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知りたい!サイエンス

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—脳にも？　意外に身近なパラサイト—

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4．免疫学的検査／C．感染症・免疫血清診断

抗アニサキス IgG・A 抗体

丸山治彦

表1　デシジョンレベル

Ig(G+A) Index	その他の検査所見	消化器症状	方　針
Index＜1.5	好酸球増多，IgE 上昇などがみられない場合	な　し	寄生虫感染の可能性は少ない．他疾患の検査実施
		あ　り	要再検査
	好酸球増多，IgE 上昇などを伴う場合		アニサキス症以外の寄生虫症のスクリーニングが必要
Index＞1.5		な　し	不顕性感染，異所寄生の可能性あり，要精査
		あ　り	アニサキス症の可能性大

■検査の意義

アニサキスはクジラなど海獣類の胃に寄生する回虫の仲間の線虫で，幼虫はサバやアジ・タラ・イカなど多種類の魚介類に寄生している．人間がこれらを刺身や鮨などの形で生食すると，経口摂取された幼虫が胃や小腸の粘膜に侵入してアニサキス症を引き起こす．

本症の代表的な病型は胃アニサキス症で，食品摂取後数時間以内に激しい腹痛，悪心・嘔吐を引き起こす．この場合，通常は内視鏡による虫体の確認と摘出が行われるので抗体検査は必要ない．しかしながら，ときに小腸下部など内視鏡による虫体の確認や摘出が困難な部位に侵入したり（腸アニサキス症），腹腔や胸腔などに迷入することが知られている（異所性アニサキス症）．そのような際には血清検査が有用である．札幌 IDL が札幌医大第一病理と共同で開発した血清中抗アニサキス IgG・A 抗体価測定用 ELISA キット（PARACHECKELISA アニサキス）は1994年に体外診断用医薬品として承認を受け（Kishimoto Clin. Lab. Group から市販），全国で同一のキットを用いての診断が可能となっている．

■デシジョンレベル（表1）

血清中抗アニサキス IgG・A 抗体価測定用 ELISA キットは96穴プレートにモノクローナル抗体を用いてアニサキス抗原を固相化し，その抗原に結合した血清中の抗体の量をペルオキシダーゼ標識二次抗体を用いて吸光度により測定する方法である．結果の判定はロットごとの変動を考慮して補正し，Index として表す．

Index＝(検体の吸光度－ブランクの吸光度)/(陰性コントロールの吸光度－ブランクの吸光度)×F

F：ロットごとに定められた補正係数

■どういうときに検査するか

胃アニサキス症では内視鏡による診断をかねた治療が可能なので，通常は免疫診断の対象とはならない．新鮮な魚介類を加熱せずに食べた後，腹痛や悪心・嘔吐などの消化器症状が数日以上持続し，細菌などによる食中毒が否定的で好酸球増多や IgE の上昇がみられた場合に，腸アニサキス症や異所性アニサキス症を疑って本検査を実施することになる．また，手術標本の病理検査で偶然虫体の断面が見つかることがあり，そのような場合にも抗体の有無を調べることがある．

■判定上の注意

この ELISA キットを用いて健常者血清を調べると約10％の陽性者があるが（図1），日本

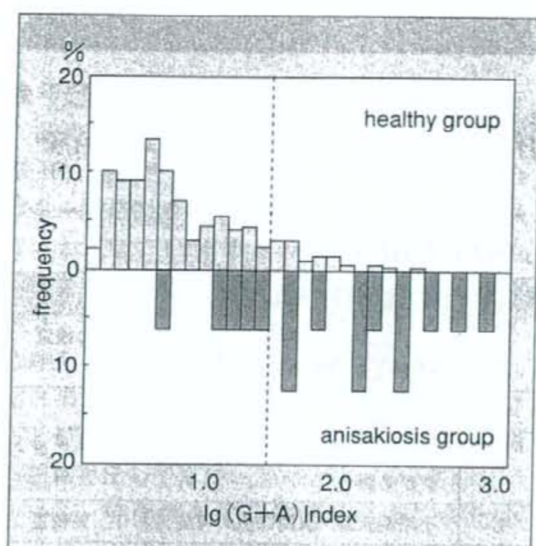

図1　健常者とアニサキス症患者の抗体価
検体数は健常者323，患者16．Index 1.5（点線）をカットオフ値としたとき，抗体陽性率は健常者9.3%，患者68.8%であった．（文献1）より転載）

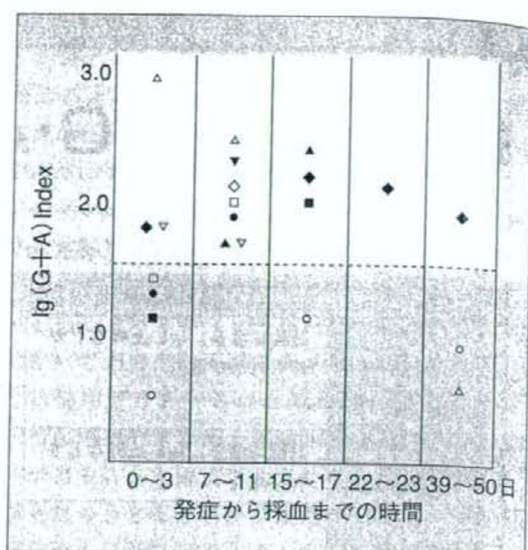

図2　抗アニサキス抗体価の経時変化
発症後7日から11日の間に測定した7例は全例陽性となった．このうち3例（■，●，□）は発症後3日以内の最初の測定のときは陰性であった．（文献1）より転載）

人の食習慣から考えて，これらの陽性者は近い過去に自覚症状なしに感染を経験し自然治癒したと考えてよいであろう．また，アニサキス症では発症直後に抗体価は必ずしも上昇しておらず，発症から1～2週後に陽性となることがある（図2）．したがって，初回検査が抗体陰性でも臨床的にアニサキス症が疑われる場合には，1～2週後に再検査することが望ましい．ただし，アニサキスはヒト体内では長期生存はできず，2週間ほどすると死滅するとされているので，感染後あまりに長期間経過すると抗体は陰性化すると考えられる．抗アニサキス抗体が陰性で，好酸球増多やIgE上昇などから寄生虫感染が疑われる場合には，寄生虫症全般についての免疫血清学的なスクリーニングが望ましい．

■参考事項

（株）エスアールエルの多項目アレルゲン特異的IgE測定用キットAlaSTATにはアニサキスが含まれており，それを利用することもできる．また，同社ではアニサキスを含む12種類の寄生虫抗原を用いたmultiple-dot ELISA法による抗寄生虫抗体スクリーニングを保険適用外検査として実施している．何らかの寄生虫の関与が疑われるときは利用するとよいであろう．この場合，陽性から疑陽性との判定が出た例については，宮崎大学医学部感染症学講座寄生虫学分野で精査を行うシステムになっている[3]．また，同社を介さずに，宮崎大学医学部寄生虫学へ直接抗体検査を申し込むことも可能である．詳細については同社に問い合わせるか，宮崎大学医学部寄生虫学のウエブサイトを参照していただきたい．

