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H.知的所有件の取得状況

1.特許取得

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別紙4　　研究成果の刊行に関する一覧表

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AXENIC CULTIVATION OF *ENTAMOEBA DISPAR* IN NEWLY DESIGNED YEAST EXTRACT–IRON–GLUCONIC ACID–DIHYRDOXYACETONE–SERUM MEDIUM

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ABSTRACT: Yeast extract–iron–gluconic acid–dihyrdoxyacetone-serum medium that allows axenic cultivation of *Entamoeba dispar* was designed based on casein-free yeast extract-iron-serum (YI-S) medium, and the usefulness of the medium was assessed. The main differences from YI-S medium are replacement of glucose by gluconic acid, addition of dihydroxyacetone and D-galacturonic acid monohydrate, and sterilization by filtration. This medium promoted the axenic growth of 5 strains of *E. dispar* (2 strains of nonhuman primate isolates and 3 strains of human isolates). In addition, to clarify the biological basis for the growth of *E. dispar* in this medium, analyses of relevant enzymes on the glycolytic pathway of the amoebae as well as of the protozoans that are the best culture supplement for amoebae are being performed.

After axenic cultivation of *Entamoeba dispar* (strain: SAW760RRcloneAR) was reported (Clark, 1995) in casein-free yeast extract–iron–serum (YI-S) medium (Diamond et al., 1995) supplemented with gastric mucin, the efficacy of this axenic culture system was assessed for other *E. dispar* strains. However, despite its utility, the only *E. dispar* strain established as an axenic amoeba in YI-S medium thus far is SAW760RR clone A.

On the other hand, when a monoxenic culture system for *E. dispar* in biosate–cysteine–starch–iron–serum (BCSI-S) medium with *Pseudomonas aeruginosa* was developed (Kobayashi et al., 1998), a significant growth-promoting effect of dihydroxyacetone (DHA) was observed. DHA is a ketotriose and as a sugar source for *E. dispar* is thought to be directly metabolized to DHA phosphate (DHAP), which is an intermediary metabolite in the Embden–Meyerhof–Parnas glycolytic pathway.

Another significant result was that during characterization of the glycolytic pathway of *Crithidia fasciculata*, an effective culture associate of *E. dispar* in BI-S-33 medium (Diamond et al., 1978), approximately 35 times as much glucose-6-phosphate dehydrogenase (G6PDH) activity as that of *Trypanosoma cruzi* (Tulahuen strain) was detected. *Trypanosoma cruzi* can be used as a growth-promoting supplement for monoxenic culture of *E. histolytica*. However, it does not promote the growth of *E. dispar*, whereas *C. fasciculata* does (S. Kobayashi, unpubl.). G6PDH activity has not been detected in axenically grown *E. histolytica* (Reeves, 1972) or *E. dispar* (S. Kobayashi, unpubl.). G6PDH is an essential enzyme not only in the pentose-phosphate pathway but also in the Entner–Doudoroff pathway, which starts with G6P. G6PDH catalyzes the transformation of G6P to 6-phosphogluconate (6PG) via 6-phosphogluconolactone, and we focused attention on the glycolytic pathway after 6PG in *E. dispar* and examined the reactions after 6PG. Both the pentose-phosphate and Entner–Doudoroff glycolytic pathways can branch from 6PG. The activity of 6PG dehydratase (EC 4.2.1.12.) (Gottschalk and Bender, 1982; Nguyen and Schiller, 1989), which is one of the enzymes in the Entner–Doudoroff pathway, was detected in the lysates of both *E. histolytica* (HM-1:IMSS clone 6) and *E. dispar* (CYNO 16:TPC), and its activity in *E. dispar* was 2.87 times greater than in *E. histolytica*. By contrast, no 6PG dehydrogenase activity in the pentose-phosphate pathway was detected in either lysate (S. Kobayashi, unpubl.). On the other hand, DHA, which has a growth-promoting effect, is thought to be metabolized to G3P. through DHAP, which is converted to glyceraldehyde-3-phosphate (G3P) by triose phosphate isomerase (EC 5.3.1.1.). Thus, if viable *C. fasciculata* actually supplies a growth-promoting substance like DHA, DHA may be preferable to viable *C. fasciculata* as a culture ingredient. On the basis of this information, we hypothesized the existence of some failure in the upstream portion of the glycolytic pathway in *E. dispar*, before DHAP or G3P production from glucose in YI-S medium. We, therefore, attempted to design an axenic culture medium for *E. dispar* by modifying the ingredients of YI-S medium, and we tested its usefulness.

MATERIALS AND METHODS

Reagents

All chemicals used in this study were of the highest quality commercially available unless otherwise stated.

Entamoeba dispar isolates

Five strains of *E. dispar* were subjected to a trial of axenic cultivation in this study. Two strains (CYNO 09:TPC and CYNO 16:TPC) from nonhuman primates were isolated in Japan (Kobayashi et al., 1998) in Robinson's medium (Robinson, 1968), and 2 strains (AS 2 IR and AS 16 IR) were isolated from humans in Iran. An *E. dispar* strain, SAW 1734R clone AR (SAW1734RclAR), was used as the reference strain. The xenic *E. dispar* strains were transferred to monoxenic culture medium with viable *C. fasciculata* and antibiotics (as in the classic approach Diamond used for *E. histolytica*, Diamond, 1983) in BI-S-33 medium. The *C. fasciculata* was then replaced with antibiotic-sensitive *P. aeruginosa* in BCSI-S medium because *P. aeruginosa* is removed easily by antibiotics and antibiotic-resistant *C. fasciculata* usually survive in test medium for axenic culture even after 2–3 subcultures, and it promotes the growth of *E. dispar*.

Yeast–iron–gluconic acid–dihydroxyacetone–serum medium

Yeast–iron–gluconic acid–dihydroxyacetone (YIGADHA) broth was prepared by first dissolving the following ingredients in 880 ml of water distilled and purified with a Milli-Q column (Millipore Co., Billerica, Massachusetts): K_2HPO_4, 1 g; KH_2PO_4, 0.6 g; NaCl, 2.0 g; yeast extract (BBL, Becton Dickinson Co., Cockeysville, Maryland), 30 g; gluconic acid, 5.0 g; DHA (Sigma Chemical Co., St. Louis, Missouri), 1.0 g; D-galacturonic acid monohydrate, 0.2 g; L-cysteine hydrochloride, 1.0 g; ascorbic acid, 0.2 g; and ferric ammonium citrate (brown), 22.8 mg.

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The pH was adjusted to 6.5 with 1 N NaOH, and the solution was sterilized by filtration (Sartorius membrane filter; 0.2-μm pore size). An 88 ml volume of YIGADHA broth was aseptically dispensed into a 100-ml, screw-capped glass bottle and stored at −30 C.

To complete the YIGADHA-serum (YIGADHA-S) medium, 2 ml of vitamin mixture #18 prepared according to the instructions for PDM-805 medium (Diamond and Cunnick, 1991), and 16 ml of heat-inactivated bovine serum was aseptically added to 88 ml of the YIGADHA broth. A 5-ml volume of the complete medium was then dispensed into 13- × 100-mm, screw-capped borosilicate glass culture tubes (Asahi Techno Glass Co., Chuo-ku, Tokyo, Japan).

Establishment of the culture of *Entamoeba dispar* with sterilized *Crithidia fasciculata*

After removing *P. aeruginosa* by washing and addition of antibiotics (polymyxin B, 130 units/ml), trophozoites of the 5 strains of *E. dispar* were transferred to YIGADHA-S medium with sterilized *C. fasciculata* (2–3 million/ml, ReF-1:PRR, ATCC no. 50083) by fixation with 10% (v/v) formalin as described in the literature (Clark, 1995). All 5 strains of *E. dispar* adapted to the culture at 35.5 C in the YIGADHA-S medium with the associate within 2 wk after 3–4 whole-medium changes by centrifugation (275 *g*, 4 min) and eventually started to grow continuously. When the same growth-promoting effect was later found with autoclaved *C. fasciculata* in Hanks' solution as with the formalin-fixed *C. fasciculata*, we switched the method of sterilizing *C. fasciculata* to autoclaving (121 C, 15 min) because of its simplicity.

When the amoebae were subcultured, 0.6–0.8 ml of the amoeba suspension (2.5 × 10^4 to 5 × 10^4/ml) was usually transferred to the fresh medium, after the cultures were chilled for 5 min in an ice-water bath. They were then inverted several times to disperse the amoebae. Subcultures were successfully prepared at intervals of 3 and 4 days.

Growth kinetics of the amoebae

At 24-hr intervals for 96 hr, the number of amoebae in 5 μl of the homogeneous amoeba suspension diluted with known volume of YIGADHA-S medium were counted under a microscope after chilling the culture tubes for 5 min in an ice-water bath. The data were then converted to number per milliliter.

Zymodeme analysis and polymerase chain reaction

Zymodeme analysis (Sargeaunt, 1988) and polymerase chain reaction (PCR) analysis (Tachibana et al., 1991; Cheng et al., 1993) of the amoeba isolates were performed to characterize the amoebae grown in the axenic culture.

RESULTS

YIGADHA-S medium was designed, and the culture associates tested could be removed with no loss of *E. dispar* viability as a result of searching for the improved basic medium and condition for axenic cultivation of *E. dispar*, based on the casein-free YI-S medium.

YI-S medium was therefore modified as follows. Glucose was replaced by GA, DHA and D-galacturonic acid monohydrate were added because of the significant growth-promoting effects under the axenic culture conditions, and the medium was sterilized by filtration and adjusted to pH 6.5 with NaOH.

