobic conditions (Inui et al. 1982) but is explainable by the presence of a quinol-dependent fumarate reductase. *Escherichia coli* possesses the family 2 DHOD and two types of quinones, ubiquinone and menaquinone. Under anaerobic conditions, menaquinone functions as electron acceptor for DHOD (fumarate reductase), despite its lower electron potential (E'_m = −74 mV; ubiquinone, +110 mV) (Newton et al. 1971). The derived hydroquinones are then re-oxidized by the membrane-bound, quinol–fumarate reductase, leading to the production of succinate. Since *E. gracilis* has rhodoquinone, a low-redox potential quinone (E'_m = −63 mV) which resembles menaquinone, and produces succinate under anaerobic conditions (Powls and Hemming 1966; Schneider and Betz 1985), it is possible that *E. gracilis* utilizes a membrane-bound, rhodoquinone–fumarate reductase under anaerobic conditions in order to produce orotate via DHOD.

Our findings provide not only the molecular basis of unique anaerobic adaptation by secondary acquisition of the family 1A *DHOD* gene in kinetoplastids leading to the establishment of parasitism in trypanosomatids, but also the different trait for establishing anaerobic de novo pyrimidine biosynthesis in the closely related euglenoids. We conclude that anaerobiosis requires appropriate electron acceptors for DHOD activity but is governed by different redox systems in euglenoids and in kinetoplastids.

Novel ACT/DHOD *Gene Fusion*

We demonstrated an *ACT/DHOD* gene fusion in *B. saliens* not observed before (Fig. 3). Both ACT and DHOD phylogenies clearly demonstrated the monophyly of kinetoplastids, indicating a common evolutionary origin of both ACT and DHOD. To know whether the separate genes have fused in bodonids or the fused genes have split in kinetoplastids, we attempted to clone the *DHOD* gene of another bodonid, *B. caudatus*. Using a RACE system, we obtained the 3′ half of the DHOD but failed to amplify the 5′ half. Because of this, it is difficult to determine from this study whether the *ACT/DHOD* gene fusion is a characteristic feature in *B. saliens* or an ancient form of the separate genes in trypanosomatids. Phylogenetic analysis of HSP90 in the bodonids revealed that they are paraphyletic and that *B. saliens* and *B. caudatus* belong to different clades (Simpson et al. 2002). If *B. caudatus* has the *ACT/DHOD* gene fusion, theoretically, a secondary gene split has to be postulated in the trypanosomatid lineage.

Why and how have the genes for the "second" and "fourth" pyrimidine-biosynthetic enzymes fused? The animal CAD (the CPS II–DHO–ACT fusion) comprises the first, second, and third enzymes of the pathway, and channeling of the substrate between the catalytic domains is postulated (Carrey 1995; Irvine et al. 1997). If the bodonid ACT/DHOD fusion exists as a bifunctional protein, the substrate channeling could not be carried out without assembly of the third enzyme, DHO. Further biochemical studies are needed to clarify the nature of the translation product of the *ACT/DHOD* gene fusion. In the pyrimidine-biosynthetic gene cluster of *T. cruzi* and *L. mexicana*, the *ACT* and *DHOD* genes are juxtaposed (Nara et al. 2003) (see Fig. 3A). Theoretically, deletion of the intervening sequence between the juxtaposed genes might yield the gene fusion, and vice versa. The evolutionary history of the trypanosomatid pyrimidine-biosynthetic gene cluster is still unknown, and thus, the pyrimidine-biosynthetic gene organization in bodonids, if any, would provide an insight into the nature of the gene clustering for all the de novo pyrimidine biosynthetic enzymes in the kinetoplastids.

In conclusion, we demonstrated the biological significance of the family 1A DHOD in *T. cruzi*. Especially, the fumarate reductase activity of this enzyme may play an essential role, coupled with pyrimidine biosynthesis, in controlling the cellular redox state. In addition to the trypanosomatids, the bodonids possess the family 1A *DHOD* gene and fumarate-dependent DHOD activity. The horizontal acquisition of the family 1A *DHOD* gene probably enabled anaerobic pyrimidine biosynthesis in a common ancestor of kinetoplastids, possibly allowing further establishment of different types of anaerobic energy metabolism in the trypanosomatid lineages that may have been unrelated to the de novo pyrimidine biosynthesis. The euglenoids may have preceded another evolutionary tract, in which the production of rhodoquinone might be a key event to utilize the family 2 DHOD under anaerobic conditions. Thus, the strategies for adaptation to anaerobic pyrimidine biosynthesis are different between kinetoplastids and euglenoids in Euglenozoa.