(URL: http://www.miyazaki-med.ac.jp/parasitology/default.html)

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CHAPTER TWENTY-FOUR

Analysis of Autophagy in the Enteric Protozoan Parasite *Entamoeba*

Karina Picazarri,* Kumiko Nakada-Tsukui,*,† Dan Sato,*,‡,§ *and* Tomoyoshi Nozaki*,†

Contents

1. Introduction 360
   1.1. Organisms 360
   1.2. Disease and clinical manifestations 361
2. Unique Features of Autophagy in *Entamoeba* 361
   2.1. Genome-based identification of genes involved in autophagy in *Entamoeba* 361
   2.2. Uniqueness of autophagy in *Entamoeba* 362
3. Analysis of Autophagy in *Entamoeba* 363
   3.1. Production of recombinant *E. histolytica* Atg8 and its antibody 363
   3.2. Quantitative analysis of kinetics and modification of EhAtg8 by immunoblot analysis 365
   3.3. Visualization of autophagy by indirect immunofluorescence assay 367
   3.4. Construction of *E. histolytica* transformants expressing an HA-tagged or Myc-fused protein of interest 368
4. Conclusion 369
Acknowledgments 370
References 370

Abstract

Entamoeba histolytica is the enteric protozoan parasite that causes human amoebiasis. We have previously shown that autophagy is involved in proliferation and differentiation in the related species *Entamoeba invadens*, which infects reptiles and develops similar clinical manifestations. Because this group of protists possesses only a limited set of genes known to participate in autophagy in other eukaryotes, it potentially represents a useful model for

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studying the core system of autophagy and provides tools to elucidate the evolution of eukaryotes and their organelles. Here we describe the methods to study autophagy in *Entamoeba*.

1. Introduction

1.1. Organisms

Entamoeba histolytica is the enteric protozoan parasite that causes human amoebiasis (Petri *et al.*, 2002; Stanley *et al.*, 2003). This anaerobic or microaerophilic eukaryote has a simple life cycle consisting of two forms: the motile, proliferative trophozoite (the active, feeding stage) (Fig. 24.1), which is responsible for the pathology of amoebiasis, and the dormant, infective cyst, which is essential for transmission. This organism lacks organelles commonly observed in other eukaryotes, such as the mitochondria, the peroxisome, and the Golgi apparatus, and is considered one of the early branching eukaryotes (Hasegawa *et al.*, 1993; Loftus *et al.*, 2005). Recent discovery of the mitochondria-related genes (e.g., Cpn60) and the mitochondrial-related remnant organelle, named the *mitosome* (Tovar *et al.*, 1999), led to the presumption that this organism secondarily lost the mitochondria (Clark, 2000). Thus, this organism potentially helps in the elucidation of important questions on the evolution of eukaryotes and organelles.

The developmental transition of the trophozoite to the cyst stage, called *encystation*, is the essential process for transmission and reinfection of the organism. Therefore, its interruption is potentially exploitable to interfere with dissemination of this organism. However, inability to induce encystation *in vitro* hampers molecular understanding of the process in *E. histolytica* (Eichinger *et al.*, 2001) . *Entamoeba invadens*, a related *Entamoeba* species that

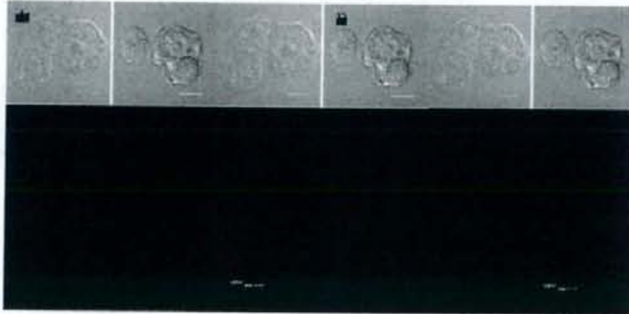

Figure 24.1 Differential interference contrast images of *E. histolytica* trophozoites. (A) two trophozoites containing numerous vacuoles. (B) A trophozoite (*right*) ingesting a Chinese hamster ovary cell (*left*).

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infects reptiles and causes similar clinical manifestations, is instead used as a model of encystation because of the ease of inducing encystation using an artificial low-osmolarity, glucose-deprived encystation medium (Sanchez *et al.*, 1994).

1.2. Disease and clinical manifestations

There have been an estimated 40–50 million cases of amoebic colitis and liver abscess, which cause 40,000 to 110,000 deaths worldwide each year (Clark *et al.*, 2000; WHO, 1997). Infection of human and other mammalian hosts occurs upon ingestion of water or food contaminated with cysts. *E. histolytica* cysts are round, usually 10–15 μm in diameter, and protected by a chitin-containing wall. After ingestion, the cyst excysts in the small intestine to release the polymorphic trophozoite, which varies in size from 10–50 μm in diameter. Highly motile trophozoites colonize the large intestine. Epidemiological studies suggested that only 3% of infected individuals develop symptoms such as amoebic colitis and dysentery (Haque *et al.*, 2001; Stanley *et al.*, 2003), while the rest of the infected individuals remain asymptomatic and are able to clear the infection without developing disease (Haghighi *et al.*, 2003; Haque *et al.*, 2006; Stanley *et al.*, 2001). However, asymptomatic carriers represent a risk of contagion, and up to 10% of them develop disease within a year after infection (Stanley *et al.*, 2003). Children (Haque *et al.*, 2001; Warunee *et al.*, 2007), immunocompromised individuals (Hung *et al.*, 2008), men who have sex with men, and mentally handicapped persons (Nozaki *et al.*, 2006) are often more susceptible to infection. The trophozoite is responsible for all clinical manifestations, including abdominal pain, tenderness, and bloody diarrhea. *E. histolytica* can also colonize organs other than the intestine via hematogenous (i.e., originating in the blood) spread of trophozoites from the colon in 5%–10% of the diarrheal/dysenteric patients. Liver abscess is the most common extraintestinal form of amoebic infection. Patients who develop liver abscess present fever, right-upper-quadrant pain, hepatic tenderness, cough, anorexia, and weight loss.

2. Unique Features of Autophagy in *Entamoeba*

2.1. Genome-based identification of genes involved in autophagy in *Entamoeba*

In *Saccharomyces cerevisiae* approximately 30 genes have been identified as involved in autophagy (Klionsky *et al.*, 2003; Suzuki and Ohsumi, 2007; Xie and Klionsky, 2007), of which 17 genes encode proteins composing the core machinery of autophagy (Suzuki and Ohsumi, 2007). Most of them are also conserved in higher eukaryotes including mammals (Mizushima *et al.*,

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2002; Xie and Klionsky, 2007). Autophagy genes have been categorized into four functional groups: regulation of autophagy induction, vesicle nucleation, vesicle expansion and completion (consisting two ubiquitin-like conjugation systems), and retrieval (Levine and Yuan, 2005). Among them, *Entamoeba* lacks genes involved in the regulation of the autophagy induction except TOR (target of rapamycin), the entire Atg12–Atg5 conjugation system, and the retrieval system, whereas genes involved in the nucleation of the isolation membrane forming the phosphatidylinositide complex (Vps15, Vps34, and Atg6/Beclin1) and the Atg8 conjugation system (Atg7, Atg3, Atg8, and Atg4) are conserved (Picazarri *et al.*, 2008).