As a result of these modifications, trophozoites of *E. dispar* started to grow continuously in the absence of viable *C. fasciculata* or *P. aeruginosa* for the first time, although supplementation with the formalin-fixed organisms was still necessary for growth.

The growth-promoting ability of *C. fasciculata* was also maintained in this YIGADHA-S culture system even after it was autoclaved (121 C, 15 min) in Hanks' solution. There was no difference between the growth-promoting effect of autoclaved *C. fasciculata* and of formalin-fixed organisms. Because

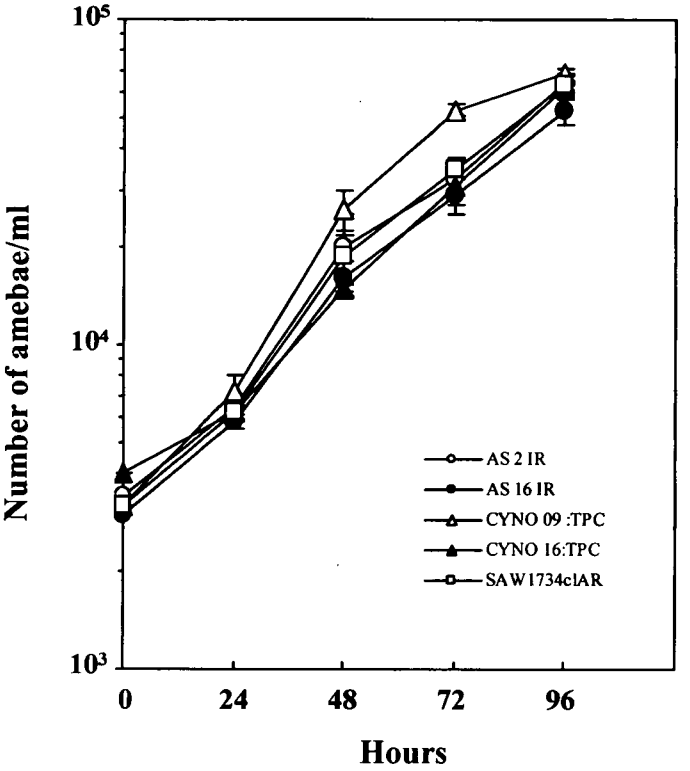

FIGURE 1. Growth kinetics of 5 strains of *Entamoeba dispar* in the 4-yr passaged cultures in YIGADHA-S medium supplemented with autoclaved *Crithidia fasciculata*. Mean numbers of amoebae in duplicate cultures are plotted.

the procedure was easier, the amoebae were maintained in the medium with autoclaved *C. fasciculata*. The growth kinetics of the 5 strains of *E. dispar* in this culture system are shown in Figure 1.

After 3 mo of maintenance in this culture system, 1 strain of *E. dispar* (CYNO 16:TPC) started to grow without any supplement, e.g., without autoclaved *C. fasciculata*. However, addition of 6PG (Sigma P-7877, 67 μg/ml), an intermediary metabolite in the Entner–Doudoroff pathway (Fig. 3), was necessary to maintain this strain without the autoclaved organisms.

After culturing with autoclaved *C. fasciculata* for 1 yr and 4 yr, respectively, other *E. dispar* strains started to grow axenically in the YIGADHA-S medium without the addition of any culture associates. The growth kinetics of 5 strains of axenically grown *E. dispar* are shown in Figure 2. The clear differences in growth kinetics between monoxenic culture (Fig. 1) and axenic culture (Fig. 2), except for the CYNO 09:TPC strain, suggest the existence of some other growth-promoting factor in autoclaved *C. fasciculata*.

The CYNO 16:TPC and CYNO 09:TPC strains were usually subcultured by transferring 1 ml of amoeba suspension to fresh YIGADHA-S medium, and the AS 16 IR, AS 2 IR, and SAW1734RclAR strains were subcultured by transferring 1 ml of amoeba suspension concentrated to an adequate density for subculture (3 × 10^4 to 6 × 10^4 amoebae/ml) by centrifugation (275 *g*, for 4 min).

The zymodemes of the 5 strains grown in both the monoxenic and axenic YIGADHA-S media were all judged to be type I. All 10 DNA samples of the 5 strains used in the PCR

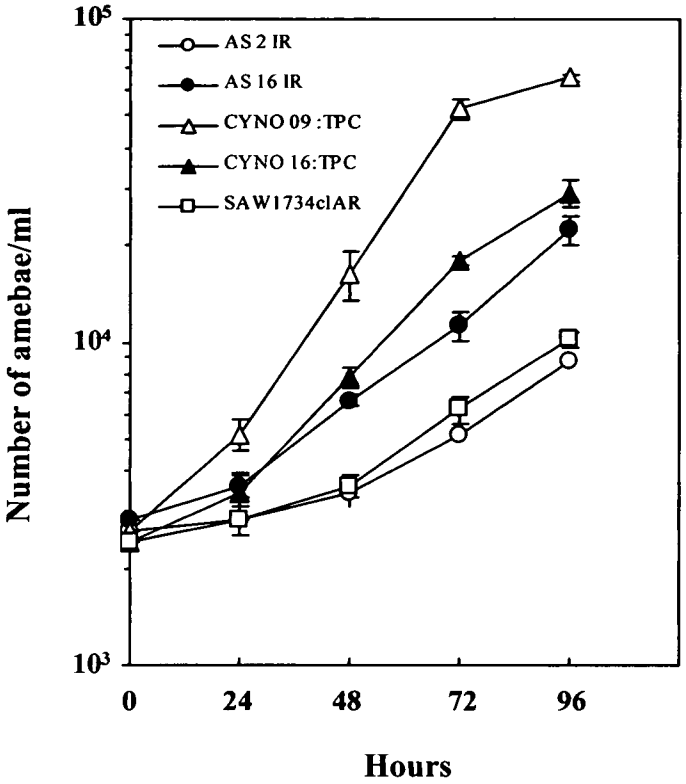

FIGURE 2. Growth kinetics of 5 strains of axenically grown *Entamoeba dispar* in 2-yr (CYNO 09: TPC), 3-yr (CYNO 16:TPC), and 2-mo (AS 2 IR, AS 16 IR, and SAW 1734RclAR) passaged cultures in YIGADHA-S axenic medium. Mean numbers of amoebae in duplicate cultures are plotted.

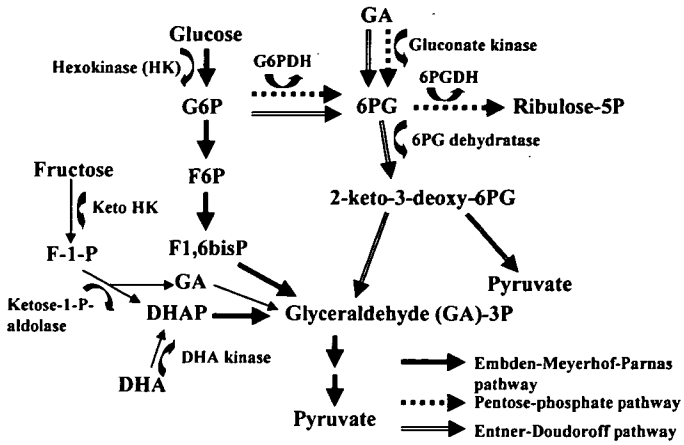

FIGURE 3. Metabolic map of 3 glycolytic pathways.

analyses described above were amplified with *E. dispar*–specific primers alone; the *E. histolytica*–specific primers did not elicit any DNA amplification. These findings are summarized in Table I.

DISCUSSION

We have previously reported a monoxenic culture system for *E. dispar* in BCSI-S medium in which glucose was replaced with starch and to which sterilized *C. fasciculata* were added after heat treatment at 56 C for 30 min followed by incubation in 1% hydrogen peroxide at 4 C for 24 hr (Kobayashi et al., 1998). The sterilized parasite appeared to be metabolically inactive as judged by the nuclear magnetic resonance spectra. Although the growth-promoting effect of *C. fasciculata* was partially lost as a result of this sterilization step, the minimum essential effect persisted, which made it possible to establish a long-term monoxenic culture of *E. dispar* with metabolically inactive *C. fasciculata*. In this study, we succeeded in producing an axenic culture system of *E. dispar* by using YIGADHA-medium designed on the basis of YI-S medium from the monoxenic culture of the amoeba with sterilized *C. fasciculata*.

During attempts to improve the culture system, it was found that by replacing some kinds of sugar from starch, the growth of *E. dispar* was promoted significantly in the BCSI-S monoxenic culture system. First, a marginal growth-promoting effect of fructose was found, although its effect was insufficient for axenic cultivation of *E. dispar*, and a more useful compound, DHA, was used. The concentration (0.1%) of DHA required for the growth of *E. dispar* in YIGADHA-S medium was very critical: DHA concentrations below 0.05% and greater than 0.15% failed to effectively promote the growth of *E. dispar*. However, despite the clear effect of DHA and fructose on the growth of *E. dispar*, the results of our biochemical assays for phosphorylation of DHA by DHA kinase, ketohexokinase, and ketose-1-phosphate aldolase, the enzymes involved in the conversion of fructose or DHA to DHAP (Fig. 3), showed no significant activity of these enzymes in the crude lysates of axenic *E. dispar* (CYNO 16:TPC) or *E. histolytica* (HM-1:IMSS clone

TABLE I. Zymodeme and PCR analyses of 5 strains of *Entamoeba dispar*.

