A Novel Heat Shock Transcription Factor Family in *Entamoeba histolytica*

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**Abstract:** The HSTF is a master molecule involved in the transcriptional control of several genes during different types of stress. This transcription factor is a very conserved protein identified in different organisms from bacterial to human. *Entamoeba histolytica* is the protozoan responsible for the human amoebiasis. This parasite is exposed to different kind of stress as changes in the pH, temperature, drugs, all that situations in where the parasite needs survive. Here we identified and isolated a novel gene family of HSTFs in the protozoan parasite *E. histolytica*. Three members that we called *Ehhstf1*, *Ehhstf2* and *Ehhstf3* compose this family. Amino acid alignments and domain architecture analysis revealed that the EhHSTFs presents a conserved DNA-binding domain composed of approximately 25 residues. Interestingly this domain is shorter than the domain of the human, mouse and yeast HSTFs. Heterologous antibodies recognized four peptides of 73, 66, 47 and 23 kDa in total extracts from trophozoites growth under normal conditions. The 73, 47 and 23 kDa peptides increased their intensity when the cells were growth at 42°C by 2 h. All results together demonstrate that the amoeba present HSTFs, which may be, controlled the gene expression of this parasite under different stress situations.

**Key words:** heat shock, HSTF, amoebiasis, transcription factor

**INTRODUCTION**

All the organisms respond to chemical, environmental or physiological stress through a transient arrest of the cell cycle that is accompanied by widespread changes in macromolecular synthesis, degradation, trafficking, overall cellular metabolism and signal transduction pathways to cope with stressful conditions until more favourable conditions are encountered [1].

The termic stress in any organism may provoke different changes, since expression and/or repression of different molecules, the destabilization of protein conformation, leading to protein unfolding and aggregation, until severe or prolonged stresses can lead irreversible protein damage provoking the cell die [2]. The heat shock stress is one of the must studied mechanisms from bacteria to mammal cells. A very knows group of proteins have been completely related to this event: the heat shock proteins (Hsps). These proteins are chaperones involved in the folding, trafficking, maturation and degradation of proteins [3]. Additionally, other important proteins involved in the heat shock response are the heat shock transcription factors (HSTF), which rapidly activate and bind to the heat shock element (HSE) present in the Hsp promoters. Then this factor induces the Hsps gene expression whose products ensure the survival of the cell during stressful conditions by providing defense against general protein damage [4].

Although the heat shock response is conserved among eukaryotes, both the number and overall sequence of HSTFs vary widely among different species. Yeast as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Kluyveromyces lactis* and in the fruit fly as *Drosophila melanogaster* appear to have a single hsf gene, most vertebrates and higher plants possess multiple hsf genes: at least three hsf genes have been isolated from human, mouse, chicken and tomato genomes, whereas *Arabidopsis thailiana* has 21 hsf genes [5-15].

Little is known about transcriptional control of its genes in *Entamoeba histolytica*, the parasite that causes amebic dysentery. Just four promoters from the *EhPgp1*, *EhPgp5*, *hgl5* and *actin* genes have been functionally characterized and until this moment have been functionally described the TATA box, the CAAT/enhancer binding elements, the GAAGCT and the
URREs sequences [16-24]. With respect to the transcription factors have been reported the sequences for the TATA box binding protein (TBP) [25], two enhancer binding proteins (EhEBP1 and EhEBP2) that binds to the URE4 sequence [26] and the p53 [27] and C/EBP-like proteins (personal communication). Recently, in the multidrug resistance EhPgp5 gene promoter from E. histolytica Nieto and coworkers identified a functional putative heat shock response element (HSE) ‘unpublished data’ suggesting that in this parasite will be present a HSTF. In this work, we performed the screening and isolation of a novel heat shock transcription factors from the amoeba.

METHODS

E. histolytica strain: Trophozoites of clone A from the pathogenic E. histolytica strain HM-1:IMSS were axenically cultured in TY1-S-33 medium [28]. For heat shock treatment, trophozoites cultured in Diamond’s TY1-S-33 medium were incubated at 42ºC for 30, 60 and 120 min.