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Inhibitory action of marine algae extracts on the *Trypanosoma cruzi* dihydroorotate dehydrogenase activity and on the protozoan growth in mammalian cells

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Abstract

Trypanosoma cruzi, the causative agent of Chagas' disease, replicates in mammalian cells and relies on the de novo pyrimidine biosynthetic pathway that supplies essential precursors for nucleic acid synthesis. The protozoan dihydroorotate dehydrogenase (DHOD), the fourth enzyme of the pathway catalyzing production of orotate from dihydroorotate, markedly differs from the human enzyme. This study was thus aimed to search for potent inhibitors against *T. cruzi* DHOD activity, and a number of methanol extracts prepared from green, brown, and red algae were assayed. The extracts from two brown algae, *Fucus evanescens* and *Pelvetia babingtonii*, yielded 59 and 58% decrease in the recombinant DHOD activity, respectively, at the concentration of 50 μg/ml. Inhibition by these extracts was noncompetitive with respect to dihydroorotate, with apparent K_i values of 35.3±5.9 and 10.3±4.4 μg/ml, respectively. Further, in an in vitro *T. cruzi*–HeLa cell infection system, ethanol-reconstituted *F. evanescens* and *P. babingtonii* extracts at the concentration of 1 μg/ml, respectively, decreased significantly the infection rate of host cells and the average parasite number per infected cell. These results imply that *F. evanescens* and *P. babingtonii* contain inhibitor(s) against the *T. cruzi* DHOD activity and against the protozoan infection and proliferation in mammalian cells. Identification of inhibitor(s) in these two brown algae and further screening of other marine algae may facilitate the discovery of new, anti-trypanosomal lead compounds.
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Keywords: *Trypanosoma cruzi*; De novo pyrimidine biosynthesis; Dihydroorotate dehydrogenase; Marine algae extracts

1. Introduction

Chagas' disease, endemic in Central and South America, is one of the important tropical diseases and is caused by infection of a flagellated protozoan, *Trypanosoma cruzi*, transmitted by sucking bugs. While medication is usually effective when given during the acute phase, once the disease enters into the chronic phase, no medication is proven effective [1]. Therefore, development of chemotherapeutic drugs is urgently needed.

Molecular identification and characterization of enzymes and metabolic pathways that are essential and distinct in *T. cruzi* provide the greater potential of the primary targets for screening a number of bioresources in vitro in the search for a new generation of chemotherapy [2]. Pyrimidine biosynthesis is an essential biological activity for supplying nucleotide precursors to RNA and DNA syntheses in all living organisms and is conducted by the de novo and salvage pathways. *T. cruzi* possesses both the pathways, but the balance between de novo and salvage activities varies in different developmental stages of the parasite; it is likely that amastigotes essentially rely on the de novo pyrimidine biosynthesis in vivo [3]. The de novo pathway comprises six sequential steps of enzymatic

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reactions. Dihydroorotate dehydrogenase (DHOD) is the fourth enzyme that catalyses orotate formation from dihydroorotate and transfers the resulting electrons to appropriate acceptors. Previously, we cloned *T. cruzi DHOD* gene that occurs in the pyrimidine-biosynthetic gene cluster [4]. Phylogenetic analysis of amino acid sequences of various DHODs revealed that *T. cruzi* DHOD differs significantly from human DHOD [5]. The former localizes in the protozoan cytosol and utilizes preferentially fumarate as electron acceptor, while the latter localizes in the inner membrane of mitochondria and uses ubiquinone as electron acceptor [6]. In addition, *T. cruzi* DHOD is responsible for approximately 40% of total cellular fumarate reductase activity. We recently demonstrated that the DHOD gene-knockout *T. cruzi* could not survive even in the presence of pyrimidine precursors, indicating that DHOD is essential in the parasite and plays important roles not only in the de novo pyrimidine biosynthesis but also in the protozoan cellular redox balance [7]. From these views, the parasite DHOD would be a good target for the development of drugs against Chagas' disease.

