GROWTH-PROMOTING EFFECT ON IRON-SULFUR PROTEINS ON AXENIC CULTURES OF *ENTAMOEBA DISPAR*

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Summary:

A growth-promoting factor (GPF) that promotes the growth of *Entamoeba dispar* under axenic culture conditions was found in fractions of mitochondria (Mt), hydrogenosomes (Hg) and chloroplasts (Cp) obtained from cells of six different protozoan, mammalian and plant species. We were able to extract the GPF from the Cp-rich leaf cells of a plant (spiderwort: *Commelina communis* L.) in an acetone-soluble fraction as a complex of chlorophyll with low molecular weight proteins (molecular weight [MW] approximately 4,600). We also found that on treatment with 0.6 % complexes of 2-mercapthoethanol (2ME), complexes of chlorophyll-a with iron-sulphur (Fe-S) proteins (e.g., ferredoxins [Fd] from spinach and *Clostridium pasteurianum*) and noncomplex rubredoxin (Rd) from *C. pasteurianum* have a growth-promoting effect on *E. dispar*. These findings suggest that *E. dispar* may lack a sufficient quantity of some essential components of Fe-S proteins, such as Fe-S center.

KEY WORDS : growth, mitochondria, hydrogenosomes, chloroplasts, iron-sulphur protein, *Entamoeba dispar*.

Résumé : LES EFFETS D'ACCÉLÉRATION DE CROISSANCE DES PROTÉINES FER-SOUFRE DANS LA CULTURE AXÉNIQUE D'*ENTAMOEBA DISPAR*

*Des facteurs d'accélération de croissance (Growth promoting factor: GPF) favorisant le développement d'*Entamoeba dispar *ont été détectés dans la composition de la mitochondrie, de l'hydrogénosome et du chloroplaste isolés à partir de six sortes de cellules issues de protozoaires, mammifères et plantes. De plus, les GPF de cellules de mésophile contenant une grande quantité de chloroplaste végétal (*Commelina communis *L.) ont pu être extraits comme une substance composée de protéines de faible masse moléculaire (≈ 4600) et de chloroplastes dans la composition acétone-soluble. À partir de ces résultats, nous avons préalablement pu découvrir des effets d'accélération de croissance dans le corps composé de protéines fer-soufre (épinard et ferrédoxine de* Clostridium pasteurianum*) et de chlorophylle-a traité au 2-mercaptoéthanol 0,6 % et du corps simple rubrédoxine de* C. pasteurianum. *Ces observations ont suggéré qu'une composante essentielle formant la protéine fer-soufre d'*E. dispar *(comme noyau fer-soufre) semblait être insuffisante.*

MOTS CLÉS : *croissance, mitochondrie, hydrogénosome, chloroplaste, protéine fer-soufre,* Entamoeba dispar.

INTRODUCTION

Entamoeba dispar grows well under xenic and monoxenic culture conditions along with enteric bacteria such as *Escherichia coli* or anaerobic bacteria such as *Fusobacterium symbiosum* (*Clostridium symbiosum* ATCC 14940) (Robinson, 1968; Diamond, 1982; Vargas *et al.*, 1990). However, even after the introduction of the axenic yeast extract-iron serum (YI-S) medium for *E. dispar* (Diamond *et al.*, 1995; Clark, 1995) the axenic cultivation of *E. dispar* remains difficult. Compared with *E. dispar*, pathogenic *E. histolytica*, which is closely related to *E. dispar*, easily adapts to the axenic culture medium (TYI-S-33) (Diamond *et al.*, 1978). Additionally, *Entamoeba histolytica* is capable of invading the mucosa of the large intestine. We have also developed a new yeast extract-iron-gluconic acid-dihydroxyacetone-serum medium (YIGADHA-S) (Kobayashi *et al.*, 2005) based on the YI-S medium and on the results of an investigation on bacterial metabolic products, and have succeeded in culturing five strains under axenic conditions. However, despite using the YIGADHA-S culture system, the axenic growth of four of five strains of *E. dispar* was found to be very poor, with the exception of one primate-derived strain (CYNO, 09: TPC) isolated from a cynomolgus monkey. A further search for useful growth promoting factors (GPFs) revealed that autoclaved (121° C, 15 minutes) bacteria and more than 20 types of protozoan, mammalian and plant cells containing mitochondria (Mt), hydrogenosomes (Hg) and chloroplasts (Cp) have a significant growth-promoting effect on *E. dispar*. However, the degree of these growth-promoting effects differs among the GPFs. In the present study, we were able to extract GPF from the Cp-rich leaf cells of a plant (spiderwort: *Commelina communis* L.) in an acetone-soluble fraction as a chlorophyll complex with low mole-

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cular weight proteins. Significant amounts of iron (Fe), sulphur (S) and molybdenum (Mo) atoms were found in the protein-chlorophyll complex fraction along with the magnesium (Mg) atom of chlorophyll.
Based on these findings, we hypothesized that some common components of Fe-S proteins present in Mt, Hg, Cp and bacteria support the growth of *E. dispar*. This is because in *E. dispar*, an essential redox Fe-S protein [ferredoxin (Fd)] is expected to be involved in energy metabolism, such as oxidative decarboxylation of pyruvate to acetyl-coenzyme A (acetyl-CoA), which is similar to that found in *E. histolytica* (McLaughlin & Aley, 1985).

MATERIALS AND METHODS

E. DISPAR ISOLATE

In this study, one human-derived strain of axenically grown *E. dispar* (AS 16 IR) (Kobayashi *et al.*, 2005) was used to determine the growth promoting effect of Fe-S proteins.

AXENIC CULTIVATION OF *E. DISPAR*

The YIGADHA-S medium (Kobayashi *et al.*, 2005) containing 15 % heat-inactivated bovine serum was used as the axenic cultivation medium for *E. dispar*.

DONOR CELLS OF MT, HG, CP AND MITOSOMES

The Mt donors are as follows: protozoan parasites: i) *Crithidia fasciculata* (ReF-1, PRR strain, ATCC 50083); ii) *Trypanosoma cruzi* (Tulahuen strain); iii) *Leishmania major* (MHOM/SU/73/5-ASKH strain, ATCC 50155); and iv) *Acanthamoeba castellanii* (ATCC 30011). v) The vertebrate cells used as Mt donors were from the mouse lymphoblast cell line (P388D1, ATCC CCL-46). The Hg donors are as follows: protozoan parasites: vi) *Trichomonas vaginalis* (KO-11 strain) (Qi *et al.*, 1995) and vii) *Trichomonas foetus* (Okamoto *et al.*, 1998). viii) Leaves of the spiderwort (*Commelina communis* L.) were used as the Cp donors. Consisting amitochondrial protozoan parasite mitosomes that are mitochondrial remnant organelles (Tovar *et al.*, 2003) was used as the mitosome donor, ix) *Giardia intestinalis* (syn. *lamblia*), (Portland-I strain, ATCC 30888). The bacterium used was *Pseudomonas aeruginosa* (PA:KEIO strain) (Kobayashi *et al.*, 1998).

PREPARATION OF CELLS

C. fasciculata and *T. cruzi* were axenically cultured in liver infusion tryptose (LIT) medium (*Gutteridge et al.*, 1969) supplemented with 10 % foetal bovine serum (FBS). After cultivation for three and seven days respectively, 2×10^7 cells of each parasite were harvested by centrifugation (650 g × 8 minutes). The cells of each parasite were washed three times by centrifugation with 10 mM phosphate-buffered saline (PBS) (pH 7.4), and each pellet was suspended in approximately eight times its volume of 50 mM Tris-HCl (pH 7.4). *L. major* was axenically cultured in Schneider's medium (Gibco™) supplemented with 15 % FBS at 26° C for four days and harvested by centrifugation (650 g × 8 minutes). *A. castellanii* was axenically cultured in peptone-yeast glucose (PYG) medium (Rowbotham, 1983; ATCC media formulations No. 712) for four days and harvested by centrifugation (650 g × 4 minutes). The P388D1 cell line was cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Taito-ku, Tokyo, Japan) supplemented with 10 % FBS for four days and harvested by centrifugation (125 g × 4 minutes). *T. vaginalis* and *T. foetus* were axenically cultured for three days in BI-S-33 medium in which the peptone components of TYI-S-33 medium (Diamond *et al.*, 1978) are substituted by Biosate (BBL, Becton Dickinson Co., Cockeysville, Maryland, USA), and harvested by centrifugation (275 g × 4 minutes). The Cp-rich fraction from the wild spiderwort (*C. communis* L.) was isolated. The leaves of wild spiderwort were picked from the private field of Keio University (Tokyo) during the flower season from June to August (2000-2003). Fresh green spiderwort leaves were stripped from the stems, washed with tap water and then with distilled water. The leaves were ground with serum-free RPMI 1640 medium (RPMI) in an earthenware mortar by using a wooden pestle. The resulting leaf cell suspension was filtered through a double thickness gauze to remove the residue. The cell suspension was then washed three times with RPMI by centrifugation (440 g × 10 minutes). *P. aeruginosa* was cultured in Biosate-Iron broth (Diamond *et al.*, 1978) for three days and harvested by centrifugation (650 g × 4 minutes). *G. intestinalis* was axenically cultured in modified BI-S-33 medium (Keister, 1983) for three days and harvested by centrifugation (275 g × 4 minutes).

PREPARATION OF MT, HG AND CP FRACTIONS

Mt, Hg and Cp rich fractions were prepared as described by Hogeboom (1955), Opperdoes *et al.* (1984) and Gorham (1955), respectively. Since the methodology for mitosome isolation has not been established, intact cells of *G. intestinalis* were tested for their growth-promoting effect without preparing the mitosome rich fraction. After washing the fractions with RPMI once by centrifugation (440 g × 10 minutes), each fraction was suspended in 2 ml of RPMI. In order to test for their growth-promoting effect under axenic culture conditions, the fractions were sterilized by autoclaving (121° C, 15 minutes), and 0.2 ml of each suspension was added into the YIGADHA-S medium (5 ml). The

medium was then inoculated with a 0.8 ml suspension of *E. dispar* (AS16IR strain = AS 16 IR) (final density: 200-2,700 amoebae/ml).

NuPAGE® Novex 4-12 % Bis-Tris gradient peptide gel electrophoresis and preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The lyophilized cell fraction of spiderwort was extracted with 20 %, 40 %, 80 % and 100 % acetone solutions (20 mg/ml) for 10 minutes at 4° C. After centrifugation (17,800 g × 10 minutes, 4° C) of each acetone extract, the supernatant was collected. The supernatants that were extracted with 80 % and 100 % acetone solutions were diluted with distilled water to obtain an acetone concentration of 50 %. Each supernatant was then concentrated by evaporation by using a SpeedVac system (SVC 100; Savant Instruments, Inc., Farmingdale, New York, USA) until evaporated to dryness. The molecular weight of the proteins in each extract was determined by NuPAGE® Novex 4-12 % Bis-Tris gradient peptide (Invitrogen Corp., Carlsbad, California, USA) gel electrophoresis.

The bands of complexes of low molecular weight protein with chlorophyll that were detected in the supernatants of 80 % and 100 % acetone extracts were isolated by preparative SDS-PAGE on a 12 % gel in a Mini Prep Cell (Bio-Rad Laboratories, Inc., Hercules, California, USA) electrophoresis unit. SDS-PAGE was performed according to the procedure described by Laemmli (1970). For NuPAGE® (Novex 4-12 % gradient peptide gel = Novex 4-12 %) each dried and concentrated supernatant of the acetone extract was redissolved in the sample buffer with 50 mM dithiothreitol and heated at 70° C for 10 minutes to reduce the disulfide bonds. For SDS-PAGE (12 % gel) each supernatant was redissolved in the sample buffer with 1 % (v/v) 2-mercaptoethanol (2ME) and boiled for four minutes. Following NuPAGE®, the gel was stained with 2D-silverstain II (Daiichi Pure Chemicals Co. Ltd., Chuo-ku, Tokyo, Japan).