2.2. Uniqueness of autophagy in *Entamoeba*

In both *E. histolytica* and *E. invadens*, the Atg8-positive structures have been identified by confocal microscopy as punctate particulate (or dotlike), vesicular, vacuolar (1–5 μm in diameter), linear, or aggregate-like structures (Fig. 24.2). The size and shape of autophagosomes in this organism is rather unique, and among the largest similar to ones containing intracellular pathogens, Group A *Streptococci* and *Mycobacterium tuberculosis* (Andrade *et al.*, 2006; Gutierrez *et al.*, 2004; Nakagawa *et al.*, 2004).

Autophagy is induced under particular conditions such as starvation, differentiation, defense, and antigen presentation in other organisms (Andrade *et al.*, 2006; Besteiro *et al.*, 2006; Nakagawa *et al.*, 2004; Nimmerjahn *et al.*, 2003). In *Entamoeba*, neither nutrient (e.g., glucose and serum) deprivation nor stress (e.g., heat and oxidative stress) induces the formation Atg8 structures in *E. histolytica*. Autophagosomes are constitutively present in the proliferative trophozoite. In *E. invadens*, where encystation can be induced *in vitro*, autophagosome formation is up-regulated at the mid-to-late logarithmic growth phase and at the early phase of encystation. In *E. invadens*, phosphatidylinositol 3-kinase inhibitors simultaneously inhibit the formation of the Atg8-postive structures and encystation in a dose-dependent manner. This observation suggests a close correlation between autophagy and encystation via phosphatidylinositol 3-kinase-mediated signaling. These data are consistent with the premise that autophagy plays a housekeeping role in *Entamoeba*, as seen in neurons where autophagy was suggested to be involved in the constant turnover of undesirable polyubiquitinated proteins (Komatsu *et al.*, 2006). This chapter describes some essential protocols to understand the function of autophagy in *Entamoeba*: immunoblot and immunofluorescence assays, as well as the creation and analysis of *E. histolytica* transformants expressing an epitope-tagged protein of interest.

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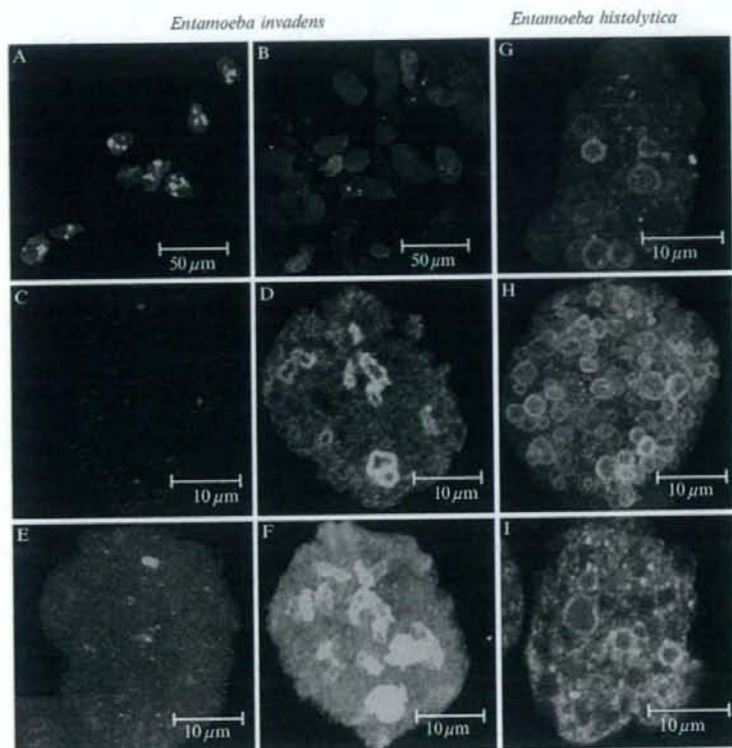

Figure 24.2 Immunofluorescence images of autophagy in *E. invadens* and *E. histolytica* on a confocal microscope. (A, B) Autophagosomes in the proliferating *E. invadens* trophozoites at 1 (logarithmic phase, A) and 2 weeks after the initiation of the culture (stationary phase, B). (C–F) Autophagosomes in the encysting *E. invadens* trophozoites at 0 (C, E) and 24 h postencystation induction (D, F). Single slices (C, E) and maximum projections of 20 slices taken at 1-μm intervals on the z-axis (E, F) are shown. (G, H) Autophagosomes in the proliferating *E. histolytica* trophozoites at days 1 (logarithmic phase, G) and 5 (stationary phase, H) (maximum projection). (I) Colocalization of Atg8 (green) and the lysosome marker, LysoTracker Red (red) in an *E. histolytica* trophozoite (day 3).

3. Analysis of Autophagy in *Entamoeba*

3.1. Production of recombinant *E. histolytica* Atg8 and its antibody

Oligonucleotide primers and conditions of PCR amplification of *EhAtg8a* cDNA have been described elsewhere (Picazarri *et al.*, 2008). Cloning of the *EhAtg8a* cDNA into the pGEX-6P-2 (GE Healthcare Bioscience,

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27-4598-01) expression vector to make pGST-EhAtg8a as well as antibody production have also previously been described in detail (Picazarri *et al.*, 2008).

3.1.1. Production of recombinant EhAtg8

1. Transform *E. coli* BL21(DE3) competent cells with pGST-EhAtg8 plasmid.
2. Select transformants on ampicillin plates. Grow a transformant overnight in 20 ml of LB containing 50 μg/ml ampicillin and use to inoculate 200 ml of the same medium. Shake at 37 °C until the O.D.$_{600}$ reaches 0.5.
3. Add 1 m*M* IPTG to the culture to induce expression of GST-EhAtg8 recombinant protein and continue shaking for 3 h at 37 °C.
4. Harvest bacteria at 6000×*g* for 10 min, wash the pellet twice with phosphate-buffered saline (PBS), pH 7.4, and resuspend in 5–20 ml of the lysis buffer (50 m*M* NaH_2PO_4, pH 8.0, 300 m*M* NaCl, 1 m*M* DTT, and complete mini protease inhibitor cocktail).
5. Sonicate the suspension using a VP-15S UltraS Homogenizer (TAITEC) or equivalent in an ice-water bucket. Occasionally examine the lysate under a light microscope with a 40x phase contrast objective to confirm the disruption of bacteria.
6. Centrifuge at 12,000×*g* for 20 min at 4 °C to remove debris.