Strain	Xenic (date)	Zymodeme*		PCR†	
		Monoxenic (date)	Axenic (date)	Monoxenic	Axenic
SAW 1734RclAR	Z-III‡ (March 1985)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*
AS 2 IR	Z-I (June 1998)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*
AS 16 IR	Z-I (June 1998)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*
CYNO 09:TPC	Z-I (April 1992)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*
CYNO 16:TPC	Z-III (April 1992)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*

* Zymodemes type I and III are classified as *E. dispar*.
† PCR analysis using 2 sets of oligonucleotide primers each (p11 plus p12 and p13 plus p14, respectively) for amplification of the DNAs of *E. histolytica* and *E. dispar*. The zymodeme and PCR analyses were performed around the same time.
‡ Data cited from Mirelman et al. (1986).

6) (data not shown). The mechanism of growth-promoting effect of DHA and fructose is not yet understood.

Pimenta et al. (2002) demonstrated an interesting morphological characteristic of *E. dispar* by electron microscopy: some concomitant bacteria cells were free and viable in the cytoplasm of *E. dispar*, without being surrounded by a distinct phagosome membrane. This suggests that some bacterial species can survive in the cytoplasm and exist in a symbiotic relationship with *E. dispar*. If the Entner–Doudoroff pathway does indeed function in glycolysis in *E. dispar*, as reported in *E. histolytica* under xenic conditions (Hilker and White, 1959), the reactions upstream in the Embden-Meyerhof-Parnas pathway (Fig. 3) including a reaction regulating the transformation of fructose-6-phosphate to fructose-1,6-bisphosphate or vice versa, usually catalyzed by 6-phosphofructokinase (6-PFK) and fructose-bisphosphatase (EC 3.1.3.11.) and affecting the both the glycolysis and glycogenesis pathways, may not function well in *E. dispar*. The regulatory reactions in *E. histolytica* are well known to be regulated by a single unique enzyme (ppi-dependent 6-PFK; EC 2.7.1.90.), and the reaction is reversible and has no apparent regulatory function (Reeves et al., 1974, 1976).

The clear growth-promoting effect of autoclaved *C. fasciculata* (or *P. aeruginosa*) indicated that they contain as yet unidentified heat-stable growth-promoting substances for *E. dispar*. If the substances can be identified, YIGADHA-S medium will be improved. Further analyses of the axenically grown *E. dispar* will enable us to further elucidate the biological properties of *E. dispar* that differ from those of pathogenic *E. histolytica*.

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Identification and Gene Expression Analysis of a Large Family of Transmembrane Kinases Related to the Gal/GalNAc Lectin in *Entamoeba histolytica*†

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We identified in the *Entamoeba histolytica* genome a family of over 80 putative transmembrane kinases (TMKs). The TMK extracellular domains had significant similarity to the intermediate subunit (Igl) of the parasite Gal/GalNAc lectin. The closest homolog to the *E. histolytica* TMK kinase domain was a cytoplasmic dual-specificity kinase, SplA, from *Dictyostelium discoideum*. Sequence analysis of the TMK family demonstrated similarities to both serine/threonine and tyrosine kinases. TMK genes from each of six phylogenetic groups were expressed as mRNA in trophozoites, as assessed by spotted oligoarray and real-time PCR assays, suggesting nonredundant functions of the TMK groups for sensing and responding to extracellular stimuli. Additionally, we observed changes in the expression profile of the TMKs in continuous culture. Antisera produced against the conserved kinase domain identified proteins of the expected molecular masses of the expressed TMKs. Confocal microscopy with anti-TMK kinase antibodies revealed a focal distribution of the TMKs on the cytoplasmic face of the trophozoite plasma membrane. We conclude that *E. histolytica* expresses members of each subgroup of TMKs. The presence of multiple receptor kinases in the plasma membrane offers for the first time a potential explanation of the ability of the parasite to respond to the changing environment of the host.

The Gal/GalNAc lectin of *Entamoeba histolytica* mediates parasite adherence to the host and signals the initiation of cytolysis (41, 44, 45, 49). It is a heterotrimer consisting of covalently linked heavy (Hgl) and light (Lgl) subunits with a noncovalently linked intermediate (Igl) subunit (9, 36, 37, 43, 46). The Igl subunit of the Gal/GalNAc lectin has two known family members, Igl1 and Igl2. The Igl subunit has sequence similarity to the variant surface protein (VSP) of *Giardia*. We have previously identified a large number of proteins in the genome of *E. histolytica* containing CXXC motifs similar to those of Igl (8). Here we show that these CXXC-rich proteins form a large family of *E. histolytica* transmembrane kinases (TMKs) with highly variable extracellular domains homologous to Igl and VSPs of *Giardia* and with cytoplasmic kinase domains.

Amebic trophozoites have been demonstrated to persist in humans for longer than 6 months (21, 22). This prolonged period of infection suggests that the amebae evade the immune system. Other protozoan parasites, such as *Plasmodium*, *Giardia*, and *Trypanosoma brucei*, are also able to infect the host for long periods in spite of inducing robust immune responses. The mechanism(s) of persistence of these organisms is thought in part to be due to the variation of surface proteins. *Plasmodium falciparum* has three families of *var* genes that are independently expressed (29). The highest variation rate of these families is 2% per generation (52). *Giardia* encodes a family of 100 to 150 VSPs whose surface expression changes at a rate of one variation every 5 to 13 generations (38). *T. brucei* has a family of over 1,000 variant surface glycoproteins that change at a rate of 10^{-2} to 10^{-7} variations per generation (13, 51).

The discovery of the large family of CXXC-containing TMKs is of interest not only for their potential role in antigenic variation but also for their role in cell signaling. *E. histolytica* must respond to a wide variety of environmental stimuli as it excysts into a trophozoite in the intestinal lumen and enters the host by invasion of the intestinal mucosal epithelium. Invasion involves attaching to the epithelium and responding to that attachment event through signaling events via the *E. histolytica* Gal/GalNAc adherence lectin that lead to host cell killing. The changing host environment should necessitate having a variety of ways of sensing and responding to the host.

Here we report sequence and expression analysis of the TMKs in laboratory-cultured trophozoites. An oligoarray and real-time PCR were used to measure the expression in cultured trophozoites of the TMK genes. We demonstrate that there are six families of TMKs, with each having one or more family members expressed. In addition, anti-TMK antibodies

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† Supplemental material for this article may be found at http://ec.asm.org/.

were used to localize the TMKs to the plasma membrane of trophozoites, consistent with their proposed function in sensing the environment.

MATERIALS AND METHODS

Identification of genes homologous to the Igl subunit of the Gal/GalNAc lectin. The genes were identified in the 7X assembly available from The Institute for Genomic Research (TIGR) and Sanger sequencing centers (http://www.tigr.org/tdb/e2k1/eha1 and http://www.sanger.ac.uk/Projects/E_histolytica/) by searching the database for homologs of Igl1. Genes with high sequence similarity to Igl1 were used to search the database and identify additional family members. Additionally, the Sanger assembly was translated in all six reading frames, and genes were identified by sequence similarity to known genes in the National Center for Biotechnology Information (NCBI) database. These sequences were then screened for genes containing sequences for three or more CXXC motifs, or kinase domains. Genes containing sequences for CXXC motifs but not kinase domains, transmembrane domains, or signal peptides were eliminated from the data set.

Identification of other virulence genes and control genes. Genes were identified by sequence similarity to genes for amoebapores, cysteine proteinases, and the Gal/GalNAc lectin Igl, Lgl, and Hgl subunits. Additionally, genes were identified by examination of the translated Sanger assembly, which had been annotated to known genes in the NCBI database. Phagocytosis genes and control genes were similarly identified. BspA genes were identified in the translated Sanger assembly and then identified by sequence similarity in the TIGR assembly.

Phylogenetic analysis of the TMK proteins. A 260-amino-acid alignment of the kinase domains of the TMK proteins was made to Hanks's kinase alignment (Protein Kinase Resource [http://pkr.sdsc.edu/html/pk_classification/pk_catalytic/pk_hanks_class.html]) using CLUSTALX (20, 61). One representative per family, called the query panel of kinases, was employed (http://pkr.sdsc.edu/html/pk_classification/pk_catalytic/query_panel.html). The alignment was manually optimized using Genedoc (39), and then sequences were analyzed using the PHYLIP v3.6 package (15) and bootstrapped using Seqboot, Protdist, Neighbor, and Consense. A subset of the sequences were then bootstrapped using Seqboot, Protpars, and Consense. The TMKs were broken into groups based on signature motifs found in the kinase domains and aligned using CLUSTALX and with manual adjustments using Genedoc.

Probes for microarray analysis. Oligonucleotide probes typically ranging from 50 to 60 bases, and optimized for standard hybridization conditions, were designed using Array Designer 2.0 software (Premier Biosoft International, Palo Alto, CA). The selected probes were then analyzed by BLAST against the 7X assembly of the *E. histolytica* genome at both TIGR and Sanger. Probes were redesigned if they contained more than 75% sequence similarity with other target sequences or had a continuous stretch of complementary sequence exceeding 15 bases (28). In some cases it was not possible to design gene-specific probes. The actin probe was predicted to hybridize to several actin genes, the Jacob probe to all three Jacob genes, the EHCP1/2 probe to genes for both *E. histolytica* cysteine proteases 1 and 2 (EHCP1 and EHCP2), the Hgl family probes to all five Hgl genes but not homolog Sp1, and the Hgl1/5 probe to Hgl1 and Hgl5 genes. The Hgl1/5, Hgl2, Hgl3, and Hgl4 probes were more than 75% similar.