Assay of the acetone extracts of spiderwort leaf cells for a GPF

One millilitre supernatants (obtained after centrifugation; 17,800 g × 10 minutes, 4° C) of the 20 %, 40 %, 80 % and 100 % acetone extracts of the lyophilized cell fraction of spiderwort (20 mg/ml) was dialyzed three times against 200-250 volumes of distilled water for 18 hours by using a Spectra/Por® 3 (3500 MWCO) dialysis membrane for 18 h; the volume of each extract was adjusted to 2.4 ml. Each extract was sterilized by filtration (Sartorius membrane filter; 0.2-µm pore size), and 0.2 ml of each extract was added to the YIGADHA-S medium to test the growth-promoting effect of the extract on *E. dispar* (AS 16 IR).

Analysis using a scanning electron microscopy (SEM)/energy dispersive X-ray analyser (EDX)-integrated analysis system

Following the preparative SDS-PAGE, the complexes of chlorophyll with low molecular weight proteins in the 80 % and 100 % acetone-soluble fractions were dialyzed in the same manner as described above. After lyophilization, the dialyzed complexes were examined using an SEM and EDX-integrated analysis system SEM-EDX III Type N/H (Hitachi Science Systems, Ltd., Hitachinaka, Ibaragi, Japan) in the Nihonbashi laboratory, Hitachi High-Technologies Co., Tokyo, Japan.

Preparation of chlorophyll-a complexes with purified iron-sulphur proteins

Purified Fd from spinach (1 µg/µl; Sigma F-3013) and *C. pasteurianum* (1 µg/µl; Sigma F-7629) and purified rubredoxin (Rd) from *C. pasteurianum* (15.7 µg/µl; Sigma R-2512) were dissolved in 0.6 % 2ME/H_2O (20 µg of Fd or 31.4 µg of Rd/4 ml of 0.6 % 2ME), and the Fd and Rd solutions were dialyzed in the same manner as described above. Purified chlorophyll-a (Sigma C-5753) was dissolved in acetone (25 µg/25 µl acetone), and after adding 4 ml of distilled water, the solution was dialyzed in the same manner as described above. The Fd and Rd solutions were mixed with dialyzed chlorophyll-a and incubated for 10 minutes at 25° C. The solutions of chlorophyll-a complexes were then sterilized by filtration, and 0.2 ml of each solution was added to the YIGADHA-S medium to test their growth-promoting effect on *E. dispar* AS 16 IR.

Statistical analysis of growth-promoting effect on *E. dispar*

All experiments for testing growth-promoting effect were repeated at least twice. The data of each experiment were subjected to one-way analysis of variance (ANOVA) with Dunnett's multiple comparison post hoc test; the levels of statistical significance were taken as $p < 0.05$ and $p < 0.01$.

RESULTS

The Mt, Hg and Cp isolated from eight different types of cells were tested to determine whether they promoted the growth of axenically grown *E. dispar* AS 16 IR. The intact cells of *G. intestinalis* and *P. aeruginosa* were also tested for the same. A growth-promoting effect was apparently observed for every fraction except for the fraction of intact cells of *G. intestinalis*. Apparently, the intact cells of *E. histolytica* (HM-1: IMSS clone 6 strain) containing mitosomes (Leon-Avila & Tovar, 2004) also did not produce a

growth-promoting effect (data not shown). Although the growth-promoting effect of the two fractions of Mt from *T. cruzi* and the Hg fraction from *T. vaginalis* retained the statistically significant difference by Dunnett's test ($p < 0.05$), the growth-promoting effect of the other six fractions containing Mt, Hg and Cp was significantly different ($p < 0.01$ or $p < 0.05$). In particular, the effect of the Cp fraction from spiderwort exceeded that of the intact cells fraction of *P. aeruginosa* as shown in Figure 1. Thus, it was concluded that some of the Mt, Hg and Cp fractions, which were obtained under appropriate conditions as well as the intact bacterial cells contain a GPF.

Acetone extraction was used to obtain a water-soluble GPF from Cp-rich leaf cells of some plant species (e.g., spiderwort, cherry and morning glory), which have sufficiently strong leaf-cell membranes that can withstand the leaf crushing and cell isolation process. Figure 2 shows the results obtained on testing the 0 %, 20 %, 40 %, 80 % and 100 % acetone-soluble fractions from lyophilized Cp-rich leaf cells of spiderwort for a growth-promoting effect. The results showed that the 20 % and 80 % acetone-soluble fractions had a stronger growth-promoting effect than the other acetone-soluble fractions (0 %, 40 % and 100 %). The growth-promoting effects of the 20 % and 80 % acetone-soluble fractions were statistically significant at $p < 0.01$ (Dunnett's test); however, the effects of the 0 %, 40 % and 100 % acetone-soluble fractions were retained at $p < 0.05$. The silverstain NuPAGE® analysis of these acetone-soluble fractions yielded a distinct dense protein band (MW 4600) that was common to the 20 %, 40 %, 80 % and 100 % acetone-soluble fractions. The intensity of the bands of the 20 % and 80 % acetone-soluble fractions were stronger than the bands of the other acetone-soluble fractions (40 % and 100 %) (Fig. 3). These results indicated that the efficacy of the growth-promoting effect was correlated with the intensity of the low molecular weight protein band (Figs 2, 3). The band of the complex of the chlorophyll with low molecular weight proteins (green colour) present in both the 80 % and 100 % acetone-soluble fractions was obtained by SDS-preparative electrophoresis; both the fractions showed a growth-promoting effect on *E. dispar* AS 16 IR (data not shown). The result showed that the band of the complex of chlorophyll with low molecular weight proteins, which was present in the acetone-soluble fractions, contained a GPF. The reduction in the intensity of the low molecular weight protein bands obtained by using the 40 % acetone-soluble fraction was considered to be caused by the incomplete solubility of chlorophyll. Some protein parts are believed to be precipitated together with the insoluble chlorophyll when extraction is performed with an intermediate concen-

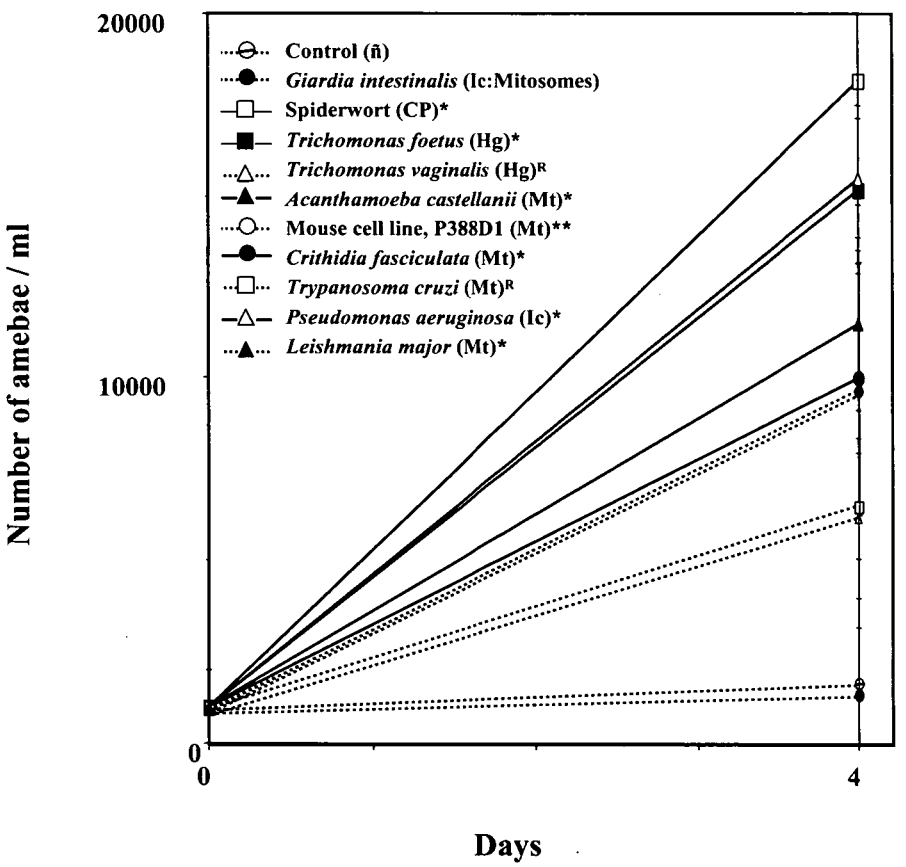

Fig. 1. – The effect of intact cells (Ic) and the fractions of mitochondria (Mt), chloroplasts (Cp) and hydrogenosomes (Hg) from ten types of cells, including bacterial, mammalian, plant and protozoan cells, on the growth of *Entamoeba dispar*.
The cellular components were sterilized by autoclaving at 121° C for 15 minutes. The growth kinetics of the *E. dispar* AS 16 IR strain in the YIGADHA-S medium are shown (mean numbers of amoebae in duplicate cultures are plotted). *, **: the mean of the growth-kinetic level was significantly higher than that of the control (*$p < 0.01$ and **$p < 0.05$ by Dunnett's test). R: the significant difference was retained by Dunnett's test ($p < 0.05$).

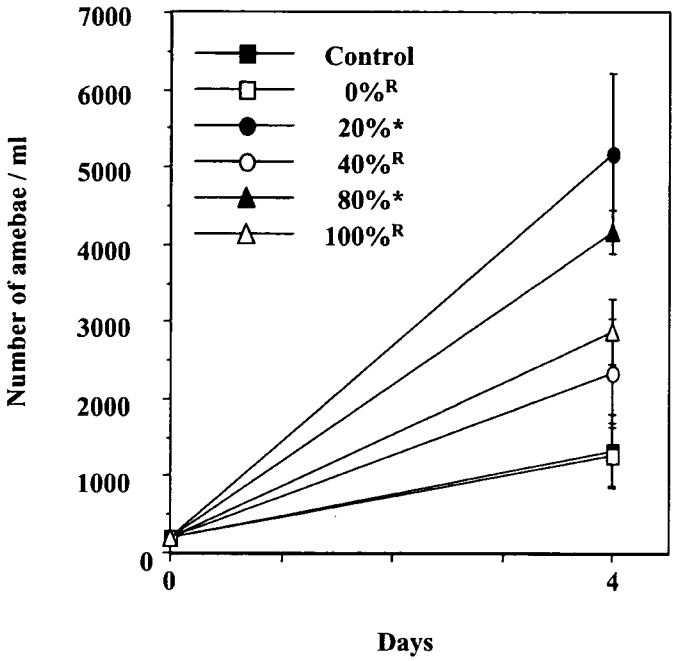

Fig. 2. – Effect of soluble fractions of spiderwort Cp-rich leaf cells extracted with five different concentrations of acetone (0 %, 20 %, 40 %, 80 % and 100 %) on the growth of *E. dispar*.
The growth kinetics of the *E. dispar* AS 16 IR strain in the YIGADHA-S medium are shown (mean numbers of amoebae in duplicate cultures are plotted). *: the mean of the growth-kinetic level was significantly higher than that of the control (*$p < 0.01$ by Dunnett's test). R: the significant difference was retained by Dunnett's test ($p < 0.05$).

tration of acetone such as 40 %. As compared to an 80 % acetone concentration, the 100 % concentration of acetone was considered to be slightly severe for the extraction of the low molecular weight proteins without decreasing the activity of a GPF. As a result, the intensity of the low molecular weight protein bands obtained by using the 40 % and 100 % acetone-soluble fraction was believed to be reduced.