3.1.2. Purification of recombinant EhAtg8 using glutathione-sepharose affinity chromatography

1. Incubate 1 volume (5 ml) of the clarified lysate with two-thirds volume of glutatione-sepharose (GE Healthcare Bioscience, 17-5279-01) slurry (50%) for 0.5 h on a rocking platform at 4 °C. Transfer the resin to a column, and wash the resin three times with 10 ml of PBS.
2. Elute GST-EhAtg8 recombinant protein with 5 ml of elution buffer (PBS containing 10 m*M* reduced glutathione) twice at room temperature.
3. Filter the eluted fractions with a 0.45-μm syringe filter and dialyze it against protease cleavage buffer (50 m*M* Tris-HCl, pH 7.0, 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT) using Slide-A-Lyzer (PIERCE, #66110; molecular weight cut off, 3500) overnight at 4 °C.
4. To remove the GST tag from the recombinant protein, mix 10 ml of the purified protein with 80 μg of PreScission protease (GE Healthcare Bioscience) and incubate at 4 °C for 4 h.
5. Add 2 ml of glutathione-Sepharose (50% slurry) to the mixture and rotate it at room temperature for 30 min. Briefly centrifuge the mixture and filter the supernatant with a 0.45-μm syringe filter.

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6. Dialyze the filtrate against 2 L of PBS for 2 h and then 3 L of PBS overnight at 4 °C. Finally, remove remaining GST tag and PreScission protease by passing the dialyzed solution through GSTrap (GE Healthcare Bioscience).

3.1.3. Purification of recombinant EhAtg8 using Mono Q anion exchange chromatography

1. Dilute the eluate from GSTrap by 5-fold with the starting buffer (50 m*M* 2, 2′-iminodiethanol, pH 8.4).
2. Preequilibrate a Mono Q HR 5/5 anion-exchange column (GE Healthcare Bioscience) with the starting buffer on an AKTA Explorer.
3. Apply the affinity-purified recombinant EhAtg8 protein onto the column with the starting buffer, and wash the column with 5 volumes of the starting buffer to remove unbound proteins.
4. Elute the proteins with a linear gradient of NaCl (0–1 *M* in 20 ml) with a flow rate of 1.0 ml/min. The recombinant EhAtg8 protein elutes at circa 150 m*M* NaCl.
5. Dialyze the eluted EhAtg8 protein against PBS at 4 °C for 4–5 h.

3.2. Quantitative analysis of kinetics and modification of EhAtg8 by immunoblot analysis

3.2.1. Preparation of amoeba lysates and separation of membrane and soluble fractions by centrifugation

1. Chill a 36-ml semiconfluent amoeba culture ($\sim 3\times10^6$ cells) cultivated in a 25-cm^2-plastic flask (Nunc Brand Products, Denmark) on ice for 5 min. Cultivate *E. histolytica* and *E. invadens* trophozoites in BI-S-33 medium [3.4% BLL Biosate Peptone (Becton, Dickinson, France; 211862), 63 m*M* D-glucose, 38.9 m*M* NaCl, 5 m*M* KH_2PO_4, 6.52 m*M* K_2HPO_4, 9.37 m*M* L-cysteine hydrochloride, 1.3 m*M* L-ascorbic acid, 0.1 m*M* Ferric ammonium citrate] (Diamond *et al.*, 1978) at 35.5 °C and 26 °C, respectively, as described subsequently (see section 3.4.1). To obtain *E. invadens* cysts, transfer the 1-week-old trophozoite culture to 47% LG medium [1.5% BLL Biosate Peptone, 17 m*M* NaCl, 2.195 m*M* KH_2PO_4, 2.86 m*M* K_2HPO_4, 3.159 m*M* L-cysteine hydrochloride, 0.565 m*M* L-ascorbic acid, 0.0428 m*M* Ferric ammonium citrate] (Sanchez *et al.*, 1994) at $\sim 6\times10^5$ cells/ml.
2. To monitor encystation, examine resistance to 0.05% Sarkosyl (Sanchez *et al.*, 1994) as follows. Centrifuge 1.2×10^5 cells at $200\times g$ for 5 min, discard supernatant, and add 200 μl of PBS containing 0.05% Sarkosyl. After incubation for 25 min, add 200 μl of PBS containing 0.44%

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Trypan blue. After 5 min, count unstained live cysts and stained dead trophozoites to calculate the percentage of cysts.

3. Harvest cells by centrifugation at 400×*g* for 5 min at 4 °C.
4. Resuspend the pellet with 10 ml of PBS containing 2% glucose, centrifuge at 400×*g* for 5 min, and carefully discard the supernatant by decanting.
5. Resuspend the pellet in 1 ml of homogenization buffer (50 m*M* Tris, pH 7.5, 250 m*M* sucrose, 50 m*M* NaCl, 200 *μM* trans-epoxysuccinyl-L-leucylamido-[4-guanidino butane] (E64)).
6. Transfer the suspension to a Dounce glass homogenizer and mechanically homogenize cells with 50–300 strokes depending on homogenizers and cell types. Occasionally examine the lysate under a light microscope with a 10–40x phase contrast objective to verify completeness of homogenization.
7. Transfer the lysate to a 1.5-ml tube, centrifuge at 700×*g* for 2 min, recover the supernatant, and discard unbroken cells in the pellet.
8. Centrifuge the supernatant at 100,000×*g* in an ultracentrifuge tube at 4 °C for 1 h.
9. Recover the supernatant and the pellet fractions separately. Carefully resuspend the pellet by pipetting with 1 ml of PBS and centrifuge to wash the pellet to minimize carryover from the supernatant.
10. Resuspend the pellet in the original volume (step 4) of the lysis buffer (50 m*M* Tris, pH 7.5, 1% Triton X-100, 1.34 m*M* E64).
11. Electrophorese 5 *μ*g of each sample by SDS-PAGE on a 13.5% polyacrylamide gel containing 6M urea.
12. Carry out immunoblot analysis using anti-EhAtg8a antibody (Picazarri *et al.*, 2008) (1:1000 dilution in Tris-buffered saline containing 0.05% Tween 20). Develop the membrane using chemiluminescence with the Immobilon Western Substrate (Millipore Corporation) according to the manufacturer's instructions.

3.2.2. Delipidation of EhAtg8 by phospholipase D and quantitation of unmodified and phosphatidylethanolamine-modified EhAtg8

1. Harvest amoeba cells from a 6-ml culture (~6×10^5 cells) as described above and resuspend and lyse the cell pellet in 50 *μ*l of lysis buffer (50 m*M* Tris, pH 7.5, 1% Triton X-100, 1.34 m*M* E64) on ice. Determine the protein concentration of the lysate, after centrifugation to remove unbroken cells.
2. Mix 10 *μ*g of the crude lysate with 2 *μ*l of 20U/*μ*l phospholipase D (Sigma-Aldrich, P8023) dissolved in 50 m*M* Tris, pH 7.5. Incubate the mixture at 37 °C for 1 h.

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3. Perform SDS-PAGE on a 13.5% polyacrylamide gel containing 6 *M* urea and transfer proteins to a nitrocellulose membrane. Conduct immunoblot analysis as described previously in section 3.2.1, step 12.
4. Visualize unmodified and phosphatidylethanolamine-modified Atg8 on a Lumi-Imager F1 workstation (Roche Applied Science) and capture the images with Lumianalyst Software. Quantify the bands with ImageJ. Unmodified Atg8 is present as a single band in *E. invadens* and doublets in *E. histolytica* around 15.0 kDa, while phosphatidylethanolamine-modified Atg8 is observed as a 14.5-kDa band in *E. invadens* and two bottom bands in *E. histolytica*.