Identification of a conserved domain in the HSTF from different organisms: HSTF genomic sequences were obtained from the GenBank and Sanger databases. Sequences alignments of the eukaryotic HSTFs were performed using ClustalW with gap penalties of 10 (http://www.ch.embnet.org/software/ClustalW.html).

Isolation of the Ehhsf genes from the E. histolytica project genome: Conserved sequence of the Human HSTF corresponding to the DNA binding domain (106 aa) was used to screen the E. histolytica databases: Sanger servers (http://www.sanger.ac.uk/Projects/E_histolytica/) and TIGR (http://www.tigr.org/tdb/e2k1/eha1/) using TBLASTN 2.0 program. To determine significant e values and identity-homology percentages, aa sequences were compared with Homo sapiens (PQ00613), Mus musculus (P38532), Gallus gallus (P38529), Xenopus laevis (P41154), D. melanogaster (P22813), K. lactis (P22121), S. cerevisiae (P10961) and A. thaliliana (P41151) related proteins using BLAST.

The molecular weight of the proteins was obtained from the ExPASy Server (http://cn.expasy.org). A neighbor Joining tree was constructed with 500 bootstrap replications using the program PHYLO_WIN.

Cloning and sequencing of the Ehhsf genes: The Ehhsf1, Ehhsf2 and Ehhsf3 genes were isolated by PCR using the sense and antisense primers Eh1S (5’-GGGGATCCATGGAGAATAAAGATA-3’), Eh2S (5’-GGGGTACCCCTTCATTTTCTTCGTTTTGTC-3’), Eh3S (5’-CGGGATCCATGGCAATGGAAATCCC-3’) and Eh3As (5’-GGGGTACCTTTATTTTACAAAAC-3’) respectively and total DNA from clone A trophozoites.

Protein Analysis: Briefly, trophozoites were washed twice, resuspended at 10^6/ml of phosphate-buffered saline (PBS) and then were disrupted by repeated freezing and thawing in liquid nitrogen. Extracts were centrifuged at 180 x g for 5 min at 4ºC, then the supernatants were separated on 12 % SDS-polyacrilamide gel. The proteins were blotted onto Hybond-C (Amersham, Little Chalfont, UK) membrane. Filters were blocked with milk and incubated overnight with antisera to human HSF1 (H-311, Santa Cruz), human C/EBPβ (Δ 198, Santa Cruz) and E. histolytica actin. Then, the bound antibody was detected with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Zymed) for the HSTF and C/EBP proteins and anti-mouse immunoglobulin (Zymed) for actin protein. Immunoblots were revealed with 4-Cl-naphtol. Protein concentration was determined using the Bradford method [29].

RESULTS

To identify a conserved sequences in the HSTFs, first, we aligned eight amonoacid hsf genes sequences from H. sapiens, M. musculus, G. gallus, X. laevis, D. melanogaster, K. lactis, S. cerevisiae and A. thaliliana using the CLUSTALW program. The multiple alignments showed the presence of a conserved sequence of approximately 106 aa, that corresponds to the DNA-binding domain located at the amino terminal of the proteins (data not shown). Additionally, we identified the hydrophobic repeats HR-A/B in the carboxi-terminal of the human, mouse, chicken and frog HSTF aa sequences and the hydrophobic repeats HR-C only in the human, mouse and chicken genes (data not shown).

In order to identify if E. histolytica contains the hsf genes in its genome, we performed a screening using the DNA-binding domain of the human hsf1 sequence (106 aa) (sp/Q00613/HSF1_HUMAN) as probe in the Sequenatation Project Genome TIGR from the amoeba and in the Sanger site (www.sanger.ac.uk/projects/E_histolytica) using the TBLASTN 2.0 program. At least 14 contings were detected, however the contings 2390555.c000134323.conting3, 2390555.c000721893.conting3 and 2390555.c000721829.conting5 presented the major
homology and identity percentages with the human HSTF1 (Table 1). When we carried out the in silico analysis of the containing 2390555.c000134323.0019, we identified an open reading frame of 510 pb, corresponding to 170 aa and generated a protein of predicted molecular weight of 20.44 kDa. We call this sequence Ehstf1 gene.