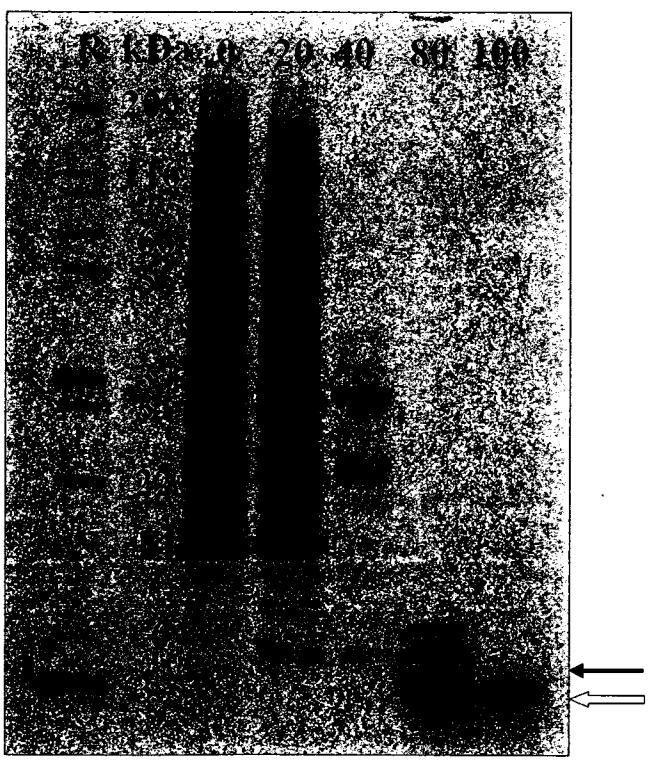

Fig. 3. – NuPAGE® (Novex 4-12 %) of five acetone-soluble fractions of spiderwort Cp-rich leaf cells extracted with different concentrations of acetone (0 %, 20 %, 40 %, 80 % and 100 %).
Molecular mass markers (M) are shown on the left. ⟵ : acetone-soluble low molecular weight protein bands were isolated from the 20 %, 40 %, 80 % and 100 % acetone-soluble fractions. ⟸: broad green band of chlorophyll.

The SEM/EDX-integrated analysis system revealed that the low molecular weight protein band contained Fe, S and Mo atoms along with the Mg atom of chlorophyll (Figs 4, 5). We believed that Fd present in Cp is a type of Fe-S protein containing Fe and S atoms. In order to confirm the growth-promoting effect of Fd, we examined the effects of purified spinach Fd (MW 12,000), *C. pasteurianum* Fd (MW 6,000) and purified *C. pasteurianum* Rd (MW 19,000) on the growth of *E. dispar* AS 16 IR. The analysis revealed that 0.6 % 2ME-treated *C. pasteurianum* Rd and each of the water-soluble chlorophyll-a complexes with 0.6 % 2ME-treated spinach Fd and *C. pasteurianum* Fd have a growth-promoting effect on *E. dispar* AS 16 IR with a statistically significant difference ($p < 0.01$) (Fig. 6). The non-complexed Fd from spinach and from *C. pasteurianum* treated with 0.6 % 2ME also had a slight growth-promoting effect when compared with the growth kinetics of the control with or without chlorophyll-a; however, these growth-promoting effects were retained ($p < 0.05$).

These results suggest that the GPF in the acetone-soluble fraction of spiderwort Cp-rich leaf cells is the complex of the chlorophyll-a with a component of an Fe-S protein, such as an inorganic Fe-S centre, released by the breakage of disulfide bonds due to treatment with 2ME. The Fe-S redox proteins are commonly found in enteric bacteria (e.g., Fd), mitochondria (e.g., Fe-S proteins in complex I and II), chloroplasts (e.g., Fd) and hydrogenosomes (e.g., Fd); however, there are diffe-

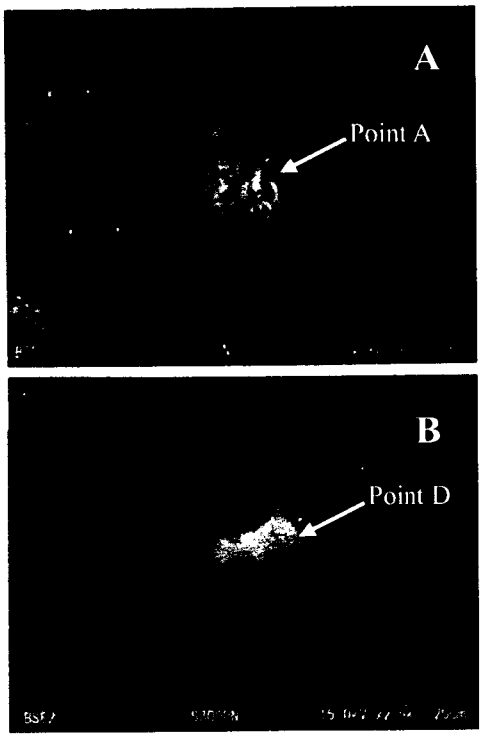

Fig. 4. – Scanning electron microscope (SEM) images of crystals observed in complexes of chlorophyll with low molecular weight proteins present in the 80 % (A) and 100 % (B) acetone-soluble fractions.
By using an SEM/energy dispersive X-ray (EDX) analyzer, Fe atoms were detected at point A (A) and S and Mo atoms were detected at point D (B).

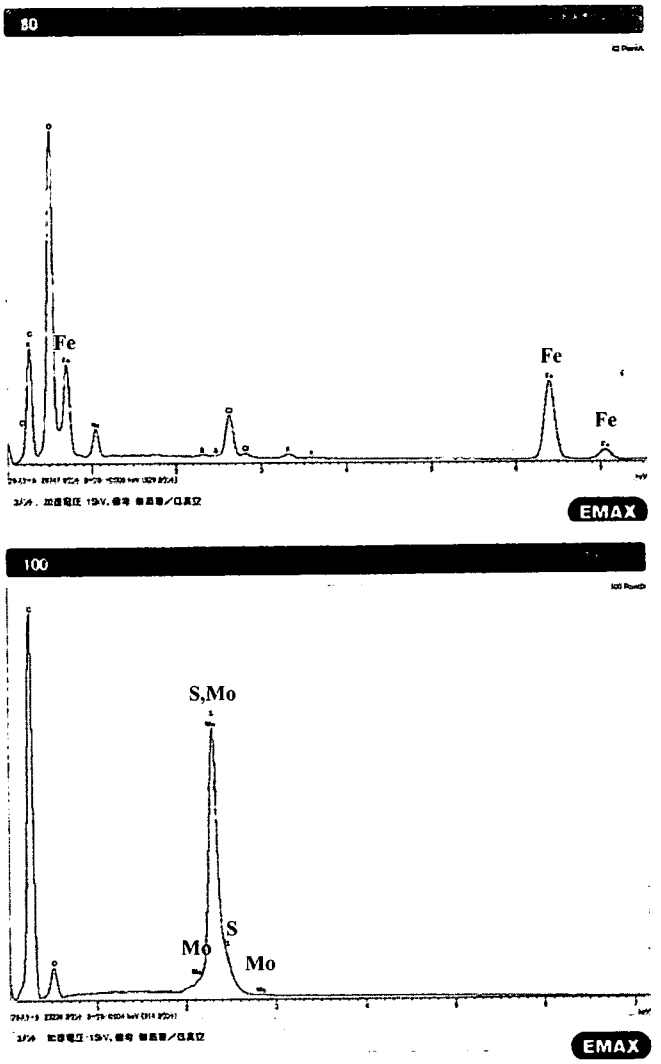

Fig. 5. – Specific peaks of the Fe atom at point A (Fig. 3) and S and Mo atoms at point D (Fig. 3) detected in the complexes of chlorophyll with low molecular weight proteins in the 80 % (A) and 100 % (B) acetone-soluble fractions by using an SEM/EDX analyzer.

rences in the chemical structure of their Fe-S centers. In *E. dispar*, Fd is considered as an essential redox protein involved in energy metabolism, similar to that in *E. histolytica*, although neither species contains Mt. These findings suggest that *E. dispar* grown under axenic culture conditions may lack a sufficient quantity of some essential component of the Fe-S proteins (e.g., Fe-S centre).

The growth-promoting effect of the GPF was tested on three other strains of *E. dispar* (SAW 1734R clone AR, AS 2 IR and CYNO 16:TPC), (Koyabashi *et al.*, 2005); it was confirmed that the GPF produced the same effect on their growth (data not shown).

DISCUSSION

Previously, we designed the YIGADHA-S medium (Kobayashi *et al.*, 2005) for axenic cultivation of *E. dispar*. It contains dihydroxyacetone (DHA) that has a significant growth-promoting effect on *E. dispar*. DHA is a ketotriose and functions as a sugar source for *E. dispar*; therefore, it is considered to be directly metabolized to DHA phosphate, which is an intermediary metabolite in the Embden-Meyerhof-Parnas glycolytic pathway. However, the growth of *E. dispar* was poor despite the presence of DHA.

In the present study, a GPF was detected in the autoclaved Mt, Hg and Cp fractions and in intact bacteria and was demonstrated to show a growth-promoting effect on *E. dispar*. However, the efficacy of each GPF from these organelles varied under different conditions. Prior to the present study, detection of a GPF from trophozoites of *E. histolytica* (HM-1:IMSS clone 6 strain) was attempted. Since *E. histolytica* is genetically closely related to *E. dispar*, it contains an abundance of Fe-S proteins, such as Fd, which are only stable under anaerobic conditions, and it contains mitochondrial remnant mitosomes (Leon-Avila & Tovar, 2004). However, neither the intact cells nor any extract from the cells could promote the growth of *E. dispar* AS 16 IR (data not shown). One reason for the failure to extract a GPF from *E. histolytica* trophozoites may be the fragility of