3.3. Visualization of autophagy by indirect immunofluorescence assay

3.3.1. Sample preparation

1. Harvest and wash amoebae from a 6-ml semiconfluent culture ($\sim 6 \times 10^5$ cells) at 4 °C as described previously.
2. Resuspend the pellet with 50 μl of PBS containing 3.7% paraformaldehyde. Incubate the mixture for 10 min at room temperature.
3. Centrifuge the mixture at 800×*g* for 3 min at room temperature, remove the supernatant fraction, and carefully resuspend the pellet by pipetting with 1 ml of PBS .
4. Centrifuge again, discard the supernatant, resuspend the pellet in 50 μl of PBS containing 0.2% saponin (PBSS), and incubate for 10 min at room temperature.
5. Wash the amoebae with PBS as described previously.
6. After discarding the supernatant, add to the pellet 100 μl of PBS containing anti-EhAtg8a antiserum (1:1000 dilution), resuspend, and incubate at room temperature for 1 h.
7. Wash the amoebae once with PBS as described previously.
8. Add 100 μl of anti-rabbit IgG conjugated with Alexa Flour 488 (1:1000 dilution) to the washed amoebae and incubate 1 h at room temperature.
9. Wash the amoebae once with PBS as described previously.
10. Resuspend the amoebae in $\sim$10 μl of the mounting medium (PBS, pH 8.0, containing 5.5 m*M* O-phenylenediamine dihydrochloride and 90% glycerol).

3.3.2. Confocal microscopy

Perform confocal microscopy on a Zeiss LSM 510 microscope or equivalent. Typically, we use a Plan-Apochromat 63x/1.4 Oil objective with an argon laser (488 nm) with appropriate configurations for Alexa Flour 488. It is important to optimize detector gain and amplifier offset with preimmune serum to eliminate background. Typically, images of 5–30 slices on the z-

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axis at 1–3 μm intervals should be captured. Obtained images can be also analyzed after creating a 3-D projection.

3.4. Construction of *E. histolytica* transformants expressing an HA-tagged or Myc-fused protein of interest

Construct a plasmid of interest using epitope (hemagglutinin or myc) tag-containing vector [e.g., pKT-3M (Saito-Nakano *et al.*, 2004) and pEhExHA (Nakada-Tsukui *et al.*, unpublished)], or a GFP- or RFP-fusion vector [e.g., pKT-MR (Nakada-Tsukui *et al.*, unpublished)], according to general recombinant DNA techniques.

3.4.1. Cell preparation

1. Inoculate an appropriate number of trophozoites of *E. histolytica* HM1: IMSS cl6 strain (Diamond *et al.*, 1972), harvested in the mid-late logarithmic growth, phase into fresh BI-S-33 medium in 25-cm^2-ml plastic flasks and cultivate for 24–30 h until the culture reaches semiconfluence ($1.5–2 \times 10^6$ cells per flask).
2. After decanting the culture medium, add 5 ml of the cold fresh medium to each flask and resuspend cells by chilling the flask on ice for 5 min. Mix suspensions and adjust the cell density to $7–9 \times 10^4$ amoebae per ml.
3. Transfer 5 ml of the suspension to a well of a 12-well flat bottom plate (Corning).
4. Seal the plate in a plastic bag containing Anaerocult A (Merck, 64271 Darmstadt, Germany). Incubate the plate at 35.5 °C for at least 30 min.

3.4.2. Lipofection

1. Right before transfection, prepare the transfection medium [TM, Opti-MEM I (Invitrogen, 31985-070), containing 56.7 m*M* L-ascorbic acid and 317 m*M* L-cysteine, pH 6.8, filter-sterilized].
2. Prepare DNA-liposome mixture as follows. Mix ~3–5 μg of DNA, sterilized with ethanol precipitation and dissolved in 30 μl of double-distilled sterile water, with 10 μl of Plus Reagent (Invitrogen, 11514015) and 10 μl of TM (mixture #1). Incubate the mixture #1 at room temperature for 15 min. Plasmids prepared using any spin column-based kits commercially available usually give comparable results.
3. Mix 20 μl Lipofectamine (Invitrogen, 183224-012) reagent (40 μg of Lipofectamine) with 30 μl of TM (mixture #2).
4. Add mixture #2 to mixture #1 (mixture #3) and incubate the mixture at room temperature for 15 min.
5. Add 400 μl of TM to the mixture #3.
6. Carefully remove BI-S-33 medium from the wells of the 12-well plate described in section 3.4.1.

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7. Add 500 μl of the DNA-Lipofectamine mixture #3 to the well. Then, incubate the plate under anaerobic conditions as described above at 35.5 °C for 5 h.
8. Place the plate on ice to detach transfected cells for 5 min, and then transfer the whole suspension to a 6-ml glass tube containing 5.5 ml of fresh BI-S-33 medium.
9. Incubate the tube overnight at 35.5 °C.

3.4.3. Drug selection

1. Remove the medium from the tube by aspiration and add 6 ml of a fresh prewarmed medium containing 1 μg/ml G418.
2. Incubate the tube at 35.5 °C for another 24 h.
3. Every 24 h replace the medium containing 1 μg/ml of G418 for 5–10 days. After 3–10 days G418-sensitive cells start to die and the cell number dramatically decreases. Keep replacing the medium containing 1 μg/ml G418 every day until G418-resistant cells become visible. It usually takes an additional 2–5 days. Once G418-resistant cells start to rapidly grow, increase G418 concentrations in a step-wise fashion with an increment of 1 μg/ml per day or per passage until it reaches 10 μg/ml G418. Adjustment of G418 concentration may be necessary depending on the plasmids used. Dead cells may not be apparent after 24 h but usually become visible after 48–72 h (dead cells often accumulate at the bottom of the tube).
4. After growth of the transfectants is established (typically at 1–2 weeks postlipofections), confirm the expression of the gene of interest by immunoblot and immunofluorescence assays. If the expression level is not sufficient, G418 concentrations may be further gradually increased to 20–50 μg/ml.

4. Conclusion

Repression (knockdown) of gene expression by siRNA or gene silencing has gradually become available in *E. histolytica* (Boettner *et al.*, 2008; Bracha *et al.*, 2006; Solis and Guillen, 2008). Gene silencing of *Atg8* and other genes involved in autophagy has recently been accomplished in *E. histolytica* (unpublished). Such reverse genetic tools should help in unequivocal assignment of a role of individual gene products. Because *Entamoeba* is a primitive eukaryote, which apparently possesses only a limited, if not minimal, set of autophagy genes identified in both mammals and yeasts, an understanding of the molecular mechanisms of autophagy in this group of organisms should contribute to the elucidation of the origin and evolution of this important cellular mechanism for protein degradation.