In the containing 2390555.c000721893.0019, we located a gene of 837 bp, that encode to protein of 279 aa and its molecular mass obtained by the Expasy program was 32 kDa. The name of this gene is Ehstf2. The third containing (2390555.c000721829.0019) contains a gene of 459 nucleotides that encode a polypeptide of 153 aa, the predicted molecular weight was of 17.59 kDa and it was called Ehstf3 gene.

In order to determine more precisely the identity of each one of the Ehstf genes, we carried out multiple alignments of each Ehstf aa sequences with the HSTFs of different organisms using the BLASTX program and the DNA-binding domain or the complete sequence of the genes. The results showed that the EhHSTF2 and EhHSTF3 present major identity and homology percentages with the different HSTF sequences (Table 2) than the EhHSTF1. However, the EhHSTF1 presents percentages further up 24% of identity and 37% of homology with the DNA-binding domain of different HSTFs (Table 2). In contrast, when we performed the alignments using the completed HSTF sequences the identity and homology percentages presented by the EhHSTFs were very low in comparison with the percentages observed for the HsHSTF or the MmHSTF between others (Table 3). Something similar we can observed with the A. thailiana hstf genes, because low values of identity and homology were obtained, particularly with the Athstf4 (Tables 2 and 3).

Table 1: Identification of E. histolytica sequences similar to the HSTF

| Conting  | Identity (%) | Homology (%) |
|----------|--------------|--------------|
| 2390555.c000721893.0019 | 34 | 52 |
| 2390555.c000721829.0019 | 52 | 74 |
| 2390555.c000134323.0019 | 33 | 54 |

The alignment of the EhHSTF with the HSTFs of different organisms showed that the region with major similitude between these sequences corresponds to the DNA-binding domain as we mentioned above (Fig 1). Interestingly, the size of this domain is smallest in the three EhHSTFs than in the other HSTF sequences, because it is conformed by 25 conserved aa instead of the 106 aa of the human, mouse and chicken domains between others (Fig 1 and 2). In addition, we observed six aa changes in this domain: one of them correspond to the third aa which is different in the three EhHSTF sequences; other three changes are located in the fourth, eighth and tenth amino acids only in the EhHSTF1, and two aa more at 11 and 25 positions are changed just in the EhHSTF2 (Fig 2).

Fig 1: Alignment of the amino acid sequences of H. sapiens, M. musculus, X. laevis, G. gallus, D. melanogaster, K. lactis, S. cerevisiae, A. thailiana with the E. histolytica HSTFs. All accession numbers are from the GenBank database. The DNA-binding domain is in bold face.

Table 1: Identification of E. histolytica sequences similar to the HSTF

| Conting  | Identity (%) | Homology (%) |
|----------|--------------|--------------|
| 2390555.c000721893.0019 | 34 | 52 |
| 2390555.c000721829.0019 | 52 | 74 |
| 2390555.c000134323.0019 | 33 | 54 |
Table 2: Amino acid identities among the DNA-binding domain of the EhHSTFs and closely related homologues from other organisms.