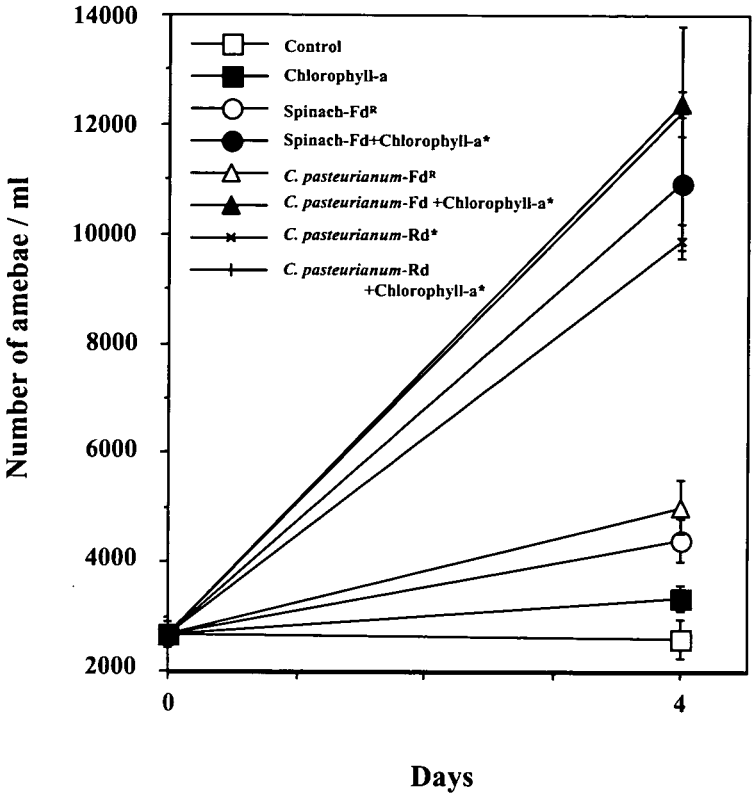

Fig. 6. - Effect of chlorophyll-a (—■—), 0.6 % 2ME-treated spinach ferredoxin (Fd) (—○—), *Clostridium pasteurianum* Fd (—△—) and *C. pasteurianum* rubredoxin (Rd) (—×—), and complexes of chlorophyll a with spinach Fd (—●—), *C. pasteurianum* Fd (—▲—) and *C. pasteurianum* Rd (—+—) on the growth of *E. dispar*, and control (—□—).
The growth kinetics of the *E. dispar* AS 16 IR strain in the YIGADHA-S medium are shown (mean numbers of amoebae in duplicate cultures are plotted). *: the mean of the growth-kinetic level was significantly higher than that of the control (*$p < 0.01$ by Dunnett's test). R: the significant difference was retained by Dunnett's test ($p < 0.05$).

the cell membrane and organelles (e.g.,mitosomes) similar to that of the spinach leaf cells. The GPF was assumed to be composed of a heat-stable substance and some other common simple inorganic substances. It was feasible to isolate the GPF from acetone-soluble fractions of Cp-rich leaf cells as bands of low molecular weight proteins complexed with chlorophyll containing Fe, S and Mo atoms.

The results of this study indicate that the protein bands may be derived from Fe-S (e.g., Fd) or Mo-Fe-S proteins [e.g., molybdoferredoxin, which is a common component of nitrogenase (present in rhizobium species)]. It appears that the GPF requires to form a complex with chlorophyll because the growth-promoting effects of purified Fd from spinach, *C. pasteurianum* and *E. histolytica* strain HM-1: IMSS clone 6 (the Fd consists of 4Fe-4S centre) on *E. dispar* AS 16 IR were not remarkable (data not shown). We found that the complex of chlorophyll-a with 0.6 % 2ME-treated Fd containing a 2Fe-2S centre from spinach and that containing 4Fe-4S centre from *C. pasteurianum* have a growth-promoting effect on *E. dispar* AS 16 IR. This effect was also observed with 0.6 % 2ME-treated Rd, which has the most simple Fe-S centre (1 Fe and 4 cysteine-residues without inorganic S), without chlorophyll-a. It is not clearly understood why complex formation must occur between chlorophyll-a and the components (Fe-S centres and cysteine residues) of bacterial and plant Fd to produce a growth-promoting effect is not clearly understood. However, it is considered that the complex of chlorophyll-a with the Fd component (MW greater than 100,000 by gel permeation chromatography) may be efficiently taken up by amoeba cells as a compact mass by a process, such as pinocytosis, and the complex may be degraded until an appropriate size of the Fe-S compound is obtained. We speculate that the Fe-S compounds are utilized as components of some essential Fe-S proteins such as amoebic Fd, which is an essential redox protein involved in oxidative decarboxylation of pyruvate to acetyl CoA in *E. dispar*, this is similar to that reported in *E. histolytica* (McLaughlin & Aley, 1985). It is reported that the amitochondrial protozoan parasite *E. histolytica*, which is closely related to *E. dispar*, contains numerous fermentation enzymes that are similar to bacterial enzymes, including the pyruvate Fd oxidoreductase, Fd and alcohol dehydrogenase E, derived from concomitant enteric bacterial DNA by horizontal transfer (Rosenthal *et al.*, 1997).

We succeeded in developing a more stable axenic cultivation system by adding the complex of chlorophyll with a component of Fe-S protein (e.g., Fd from spiderwort) treated with 2ME to the YIGADHA-S medium. We achieved an improved axenic cultivation system *for E. dispar* by using cellular components of protozoan, mammalian and plant cells as well as intact bacteria. However, we recognize that it is difficult to develop an axenic cultivation system for *E. dispar* that is at par

with the well-established cultivation system of *E. histolytica* for use in biological studies such as biochemical and immunological studies, etc.

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REVIEW ARTICLE

The Diversity of Clinical Isolates of *Entamoeba histolytica* in Japan

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In Japan, amebiasis is domestically transmitted by two major populations: male homosexuals and mentally handicapped persons, which is remarkably different from most other developed countries where *Entamoeba dispar* infection is predominantly observed. Here we briefly summarize epidemiology of amebiasis in Japan. We also review our current understanding of the diversity of *Entamoeba histolytica* clinical isolates in Japan, based on polymorphic genetic markers, clinical representations, and *in vivo* virulence, using an animal model. © 2005 IMSS. Published by Elsevier Inc.

Key Words: Amebiasis, Epidemiology, Heterogeneity, Sexually transmitted disease, Mentally handicapped persons.

Peculiarities of Amebiasis Endemic in Japan

The most unusual characteristic of amebiasis in Japan is that imported cases comprise only a minor proportion of all cases discovered in the country and cases attributable to domestic transmission dominate (1). We have two major populations infected by amebiasis: male homosexuals and mentally handicapped persons in institutions. We have approximately 500–600 cases of amebiasis reported to the Ministry of Health, Labour and Welfare, including three to four deaths annually. Approximately 90% of the reported cases are male. About 80% of cases have neither a history of traveling in endemic countries nor are they mentally handicapped. Thus, most of the reported cases are likely male homosexuals or bisexuals. Several groups previously reported a very high incidence of amebiasis among male homosexuals based on stool examination and serological tests (2–5). We recently observed sporadic cases of amebiasis transmitted through heterosexual intercourse (1) with an example of female commercial sex workers. Mentally handicapped persons are also severely affected by the disease (6–9). Mass infections of institutionalized mentally handicapped persons were often discovered during an onset of outbreaks as previously reported (6–9).

Recent Survey of Amebiasis in Institutions in Japan

Our recent survey to examine 484 individuals from six institutions [Institute B, C, D (10), E (11), and two other institutes (unpublished)] by a combination of microscopy, antigen capture ELISA, PCR, and serological tests showed that institutions were severely affected by amebiasis. Microscopic demonstration and antigen capture ELISA showed 9.7 and 12.3% overall positive, respectively. Serological tests, e.g., gel diffusion precipitin test and ELISA using whole parasite lysate as antigen showed 4.8 and 31.2% seropositive (unpublished). The positive rates of stool examination and serology varied significantly among institutions, suggesting that the intensity of infection varies among institutions or parasite strains spreading in each institution and vary in virulent competence (see below). Importantly, these cases are often unreported or underreported for several social reasons. In fact, the cases reported to the Ministry of Health, Labour, and Welfare included no cases of mentally handicapped persons. Thus, the number of amebiasis cases in Japan is largely underestimated. A very high incidence of *E. histolytica* infection in male homosexuals and mentally handicapped persons has not been reported in other developed countries (12–15) except for sporadic cases (16,17) and may be unique to Japan.

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Molecular Basis of the Diversity of *E. histolytica* Isolates in Japan

Genetic diversity among approximately 40 clinical strains isolated from either male homosexuals or mentally handicapped persons in Japan was determined by high-resolution genotyping based on nucleotide sequencing of two protein-coding (SREHP and chitinase) and non-coding regions (locus 1–2 and 5–6) (10,11). Remarkable differences existed in the degree of heterogeneity of genotypes between the two groups. The strains isolated from male homosexuals are extremely heterogeneous; all the isolates derived from male homosexuals showed distinct genotypes. In contrast, isotypes from mentally handicapped persons were less heterogeneous. Isolates obtained from an institute showed an identical genotype. Moreover, one genotype was detected at three institutions at different times (see below). Because the intensity of infection is low prior to mass infection, probably due to previous mass treatment, mass infection was likely caused by a single source. One genotype was isolated from three institutions [Institutions B, A (10) and E (11)] located in three prefectures (Kanagawa, Shizuoka, and Yamagata) in 1994, 2001, and 2002, respectively. The emergence of the same genotype coincided with the movement of a single infected individual. This case is a good example to demonstrate that molecular fingerprinting is indeed a very reliable tool to determine a source of infection and a route of transmission. In addition, this case also raised serious concern on the effective treatment of amebiasis.

The genotypes of Japanese isolates were distinct from four representative reference strains used worldwide (HM1:IMSS cl6, SAW755, SAW1627, and SAW1453) (10). In addition, none of 34 isolates from Thailand, Bangladesh, Cambodia, and Indonesia showed genotypes identical to Japanese isolates (11). Thus, the origin of Japanese strains is not understood and should be investigated in future studies.

Diversity of Clinical Manifestations and *In Vivo* Virulence among Japanese Strains

From a clinical point of view, heterogeneity of virulence attributable to genetic polymorphisms of the parasite likely exists. For instance, when we compared parasitological and serological results between Institutions D and E, which showed a similar rate of infection, notable differences in the serological marker for tissue invasion were observed. While parasitological stool examination gave a similar level of positive rate (28–30% positive) in these institutions, the seropositiveness evaluated by gel diffusion precipitin test significantly differed (0 or 16% in Institute D or E, respectively). The mean value of ELISA titer of the infected individuals was also significantly different between the two institutions (optical density at 405 nm of 0.13 or 0.50, respectively). The premise that this is not due to different rates of infection was also supported by the fact that the positive serology rate by ELISA was comparable between the two institutions (54–67%). These data strongly argue for the presence of genetic polymorphisms leading to distinct clinical manifestations.

Experimental animal infection using five isolates categorized into three representative genotypes from mentally handicapped persons also supported this premise. Hamsters were challenged with a direct inoculation of 5×10^4 trophozoites of KU13 (Institution A), KU19 (B), KU26 (C), KU27 (D), and KU33 (E), cultivated monoxenically with *Crithidia fasciculata* (18) to the liver, and abscess formation was evaluated a week later. All strains except for KU27 developed liver abscesses, while KU27 failed to cause abscess even in repeated attempts using a 4-times higher number of amebas (unpublished). These data agreed with the clinical manifestations in the patients infected with these strains and were consistent with the premise that a spectrum of virulence exists among the strains. *In vitro* virulence is conveniently assessed with the parasite's capacity to destroy a monolayer of mammalian cells (19). KU27 was incapable of destroying the monolayer of HeLa and Chinese hamster ovary cells, similar to *E. dispar* trophozoites (unpublished).

These avirulent phenotypes of KU27 are associated with a specific genotype of locus 1–2 type C and SREHP type A, neither of which was found among isolates examined in our laboratory, except two other isolates from the same institution (KU28 and KU29). Whether or not this specific marker is associated with the avirulent phenotype is not known. There is also no causal connection between this particular SREHP type and a lack of virulence. There are no notable differences in clinical manifestations of amebiasis between Japan and other countries. Hepatic, pulmonary, and brain abscesses are seen 5–20% of cases (almost exclusively in male). None of the four genetic markers was found to be associated with a tissue tropism.