The oligoarray had probes to genes for amoebapores A, B, and C and homologs (32, 67), BspA homologs (25), actin, intergenic regions, L37a from mouse and human, chitin synthase (10), chitinase (11), Jacob (16), Jessie1-3 (65), EHCPs (6), EhRabs (53, 54), Vps26, Vps35, glycerate dehydrogenase (3), methionine gamma-lyase (62), phosphoglycerate dehydrogenase (2), Ebp1 and Ebp2 (55), L10 (7), ribosomal gene Sa, indigoidine synthase homolog (50), Hgl1-5 (35, 48, 58), Igl1 and Igl2 (8), Lgl1-6 (36, 59), Sp1 (an Hgl homolog), ferredoxin (26), Ariel1 (34), an HMW1 homolog (19), serine-rich *E. histolytica* protein (56), TMK genes, and other hypothetical surface genes (Table S1 in the supplemental material). TMK genes for which we did not generate a specific probe are shown in Table S2. Jacob is an amebic cyst wall glycoprotein expressed during encystation (16). EHCP1 and EHCP2 are highly homologous cysteine proteinases (6). Sp1 is a homolog of Hgl, recently identified in the TIGR database (B. Mann, personal communication).

Probe synthesis and microarray printing. A 200-nmol quantity of each probe (typically ranging from 50 to 60 bases optimized for standard hybridization conditions) was synthesized on an ABI 3900 DNA synthesizer (Applied Biosystems, Foster City, CA). The probe oligonucleotides were dissolved in 50% dimethyl sulfoxide at a concentration of 0.25 mg/ml and arranged in 96-well microtiter plates. The panel of probes, including control (housekeeping) oligonucleotides, was printed with two spot replicates on Corning UltraGAPS coated slides using an Affymetrix 417 arrayer (Affymetrix, Santa Clara, CA). Slide quality control was analyzed by hybridizing two randomly selected slides per batch (up to 40 slides/batch) with Cy3-labeled universal oligonucleotide probes. The hybridized slides were then washed and scanned with a ScanArray 4000 scanner (PerkinElmer Life Sciences Inc., Boston, MA). If the spotting quality standards were met, the batch was deemed satisfactory for analysis.

Ameba culture. Trophozoites of *E. histolytica* strain HM1:IMSS were grown axenically at 37°C in TYI-S-33 medium (12) with 100 U/ml of penicillin and 100 μg/ml of streptomycin sulfate (Invitrogen, Carlsbad, CA). For growth curve analysis, amebae were grown until they became nonadherent but still viable (144 h), seeded into T25 flasks with 300,000 ameba per flask (Corning Life Sciences, Corning, NY), and grown for 12 to 144 h.

Erythrophagocytosis. Human erythrocytes were isolated using Mono-Poly resolving medium (ICN Biomedicals, Aurora, OH) according to the manufacturer's directions. Erythrocytes were washed twice in 10 mM HEPES (pH 7.0), 140 mM sodium chloride, and 0.1% bovine serum albumin and then resuspended in the same buffer until use. One million log-phase trophozoites were grown in 50 ml of TYI-S-33 medium for 24 h in the presence or absence of 24 million erythrocytes per ml of medium.

Isolation of RNA. Amebae were lysed with 2 ml of buffer RLT containing β-mercaptoethanol (the first component of the RNeasy kit from Qiagen, Valencia, CA). Samples were processed immediately or flash-frozen in liquid nitrogen and stored at −80°C until processing using QIAshredders, followed by the RNeasy mini kit, including all optional steps and a 5-min incubation with buffer RW1. Samples were treated on the columns with RNase-free DNase from Qiagen according to the manufacturer's directions (Qiagen). Samples were analyzed for residual DNA contamination by PCR using primers for Jacob (conditions are described below). Samples that contained residual DNA were retreated with DNase I (Roche, Indianapolis, IN) for 1 h at 37°C in a 100-μl total volume with 10 μl of 10× DNase I buffer (100 μM Tris [pH 7.5], 25 mM $MgCl_2$ and 5 mM $CaCl_2$) and 3 μl DNase I, repurified on RNeasy columns and rescreened for residual DNA contamination. The Agilent BioAnalyzer (Agilent Technologies, Palo Alto, CA) was used to assess RNA quality. The results were inspected to ensure that both ribosomal peaks were intact and that no degradation had occurred. Acceptable 260/280 ratios ranged from 1.8 to 2.1.

Sample labeling, hybridization, and scanning. DNA oligoarray assay of gene expression used cohybridization of two fluorescently labeled cDNA targets, prepared from different samples. For routine oligoarray expression analysis, a previously described (24) indirect labeling procedure was used. Approximately 10 μg of RNA per sample and random hexamers were used for synthesis of cDNA containing amino-allyl-labeled nucleotides. The newly synthesized cDNA was then labeled by a covalent coupling of an appropriate cyanine fluor (CyDye postlabeling reactive dye pack; Amersham Biosciences Corp., Piscataway, NJ). In a typical oligoarray assay, the cDNA of one preparation (control) was labeled with Cy5, while the second cDNA (experiment) was labeled with Cy3. Both reactions were purified with a QIAquick PCR purification kit (Qiagen) for removal of the uncoupled dye. The labeling efficiencies of the purified target preparations were examined by spectrophotometry, as well as by calculations of the mass of cDNA and Cy5 or Cy3 dye incorporation. The nucleotide-to-dye molecular ratios were considered suitable for oligoarray experiments with a ratio of less then 50 nucleotides/dye molecule. Both targets were equalized based on the total amount of dyes incorporated before hybridization (24). The samples were mixed, dried by speed vac, and dissolved in hybridization buffer solution (50% formamide, 5x SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% sodium dodecyl sulfate [SDS]). The cDNA-containing hybridization solution was then denatured, applied to the oligoarray (prehybridized in 5x SSC, 0.1% SDS and 1% bovine serum albumin), and hybridized at 42°C for 18 h. Following 5-min washes in 2× SSC–0.1% SDS and in 0.1× SSC, the slide was scanned using a ScanArray 4000 scanner (PerkinElmer, Wellesley, MA). Both Cy5 and Cy3 images of one experiment were analyzed with QuantArray 3.0 microarray analysis software. Normalization to median between both channels was used.

RT-PCR primer design. Real-time PCR (RT-PCR) primers (Table 1) were designed using Beacon Designer 2.0 (Premier Biosoft International, Palo Alto, CA). RT-PCR primers and oligoarray probes were designed independently; thus, the PCR fragment and the oligoarray probe represented different regions of the same gene. Each primer was analyzed against the TIGR *E. histolytica* database, and any primer that had significant sequence similarity to multiple genes was rejected. Thus, both the forward and reverse primers were specific for one gene, except actin, Jacob, and Hgl, which detected all family members and/or alleles in the genome. Optimal annealing conditions (determined by gradient PCR) were

TABLE 1. RT-PCR primers

Gene product	Primer sequence		Annealing temp (°C)
	Forward	Reverse	
RNA Pol II	AAAGAAGGTGTTACTGTAGACGTAGGG	ATCTGAACGGACACGGACATGAC	66
RNA Pol II L	CTAATAAACCAAAGCCAATGGGATTCTCTC	GCTGGATTATATGGTGAAGGACCTGAAC	66
RNA Pol II 13	GTCCCGACTGTCATCAAATAATGCTTCC	ACACAGAAACTTGTTGGGTCTTCAGC	66
Jacob	CAAAGGAGTTCAAATGGGATGTGTTAG	TTATTTGGTGTAGGAGTTGGTAATGGG	66
Tmk 19	TTGTAGAAAGGATTGTATGTGTGAAGATGG	AGCACATCCTAAACAATCTGAACGATAC	66
Tmk 21	TTTGCTTTGGAAGTGGGACATATTGTAACG	TCCACGTTCCCCATTTCCATCCATTTC	66
Tmk 31	CCTTATGGATGTCTTCGTTGTATGACTGG	AGTTGAGTCATTAACCTTCATTCCAAATTCAC	66
Tmk 63	GCTGAAGGAACACCCACATACGG	AGAAATAATAGCCATCAGCACATGACAAAC	66
Tmk 65	TGTAGTTAATGGGTCAGGGTATAAG	GCTCGTTGTTCTTCAGACATAAG	64
Tmk 71	TGTTGAAGGAGAAAAGAAAATGAATGG	CTGGTTGAGAAGGATTATGTATAGAGTC	62
Tmk 75	ACTCTGTTATGACTTGGGAAGATCCTTATC	GGTTCATCACACCAACACTCACATATAAG	66
Tmk 79	CCTGATGTTTCTAATCTTGTTGGACTTC	GCAAGTTCAAGTTCCTGATGAGAATATC	66
Tmk 80	CGCCTTTAGCCCACCCTCTTTTATTG	ACTGTCTGTTGTTGATTCACTGTATCTTCTC	66
Tmk 96	AATGGGTGTGCTGTTTGTCA	AAGCAACACACTTCGCGTCT	64
Tmk 98	TCTGCTGAAGGGTCTGTGGGTTCTC	CGGCTTTGACGGGTGTTTGTGAATTATG	66
SA	ACTTGCTGCTCGTGTCATTGC	TTAGTGAATGATCCTGGTGTGAATCTTC	66
Actin	GCACTTGTTGTAGATAATGGATCAGGAATG	ACCCATACCAGCCATAACTGAAACG	62
Hgl	GGCGGATCCTGTGGTGGAGATTCTACA	CATCACCAACTGCTTGAA	59

used to ensure specificity, and any PCR primer pair that produced more than one melt peak was discarded. PCR products that produced single melt peaks were analyzed by gel electrophoresis in 1.5% agarose–Tris-borate-EDTA, and if multiple bands were observed, the primer pair was discarded. Finally, all PCR products were sequenced using the forward amplification primer to verify specificity.