| EHHS1  | EHHS2  | EHHS3  | HhHS1  | HhHS2  | HhHS4  | GgHS1  | GgHS2  | GgHS3  | MmHS1  | MmHS2  | MmHS4  | AhHS1  | AhHS2  | AhHS3  | AhHS4  |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| 100%   | 0%     |        |        |        |        |        |        |        |        |        |        |        |        |        |        |
| EHHS2  | 25%    | 100%   | 39%    | 0%     |        |        |        |        |        |        |        |        |        |        |        |
| EHHS3  | 28%    | 62%    | 100%   | 41%    | 78%    | 0%     |        |        |        |        |        |        |        |        |        |
| HhHS1  | 26%    | 43%    | 43%    | 100%   | 40%    | 61%    | 62%    | 0%     |        |        |        |        |        |        |        |
| HhHS2  | 28%    | 42%    | 40%    | 65%    | 100%   | 40%    | 62%    | 49%    | 0%     |        |        |        |        |        |        |
| HhHS4  | 25%    | 40%    | 42%    | 76%    | 61%    | 100%   | 39%    | 59%    | 59%    | 83%    | 75%    | 0%     |        |        |        |
| GgHS1  | 25%    | 43%    | 44%    | 91%    | 65%    | 74%    | 100%   | 40%    | 61%    | 61%    | 92%    | 80%    | 81%    | 0%     |        |
| GgHS2  | 28%    | 41%    | 39%    | 58%    | 78%    | 59%    | 58%    | 100%   | 37%    | 57%    | 65%    | 73%    | 94%    | 75%    | 73%    |
| GgHS3  | 26%    | 43%    | 41%    | 58%    | 58%    | 58%    | 58%    | 57%    | 59%    | 59%    | 100%   |        |        |        |        |
| MmHS1  | 26%    | 43%    | 43%    | 100%   | 65%    | 76%    | 51%    | 58%    | 58%    | 100%   |        |        |        |        |        |
| MmHS2  | 40%    | 61%    | 62%    | 100%   | 66%    | 83%    | 72%    | 73%    | 73%    | 0%     |        |        |        |        |        |
| MmHS4  | 25%    | 40%    | 43%    | 76%    | 61%    | 100%   | 74%    | 59%    | 59%    | 76%    | 62%    | 100%   |        |        |        |
| AhHS1  | 38%    | 59%    | 59%    | 83%    | 75%    | 100%   | 81%    | 75%    | 77%    | 83%    | 75%    | 0%     |        |        |        |
| AhHS2  | 35%    | 33%    | 35%    | 47%    | 42%    | 42%    | 46%    | 40%    | 42%    | 47%    | 47%    | 42%    | 42%    | 100%   |
| AhHS3  | 30%    | 31%    | 33%    | 43%    | 43%    | 43%    | 43%    | 43%    | 43%    | 43%    | 41%    | 74%    | 100%   |
| AhHS4  | 30%    | 35%    | 34%    | 41%    | 38%    | 40%    | 39%    | 35%    | 43%    | 41%    | 48%    | 40%    | 59%    | 61%    |

Top: identity percentages
Bottom: homology percentages

Table 3: Amino acid identities among the EhHSTFs and related homologues from other organisms.

| EHHS1  | EHHS2  | EHHS3  | HhHS1  | HhHS2  | HhHS4  | GgHS1  | GgHS2  | GgHS3  | MmHS1  | MmHS2  | MmHS4  | AhHS1  | AhHS2  | AhHS3  | AhHS4  |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| 100%   | 0%     |        |        |        |        |        |        |        |        |        |        |        |        |        |        |
| EHHS2  | 7%     | 100%   | 13%    | 0%     |        |        |        |        |        |        |        |        |        |        |        |
| EHHS3  | 8%     | 18%    | 100%   | 25%    | 0%     |        |        |        |        |        |        |        |        |        |        |
| HhHS1  | 7%     | 7%     | 4%     | 100%   | 11%    | 11%    | 8%     | 0%     |        |        |        |        |        |        |        |
| HhHS2  | 6%     | 5%     | 4%     | 26%    | 100%   | 11%    | 11%    | 7%     | 40%    | 0%     |        |        |        |        |        |
| HhHS4  | 7%     | 6%     | 6%     | 36%    | 22%    | 100%   | 7%     | 5%     | 39%    | 22%    | 39%    | 0%     |        |        |        |
| GgHS1  | 7%     | 6%     | 5%     | 28%    | 91%    | 23%    | 26%    | 100%   | 4%     | 44%    | 37%    | 43%    | 0%     |        |        |
| GgHS2  | 10%    | 10%    | 7%     | 44%    | 84%    | 43%    | 43%    | 100%   | 6%     | 44%    | 37%    | 43%    | 0%     |        |        |
| GgHS3  | 10%    | 10%    | 7%     | 44%    | 84%    | 43%    | 43%    | 100%   | 6%     | 44%    | 37%    | 43%    | 0%     |        |        |
| MmHS1  | 14%    | 12%    | 7%     | 30%    | 31%    | 29%    | 31%    | 0%     | 4%     | 49%    | 37%    | 43%    | 0%     |        |        |
| MmHS2  | 11%    | 11%    | 6%     | 32%    | 44%    | 41%    | 80%    | 44%    | 28%    | 0%     |        |        |        |        |        |
| MmHS4  | 5%     | 7%     | 9%     | 29%    | 94%    | 22%    | 28%    | 74%    | 17%    | 23%    | 17%    | 23%    | 17%    | 100%   |
| AhHS1  | 11%    | 11%    | 6%     | 32%    | 44%    | 41%    | 80%    | 44%    | 28%    | 0%     |        |        |        |        |        |
| AhHS2  | 8%     | 8%     | 5%     | 15%    | 13%    | 15%    | 13%    | 13%    | 13%    | 13%    | 13%    | 13%    | 13%    | 13%    | 13%    |
| AhHS3  | 10%    | 10%    | 7%     | 44%    | 37%    | 43%    | 0%     | 4%     | 49%    | 37%    | 43%    | 0%     |        |        |        |
| AhHS4  | 15%    | 15%    | 8%     | 44%    | 37%    | 43%    | 0%     | 4%     | 49%    | 37%    | 43%    | 0%     |        |        |        |