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Impact of intestinal colonization and invasion on the *Entamoeba histolytica* transcriptome

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Abstract

A genome-wide transcriptional analysis of *Entamoeba histolytica* was performed on trophozoites isolated from the colon of six infected mice and from in vitro culture. An Affymetrix platform gene expression array was designed for this analysis that included probe sets for 9435 open reading frames (ORFs) and 9066 5′ and 3′ flanking regions. Transcripts were detected for >80% of all ORFs. A total of 523 transcripts (5.2% of all *E. histolytica* genes) were significantly changed in amebae isolated from the intestine on Days 1 and 29 after infection: 326 and 109 solely on Days 1 and 29, and 88 on both days. Quantitative real-time reverse transcriptase PCR confirmed these changes in 11/12 genes tested using mRNA isolated from an additional six mice. Adaptation to the intestinal environment was accompanied by increases in a subset of cell signaling genes including transmembrane kinases, ras and rho family GTPases, and calcium binding proteins. Significant decreases in mRNA abundance for genes involved in glycolysis and concomitant increases in lipases were consistent with a change in energy metabolism. Defense against bacteria present in the intestine (but lacking from in vitro culture) was suggested by alterations in mRNA levels of genes similar to the AIG1 plant antibacterial proteins. Decreases in oxygen detoxification pathways were observed as expected in the anaerobic colonic lumen. Of the known virulence factors the most remarkable changes were a 20–35-fold increase in a cysteine proteinase four-like gene, and a 2–3-fold decrease in two members of the Gal/GalNAc lectin light subunit family. Control of the observed changes in mRNA abundance in the intestine might potentially rest with four related proteins with DNA binding domains that were down-regulated 6–16-fold in the intestinal environment. In conclusion, the first genome-wide analysis of the transcriptome of *E. histolytica* demonstrated that the vast majority of genes are transcribed in trophozoites, and that in the host intestine trophozoites altered the expression of mRNAs for genes implicated in metabolism, oxygen defense, cell signaling, virulence, antibacterial activity, and DNA binding.

Keywords: *E. histolytica*; Oligonucleotide array sequence analysis; Gene expression profiling; Entamoeba/parasitology; Protozoan metabolism; Computational biology

1. Introduction

The early branching eukaryote *Entamoeba histolytica* is the etiologic agent of amebic dysentery and liver abscess. These diseases result in substantial morbidity and mortality in the developing world. To colonize and invade humans, trophozoites

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must adapt to the complex anaerobic environment of the colon. *E. histolytica* is an aerotolerant anaerobe that derives energy in vitro from the glycolytic and fermentation pathways. It has undergone a secondary loss of mitochondria, retaining only a remnant organelle that lacks a mitochondrial genome [1]. Energy is acquired from glycolytic and fermentation pathways that include many laterally transferred genes of bacterial origin [2,3].

The ability of *E. histolytica* to cause disease in animal models requires not only an energy source for the parasite but also virulence factors that mediate contact-dependent killing and phagocytosis of host cells and intestinal invasion. Virulence factors include cysteine proteases, the galactose/*N*-acetyl D-galactosamine-inhibitable (Gal/GalNAc) adherence lectin, and amebapore pore-forming proteins [4,5].

To date our understanding of virulence has been limited by the inability to study parasite gene expression in vivo at a comprehensive genome-wide scale. In order to identify pathways and genes that are involved in intestinal adaptation and invasion, we designed a gene expression array representing 9435 open reading frames (ORFs). This array was used to perform a transcriptional analysis of *E. histolytica* isolated from mouse ceca at early and late time points of infection. The changes in mRNA abundance and their potential impact on the biology of the parasite during intestinal colonization and invasion are discussed.

2. Materials and methods

2.1. Cultivation of E. histolytica

E. histolytica strain HM1:IMSS trophozoites were grown at 37 °C in TYI-S-33 medium containing penicillin (100 U/ml) and streptomycin (100 μg/ml) (GIBCO/BRL) [6]. To cultivate amebae from the intestine, infected mouse cecal contents were added to amebic growth medium and amebae were counted on Day 4.

2.2. Mouse model of amebic colitis

CBA/J mice were infected by luminal injection of 2×10^6 trophozoites into the ceca, exposed by laproscopic surgery [7]. Infected mice were sacrificed at Day 1 or Day 29 after infection.

2.3. RNA isolation

Approximately $2.4–5 \times 10^5$ *E. histolytica* trophozoites from culture were lysed by the addition of 0.8 ml of Trizol reagent (Invitrogen), and total RNA isolated by the manufacturer's directions. RNA from infected mice was purified from cecal contents of infected mice immediately after sacrifice. Cecal contents were washed in ice cold PBS and collected by centrifugation for 10 s at 14,000 rpm. One milliliter of Trizol (Invitrogen) was added to the cecal contents and an initial RNA preparation performed according to manufacturer's directions. Total RNA was treated with DNAse I (Roche Applied Science) to remove contaminating genomic DNA. RNA greater than 200 nucleotides in length was separated from total RNA by the RNeasy protocol (Qiagen). Control RNA was isolated from the ceca of mock-infected CBA mice by an identical protocol to test for cross-hybridization of the array with murine transcripts.

2.4. Design of Affymetrix custom array

A custom array (E_his-1a520285) was designed using information generated from the *E. histolytica* genome sequencing project release date 12/08/04 [3]. The ORF probe sets were preferentially selected from the 600 bases proximal to the 3′ end of the *E. histolytica* sequences. Probe sets consisted of 8–16 25mer oligonucleotide probes. Probe pairs consisted of a perfect match to the available sequence, and a control probe, which contained a mismatch at position 13. Probe sets were generated to 7712 sequences that represented 9435 ORFs, and consisted of both (_at) probe sets that represent single gene and (_x_at) probe sets. The latter may cross-hybridize in an unpredictable manner with sequences other than the main target. Some sequences were represented by both (_at) and (_x_at) probe sets, therefore the total number of probe sets for ORF's was 11,397. As 1723 predicted genes in the *E. histolytica* genome were identical to other ORF's on the chip, or were so highly similar in sequence as to make it impossible to design a unique probe set, these genes were represented by a probe set with the suffix (_s) and the additional represented genes are listed in Supplemental Table 1. Probes to highly repetitive sequences (LINE and SINE elements, rRNA, and tRNA genes) were not included in the array. *E. histolytica* sequences similar to *E. coli*, mouse or human sequences were also removed. This "pruning" process deleted approximately 5% (505) of the *E. histolytica* ORFs from the designed array.

Probe sets spotted on the Affymetrix array represented both ORF and selected UTR regions. The UTR probes were not used in the analysis described in this paper but are included in the MIAME-compliant data sets (supplemental data) [8]. In the design of the UTR probes 1–15 probe pairs were chosen with a desired gap between probes of 20–70 bp from available sequence information. In cases where the UTR sequence was >1000 bp in length, probe sets were chosen from the first and last 500 bases. These sets were distinguished by -a and -b suffixes. There are 12,777 UTR probe sets, which represented 9066 5′ and 3′ UTR's. Seven hundred and forty-one intergenic sequences were not represented on these arrays due to lack of space on the array.

2.5. Affymetrix DNA chip hybridization and analysis

Isolated total RNA was checked for integrity and concentration using the Agilent Bioanalyzer 2100 RNA nanochip Assay (Agilent Technologies). Fifty nanograms of total RNA was converted into cDNA using the SPIA™ isothermal linear RNA amplification system, which employs a single chimeric primer, DNA polymerase with strand displacement activity and RNase H (NuGen Technologies). RNase H cleaves the RNA portion of the heteroduplex at one end of the double-stranded cDNA, thus generating a unique partial duplex cDNA with a single-stranded DNA tail at the 3′ end of the second strand cDNA.

This tail is the priming site for the SPIA(tm) amplification step. The sequence of the SPIA(tm) amplification primer, a chimeric DNA/RNA primer, is complementary to the sequence of the single-stranded 3′ end of the second strand cDNA in the partial duplex, which is extended along the template DNA by DNA polymerase. Strand displacement DNA synthesis results in displacement of the prior primer extension product away from the template DNA. This cycle of primer binding, extension, displacement and cleavage results in the generation of the amplified product.

The resulting ~5 μg of biotinylated cDNA was hybridized to the E_his-1a520285 custom array (Affymetrix). In cases where the proportion of amebic RNA was less than 50% of total RNA (as determined by the ratio of amebic to mouse rRNA), and when labeling RNA derived from the sham infected CBA mouse (as a control), two separate labeling reactions were performed and pooled for the hybridization. The arrays were washed, stained with streptavidin-phycoerythrin (Molecular Probes), following the standard Affymetrix protocol for eukaryotic targets (Affymetrix). The arrays were scanned with an Affymetrix Gene Chip scanner 3000I and report files were generated to determine the percentage of present calls of each array.

In experiments using amebic mRNA isolated from the cecum of mice, only microarray hybridizations with low background hybridization values and raw probe set intensities with an average signal of greater or equal to 180 were included in the analyses (see Supplemental Table 2). To test for cross-hybridization with murine transcripts, the arrays were hybridized to mouse RNA and to cecal contents from mock-infected CBA mice.

Raw data from the arrays was normalized at probe level using gcRMA algorithm [9]. The detection calls (present, marginal, absent) for each probe set was obtained using the GCOS system (http://www.affymetrix.com/products/software/specific/gcos.affx). Only genes with at least one present call across all the compared hybridizations were kept for downstream statistical analyses. Significance of gene expression was determined using the significance analysis of microarrays (SAM) statistical program (http://www-stat.stanford.edu/~tibs/SAM/). Genes significantly modulated in vivo in the intestine were defined by the following criteria: a calculated q-value of less than 0.05 from SAM analysis; probe sets with a normalized signal intensity greater than 50; and changes greater than two-fold compared to cultured amebae. The normalized intensity value for each probe set, probe set ID, gene ID, GenBank #, fold difference between conditions and statistical significance from SAM analysis are shown in Supplemental Table 4. Additional GO terms and pfam domains were provided in each list of the identified differentially expressed genes and the data is shown in Supplemental Table 5. The whole dataset was deposited at VBIExpress (http://vbiexpress.vbi.vt.edu/VbiExpress/public), which follows MIAME guidelines.

2.6. qRT-PCR

Reverse transcription real-time PCR (qRT-PCR) was used to independently measure mRNA abundance. The Superscript II enzyme (Invitrogen) was used according to the manufacturer's directions, with qRT-PCR analysis of the amebic transcripts performed in an Opticon II (MJ Research) machine. The reverse transcription of both sense and antisense RNA was performed using random sequence hexamers. The cDNA was subjected to 40 amplification cycles with HotStar Taq (Qiagen) Opticon. Primers were designed to amplify 100–300 base pairs using the *E. histolytica* Genome Sequencing Project and the Primer3 program (Supplemental Table 4) [3]. The fluorescent dye SYBR Green I (Molecular Probes) was used to detect the amplified cDNA. Continuous SYBR Green I monitoring during amplification using the MJR Opticon II machine was done according to the manufacturer's recommendations. All real-time amplification reactions were performed in duplicate and the resulting fluorescent values averaged. In all experiments utilizing qRT-PCR the cycle threshold values (C_T, the cycle number at which fluorescence exceeds the threshold value) were linked to the quantity of initial DNA after calibration of the amplification efficiency of the primer pair utilized [10]. To identify the most stably expressed transcripts to determine the quantity of amebic RNA isolated from the mouse ceca, the geNORM program of Vandesompele et al. [11] was used to compare a data set of "housekeeping" genes. This allowed us to empirically determine the most stably expressed of the *E. histolytica* transcripts (RNA pol II L, TSA, and cysteine protease 19). The geographic mean of the levels of these transcripts was used to compensate for the variation in the amount of amebic mRNA isolated from the infected mouse. Statistical significance was determined using either the Student's t-test or Welch's approximate t-test, which does not assume equal variance of in the two populations (InStat 2.03 program (GraphPad Software)).