Real-time PCR validation of oligoarray results. RNA was reverse transcribed using iQsuperscript (Bio-Rad, Hercules, CA) according to the manufacturer's directions. cDNA levels were measured using RiboGreen (Molecular Probes, Eugene, OR) with DNA of known quantity as a standard in a SPECTRAmax Gemini EM fluorescent plate reader, according to the manufacturer's directions. Before proceeding to analysis of cDNA samples, a no-reverse-transcriptase control PCR was done using primers to the cyst-specific gene Jacob to verify that there was no residual DNA contamination of the samples. All samples were analyzed in duplicate, and all time points were analyzed in triplicate. Each time point was thus represented in six wells during the real-time PCR assays, and the six wells were averaged after normalization to the RNA polymerase II gene's average (47). Two sequentially performed growth curves were analyzed in the real-time PCR assays to ensure reproducibility. All real-time PCR assays were quantitative to allow direct comparison of gene expression levels.

A PCR master mix consisted of 1,100 μl of iQSYBRGreen super mix (Bio-Rad, Hercules, CA), 1,100 μl of distilled H_2O, 88 μl of forward primer (50 pmol/μl), and 88 μl of reverse primer (50 pmol/μl). To each well containing 2 μl of cDNA was added 25 μl of master mix. Duplicate assays were performed on each sample. Each assay included standards, no-DNA-control wells, and no-RT-control wells. The cycling conditions were 95°C for 5 min; 30 cycles of 95°C for 30 s, annealing for 30 s (see Table 1 for annealing temperatures), and 72°C for 30 s; and 1 cycle of 72°C for 2 min 30 s followed by a 90-step melt curve increasing 0.2°C with a 5-s hold.

TABLE 2. Properties of the domains of the *E. histolytica* transmembrane kinase groups

Group[a]	Transmembrane kinase no.	Size[b]	No. of amino acids, TM to kinase[c]	Signature motif in kinase	No. of amino acids, kinase to end[d]	No. of motifs[e]			
						CXC	CXXC	CXXXC	CXXCXXGYY
A	4, 17, 23, 25, 52, 53, 55, 61, 65, 68, 69, 72, 85	517–532	220	CC(I/V)KITDFGTSR[f]	40	4	4/5	3/4	3
B1	5, 12, 43, 76, 86, 91, 95, 100, 101, 103, 104	897–916	135	KLTDFGS(A/S)R[g]	0	1	25	2	9
B2	2, 8, 10, 11, 14, 15, 31, 36, 41, 62, 74, 75, 77, 87, 88, 92, 94, 105	822–1762	133	KLTDFGS(A/S)R[h]	0	0	90	0	25
B3	21, 28, 29, 30, 32, 35, 37, 38, 42, 48, 51, 96	830–2117	145	KLTDFGS(A/S)R	0	0	24	0	7
C	9, 13, 39, 60, 63, 71	547–624	160	C(A/G)KLTDFGTC[i]	59–82	0	33	0	11
D1	3, 18, 40, 56, 70, 79	520–619	234	PITAKVTDFGTS	63	3	5	3	7
D2	19, 27, 44, 46, 50, 57, 64, 67, 82, 97, 98	399–614	233	V(T/V)(C/X)KV(T/S)DFGTS	55	4	5	3	9
E	22, 54, 66	401–412	150	AKLSDFGTSR	60–97	1	0	0	0
F	34, 45, 59, 80	231–368	24	VKVSDFGLS and WXAPE	0	0	0	0	0

[a] Group G transmembrane kinases are diverse and include kinases 1, 6, 16, 24, 47, 49, 73, 79, with no common signature motif or other features within the group or with other groups.

[b] Number of amino acid residues in the extracellular domain.

[c] Amino acids from the C terminus of the transmembrane domain to the N-terminal glycine of the kinase domain.

[d] Amino acids from 10 residues after the conserved arginine to the stop codon.

[e] Number of times each motif is found in the protein sequence. The number of CXC, CXXC, CXXXC, and CXXCXXGYY motifs may vary between individual family members. Values with a slash indicate that some family members have 4 or 5, or 3 or 4, of the motif.

[f] Group A TMKs can additionally be identified by a moderately conserved (K/R)XXXDI(E/N)I(Y/F)KQQQPXYYYYIXGSXXXPXKXX(K/R)Y motif C-terminal to the transmembrane domain.

[g] Group B1 TMKs can be identified by a KRKEKKEREKTTIFKTITQSNI(K/R)FI(S/P)LGDG sequence after their transmembrane domain.

[h] Group B2 TMKs can be identified by a PV(N/G)(Q/E/K)E(S/T)(K/R)DL(L/I)CIGNXXKXXXKVQ sequence after their transmembrane domain.

[i] Group C TMKs can be identified by a RRR(K/R)XXKXXXXXIKPF(H/K)VSSD(L/V)ELXLL sequence following the transmembrane domain.

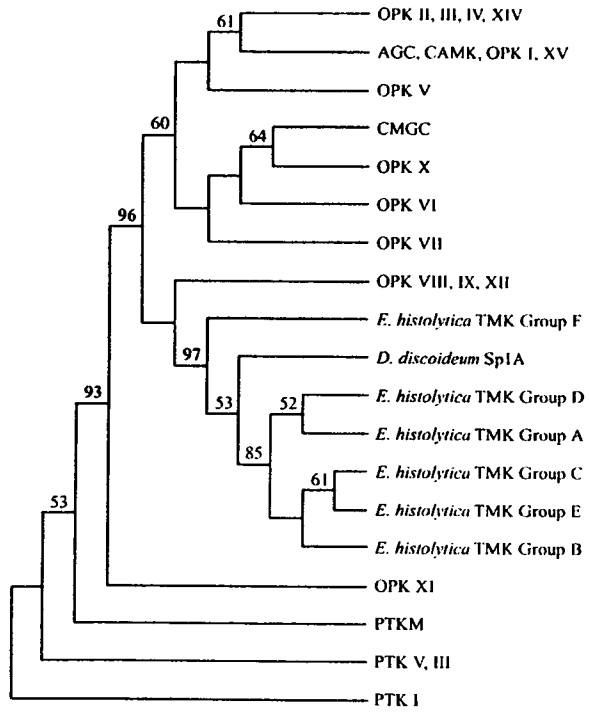

FIG. 1. Phylogenetic and sequence analysis of the kinase domain of *E. histolytica* transmembrane kinases. CMGC is the cyclin-dependent kinase, mitogen-activated protein kinase, glycogen synthase kinase, and CDK-like kinase family. CAMK is the calmodulin-dependent kinase family. AGC is the family of protein kinases A, G, and C. TMKs were aligned to Hanks's alignment (20) using CLUSTALX (61). The aligned sequences were bootstrapped using Seqboot, Protpars, and Consense (15). Significant bootstrap values are shown in bold, and bootstrap values above 50 are shown. The GenBank numbers for the TMKs are in Tables S1 and S2. SplA is GenBank accession no. U32174 (40).

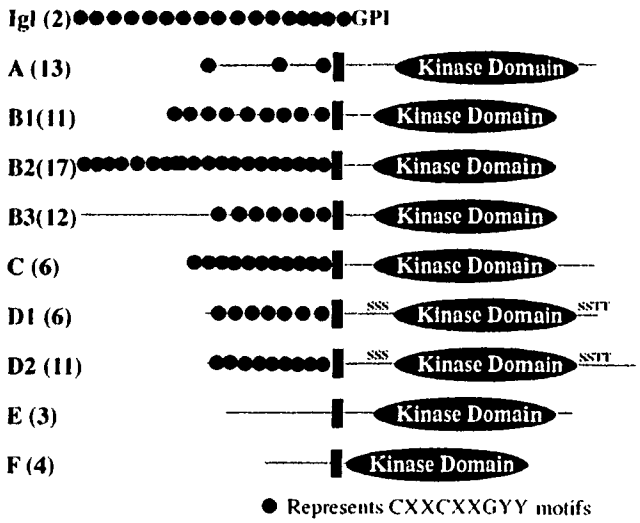

FIG. 2. Diagram of Igl and the TMKs. This diagram shows the approximate sizes of the different proteins and the distribution of the CXXCXXGYY motifs in the extracellular domain (indicated by a black circle). The CXXCXXGYY motifs are part of a larger motif, CXXCXXG(Y)(Y/F)(L/V/F/Y/M)-Polar-Polar, which also can begin with CXC instead of CXXC. GPI represents a putative GPI anchor. A black rectangle indicates a transmembrane domain, and a black oval indicates a putative kinase domain. The serine (SSS)- and serine/threonine (SSTT)-rich regions found in groups D1 and D2 are shown. Numbers in brackets indicate numbers of known family members.

Production of anti-ΔTMK96 rabbit serum production. The kinase region of Tmk96 (ΔTMK96) was PCR amplified with the primers 5′-CAATTTAGAGAA GGAATTCCT-3′ (5′ primer) and 5′-TCACATTAATTGAAGATGTTTTAAA ACAACA-3′ (3′ primer). This 1,000-bp fragment was cloned into TOPO NT/T7 (Invitrogen), in frame with an amino-terminal six-His tag via TA cloning. Bacteria were grown at 37°C to an optical density at 600 nm of 0.5 and induced with isopropyl-β-D-thiogalactopyranoside for 4 h, and the recombinant protein was purified with nickel agarose beads (Qiagen). Antibodies were raised to this purified recombinant protein by a 90-day protocol including three inoculations of New Zealand White rabbits with recombinant ΔTMK96 (Covance, Princeton, NJ), and the antibodies were purified from serum with a protein A column.