Top: identity percentages
Bottom: homology percentages
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Fig 2: Alignment of the deduced amino acid sequences from the EhHSTFs. Gaps are indicated by dashes. Identical amino acid residues are in black squares. Homologous amino acid residues are in gray squares. Underlined sequence corresponds to the DNA-binding domain. Arrowheads show the amino acid changes in the EhHSTFs DNA-binding domain.

The phylogenetic tree constructed using the alignment of the EhHSTFs and the HSTF from other organisms showed a closer relationship of the EhHSTF2 and EhHSTF3 proteins, while the EhHSTF1 exhibits high similarity with the HSTF1 from A. thaliana (Fig 3) and appears to be derive from the same root than the HSTF1 from human, mouse, chicken, frog and fruit fly (Fig 3). However, we can observed that all the HSTF have a common ancestry may be by these reason all these factors present a high similarity in their DNA-binding domain.

These results suggest that the protozoan parasite E. histolytica presents at least three putative Ehhsf genes which contains a very conserved DNA-binding domain and that they could also bind to the HSEs located in the gene promoters from this parasite.

Isolation of the Ehhsf: To demonstrate the physical presence of the Ehhsf genes in the amoeba, we realized different PCR reactions. Briefly, based on the Ehhsf sequences obtained from the screening in the TIGR database from E. histolytica, we designed specific oligonucleotides to amplify the entire Ehhsf1, Ehhsf2 and Ehhsf3 genes (see Methods). The PCR reactions were performed using the sense and antisense oligonucleotides specific for each gene previously described and total DNA from the trophozoites of E. histolytica as template. As expected a DNA fragments of 510, 837 and 459 bp corresponding to the Ehhsf1, Ehhsf2 and Ehhsf3 genes respectively were successfully amplified. These DNA fragments were cloned, sequenced and found to be identical to the assembled sequences (data not shown).

Detection of homologous HSTF in total extracts from E. histolytica: We carried out Western blot assays using total extracts from E. histolytica and heterologous antibodies against anti-human HSTF1 thus in order to identify if some amoeba proteins are recognized by these heterologous antibodies. The results revealed the presence of four bands of 73, 66, 47 and 23 kDa in the total extracts from the trophozoites growth with out heat shock stress. However when the trophozoites were incubated at 42°C by different times the intensity of the bands change (Fig 4A). The 73, 47 and 23 kDa peptides increased their intensity particularly when the trophozoites were incubated at 42°C by 2 h, whereas the band of 66 kDa was diminishing their intensity according to the increased incubation time. As controls we performed other immunoblottings with the same membrane using other heterologous anti-C/EBP antibodies or the anti-actin from E. histolytica. The anti-C/EBP reveals an expected peptide of 65 kDa (Fig 4B) [22], while the anti-actin antibodies reveals also an expected band of 44 kDa (Fig 4C). The results suggest that in the total extracts from E. histolytica are present HSTFs-like, because the heterologous antibodies against the human HSTF1 recognized four peptides.

Fig 3: Phylogenetic tree of the DNA-binding domains present in the HSTF. DNA-binding domains from E. histolytica and other organisms were aligned using CLUSTALX1.8 program. The EhHSTFs are in bold face. The phylogenetic tree was then inferred by Neighbor-joining analysis (NEIGHBOR program).