3. Results and discussion

3.1. Amebic burden in infected mice

Chronic non-healing amebic colitis in the cecum of C3H and CBA mice occurs after intracecal injection of *E. histolytica* trophozoites [7]. We measured the number of amebae residing in the cecal lumen at 1, 4, 9 and 29 days after infection by determining the level of the RNA polymerase II L protein mRNA by qRT-PCR (Fig. 1A), and by semi-quantitative amebic culture (Fig. 1B). The *E. histolytica* transcriptome was measured at Days 1 and 29 after infection for two reasons, first that the amebae were most abundant then, and because these represented early and late time points in the adaptation to intestinal growth.

3.2. E. histolytica intestinal transcriptome

To identify changes in gene transcription that occurred in vivo, amebic RNA was isolated from the cecal lumen of three mice early in infection (Day 1) and three mice late in infection (Day 29), and from the cultured parasites used to initially infect the mice. Transcripts were detected for more than 80% of the ORF probes sets on the array with cDNA generated from in vitro cultivated amebae (Supplemental Table 5). A total of 523 transcripts (5.2% of all *E. histolytica* genes) were signif-

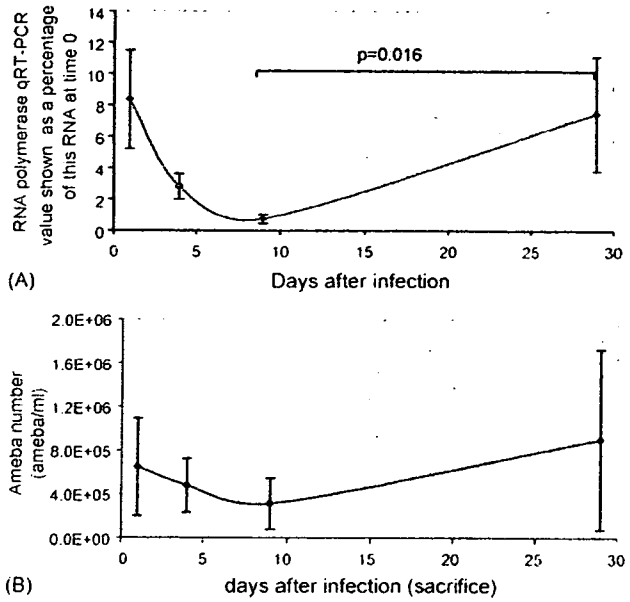


Fig. 1. Burden of *E. histolytica* in the Intestine: (A) cecal contents were washed in ice-cold PBS, and collected by centrifugation mRNA levels were measured by qRT-PCR of a relatively invariant mRNA RNA polymerase II L. *y*-axis shows RNA polymerase II L transcript levels relative to the RNA polymerase II L mRNA in the caecum right after injection (100%) and (B) semi-quantitative culture measurements of the abundance of *E. histolytica* in the cecum at Days 1–29 after infection by intracecal injection of trophozoites.

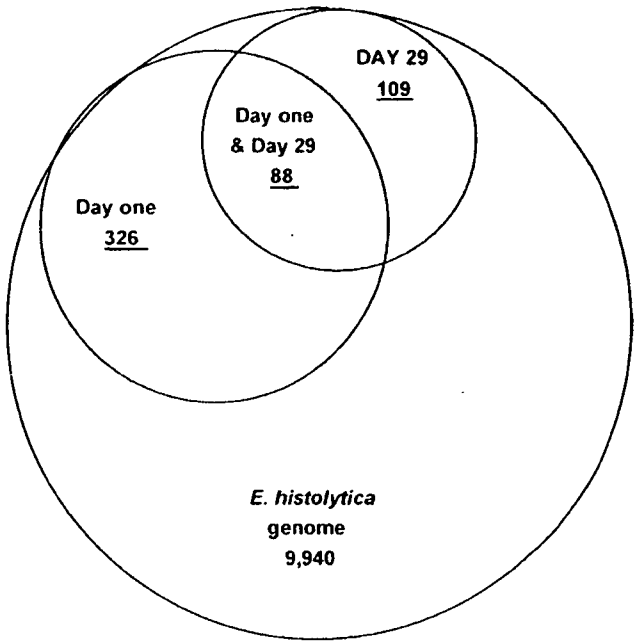


Fig. 2. Venn diagram of differentially expressed transcripts at 1 and 29 days after infection. Transcripts were included on the basis of a ≥two-fold difference and a *q*-value of ≤0.05. Some transcripts, however, while statistically changed at only Day 1 or 29, were still over two-fold different at the other day although no longer statistically significant (see Supplemental Table 5).

icantly modulated in vivo in the intestine as defined by the following criteria: probe sets with a normalized average signal intensity greater than 50 in at least one of the compared conditions; changes greater than two-fold compared to cultured amebae; and a calculated *q*-value of less than 0.05 from SAM analysis. Compared to cultured amebae, 326 of these transcripts were significantly changed only at Day 1, 109 were significantly changed only at Day 29, and 88 were changed at both time points (Fig. 2). Approximately 45% of these genes were annotated as hypothetical or of unknown function. The modulated genes were grouped into broad categories based on the genome annotation (Fig. 3) and discussed below.

3.3. Verification of the microarray data

To validate the changes in mRNA abundance detected by microarray analysis, RNA was isolated from the ceca of an additional three mice from Day 1 to Day 29. qRT-PCR was performed on six differentially expressed genes and five invariant genes, based on Affymetrix analysis. qRT-PCR data agreed with the microarray data for 10/11 transcripts, and was statistically significant in 9/11 of the genes tested (Table 1). The only transcript that gave inconsistent expression data between qRT-PCR and microarrays encoded cysteine protease 9 (CP9). The cysteine proteases are part of a large gene family and, while probes were carefully chosen, it is possible that cross-hybridization with the other highly modulated CP transcripts may account for this result. Overall we concluded that there was an excellent correlation between the array data and the qRT-PCR results.

3.4. Day 1 versus Day 29 adaptation to the intestine

At Day 1 there was a general down-regulation of genes involved in metabolism and protein translation. The mRNA levels of many of these genes returned to levels observed in cultured amebae by Day 29. We considered if this could be a reflection of an initial stress response on Day 1 to the gut environment. In fact two known stress-related transcripts (an Hsp70 and an Hsp90 gene) had significantly increased expression at Day 1. However, transcripts from two other members of the Hsp70 heat shock protein family, as well as two different transcripts encoding DNAJ proteins, and a RAD52 mRNA, were decreased in abundance at Day 1. Interestingly none of the transcripts found to be modulated in response to in vitro heat shock by Bruchhaus et al. were significantly changed in vivo at either Day 1 or Day 29 [12]. We concluded that changes in gene expression at Day 1 but not Day 29 were likely to be at least in part due to the initial stress of intestinal adaptation on the first day after infection.

3.5. Cysteine proteinase gene expression

Cysteine proteinases are hypothesized to facilitate invasion by degrading the gut extracellular matrix and inhibition of the their expression with antisense RNA or with chemical inhibitors blocks in vivo virulence. Twenty of the 29 cysteine proteinase genes were expressed above background, and of these 12 had been previously described [12]. The expression of 11 of 12 previously identified genes was consistent with prior studies (Table 2). The CP1 gene that is unique to *E. histolytica* (absent in the closely related but nonpathogen *E. dispar*) increased two-

Table 1
Verification of array data by qRT-PCR

Locus represented by probe set	Length of time in vivo (days)	Array[a]		qRT-PCR[b,c]		Gene annotation
		Fold difference	SAM q value	Fold difference	p value (t-test)	
34.m00259	29	8.29	0	6.2	0.0006	Hypothetical protein
56.m00149	29	5.45	0	23.2	0.017	Calcium binding protein[d]
395.m00028	1	14.65	0.0165	2.2	0.01	EhMGL1[e]
347.m00051	1	2.57	0.0482	1.49	0.64	Tryptophanase[f]
613.m00022	1	−1.86	0.016	−2.37	0.01	URE3-BP
2.m00532	1	2.94	0.016	0.94	0.84	Cysteine protease 9[g]
147.m00095	1	1.07	0.51	1.02	0.87	RNA polymerase II L[h]
1.m00673	1	−1.62	0.09	−1.1	0.73	Lgl5[i]
176.m00112	29	1.22	0.68	1.2	0.55	TSA[j]
344.m00043	29	1.12	0.7	−1.03	0.96	RNA polymerase II subunit 13[h]
152.m00118	29	1.14	0.7	1.05	0.85	Cysteine protease 19[g]

[a] Change in transcript levels calculated from the results of Affymetrix array hybridization of cDNA derived from in vitro and in vivo amebae (fold increase and statistical significance).
[b] Values were compared after normalization using the geographic mean of empirically determined invariant genes (RNA pol II L, TSA, and cysteine protease 19) after correction for the efficiency of amplicon amplification.
[c] Change in transcripts determined by qRT-PCR for three mice (fold increase and statistical significance).
[d] EhCaBP has been previously described by Sahoo et al. [36].
[e] EhMGL1 has been previously described by Tokoro et al. [13].
[f] Tryptophanase has been previously described by Anderson et al. [30].
[g] Cysteine protease 9 and 19 have been previously described by Bruchhaus et al. [12].
[h] RNA polymerase II L and 13 have been previously described and used as internal controls by Beck et al. [33].
[i] Lgl5 is a novel member of the family of genes which encode the light subunit of the Gal/GalNAc lectin [5].
[j] TSA has been independently described by two groups [21].

Table 2
Cysteine protease transcripts

Probe set	Day 1 in vivo		Day 29 in vivo		In vitro ameba	Annotation of gene represented by probe set[a]
	Fold difference	SAM q value	Fold difference	SAM q value	Average normalized probe value	
10.m00362_at	**28.33**	**0**	**35.41**	**0**	**82**	**Cysteine proteinase 4[b] related**
2.m00545_at	**9.4**	**0**	**6.44**	**0.11**	**97**	**Cysteine protease 6[b] related**
2.m00532_at	**2.94**	**0.02**	**2.09**	**0.41**	**72**	**Cysteine protease 9[b]**
242.m00078_s_at[c]	**1.7**	**0.09**	**2.38**	**0.04**	**5403**	**Cysteine protease 1[b]**
79.m00156						
109.m00099_at	N/A	N/A	2.08	0.65	8	Cysteine protease 14-related[b]
222.m00084_at	1.41	0.44	−1.04	0.64	8	Cysteine protease 16[b]
152.m00118_at	1.24	0.44	1.14	0.7	853	Cysteine protease 19[b]
180.m00101_at	1.2	0.48	1.01	0.7	278	Cysteine protease 17[b]
65.m00146_at	1.11	0.51	1.77	0.53	29.5	OTU-like cysteine protease, putative
3.m00597_at	1.06	0.51	1.2	0.69	142	Calpain-like cysteine protease, putative
24.m00271_at	1.05	0.51	−1.11	0.6	23	Cysteine protease 10[b]
71.m00132_at	−1.08	0.39	−1.1	0.6	25.9	Cysteine protease, putative
2.m00493_at	−1.25	0.3	1.47	0.6	13.55	Peptidase, putative
191.m00117_at	−1.48	0.09	−1.2	0.47	7587	Cysteine protease 5[b] related
260.m00068_at	−1.49	0.12	1.09	0.7	910	Cysteine protease, putative
446.m00031_at	−1.62	0.09	−1.5	0.28	38	Cysteine protease 13[b]
97.m00133_at	−1.95	0.06	−1.62	0.4	38	Cysteine protease, putative cp112[d]
501.m00019_s_at	**−14.31**	**0.01**	**−4.88**	**0.02**	**2994**	**Cysteine protease 8[e]**

[a] Genes in bold demonstrate statistically significant change.
[b] In vitro expression previously determined by Bruchhaus et al. [21].
[c] Probe set also represents the 79.m00156 open reading frame which differs by one nucleotide from 242.m00078. This causes a change in the terminal 3′ of the open reading frame and hence an extension of 57 nucleotides.
[d] Gene described by Garcia-Rivera et al. [41].
[e] Probe set also represents the 358.m00031 open reading frame, which differs by three nucleotides from 501.m00019.