Recently, marine algae have been highlighted as resources that contain a variety of biologically active compounds, with antibacterial, antitumor, and immunostimulating activities [8–11]. The present study was aimed to screen various marine algae for their inhibitory effects on the recombinant *T. cruzi* DHOD activity. Two methanol extracts from brown algae, *Fucus evanescens*, and *Pelvetia babingtonii*, showed significant decreases in the DHOD activity in noncompetitive manner with respect to the substrate, dihydroorotate. Further, these two extracts yielded significant inhibitions of the *T. cruzi* infection and proliferation in cultured mammalian cells.

2. Materials and methods

2.1. Parasite and host cells

HeLa cells, a human cancer cell line, were infected by the mammalian stages of *T. cruzi* Tulahuen strain [12]. Time courses of *T. cruzi* infection and proliferation in HeLa cells were as described [13]. HeLa cells or the cells infected with *T. cruzi* were inoculated at an initial cell density of $3–5\times10^5$/ml into Eagle's Minimum Essential Medium (MEM, Sigma-Aldrich Japan) supplemented with 10% fetal bovine serum (FBS) in 25-cm^2 culture flasks and subcultured every 3–4 days at 37 °C and at 5% CO_2 in air. Trypomastigotes, the infective form of *T. cruzi*, were collected from the preceding subculture of the infected HeLa cells. The medium of this subculture was recovered into 15-ml polypropylene tubes and centrifuged at $800\times g$ for 5 min at 4 °C to remove the host cells and cell debris. The resulting supernatant was then centrifuged at $1500\times g$ for 10 min at 4 °C and the pellet containing trypomastigotes was washed three times with 10 ml of MEM by repeated suspension and centrifugation. The purified trypomastigotes were counted on an improved Neubauer hemacytometer and verified to contain less than 10% of amastigotes, the replicating form of the parasite. Epimastigotes, the insect stage of *T. cruzi*, were routinely subcultured every 1 week in a serum-free medium (5.0 ml), GIT (Nippon Seiyaku, Tokyo), containing 10 μg/ml of hemin (Sigma-Aldrich Japan) by seeding epimastigotes at an initial density of 5×10^5/ml in tightly capped 25-cm^2 culture flasks at 26 °C.

2.2. Preparation of algae extracts

Seventy-nine different marine algae samples consisting of one seaweed, six green algae, 20 brown algae, and 52 red algae were collected along the coastline of Japan (Table 1). Preparation of algae extracts was essentially as described [14]. Briefly, wet alga sample (5.0 g) was homogenized with four volumes of phosphate-buffered saline (PBS, pH 7.2). After sedimentation by centrifugation, the pellet was suspended in four volumes of methanol by thorough pipetting and filtrated through a disposable filter unit with a pore size of 0.22 μm and stored at −20 °C until use. This preparation is designated as methanol extract. Methanol extracts from two brown algae, *F. evanescens* and *P. babingtonii*, were dried up using an evaporator and the resulting pellets were dissolved in ethanol to give a concentration of 20 mg/ml, followed by storage at −20 °C until use. These are designated as ethanol extracts and used to examine their effects on *T. cruzi* infection and proliferation in cultured mammalian cells.

2.3. Preparation of the recombinant T. cruzi DHOD

Preparation of the recombinant *T. cruzi* DHOD was carried out as described with minor modifications [6]. Briefly, the open reading frame of *T. cruzi DHOD* gene carried by the recombinant phage DNA (clone No. 4, in Ref. [15]) was subcloned into an expression vector, pET28a (Novagen). The strain BL21 (DE3) of *Escherichia coli* (Novagen) was transformed by the resulting plasmid DNA. Induction of expression was carried out by 1 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries, Tokyo) for 2 h. The recombinant DHOD having His6-tag at its N-terminus was purified from the cytosolic fraction of the bacteria using a column packed with TALON® metal affinity resins (CLONTECH Laboratories). The column was washed with 100 mM NaCl/5 mM imidazole/40 mM Tris, pH 8.0, and the bound proteins were eluted with 100 mM NaCl/100 mM imidazole/40 mM Tris, pH 8.0. This buffer in the protein eluate was exchanged with 50 mM potassium phosphate, pH 8.0, using a PD-10 column (Amersham Bioscience). The purity of this affinity-purified recombinant DHOD was more than 95% when judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The specific activity and kinetic constants of the recombinant DHOD