Fig 4: EhHSTF-like detected with the anti-humanHSTF1 on an immunoblot. Lanes 1, total extracts from parasites growth with out heat shock stress; lanes 2, 3 and 4, total extracts from parasites growth 30, 60 and 120 min at 42°C respectively. Immunoblots were performed using anti-human HSTF1 (A), anti-human C/EBP (B) and anti-E. histolytica actin (C).
DISCUSSION

The cells possess a conserved response to heat shock stress. This response involves the rapid activation of a HSTF, which regulates the transcription of constitutive and stress-inducible mRNA synthesis of various genes. The HSTF is a central regulator for the expression of different proteins during the heat shock response and to exposition to a variety of chemical and physiological stresses. This protein has been identified in different organisms where the role of some of them has been elucidated [30].

In *E. histolytica* have been described the presence of Hsps [31] and recently have been identified a putative ‘unpublished data’ suggesting that the amoeba presents a HSTF in their genome. To address this possibility, we performed different alignments, firstly to identify a conserved region in the HSTFs described in other organisms, and this region was the DNA-binding domain. Then, we performed a screening in the Genome Project of *E. histolytica* using this domain as probe. Our analysis revealed the presence of three *hstf* genes (*Ehhstf1*, *Ehhstf2*, *Ehhstf3*) in this parasite, as has been documented for human, mouse, chicken and tomato [9-14]. Higher eukaryotic cells possess multiple distinct HSTF isoforms, encoded by different genes. This diversity is further increased through differential splicing, responses to distinct stresses, and preferences for binding to distinct arrangements of HSEs [30]. The existence of multiple HSTF species in any organisms suggest that the HSTF isoforms may have specialized functions that can be triggered by distinct stresses or may activate specific target genes and that the organisms requires of a coordinately co-regulation of different *hsp* genes [1, 3, 32]. Something similar could be occurring in *E. histolytica*, since this parasite is exposed to hard ambient conditions as gastric juices, changes in the temperature, pH and humidity depending of the organ or tissue invaded [33], until the contact with immune system cells and different bacteria species of the intestinal flora. All that situations will be generating the expression or repression of different molecules that permit to the amoeba survive.

On the other hand, the EhHSTFs sequences showed a very conserved DNA-binding domain among the HSTFs cloned from human, mouse, yeasts, frog and plant as *A. thaliana*. However, this domain in the three EhHSTFs is very short (25 aa) in comparison with the observed in the HSTFs of other organisms (∼106 aa).

Additionally, we also observed some differences in this DNA-binding domain in the EhHSTFs. There are six amino acid residues changed, this finding will be implicated in the EhHSTFs affinity, stability and specificity to recognize and bind to the HSEs of different *E. histolytica* promoter genes. Moreover, these punctual differences may allow a wide range of distinct interactions of the DNA-binding domain of the EhHSTFs with the HSEs or other sequences located at the amoeba promoters. In *S. cerevisiae* has been demonstrated that a single amino acid substitution in the DNA-binding domain of yeast HSTF alters the specificity of HSTF on different promoters [34-35].

Despite sequence divergence, that we observed in the alignment of complete amino acid sequences of different HSTFs, all members of the HSTF family have the highly conserved feature: a DNA-binding domain and the EhHSTFs showed these conserved domain. Conservations of homologous HSTF proteins in organisms as diverse as protozoa, plants, chicken, mouse and human suggest that they could play important and similar roles in the biology of eukaryotes. All that thereby influencing the level of transcriptional activation, and ultimately fine-tunes the nature of the heat shock response and other kind of stresses.

On the other hand, we demonstrate that heterologous antibodies against the human HSTF recognized four bands in total extracts from trophozoites growth with out or with termic stress. Although the molecular weight of the peptides recognized do not precisely correspond to the molecular weight of the predicted EhHSTF, in the literature has been reported that these proteins may occur posttranslational modifications as phosphorylation provoking changes in their molecular weights [36]. Perhaps, have been reported that the HSTF requires being homotrimerized to binds to the HSE [37]. In *E. histolytica* we do not know yet if this mechanism also occurs, however under both control and heat shock conditions we observed four proteins, where the 47 and 66 kDa could be homodimers or trimers of these factors.

What is the role of the EhHSTFs? How they control the gene expression in the amoeba? Why this parasite presents three HSTFs? These questions require to be answered.

Finally, *E. histolytica* presents three *Ehhstf* genes, however, their conclusive inclusion as authentic
members would require further functional characterization.

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