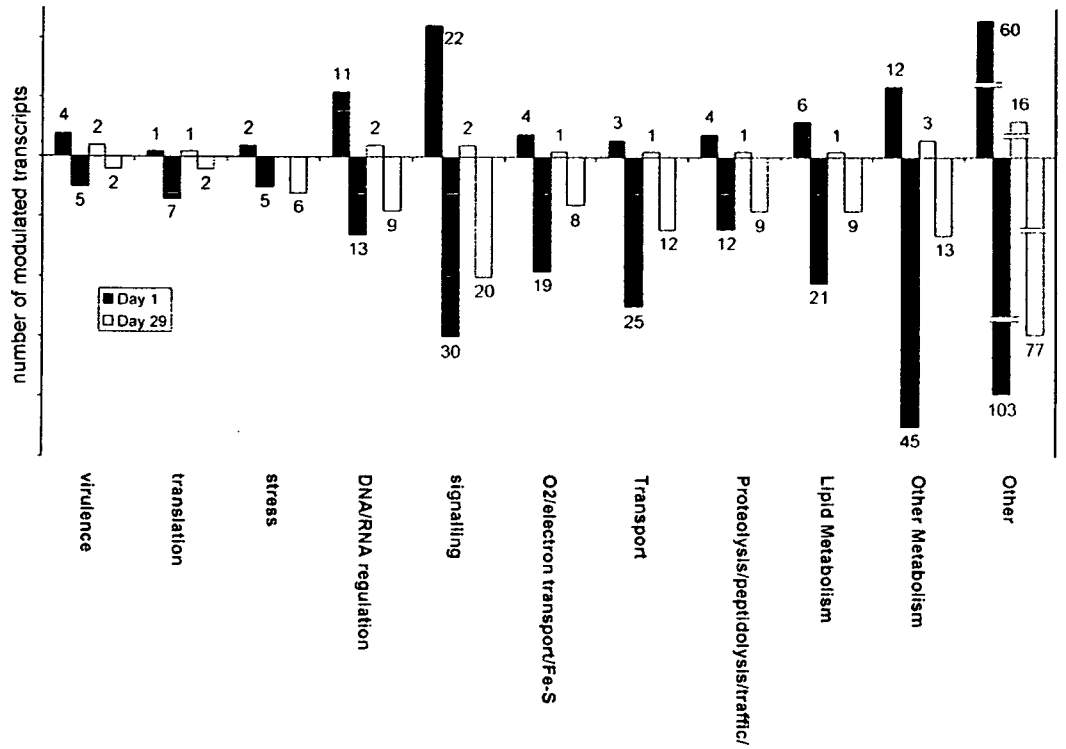


Fig. 3. Distribution of changed transcripts into functional categories. Differentially expressed genes were categorized by functional group. The changed transcripts were assigned to a putative function on the basis of gene annotation. Genes of unknown function were further annotated and categorized on the basis of Pfam/COG families and then loosely grouped (shown in more detail in Supplemental Table 5). The functional group is shown on the *x*-axis, and number of genes modulated in either a positive or negative direction is indicated on the *y*-axis. Transcripts changed at Day 1 are shown as solid bars and Day 29 modulated genes are shown as white bars. The exact number of changed transcripts is shown beside each column.

fold. The transcript from a CP4-like gene (CP10.m00362 gene) was expressed at low levels in vitro, but exhibited a 20–35-fold increase at Days 1 and 29. The CP6-like cysteine protease (CP2.m00545) was 10-fold increased at Day 1. In contrast, the CP8 transcript was significantly decreased in vivo (Table 2). The changes in transcript levels of specific CP genes suggest non-redundant functions for the individual proteases in the intestinal life of the parasite.

3.6. *Laterally-transferred metabolic enzymes*

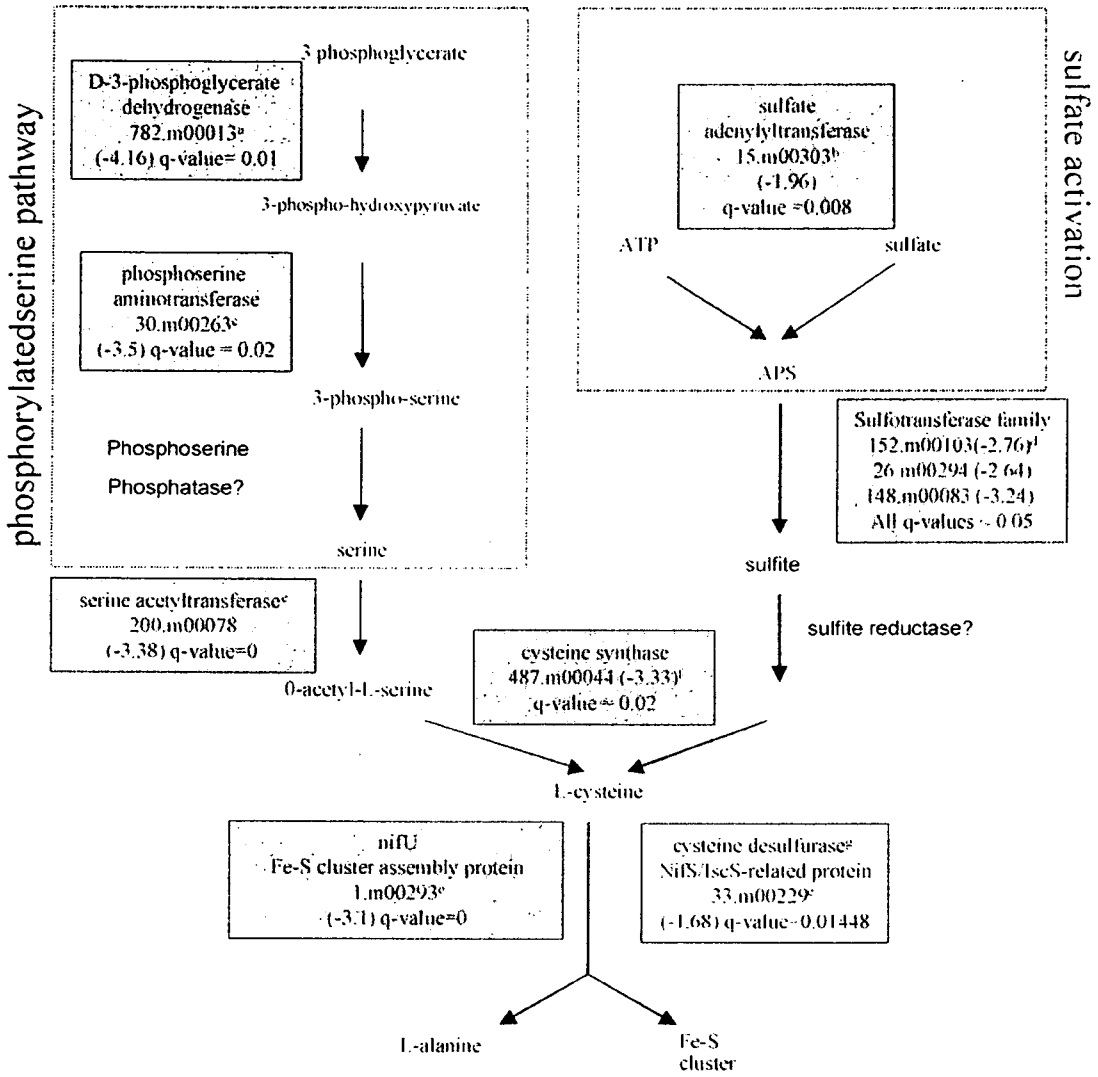
A number of important metabolic enzymes appear to have been acquired by lateral gene transfer from prokaryotes. These genes encode many of the enzymes involved in serine and cysteine biosynthesis, degradation of sulfur-containing amino acids (Fig. 4), and energy metabolism [13–16]. The results of the array hybridization indicated that 84 of 96 laterally transferred genes had detectable transcripts, with 11 decreased and 6 increased at least two-fold in vivo (Table 3).

3.7. *Oxygen detoxification*

Ameba colonize the anaerobic lumen of the gut. In this environment the proteins necessary for oxygen defense would likely not be required. *E. histolytica* is an aerotolerant anaerobe that uses an iron–sulfur ferredoxin to oxidize pyruvate as the end-electron acceptor. The levels of ferredoxin 2 [17] and iron–sulfur flavoproteins (328.m00064) transcripts were decreased, as was the mRNA of almost all genes encoding enzymes involved in the synthesis of iron–sulfur [Fe–S] clusters [18] including the sulfur assimilatory de novo cysteine biosynthetic pathway [14] (Fig. 4). One exception to the down-regulation of iron–sulfur transcripts was the mRNA of a protein with a strong match (99.8% aligned with a 770 Score, $E=0.0$) to the hybrid cluster protein (8.m00410; pfam HCP,cd01914.1). Hybrid cluster proteins incorporate both a [4Fe–4S] cubane cluster and a [4Fe–2S–2O] iron–sulfur cluster. Hybrid cluster proteins are thought to be involved in nitrate and/or nitrate respiration and have only been detected in facultative anaerobes cultivated in anaerobic conditions [19,20]. Also increased were thioredoxin (212.m00092) and glutamate synthase (78.m00152), both electron transport proteins lacking Fe–S clusters. The transcripts of other proteins involved in the detoxification of reactive oxygen metabolites were unchanged in vivo [21–24]. We concluded that adaptation to the intestine was associated with an overall decrease in the abundance of mRNAs encoding for oxygen detoxification proteins.

3.8. *Energy metabolism*

The lack of mitochondria necessitates that *E. histolytica* derive most of its ATP from glycolysis and fermentation. It was therefore notable that the transcripts of the genes encoding the glycolytic pathway were overall decreased at Day 1 but



[a] D-3-phosphoglycerate dehydrogenase has been previously described [14].
[b] sulfate adenylyltransferase has been previously described [14].
[c] phosphoserine aminotransferase has been previously described [14].
[d] the transcript of 152.m00103 is significantly increased at day 29.
[e] 200.m00078 (SAT1) has been previously described [14]. A second minor transcript. (253.m00083) encoding the same enzyme is significantly increased at day 1 and 29.
[f] 487.m00044 (CS2) has been previously described by Nozaki et al. [40].
[g] A second transcript annotated as cysteine desulfurase 46.m00208 was also down-regulated in vivo (-6.09; q-value=0). This gene may be a molybdenum cofactor sulfurase.
[h] 33.m00229 (NifS) and 1.m00293 (nifU) have been previously described [40].