Western blots using ΔTMK96. Soluble proteins were extracted from amebae by harvesting 5×10^7 trophozoites by incubation on ice for 10 min, followed by centrifugation ($200 \times g$ at 4°C for 5 min). The amebae were lysed in 10 mM sodium phosphate buffer with protease inhibitor cocktail I (Sigma, St. Louis, MO) per the manufacturer's directions. Membranes were then cleared by centrifugation ($100,000 \times g$ at 4°C for 1 h). Whole-cell lysates were prepared by sonication of 10^6 amebae in three 5-min pulses on ice. Large intact particles were eliminated by centrifugation ($20,000 \times g$ at 4°C for 30 min). All samples were then separated on 10% polyacrylamide gels and then electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Nonspecific binding was blocked by preincubation with Tris-buffered saline with 5% bovine serum albumin and 0.05% Tween 20 (TBST). In order to detect proteins on the blot, either anti-ΔTMK96 rabbit serum (a dilution of 1:5,000) or preimmune serum (1:2,500) was added in TBST for 1 h at room temperature. Interactions were detected by the addition of peroxidase-conjugated goat anti-rabbit IgG (Sigma) and development with ECL (Amersham) per the manufacturer's directions.

Confocal microscopy. *E. histolytica* trophozoites (10^6) were bound to glass coverslips in a 24-well plate for 30 min at 37°C in TYI-S-33 medium. Adherent amebae were washed twice in phosphate-buffered saline (PBS) and fixed in 3% paraformaldehyde for 30 min at room temperature. To make the plasma membrane permeable, amebae were treated with 0.2% Triton X-100 in PBS for 1 min. Nonspecific binding was blocked by incubation with 20% goat serum and 5% bovine serum albumin (Sigma) in PBS for 1 h at 37°C. After incubation with either the anti-ΔTMK96 rabbit polyclonal antibody (200 μg/ml) or anti-Gal/GalNAc lectin antibody (6 μg/ml) for 1 h at 37°C, the coverslips were washed twice before Cy3-conjugated goat anti-rabbit secondary antibodies (Jackson Laboratories, Bar Harbor, ME) were added at a 1:160 dilution for 1 h at 37°C. The coverslips were washed twice and mounted on slides with Gel/Mount (Biomeda, Foster City, CA). Confocal images were visualized using a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY).

RESULTS

Phylogenetic analysis of the *E. histolytica* TMK family. Previously we reported a family of CXXC-containing proteins with sequence similarity to Igl1 and Igl2 (8). With the completion of the genome project, it became apparent that the majority of these CXXC-containing proteins were members of an approximately 80-gene family with intracellular kinase domains. The extracellular domains shared sequence similarity to Igl and to VSPs of *Giardia*. The kinase domain of these proteins in most cases contained all five conserved kinase motifs: the key conserved glycine-rich motif, the K residue, and the HRDL, DFG, and APE motifs (Table 2). However, a few lacked the glycine-rich motif (Tmk01, Tmk24, Tmk45, and Tmk73), which helps coordinate the second phosphate of ATP (14). The glycine, K, and DFG motifs are implicated in the binding and orientation of ATP. The HRDL motif is involved in catalysis. The APE motif is responsible for anchoring the substrate and thereby influencing the specificity of interaction with the protein that is being phosphorylated (14, 20, 60). Despite the conservation of the essential kinase motifs, overall the kinase domains were

TABLE 3. Consensus sequence comparison of subdomains of malian tyrosine kinases, serine/threonine kinases, and the *E. histolytica* transmembrane kinase groups

Kinase	Consensus sequence in subdomain[a]				
	I	II	III	IV	V
Tyrosine	LGxGxFGxVxxG	VA(I/V)K	ExxxM(R/K)	GxCooo	GxL(F/Y)lx
Ser/Thr	LGxGxFGxVxxx	VAIK	Exxxx(K/R/H)	Gxxxxx	GXX(Y/F)(L/I)H
A	IAEGAMG-VY-G	VA(I/V)K	ExxxLK	GSVTYI	GSLgeYOR
B1	IGEGsFGIVYVG	VAIK	Ee(S/N)EDK	GAVFIP	GSIQDIMNK
B2	oGEGsFGoVY(K/o)G	VAIK	EVxMLDK	Gavflp	GSLxxooxK
B3	oGEGsFGoV(Y/F)KG	VAIK	EoxmLdK	Gavoop	GSoxxoo(x)K
C	IGEGAFGMVFRG	VAIK	EQEKEF	GAVYTE	GS(M/L)SKOWxK
D1	IGGGTFGIVYRA	VAVK	EAEMMER	GSVoTx	GSLRKFMK
D2	IGxGTFGoVY(K/R)(G/A)	VA(V/I)K	ExxooEx	Gsvxxx	GSLRK(Y/f)ok
E	IGEG(S/T)FgiV(Y/F)xG	VAIK	ExFxxE	Gxxxox	GSoKxoY
F	ogxg(S/T)xgxvwxa	oaVK	evxlmK	Gsgxdx	gxossoL

[a] Subdomain is according to Hanks's alignment of kinases (20). Underlined in tyrosine and serine/threonine kinases indicate highly conserved residues. Letters in uppercase are conserved. Letters in lowercase indicate moderate conservation. x, any residue; o, hydrophobic residue; —, sequence of varying length.

divergent from other known kinases. The TMK family branched closely with the other protein kinase group IX (OPK IX), which are the TGFβ receptor and activin family of serine/threonine kinases, OPK VIII, which are a Raf family of serine/threonine kinases, and OPK XII, which are the casein kinase I family of kinases (Fig. 1). It was not possible to determine by sequence analysis alone if the TMKs were serine/threonine or tyrosine kinases. Spl A from *Dictyostelium discoideum* was the most closely related kinase identified by sequence similarity in the NCBI database that has been functionally characterized. The Spl A kinase has been shown to be a dual-specificity kinase that phosphorylates both tyrosine and serine/threonine residues (40). The Spl A kinase was found to be phylogenetically within the TMK family (Fig. 1). Unlike the TMK family, Spl A is a cytoplasmic kinase rather than a transmembrane kinase. Two TMKs (Tmk58 and Tmk89) clearly did not group with the TMK family but grouped closely with other protein kinase groups VI and VII (data not shown), suggesting that these may represent serine/threonine transmembrane kinases that have evolved separately from the TMK family.

The TMK family members were grouped based on sequence and phylogenetic analysis of their cytoplasmic kinase domains. A specific signature motif between the conserved HRDL and APE motifs was identified for each family (Table 2). The motif was 15 to 24 amino acids C-terminal to the histidine in the HRDL motif. The motif sequences were CC(I/V)KITDF GTSR (group A), KLTDFGS(A/S)R (group B), C(A/G)KLT DFGTC (group C), PITAKVTDFGTS (group D1), V(T/V) (C/X)KV(T/S)DFGTS (group D2), AKLSDFGTSR (group E), and VKVSDFGLS with a conserved tryptophan two resi-

TABLE 4. Significantly changed genes during growth

Growth period[a]	Probe(s)[b]	Protein	Group	Change	*P* value
Early (12 h p.i.) to mid-log (48 h p.i.)	1, 91, 99	Actin		Increased 2.17-fold	<0.05
	190	Lg13	Gal/GalNAc lectin	Increased 1.37-fold	<0.05
Late log (96 h p.i.) to nonadherent (144 h p.i.)	1, 91, 99	Actin		Decreased 1.88-fold	<0.01
	321	EHCP1&2	Cysteine proteinase	Decreased 1.79-fold	<0.05
	320	EHCP20	Cysteine proteinase	Increased 1.37-fold	<0.05
	88	Hgl family	Gal/GalNAc lectin	Decreased 1.58-fold	<0.01
	189	Lgl1	Gal/GalNAc lectin	Decreased 2.87-fold	<0.05
	280	Lgl4	Gal/GalNAc lectin	Decreased 2.28-fold	<0.01
	232	Lgl5	Gal/GalNAc lectin	Decreased 4.26-fold	<0.05
	224	ENT3593	Unknown—surface	Increased 1.61-fold	<0.05
	145	TSA	Thiolredoxin	Decreased 1.65-fold	<0.05
	239, 277	SREHP	Surface protein	Decreased 3.08-fold	<0.05

[a] Three hybridizations (biological replicates) were analyzed in two dye experiments comparing 12 h to 48 h, 48 h to 96 h (no significant differences were found), and 96 h, to 148 h postinoculation (p.i.).
[b] Probe numbers are from Table S1 in the supplemental material.