Table 1
List of marine algae used in this study

Seaweeds (1)	1. *Phyllospadix iwatensis*	
Green algae (6)	2. *Chaetomorpha aerea*	3. *Chaetomorpha moniligera*
	4. *Entermorpha intestinalis*	5. *Enteromorpha linza*
	6. *Ulothrix flacca*	7. *Ulva pertusa*
Brown algae (20)	8. *Alaria crassifolia*	9. *Alaria praelonga*
	10. *Analipus japonicus*	11.*Coilodesme japonica*
	12. *Costraia costata*	13. *Cystoseira hakodatensis*
	14. *Dictyopteris divaricata*	15. *Dictyota dichotoma*
	16. *Fucus evanescens*	17. *Hydroclathrus clathratus*
	18. *Laminaria japonica*	19. *Laminaria longissima*
	20. *Laminaria ochotensis*	21. *Laminaria religiosa*
	22. *Pelvetia babingtonii*	23. *Pseudochorda nagaii*
	24. *Sargassum confusum*	25. *Sargassum horneri*
	26. *Sargassum thunbergii*	27. *Undraia pinnatifida*
Red algae (52)	28. *Ahnfeltia fastigiata*	29. *Ahnfeltiopsis flabelliformis*
	30. *Alatocladia modesta*	31. *Bossiella cretacea*
	32. *Capopeltis affinis*	33. *Ceramiun boydenii*
	34. *Ceramiun japonicum*	35. *Ceramiun kondoi*
	36. *Chondrus armatus*	37. *Chondrus crispus*
	38. *Chondrus nipponicus*	39. *Chondrus ocellatus*
	40. *Chondrus pinnulatus*	41. *Chondrus yendoi*
	42. *Chondrus yendoi f. subdichotomus*	43. *Chondria crassicaulis*
	44. *Chordaria flagelliformis*	45. *Chrysymenia wrightii*
	46. *Constatinea rosa-marina*	47. *Corallina pilulifera*
	48. *Dasya sessilis*	49. *Desmarestia ligulata*
	50. *Desmarestia virdis*	51. *Farlowia mollis*
	52.*Gelidium elegans*	53. *Gloiopeltis furcat*
	54. *Gracilaria vermiculophylla*	55. *Grateloupia divaricata*
	56. *Grateloupia okamurae*	57. *Grateloupia turuturu*
	58. *Laurencia nipponica*	59. *Lomentaria hakodatensis*
	60. *Mastocarpus pacificus*	61. *Masudaphycus irregulae*
	62. *Mazzaella japonica*	63. *Nemalion vermiculare*
	64. *Neodilsea yendoana*	65. *Neoholmesia japonica*
	66. *Neohypophyllum middendorfii*	67. *Neoptilota asplenioides*
	68. *Neorhodomela aculeate*	69. *Odonthalia corymbifera*
	70. *Odonthalia macrocarpa*	71. *Pneophyllum zostericola*
	72. *Polysiphonia morrowii*	73. *Polysiphonia senticulosa*
	74. *Porphyra variegata*	75. *Ptilota filicina*
	76. *Ptilota phacelocarpoides*	77. *Rhodomela teras*
	78. *Symphyocladia latiuscula*	79. *Tichocarpus crinitus*

did not differ significantly, with the presence or absence of His6-tag [6]. The final DHOD preparation (18.0 mg), obtained from 500 ml of the transformant *E. coli* culture, was stored at −80 °C until use.

2.4. DHOD assay

DHOD activity was measured by orotate production [16] in the presence of dihydroorotate as substrate and of fumarate as electron acceptor. The algae extract or methanol (control) (10 μl each) was added to 1.0 ml of the reaction mixture containing 1 mM dihydroorotate/1 mM fumarate/50 mM potassium phosphate, pH 7.0, and pre-incubated at 37 °C for 5 min. Reaction was started by addition of 2 μg of the recombinant DHOD and the enzyme activity was monitored spectrophotometrically at 290 nm (ϵ=5.92 $mM^{-1}cm^{-1}$). Methanol at the final concentration of 1.0% had no inhibitory effect on the DHOD activity.