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Isoform-dependent feedback regulation of serine O-acetyltransferase isoenzymes involved in L-cysteine biosynthesis of *Entamoeba histolytica*☆

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ABSTRACT

Serine acetyltransferase (SAT; EC 2.3.1.30) catalyzes the CoA-dependent acetylation of the side chain hydroxyl group of L-serine to form O-acetyl serine, in the first step of the L-cysteine biosynthetic pathway. Since this pathway is selectively present in a few parasitic protists and absent in mammals, it represents a reasonable target to develop new chemotherapeutics. *Entamoeba histolytica* apparently possesses three SAT isotypes (EhSAT1-3) showing 48–73% mutual identity, a calculated molecular mass of 34.4–37.7 kDa, and an isoelectric point of 5.70–6.63. To better understand the role of individual SAT isotypes, we determined kinetic and inhibitory parameters of recombinant SAT isotypes. While the three SAT isotypes showed comparable K_m and k_{cat} for L-serine and acetyl-CoA, they showed remarkable differences in their sensitivity to inhibition by L-cysteine. The K_i values for L-cysteine varied by 100-fold (4.7–460 μM) among SAT isotypes (EhSAT1 < EhSAT2 < EhSAT3). Consequently, these EhSAT isotypes revealed remarkable differences in activity in the presence of physiological L-serine and L-cysteine concentrations. We propose that multiple SAT isotypes with different properties may play complementary roles in the regulation of the cysteine biosynthetic pathway in *E. histolytica* under different conditions, e.g. during colonization of the intestine and tissue invasion.

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1. Introduction

The L-cysteine biosynthetic pathway plays a key role in the sulfur assimilatory cycle in nature. Inorganic sulfur is incorporated from the extracellular milieu or the soil by microorganisms or plants, respectively, reduced, and fixed into L-cysteine, the first reduced sulfur-containing organic compound [1]. L-Cysteine is used as a sulfur donor for synthesis of methionine and sulfur-containing secondary metabolites, or, alternatively, incorporated into proteins, glutathione, and iron–sulfur clusters. The cysteine biosynthetic pathway consists of several enzymatic reactions [1–4]. In the first committed reaction of the two last steps, serine acetyltransferase (SAT, EC 2.3.1.30) catalyzes the CoA-dependent acetylation of the side chain hydroxyl group of L-serine to form O-acetylserine (OAS) [5]. Cysteine synthase [CS; OAS (thiol) lyase; EC 4.2.99.8] subsequently catalyzes β-replacement of the acetyl moiety on OAS with sulfide to form L-cysteine. In plants, these two enzymes form a heteromeric complex ("cysteine synthase complex"), and play a key role in cross-talk, via the generation of OAS, between sulfur assimilation and carbon and nitrogen metabolism [6]. L-Cysteine potentially inhibits its own synthesis by negative feedback of SAT. In plants, the nature of SATs varies in cellular compartmentalization and sensitivity to L-cysteine inhibition [7,8]. For instance, cytosolic SAT from *Citrullus vulgaris* (watermelon) [7], and *Arabidopsis thaliana* (SAT-c) [8,9] are highly sensitive to feedback-inhibition by L-cysteine at the physiological concentrations (3 μM). In contrast, the plastid SAT (SAT-p) [10,11], and mitochondrial SAT (SAT-m) [12–14] isoforms from *A. thaliana* are insensitive to L-cysteine inhibition [8].

Other than bacteria and plants, where cysteine biosynthesis has been well conserved, only a limited lineages of parasitic protists such as *Entamoeba histolytica*, *Trichomonas vaginalis*, and

Abbreviations: SAT, serine O-acetyltransferase; EhSAT, *Entamoeba histolytica* serine O-acetyltransferase; IPTG, isopropyl β-D-thio galactopyranoside; E64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; PCR, polymerase chain reaction.

☆ *Note:* The nucleotide sequence data of *E. histolytica* SAT1, SAT2 and SAT3 reported in this paper has been submitted to the DDBJ data bank with accession numbers, AB023954, AB232374, and AB232375, respectively.

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Trypanosoma cruzi possess the cysteine biosynthetic pathway. *E. histolytica* is the enteric protozoan parasite that causes amoebic colitis and extra intestinal abscesses (hepatic, pulmonary and cerebral) in inhabitants of endemic areas and is estimated to cause severe disease in 48 million people, killing about 70,000 each year [15]. While *E. histolytica* synthesizes L-cysteine via sulfur assimilatory pathway like plants and bacteria, it lacks, based on the genome information, both forward and reverse trans-sulfuration pathways [16,17], which makes this organism, together with *T. vaginalis*, very unique. In this organism, L-cysteine is essential for the synthesis of various proteins and the Fe–S cluster of Fe–S proteins such as ferredoxin and pyruvate:ferredoxin oxidoreductase. L-Cysteine has also been shown to be necessary for growth, survival, attachment, and anti-oxidation in this parasite [17,18].

The recent disclosure of the whole genome [19] revealed that *E. histolytica* possesses three isoenzymes each of CS and SAT [18,20]. We previously demonstrated the biochemical features of two CS isoforms (EhCS1and EhCS2) [16,21], and one SAT isoform, EhSAT1 hereinafter, [17]. In the present study, we biochemically characterized the two remaining SAT isotypes (EhSAT2 and EhSAT3), and compared kinetic parameters as well as their feedback-regulatory mechanisms.

2. Materials and methods

2.1. Chemicals

Acetyl-CoA, L-serine, Na_2S, glucose, ninhydrin reagent, trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64) were purchased from Sigma–Aldrich (Tokyo, Japan). All other chemicals of analytical grade were purchased from Wako (Tokyo, Japan) unless otherwise stated.

2.2. Microorganisms and cultivation

Trophozoites of the *E. histolytica* clonal strain HM1: IMSS cl 6 were maintained axenically in Diamond's BI-S-33 medium at 35.5 °C as described previously [22,23]. Trophozoites were harvested in the late-logarithmic growth phase for 2–3 days after inoculation of one-thirtieth to one-twelfth of the total culture volume. After the cultures were chilled on ice for 5 min, trophozoites were collected by centrifugation at 500 × g for 10 min at 4 °C and washed twice with ice-cold PBS, pH 7.4. *Escherichia coli* BL21 (DE3) pLysS strain was purchased from Invitrogen (Tokyo, Japan).