TABLE 3—*Continued*

Consensus sequence in subdomain[a]						
VIa	VIb	VII	VIII	IX	X	XI
GMxYlx	o(I/V)HRDLaar N(c/l)oo	O-KixDFGl	PIW(K/R) WXAPEOO	SDVW(S/A)(F/Y) GoooWE–Pyp	—	CWxxxxxRPxF
(G/A)ox (Y/F)oH	ooHRDoKPxNOOO	KooDFGo	xxxx(Y/F) xAPEoo	XDoW(G/S) oGoooe–PFx	—	xxxxxxxRxxx
GMxFLH	IoHLDLKPDNLLVN	KITDFGT	GTPoYxAPExY	(g/s)DV(Y/F)(s/a) (F/Y)AotaWEoFYqxEPYK	—	CWkQxxxeRpxF
GISYLH	iLHRDLIKPDNFLVV	KLTDFGS	GTPKYMAPEvL	SDIYSFSITMLQiiTWQDPFP	—	SWQQEPKERITI
GIxYLH	ILHRDLIKPDNoLoo	KLTDFGS	GTPxYMAPEoL	ADIySFaotmY(e/q) xoxWxxPyP	—	(s/c)WxqxPxxRox
GIxYLH	ILHRDLIKpdNoLoo	KLTDFGS	GTPxYMAPEoL	ADI(y/f)sFaotmyEo oxWxxa(y/f)p	—	(s/c)WxxxPxxRox
ALX(Y/F) LH	IIHRDVKGENoLo (Y/F)	KLTDFGT	GTPTYMAPECL	vDVYAYGIVLYET(y/f) xExxAYxxDERFNQPWM	—	CwxQxxxxRPxF
GMEYLH	ILHRDLKTDNVLVY	KVTDFGT	GTPVYMAPEI (s/t)	SDVYSFAICMLEIWLGRDPYDP	—	(a/s)WxHxPS (E/D)RPTF
GMxYLH	IoHrDLKtDNVLvo	KV(T/S) DFGT	GTPmYMAPEoh	SDVYSFAICoLEIWoxxxPY (d/p)	—	CWxxxPxxRPxF
GMxFLH	IIHRDLKPDNoLoo	KLSDFGT	GTPxYMAPEoo	oDV(F/Y)s(F/Y)AoVx (Y/F)EoosrkoPYs	—	CWaxDPxxRPxF
GMoYLH	IoHxdLksxNoLVx	KvSDfgl	gtlxWxAPEoL	xDvYS(F/Y)gIomWEoot rxxP(Y/F)x	—	CWxxxpexrpxf

dues N-terminal to the APE motif (group F) (Table 2 and Fig. 2). Tmk58 and Tmk89, which did not group with the rest of the TMKs, contained an ITDFGLAKK motif. Group G TMKs lacked a conserved motif in the kinase domain and also lacked one or more of the conserved kinase domain motifs. Sequence similarity between family members was not limited to the kinase domain. Additional motifs were found in some families that can be used to identify group members between the cytoplasmic and kinase domain (Table 2 footnotes). Additionally, group D TMKs had a serine-rich region N-terminal to their kinase domain and a serine/threonine region C-terminal to their kinase domain. Serine/threonine rich regions are often found in kinases and typically regulate kinase activity (30). The kinase domains of many of the *E. histolytica* TMKs were a mix of serine/threonine and tyrosine kinase signature motifs, further complicating efforts to predict their kinase activity (Table 3). However, the sequence similarity to SplA of *D. discoideum* suggests that they likewise may be dual-specificity kinases.

An overview of the sequence motifs of the different subfamilies of kinases is shown in Table 2 (all domains except kinase domains), Table 3 (kinase domains), and Fig. 2. The TMKs had extracellular domains of 36 to 2,117 amino acids (with 11 to 98% similarity between family members), a transmembrane domain, and a kinase domain. Groups B, C, and D shared significant sequence similarity in their extracellular domain to Igl1 (Fig. 2). Most extracellular domains were rich in CXC/CXXC/CXXXC motifs. Almost all of the TMKs had at least one CXXCXXGYY motif beginning approximately 25 amino acids N-terminal to the transmembrane domain. Like Igl1 most of the TMKs had many additional CXXCXXGYY motifs (Table 2 and Fig. 2). A very similar C(D/E)XCXXG(Y/F)(Y)(G) motif was found in Igl, VSP of *Giardia* (1, 38), and laminin LE domains (27, 57, 63). In VSP of *Giardia* the CXXC motifs have not been crystallized but have been shown to bind zinc (38), to have their N terminus at the host parasite interface (38), and have nonreactive cysteines (42). In laminin the LE domains form a rod-like structure of mini-globular folds (57, 63). This is consistent with the CXXCXXGYY motifs forming a linear array of mini-globular folds. By analogy to the laminin LE domains, the TMKs may have a rod-like structure.

Gene expression analysis of *E. histolytica* during growth. To characterize the expression profile of these genes, we constructed an oligoarray of the TMK genes, Gal/GalNAc lectin genes, and other putative surface virulence genes. Few changes in gene expression were seen when early phase (12 h) was compared with mid-log phase (48 h) and late log phase (96 h) was compared with nonadherent phase (144 h) (Table 4). When mid-log-phase (48 h) and late-log-phase (96 h) amebic cultures were compared, very little change was seen (data not shown). *actin* was clearly growth regulated (Table 4), as were the Gal/GalNAc lectin *hgl* genes, many but not all of which decreased significantly during late log phase (Table 4 and data not shown). This decrease is consistent with previous observations of *hgl1*, *hgl2*, and *hgl3* gene expression (48). Expression of known genes was, in general, consistent with RT-PCR, Western, and/or Northern analysis (data not shown). We concluded that *E. histolytica* did not appear to growth-phase regulate expression of most putative virulence genes when grown under lab culture conditions.

Expression analysis of the TMKs. The expression profile of 67 of the TMK genes during mid-log phase (72 h) is shown in Fig. 3. At this time point, 19 genes (group A, *tmk61*, *tmk65*, and *tmk72*; group B2, *tmk02*, *tmk08*, and *tmk74*; group B3, *tmk21* and *tmk28*; group C, *tmk39* and *tmk63*; group D1, *tmk40* and *tmk56*; group D2, *tmk19*, *tmk44* and *tmk46*; group E, *tmk22* and *tmk54*; group F, *tmk59*; group G, *tmk06*) (Fig. 3) showed hybridization values in trophozoites significantly greater than that for the cyst-specific transcript *jacob* ($P < 0.05$).

Analysis of TMK expression by RT-PCR. Real-time PCR was conducted on *jacob*, *tmk31*, *tmk80*, *tmk96*, and *tmk98*, all of

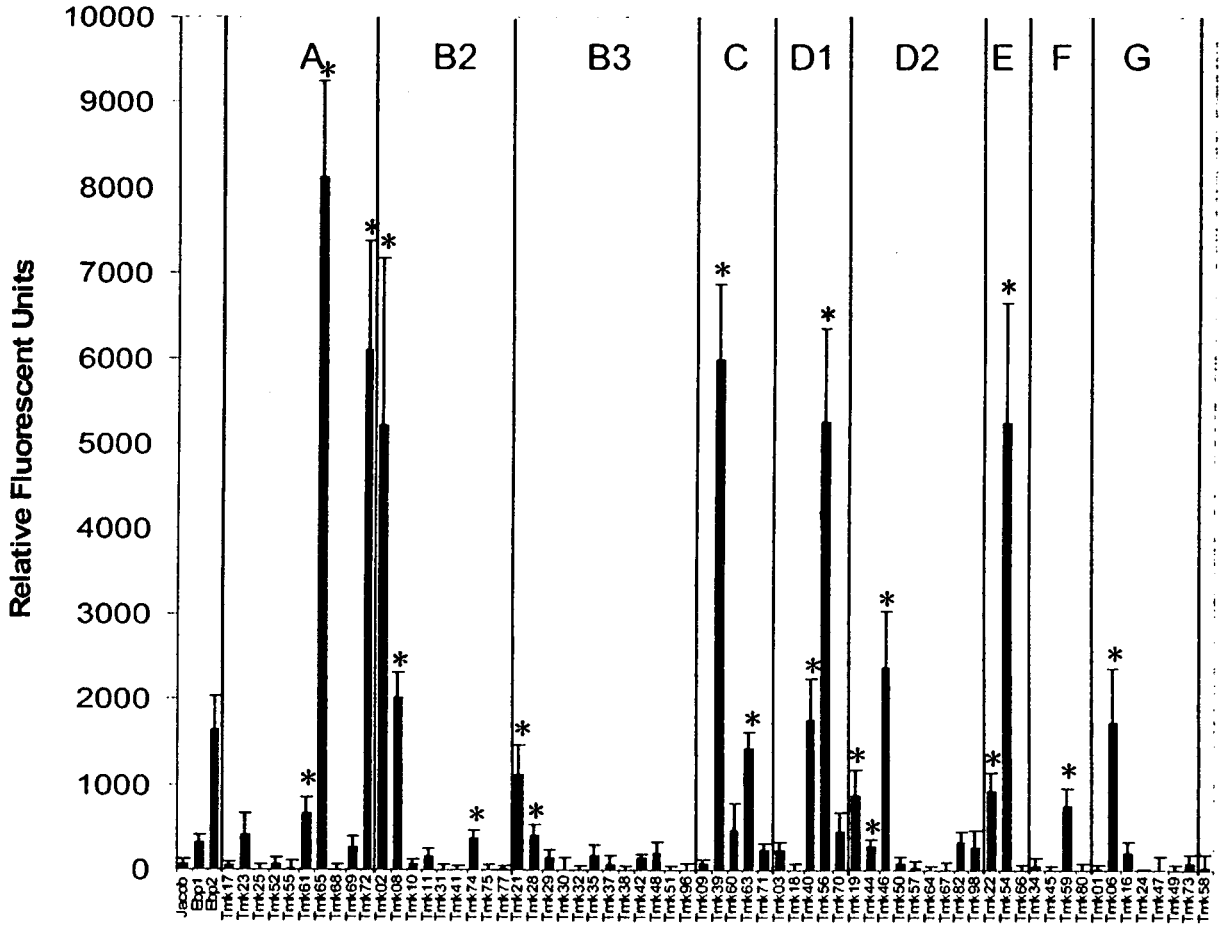

FIG. 3. Expression of TMKs during log-phase culture as determined by oligoarrays. The TMKs were classified into groups: A, B2, B3, C, D1, D2, E, F, G, and "other" (*tmk58*). Expression of *jacob* (encoding a cyst protein not expected to be expressed in trophozoites), *ebp1*, and *ebp2* genes is shown for reference. TMK genes expressed at a higher level than *jacob* ($P < 0.05$) are indicated by an asterisk and were as follows: group A, *tmk61* (391.t00004-AAFB01000774, 279.t00010-AAFB01000993), *tmk65* (62.t00013-AAFB01000240), and *tmk72* (302.t00003-AAFB01000819); group B2, *tmk02* (70.t00014-AAFB01000264), *tmk08* (10.t00040-AAFB01000051), and *tmk74* (6.t00088-AAFB01000031); group B3, *tmk21* (42.t000019-AAFB01000175), and *tmk28* (66.t00027-AAFB01000251); group C, *tmk39* (359.t00009-AAFB01000933), and *tmk63* (20.t00067-AAFB01000094); group D1, *tmk40* (65.t00015-AAFB01000247), and *tmk56* (5.t00091-AAFB01000028); group D2, *tmk19* (135.t00017-AAFB01000458), *tmk44* (159.t00012-AAFB01000511), and *tmk46* (131.t00015-AAFB01000449); group E, *tmk22* (12.t00043-AAFB01000464) and *tmk54* (75.t00011-AAFB01000285); group F, *tmk59* (304.t00008-AAFB01000821); and group G, *tmk06* (274.t00010-AAFB01000764). Error bars represent the standard error of the mean of three hybridizations (biological replicates).