2.5. Inhibition of T. cruzi growth in HeLa cells

In vitro infection of the host HeLa cells by *T. cruzi* trypomastigotes was as essentially described [13]. In brief, a round coverslip was placed into each well of 24-well plate. Exponentially growing HeLa cells were inoculated into 24 wells (5×10^3 cells in 1 ml per well), followed by incubation for 2 days, and infected with 5×10^4 trypomastigotes. Ten microliters of ethanol extract from brown algae, *F. evanescens* or *P. babingtonii*, or ethanol alone (control) was added to each well immediately after the addition of trypomastigotes. Four days later, the host cells attached to the coverslip were washed 3 times with PBS, pH 7.2, fixed with methanol, and stained with Diff-quick (Kokusai Seiyaku). The percentage of the infected cells and the average number of amastigotes per infected cell were determined microscopically, in which more than 200 host cells were analyzed in each of four microscopic fields randomly selected. Cytotoxicity by algae extracts against the host cell was observed at the concentration of 100 μg/ml. To determine the growth inhibition of *T. cruzi* epimastigotes, the protozoan cells (5×10^5/well) were placed into each well containing GIT medium (1 ml) on 24-well plates, followed by addition of 10 μl of the alga ethanol extract or ethanol alone (control) and by incubation at 26 °C for 5 days. Ethanol at the final concentration of 1% had no inhibitory effect on *T. cruzi* infection and growth in mammalian cells and on the growth of epimastigotes. The living parasites were counted on an improved Neubauer hemacytometer. Statistical analysis was carried out using Fisher's PLSD methods for Post-Hoc test using the program StatView™ version 4.0 (Abadus Concepts).

3. Results

3.1. Effects of algae extracts on the T. cruzi DHOD activity

To examine the inhibitory effects of marine algae extracts on the *T. cruzi* DHOD activity, we established an assay system using the recombinant DHOD that displays enzymatic properties essentially identical to the native enzyme's properties [6]. Table 1 shows a variety of marine algae used in this study and classified them into four different groups. Fig. 1 shows that the methanol extracts from two brown algae, *F. evanescens* and *P. babingtonii*, decreased the DHOD activity to 41 and 42% of the control, respectively, at the concentration of 50 μg/

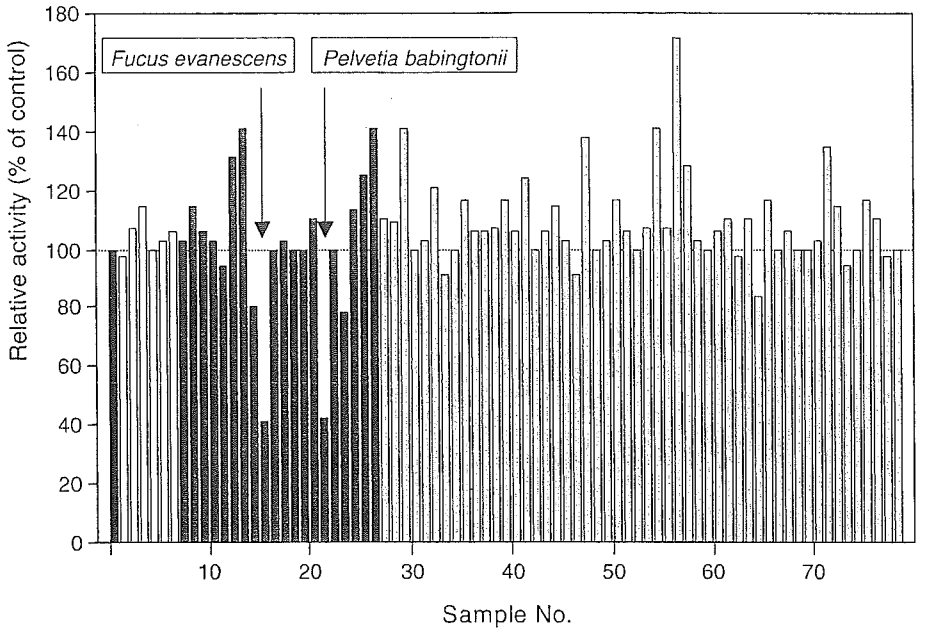

Fig. 1. Effect of marine algae extracts on *Trypanosoma cruzi* DHOD activity. The extract from seaweed (black bar), green (open bar), brown (dark gray bar), or red (light gray bar) alga was added to the DHOD assay mixture at the concentration of 50 μg/ml. The numbers affixed to *x*-axis correspond to those algae numbers in Table 1. *Y*-axis represents DHOD activity relative to the control in that methanol, but not extract, was added. A typical result of three separated experiments was shown.

ml. Most of other algae extracts did not affect the enzyme activity, but some (e.g., *Grateloupia turuturu*) elevated the activity.