2.3. Construction of plasmids

Standard techniques were used for cloning and plasmid construction as previously described [24]. Genes encoding *E. histolytica* SAT1, SAT2, and SAT3 (EhSAT1-3) were cloned to produce a fusion protein containing a histidine-tag (provided by the vector) at the amino terminus. The cDNA corresponding to an open reading frame of EhSAT1-3 of 305, 311, and 336 amino acid residues with a calculated molecular mass of 34.4, 34.8, and 37.7 kDa, respectively, were amplified by PCR using the *E. histolytica* cDNA library [16] as a template and oligonucleotide primers. The sense and antisense oligonucleotide primers used for EhSAT1, EhSAT2 and EhSAT3 were: 5′-CCT**GGATCC**GATGGACAATTACATTTATTCAATTGCACAT-3′ and 5′-CCA**GGATCC**TTAAATCGATGGTGAATTTGCTAAAGGAGAT-3′ (EhSAT1); 5′-CCT**GGATCC**GATGGATTTACTTGTTTCAAAAATTTCAAAA-3′ and 5′-CCA**GGATCC**CTAATTTAAAGGAGAGTTTATTGGACTTATT-3′ (EhSAT2); 5′-CCT**GGATCC**GATGAGTTCTTTACTACAACAAACATCTCAG-3′ and 5′-CCA**GGATCC**TTATTGTTGACAAGATACAAAACAATCTAA-3′ (EhSAT3), respectively, where bold letters indicate BamHI restriction sites. PCR was performed with platinum pfx DNA polymerase (Invitrogen) and the following parameters: an initial incubation at 94 °C for 2 min; followed by the 30 cycles of denaturation at 94 °C for 15 s, annealing at 50, 45, or 55 °C for EhSAT1-3, respectively, for 30 s, and elongation at 68 °C for 1 min, and a final extension at 68 °C for 10 min. The PCR fragment was digested with BamHI and electrophoresed, purified with Gene clean kit II (BIO 101, Vista, CA, USA), and ligated into BamHI-digested pET-15b (Novagen, Darmstadt, Germany) in the same orientation as the T7 promoter to produce pET-EhSAT1, pET-EhSAT2 and pET-EhSAT3. The nucleotide sequences of the cloned EhSAT1, EhSAT2, and EhSAT3 were verified by sequencing to be identical to the putative protein coding region of 200.m00078, 253.m00083, and 13.m00319, respectively.

2.4. Bacterial expression and purification of recombinant E. histolytica SAT isotypes

The above mentioned plasmids were introduced into *E. coli* BL21 (DE3) pLysS cells by heat shock at 42 °C for 1 min. *E. coli* BL21 (DE3) harboring pET-EhSAT1, pET-EhSAT2 or pET-EhSAT3 were grown at 37 °C in 100 ml of Luria Bertani (LB) medium in the presence of 100 μg/ml ampicillin. The overnight culture was used to inoculate 500 ml of fresh medium, and the culture was further continued at 37 °C with shaking at 180 rpm. When A_{600} reached 0.6, 0.4 mM of isopropyl β-D-thio galactopyranoside (IPTG) was added, and cultivation was continued for another 4 h at 30 °C. *E. coli* cells from the induced culture were harvested by centrifugation at 4050 × g for 20 min at 4 °C. The cell pellet was washed with PBS, pH 7.4, re-suspended in 15 ml of the lysis buffer (50 mM Tris–HCl, pH 8.0, 300 mM NaCl, and 10 mM imidazole) containing 0.1% Triton X-100 (v/v), 100 μg/ml lysozyme and 1 mM phenylmethyl sulfonyl fluoride (PMSF) incubated at room temperature for 30 min, sonicated on ice and centrifuged at 25,000 × g for 15 min at 4 °C. The supernatant was mixed with 2 ml of 50% Ni^{2+}-NTA His-bind slurry, incubated for 2 h at 4 °C with mild shaking. The recombinant EhSAT (rEhSAT)-bound resin in a column was washed three times each with buffer A [50 mM Tris–HCl, pH 8.0, 300 mM NaCl, and 0.1% Triton X-100, v/v] containing 10–50 mM of imidazole. Bound proteins were eluted with buffer A containing 100–300 mM imidazole.

After the integrity and the purity of rEhSAT proteins were confirmed with 12% SDS-PAGE analysis, followed by Coomassie Brilliant Blue staining, they were extensively dialyzed twice against the 300-fold volume of 50 mM Tris–HCl, 150 mM NaCl, pH 8.0 containing 10% glycerol (v/v) and the Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany) for 18 h at 4 °C. The dialyzed proteins were stored at −80 °C with 20% glycerol in small aliquots until further use. The purified proteins remained fully active after >3 months under these conditions. Protein concentrations were spectrophotometrically determined by Bradford method using BSA as a standard as previously described [25].

2.5. Kinetic studies of recombinant SAT isotypes

The SAT activity was determined by two methods, by either monitoring the decrease in A_{232} due to the cleavage of the thioester bond of acetyl-CoA [26] or the coupling reaction with CS [27], followed by the colorimetric ninhydrin assay. For the thioester-bond cleavage assay, the standard mixture contained 50 mM Tris–HCl (pH 8.0), 0.1 mM acetyl-CoA, 1 mM L-serine and 2.5 μg of rEhSAT. The reaction was initiated by the addition of L-serine and carried out at 25 °C for 3–5 min. The decrease in absorbance at 232 nm was

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monitored on a spectrophotometer (Shimadzu, UV 2550) equipped with an automatic cell changer. The K_m and V_{max} values were estimated with Hanes–Woolf and Line weaver–Burk plots. Kinetic studies were performed using 5–7 concentrations of both acetyl Co-A and L-serine. All steady-state kinetic parameters are the means of three to five independent experiments where two or three different preparations of recombinant SATs were used for measurements performed on at least three non-consecutive days. The coupling reaction was used only when SAT activity of the recombinant enzymes was initially demonstrated. The standard assay mixture contained 50 mM Tris–HCl (pH 8.0), 0.4 mM acetyl-CoA, 4 mM L-serine, 5 mM Na_2S, 10 mM DTT, 0.5 μg of recombinant EhCS3 and 2.5 μg of rEhSAT, in a final volume of 100 μl. The reaction was performed at 37 °C for 15–30 min, and the amount of L-cysteine was determined as described [28].

2.6. Amino acid comparison and phylogenetic analysis

Amino acid sequences of SAT from 48 other organisms that showed significant similarity to EhSATs were obtained from the DDBJ/EBI/GenBank database by using blastp search. Sequence alignments of these proteins were generated using CLUSTAL W program [29]. The alignment obtained by CLUSTAL W was inspected and manually corrected using Genedoc program (www.psc.edu/biomed/genedoc). All gaps were removed and unambiguously aligned 245 conserved sites were selected and used for phylogenetic analyses. The neighbor-joining (NJ) and maximum parsimony (MP) methods were performed using MEGA4 program [30]. A final phylogenetic tree of 35 sequences was drawn by using MEGA4. The branch lengths in these trees were obtained from the neighbor-joining analysis with bootstrap values in 100 replicates.