which were predicted by oligoarray not to be expressed, and *tmk19*, *tmk21*, *tmk63*, *tmk65*, *tmk71*, *tmk75*, *sa*, *hgl*, and *actin*, all of which were predicted to be expressed. Gene expression was monitored sequentially in trophozoites in laboratory culture over a 12-day period. To allow comparison between time points, results were normalized to the average of three RNA polymerase II genes (Fig. 4). All real-time PCR results of the TMK genes were consistent with the oligoarray results. Significant variations in expression during laboratory culture were observed for *tmk19*, *tmk63*, and *tmk79* ($P < 0.01$) (Fig. 4D, 4F, and 4I).

Expression analysis of the TMKs during erythrophagocytosis. One million trophozoites were grown in 50 ml of medium with or without a vast excess of erythrocytes (24 million/ml of medium) for 24 h. We did not observe significant changes in TMK gene expression during erythrophagocytosis (data not shown).

Detection of expression of TMK family members with polyclonal antibodies. The kinase domain of Tmk96 was expressed in *Escherichia coli* and used to generate polyclonal antibodies. Multiple trophozoite proteins were detected with polyclonal anti-kinase domain antisera (Fig. 5A). No bands were observed with preimmune sera (data not shown). The recognition of multiple proteins by the antisera raised against the Tmk96 kinase domain was not surprising given that the kinase domain is conserved between different TMKs. The predicted TMKs vary in size from 482 to 2,577 amino acids, and Tmk96 is in the largest subfamily (group B) of TMKs with multiple members expressed. Of the TMK genes that we detected with expression significantly above *jacob* the size range was from 686 to 2,577 amino acids (78 kDa to 294 kDa not accounting for potential posttranslational modifications). The smallest proteins observed on Western blots were of a mass consistent with that of a typical kinase domain (270 amino acids) and may represent

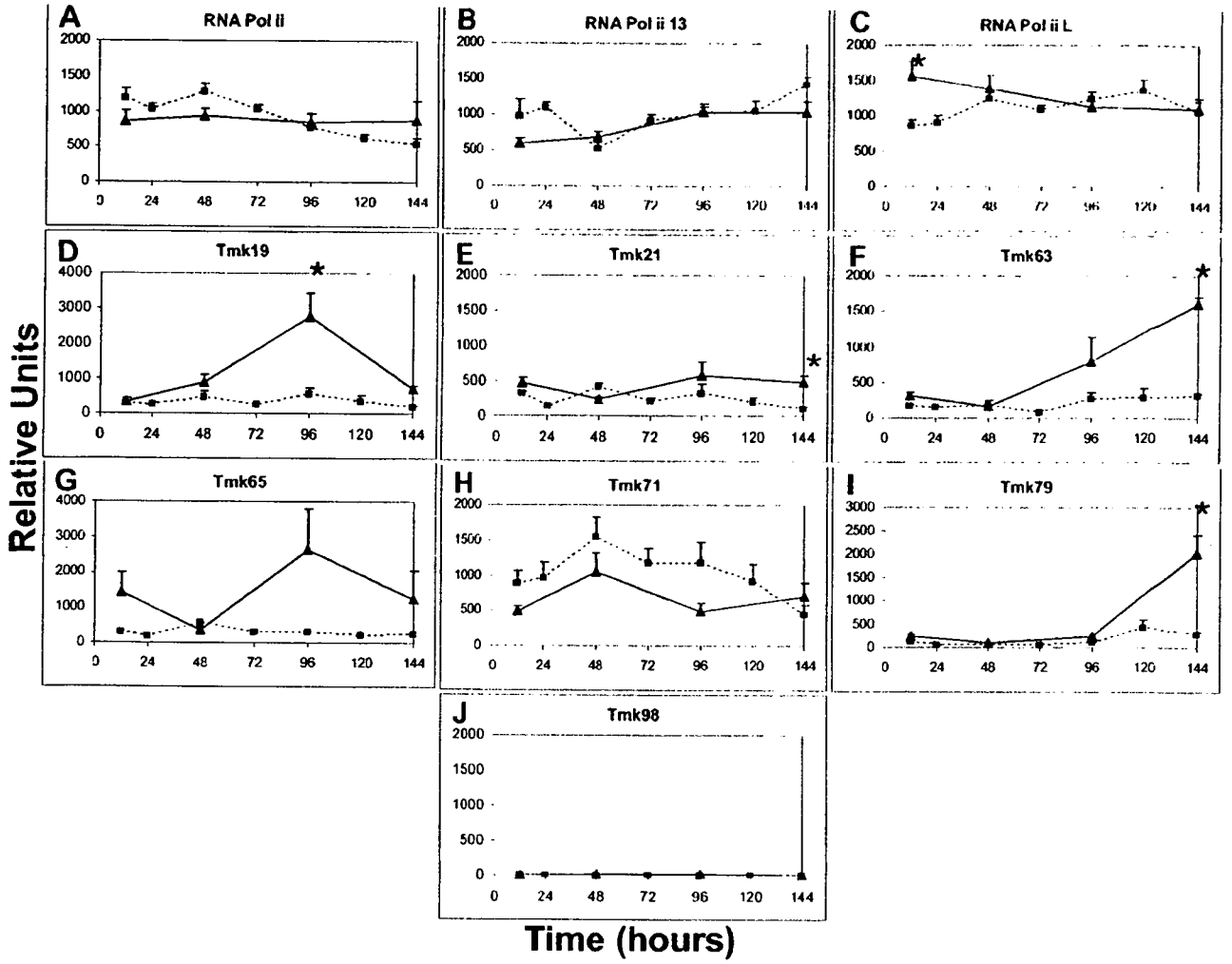

FIG. 4. Expression of TMK family genes during culture as determined by real-time PCR. Quantitative real-time PCR was performed on (A) the RNA polymerase II gene (*rna pol ii*) (27.t00035-AAFB01000114), (B) the RNA polymerase II 13 gene (*rna pol ii 13*) (344.t00001-AAFB01000903), (C) the RNA polymerase II L gene (*rna pol ii l*) (147.t0005-AAFB01000482), (D) *tmk19* (135.t00017-AAFB01000458), (E) *tmk21* (42.t00019-AAFB01000175), (F) *tmk63* (20.t00067-AAFB01000094), (G) *tmk65* (62.t00013-AAFB01000240), (H) *tmk71* (268.t00007-AAFB01000754), (I) *tmk79* (71.t00002-AAFB01000266), and (J) *tmk98* (361.t00001-AAFB01000937). Two sequential growth curves are shown. For growth curve A (triangles and solid line), samples were collected at 12, 48, 96, and 144 h postinoculation. For growth curve B (squares and dashed line), samples were collected at 12, 24, 48, 72, 96, 120, and 144 h postinoculation. Triplicate samples were collected at each time point. Culture B was established by transferring 300,000 amebae from culture A at 144 h. The standard errors of three biological samples, with each sample analyzed in duplicate, are shown. To allow comparison between time points, data for the TMKs were normalized to the average of RNA polymerase II, RNA polymerase II L, and RNA polymerase II 13. The average expression of these three genes was defined as 1,000 units of expression.

TMKs that do not have an extracellular domain or whose extracellular domain has been cleaved off.

Localization of TMK kinase domains to plasma membrane microdomains. Permeabilized amebae showed a focal plasma membrane staining pattern with anti-TMK kinase domain antibodies (Fig. 5B). No staining was seen with nonpermeabilized cells or with permeabilized cells stained with preimmune sera (data not shown). We concluded that the kinase domain was on the cytoplasmic side of the plasma membrane. The TMKs therefore appeared to be typical type I transmembrane proteins with an amino-terminal signal sequence and a predicted transmembrane domain preceding the kinase domain. The focal staining pattern contrasted with the uniform plasma membrane staining pattern seen with anti-Gal/GalNAc lectin antisera (Fig. 5C).

DISCUSSION

The most important finding of this work is the identification of a large family of over 80 transmembrane kinases in *E. histolytica*. Although eukaryotic-type transmembrane kinases are found in organisms from bacteria to humans (4, 64, 68), large families of TMKs have been previously described only in multicellular organisms (20). For example, we were not able to find any TMKs in the partially completed genome of the ameba *D. discoideum* (17), and only a few TMKs were found in the apicomplexan *P. falciparum* (4). The TMKs were also found in *Entamoeba invadens*, *Entamoeba dispar*, and *Entamoeba moshkovskii*, indicating that this family is conserved within *Entamoeba* (data not shown) (66). Multiple TMKs were observed to be expressed at the mRNA and protein levels,