3.2. Mode of inhibition of the T. cruzi DHOD activity by algae extracts

Fig. 2 shows the double reciprocal plots of the *T. cruzi* DHOD activity as a function of increasing concentrations of dihydroorotate in the presence of different concentrations of *F. evanescens* and *P. babingtonii* extracts. Inhibition of DHOD activity by these extracts was noncompetitive with respect to the substrate, dihydroorotate, with apparent K_i values of 35.3±5.9 μg/ml and 10.3±4.4 μg/ml, respectively.

3.3. Effects of algae extracts on T. cruzi infection and growth in mammalian cells

We attempted to evaluate whether the *F. evanescens* and *P. babingtonii* extracts affect the *T. cruzi* infection and proliferation in HeLa cells in vitro. The solvent of these extracts was exchanged from methanol to ethanol in order to reduce the cytotoxicity to HeLa cells. Compared with the methanol extracts, the ethanol-reconstituted extracts also decreased the *T. cruzi* DHOD activity down to an equivalent level (data not shown). The *F. evanescens* and *P. babingtonii* extracts decreased significantly the infection rate of HeLa cells and the amastigote growth even at the low concentration of 0.1 μg/ml (Table 2). These results indicate

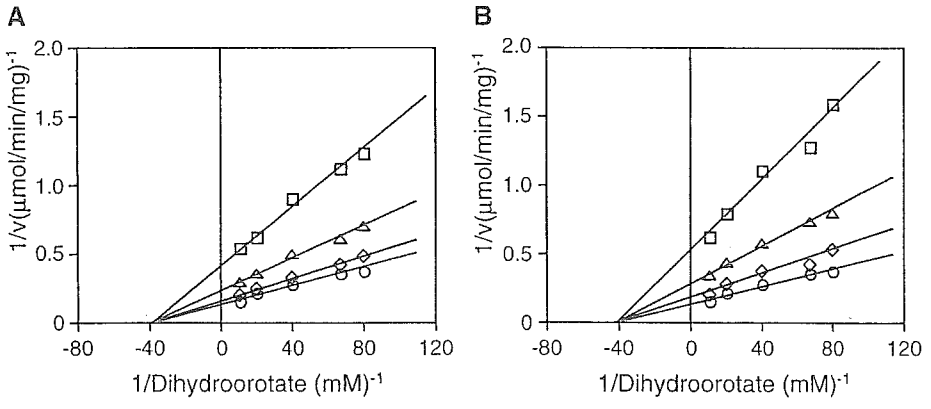

Fig. 2. Lineweaver–Burk plots of *Trypanosoma cruzi* DHOD activity as a function of increasing concentrations of dihydroorotate in the presence of methanol extracts from *Fucus evanescens* (A) and *Pelvetia babingtonii* (B). The DHOD assay was conducted as described in Materials and methods in the presence of 1 mM fumarate as electron acceptor. The concentrations of the extracts were at 0 (○), 25 (◇), 50 (△) and 100 (□) μg/ml. A typical result of three separated experiments was shown.

Table 2
Effects of ethanol-reconstituted algae extracts on the rate of *Trypanosoma cruzi* infection of HeLa cells and on the average number of amastigotes per infected cell

Algae	Conc. (μg/ml)	Infection rate (%)	Amastigote number/cell
Ethanol (control)	0	10.3±3.0	9.3±0.8
Fucus evanescens	0.1	4.8±1.0[a]	7.9±2.2
	1	4.7±2.4[a]	5.6±0.7[b]
	10	3.9±1.1[a]	6.3±1.1[b]
Pelvetia babingtonii	0.1	4.3±1.5[a]	7.1±1.6
	1	3.7±0.9[a]	6.8±1.9
	10	3.4±1.9[a]	5.4±1.3[b]

Data represent the mean±S.D. of three separate determinations. [a,b]Significantly different from the corresponding controls ($p<0.05$).

that the ethanol-reconstituted *F. evanescens* and *P. babingtonii* extracts contain inhibitory substance(s) on the host-cell-infection rate and on the intracellular growth of *T. cruzi*. At a high concentration of these brown algae extracts (100 μg/ml), host cells were damaged and detached from the coverslip after 4-days cultivation (data not shown).