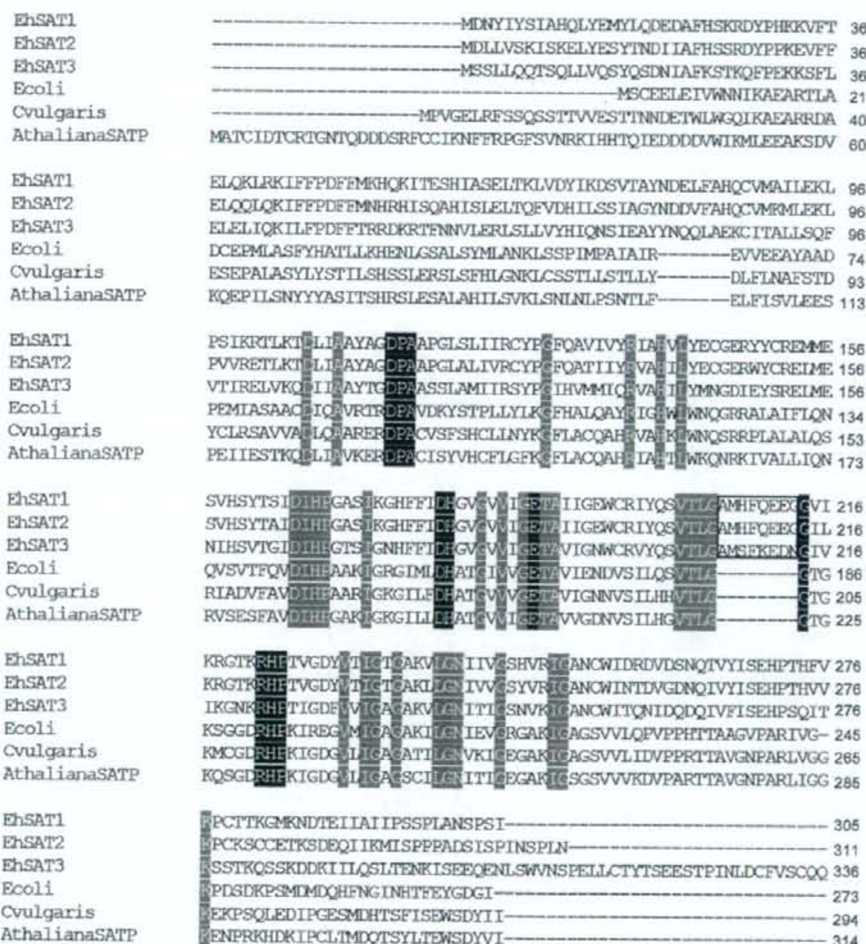

Fig. 1. Complete protein sequences of *E. histolytica* and other organisms were aligned using the CLUSTAL W program (http://clustalw.ddbj.nig.ac.jp/top-e.html). Accession numbers of these sequences are: *Escherichia coli* (NP415427), *Citrullus vulgaris* (D85624), *Arabidopsis thaliana SAT-p* (Q42538), *E. histolytica EhSAT1-3* (AB023954, AB232374 and AB232375). *Dashes* indicate gaps. Highly conserved residues that were shown to participate in the binding to substrates (acetyl-CoA and L-serine) and L-cysteine based on crystal structures of *E. coli* SAT [31,32] are highlighted in *black*. Other amino acids conserved among these organisms are highlighted in *grey*. An insertion unique to the amoebic SATs is *boxed*.

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3. Results

3.1. Identification of genes and their encoded proteins of three SAT isotypes from E. histolytica

We identified genes encoding three SAT isotypes by homology search against the *E. histolytica* genome database [19] using SAT protein sequences from bacteria, yeast, and plants. We designated them as *EhSAT1*, *EhSAT2*, and *EhSAT3* genes [corresponding to 200.m00078 (AB023954, XM_645673 and XP_650765) [17], 253.m00083 (AB232374, XM_644909 and XP_650001), and 13.m00319 (AB232375, XM_651281 and XP_656373), respectively]. *EhSAT1*, *EhSAT2*, or *EhSAT3* gene contains a 918, 936, or 1011-bp open reading frame which encodes the protein of 305, 311, or 336 amino acid residues with a predicted molecular mass of 34.4, 34.8, or 37.7 kDa and p*I* of 6.63, 5.99, or 5.70, respectively.

3.2. Features of the deduced amino acid sequences of E. histolytica SATs

The amino acid sequence of EhSATs showed 21–42% identities to SAT from archaea, bacteria, and plants. SAT from the square archaeon *Haloquadratum walsbyi* showed the highest (39–42%) amino acid identities to EhSATs. The identity to SAT from other organisms was significantly lower than *H. walsbyi* SAT. For instance, EhSAT3 showed only 27, 24, or 19% identity to *E. coli SAT*, watermelon SAT-c, or *A. thaliana* SAT-p, respectively. The identity between EhSAT1 and EhSAT2, between EhSAT1 and EhSAT3, or between EhSAT2 and EhSAT3 was 73%, 48%, or 48%, respectively. All EhSATs were devoid of the amino-terminal transit peptide found in plastid or mitochondria-located SAT from plants [26,27], which suggests that amoebic SAT genes encode cytosolic proteins. Multiple alignment of 48 SAT sequences was produced by Clustal W, and comparison of only representative SAT sequences from *E. coli*,

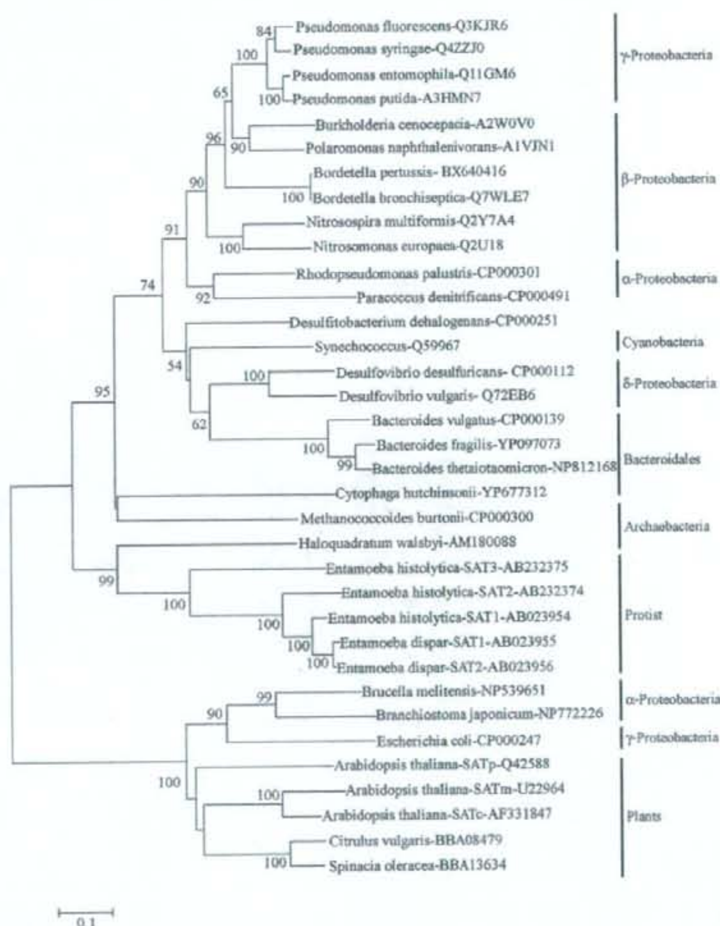

Fig. 2. Phylogenetic reconstruction of SAT proteins from a variety of organisms. A phylogenetic tree of 35 SAT proteins was constructed by using CLUSTAL W program and drawn with MEGA4 program. The number at the nodes represents the bootstrap value in percentage of 1000 replicates. The scale bar indicates 0.1 substitutions at each amino acid position. Species names and accession numbers of the sequences are also indicated.