Fig. 4. Modulation of transcripts encoding enzymes involved in iron–sulfur cluster biosynthesis. Transcripts significantly modulated in vivo are shown in shaded boxes. Gene name is followed by the locus represented by the microarray probe set, fold change in vivo, and significance (q-value) [14,40].

not Day 29 (Fig. 5). Diminution of mRNA levels for glycolytic enzymes with low catalytic efficiencies (that could therefore be pathway regulatory points) included one of the two pyruvate phosphate dikinase genes (−1.91 q-value = 0.05) and fructose-1,6-bisphosphate aldolase (−1.82 q-value = 0.0078) [25]. The PPi-dependent phosphofructose kinase (PFK) gene transcript was also significantly down-regulated (−2.03 q-value = 0.039). This enzyme, which accounts for most of the PFK activity of cultured *E. histolytica* trophozoites employs inorganic pyrophosphate as the phosphoryl donor to generate fructose-1,6-bisphosphate, and therefore increases the net energy generated by this pathway [26]. The transcript of a second gene encoding the ATP-requiring phosphofructose enzyme was expressed at low levels in culture, and was unchanged in vivo [27]. The potential down-regulation of the glycolytic pathway at Day 1 suggested by these results, could reflect a shift from rapid growth made possible by the abundant nutrients of tissue culture, to a more parsimonious existence in the resource poor colon [28]. An adaptation to slower growth is also suggested by the decrease in mRNA for the glucose ribose porter (53.m00214)

Table 3
Significantly changed transcripts from the laterally transfered genes

Probe set	Day 1 in vivo		Day 29 in vivo		Annotation of gene represented by probe set
	Fold difference	SAM q value	Fold difference	SAM q value	
395.m00028_s_at[a]	14.65	0.02	1.3	0.7	Methionine gamma-lyase
6.m00425_at	5.93	0.01	2.66	0.02	Endo-1,4-beta-xylanase
36.m00222_s_at[b]	3.01	0.01	1.42	0.6	Aminotransferase
78.m00152_s_at[c]	2.86	0.02	1.89	0.14	Glutamate synthase small subunit
347.m00051_at	2.64	0.04	1.93	0.6	Tryptophanase
189.m00093_x[d]_at	2.48	0	1.97	0.14	D-Hydantoinase
22.m00291_at	−1.15	0.36	−2.01	0.02	Aspartate ammonia-lyase
91.m00198_at[e]	−2.03	0.04	1.44	0.7	Pyrophosphate-dependent phosphofructokinase
25.m00254_at	−2.08	0.05	−1.65	0.35	Nicotinate phosphoribosyltransferase
1.m00704_at	−2.65	0	−1.2	0.53	Aspartate-ammonia ligase
289.m00068_at	−2.66	0.02	−2.83	0.05	Amidohydrolase
10.m00331_at	−2.96	0.01	−2.06	0.09	(2*r*)-Phospho-3-sulfolactate synthase
21.m00283_at[f]	−3.1	0	−1.18	0.57	Fe–S cluster assembly protein NifU
200.m00078_s_at[g]	−3.38	0	−5.95	0	Serine acetyltransferase
30.m00263_s_at[h]	−3.5	0.02	−1.22	0.53	Phosphoserine aminotransferase
782.m00013_s_at[i]	−4.16	0.01	−4.79	0	D-3-Phosphoglycerate dehydrogenase
103.m00185_at[j]	−10.53	0	−4.88	0.07	Fe-hydrogenase

[a] 395.m00028_s represents 202.m00088 with which it is identical and 132.m00106 from which it differs by one nucleotide. This gene has been previously described by Tokoro et al. as EhMGL1 [13].

[b] 36.m00222_s_at also represents 36.m00207 with which it differs by a 70 nt internal deletion.

[c] 78.m00152_s_at also represents 3.m00589 with which it differs by a 45 nt internal deletion.

[d] (_at) probe sets represent single genes, s_at. probe sets recognize two or more genes with which the probes are an exact match, _x_at probe sets may cross-hybridize in an unpredictable manner with sequences other than the main target.

[e] This gene has been previously described by Deng et al. [26].

[f] 21.m00283; this gene has been previously described [14].

[g] 200.m00078_s also represents 141.m00079 with which it is identical. This gene has been previously described by Nozaki et al. as SAT1 [14].

[h] 30.m00263_s_at represents 201.m00117 with which it is identical and 253.m00078 which differs from the other two genes by 9 nucleotides in 963 nts and whose ORF terminates 114 nts sooner than that of the other transcripts. This gene has been previously described [14].

[i] 782.m00013_s also represents 130.m00118 and 130.m00121. 130.m00118 differs by a one base pair insertion 12 nt from the terminal of 782.m00013 which changed the ORF 130.m00118 extend an additional 258 nt. 130.m00118 and 130.m00121 ORFs are identical. This gene has been previously described [14].

[j] 103.m00185 has been previously described as iron-dependent hydrogenase 2 by Nixon et al. [15].

and several tRNA synthetase genes, although no changes were observed for DNA replication and nucleotide synthesis genes.

An alternative hypothesis is that different pathways for energy generation are utilized in vivo. Zuo et al. had suggested that in the absence of glucose *E. histolytica* may catabolize amino acids; however, no consistent increase in the transcripts involved in this process was observed [29–30]. In contrast, an increase in the transcripts of two putative lipases and phospholipases suggested that this alternative energy source may be preferentially utilized in vivo.

3.9. Intracellular signaling proteins

Several potential DNA and RNA binding protein gene mRNA levels were modulated in response to the intestinal environment (Table 4). The EhEBP 1 transcription factor, an enhancer of Gal/GalNAc lectin *hgl5* subunit transcription, was down-regulated two-fold at Day 1 [31]. Four DNA binding proteins with 6–16-fold changes at Day 29 (111.m00140, 227.m00086, 143.m00093, 20.m00272) shared similarity at the protein level (71–83%). One of the other hypothetical transcripts increased at Day 29 (seven-fold) encoded a basic region leucine zipper domain. The pre-mRNA splicing factor PrP17 mRNA necessary for the splicing of introns greater than 200 nucleotides in *S. cerevisiae* and *S. pombe* was also increased [32]. A second potential RNA binding protein (91.m00190), similar to a *P. falciparum* protein RNA binding protein, was down-regulated two-fold at both Days 1 and 29.

Transmembrane receptor kinases (TMK) are key mediators of signal transduction in eukaryotic cells. *E. histolytica* is remarkable among protists for possessing over 80 putative TMKs. The *E. histolytica* TMK's have been grouped into six subfamilies (A–F) based on the kinase domain and motif structure of the extracellular domains [33]. Members of each of the subfamilies were discovered to be expressed in vitro and in vivo. Transcripts from eight TMKs were modulated in vivo (Table 5). Transcripts from 9 non-receptor *E. histolytica* kinases, and 10 phosphatases genes were also modulated. The transcript of phosphoinositide 3-kinase (9.m00406), implicated in phagocytosis, was one of the kinases decreased at Day 29 [34,35]. The mRNA of several calcium binding proteins were also changed in abundance. One of the up-regulated transcripts in vivo was EhCaBP [36], while transcripts of the potential calcium storage proteins Grainins 1 and 2 [37] were down-regulated. We concluded that the adaptation to the intestinal environment was accompanied, and likely in part mediated by, alterations in mRNA abundance of key signaling molecules.

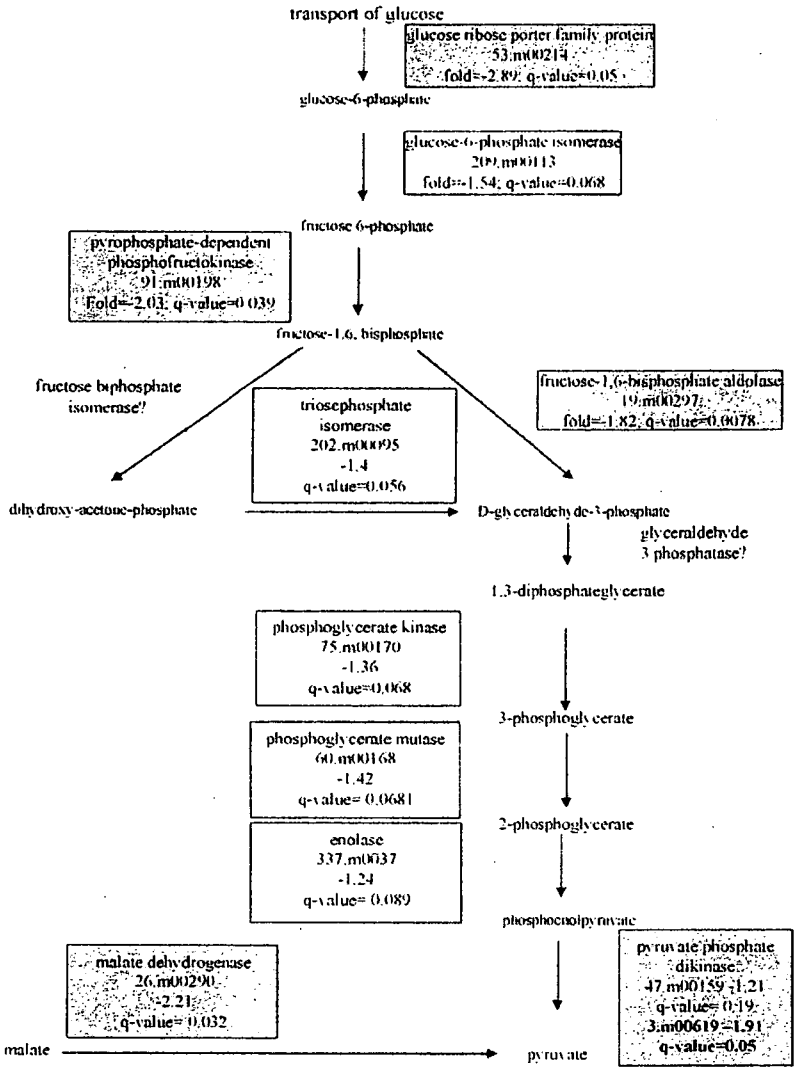


Fig. 5. Changes in expression of the genes encoding enzymes of the glycolytic pathway. Schematic representation of the *E. histolytica* glycolytic pathway. Transcripts significantly modulated in vivo are shown in shaded boxes. Gene name is followed by the locus represented by the microarray probe set, fold change in vivo and significance (*q*-value).

3.10. Other virulence genes

Of the proteins known to be important in *E. histolytica* virulence few changes in transcript levels were observed. This is perhaps not surprising because all of these factors were identified and characterized in vitro using cultured trophozoites. Among virulence factors that were altered was the Gal/GalNAc lectin, essential for ameba adherence and contact mediated cytolysis of host cells. The Gal/GalNAc lectin light and heavy subunits are each encoded by five member gene families, while the intermediate subunit is encoded by two different genes [5]. There were no statistically significant changes in the heavy and intermediate transcripts; however, two members of the light subunit gene family were statistically down-regulated (*lgl2* two-fold at Day 1 and *lgl3* four-fold at Day 1 and Day 29). In addition to these changes in known virulence factors, it is interesting to speculate if novel virulence factors may be among the hypothetical genes with altered in vivo expression.

3.11. Membrane transport

Nutrient acquisition may be an important aspect of virulence. Many of the genes with putative transport functions were down-regulated, including several members of the major facilitator superfamily, and two members of the ABC transporter family. Exceptions were increases (10-fold at Day 1 and 8-fold at Day 29) of a transcript encoding a potential amino-acid transporter (205.m00085), and the significant reversal of the down-regulation of one of the ABC transporter protein transcripts (41.m00219) (three-fold decrease at Day 1 but 1.3-fold increase at Day 29).

3.12. Endocytosis and vesicular trafficking

E. histolytica genes involved in motility and phagocytosis were also modulated. Rho family GTPases, a SNARE pfam domain-containing genes (141.m00087), Syntaxin A, and a