3.4. Effects of algae extracts on the growth of T. cruzi epimastigotes

We tested the inhibitory effect of algae extracts on the growth of *T. cruzi* epimastigotes in a serum-free medium GIT. The ethanol-reconstituted *F. evanescens* and *P. babingtonii* extracts showed a significant but weak inhibitory effect on the epimastigote proliferation (Table 3). These extracts did not cause morphological changes and cell death of the parasites at the concentration of 0.1 and 1 μg/ml, suggesting that they may lower the protozoan growth when added to the culture medium (data not shown). These results suggest that the *F. evanescens* and *P. babingtonii* extracts contain an inhibitory activity on the growth of *T. cruzi* epimastigotes.

4. Discussion

In the present study, we demonstrated inhibitory effects of extracts from two species of brown algae, *F. evanescens* and *P. babingtonii*, on the *T. cruzi* DHOD activity (Fig. 1). Kinetic analysis revealed that the inhibition by these extracts was noncompetitive with the substrate dihydroorotate (Fig. 2), suggesting the direct interaction between DHOD and substance(s) in these extracts. Recently, DHOD have been highlighted as a target of chemotherapy for cancers, rheumatoid arthritis, and infectious diseases [17–20]; brequinar sodium (DuP 785; NSC 368390; 6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinoline-carboxylic acid sodium salt) is a well-characterized, anticancer drug candidate and inhibits mammalian DHOD [17]. Brequinar and its core moiety, 2-phenyl 5-quinoline carboxylic acid (PQC), are competitive inhibitors against ubiquinone [21]. In contrast, leflunomide (A77 1726) is an immunomodulatory drug and represses proliferation of T-lymphocytes by inhibiting DHOD, resulting in depletion of pyrimidine pools [19]. Inhibition of DHOD by leflunomide is noncompetitive with ubiquinone. With respect to the substrate dihydroorotate, both brequinar and leflunomide were noncompetitive [21].

Whether these compounds inhibit the *T. cruzi* DHOD activity is unknown, however, it should be pointed out that the protozoan enzyme differs from the mammalian enzyme, particularly in their subcellular localization and requirements for cofactors and electron acceptors. The *T. cruzi* DHOD localizes in the cytosol, utilizes fumarate, much more suitable than ubiquinone, as electron acceptor [6], and is called family-1A-type enzyme, together with *Saccharomyces cerevisiae* and *Lactococcus lactis* A enzyme, whereas the human DHOD is mitochondrial membrane-linked, utilizes ubiquinone as electron acceptor, and is called family-2-type enzyme [5]. From these views, the inhibitors of the mammalian DHOD may not inhibit the trypanosomatid DHOD effectively. Interestingly, *Plasmodium falciparum* DHOD is categorized into family-2 type but displays enzymatic properties distinct from the human enzyme; *P. falciparum* DHOD is more than 1000-fold resistant to the derivatives of A77 1726 when compared with the human DHOD, suggesting species-specificity of DHOD inhibition [22].

In contrast, our findings that two brown algae extracts contain noncompetitive inhibitor(s) against *T. cruzi* DHOD (Figs. 1 and 2) may provide greater potential to develop anti-trypanosomal drug(s). Since the structure and affinity of active sites of DHODs would differ in *T. cruzi* and human each other, as suggested by kinetic analyses, inhibitor(s) in these marine algae extracts could afford a selective inhibition to the *T. cruzi* DHOD. The *F. evanescens* and *P. babingtonii* extracts also resulted in the inhibition of the host-cell-infection rate and the *T. cruzi* growth in an in vitro infection system (Table 2). Reduction of the infection rate may reflect the anti-trypomastigote activity and/or probably reflect the decreased transformation from trypomastigotes into amastigotes inside the host cells. Since trypomastigotes do not divide, the inhibition of the infection rate with these algae extracts may have resulted from the reduced invasion and/or transformation. The amastigote number that repre-

Table 3
Effects of ethanol-reconstituted algae extracts on the growth of *Trypanosoma cruzi* epimastigotes

Algae	Conc. (μg/ml)	Cell count ($\times 10^{-5}$)
Ethanol (control)	0	23.2±2.4
Fucus evanescens	0.1	20.0±2.1[a]
	1	16.4±2.1[a]
Pelvetia babingtonii	0.1	20.1±2.0[a]
	1	17.4±2.7[a]

Results represent the mean±S.D. of four separate determinations. [a]Significantly different from control ($p<0.05$). The initial cell density of epimastigotes was 5×10^5/well.