Running title: *Entamoeba histolytica* genome

Structure and Content of the *Entamoeba histolytica* Genome

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ABSTRACT

The intestinal parasite *Entamoeba histolytica* is one of the first protists for which a draft genome sequence has been published. Although the genome is still incomplete, it is unlikely that many genes are missing from the list of those already identified. In this review we summarise the features of the genome as they are currently understood and provide previously unpublished analyses of many of the genes.

1. INTRODUCTION

Entamoeba histolytica is one of the most widespread and clinically important parasites, causing both serious intestinal (amoebic colitis) and extraintestinal (amoebic liver abscess) diseases throughout the world. A recent WHO estimate (WHO, 1998) places *E. histolytica* second after *Plasmodium falciparum* as causing the most deaths annually (70,000) among protistan parasites.

Recently a draft of the complete genome of *E. histolytica* was published (Loftus *et al.*, 2005) making it one of the first protist genomes to be sequenced. The *E. histolytica* genome project was initiated in 2000 with funding from the Wellcome Trust and the National Institute of Allergy and Infectious Diseases to the Wellcome Trust Sanger Institute and The Institute for Genomic Research (TIGR) in the UK and the USA, respectively. The publication describing the draft sequence concentrated on the expanded gene families, metabolism and the role of horizontal gene transfer in the evolution of *E. histolytica.* In this review we summarise the structure and content of the *E. histolytica* genome in comparison to other sequenced parasitic eukaryotes, provide a description of the current assembly and annotation, place the inferred gene content in the context of what is known about the biology of the organism, and discuss plans for completing the *E. histolytica* genome project and extending genome sequencing to other species of *Entamoeba.*

1 The fact that the genome sequence is still a draft has several important

2 consequences. The first is that a few genes may be missing from the sequence

3 data we have at present, although the number is likely to be small. For example,

4 at least one gene (amoebapore B) is not present in the genome data despite it

5 having been cloned, sequenced and the protein extensively characterised well

6 before the start of the genome project. The second consequence is that the

7 assembly contains a number of large duplicated regions that may be assembly

8 artifacts, meaning that the number of gene copies is over-estimated in several

9 cases. These problems cannot as yet be resolved but should be eventually as

10 more data becomes available. Nevertheless, it is important to remember these

11 issues when reading the rest of this article.

12

13 As the number of genes in *E. histolytica* runs into several thousands it is not

14 possible to discuss all of them. However, we have generated a number of tables

15 that identify many genes and link them to their entries in GenBank using the

16 relevant protein identifier. Only a few tables are included in the text of this

17 review, but the others are available on line as supplementary material -

18 http://pathema.tigr.org/pathema/entamoeba_resources.shtml. The *E. histolytica*

19 genome project data are being 'curated' at TIGR and it is on that site that the

20 most current version of the assembled genome will be found. The 'Pathema'

21 database will hold the data and the annotation (http://pathema.tigr.org/). The

22 gene tables are also linked to the appropriate entry in the Pathema database and

23 the links will be maintained as the genome structure is refined over time.

24

25 Reference is made throughout the text to other species of *Entamoeba* where

26 data are available. *Entamoeba dispar* is the sister species to *E. histolytica* and

27 infects humans without causing symptoms. *Entamoeba invadens* is a reptilian

28 parasite that causes invasive disease, primarily in snakes and lizards, and is

29 widely used as a model for *E. histolytica* in the study of encystation although

30 the two species are not very closely related (Clark *et al.*, 2006b). Genome

31 projects for both these species are underway at TIGR and it is anticipated that

high quality draft sequences will be produced for both in the near future. It is
hoped that the *E. dispar* sequence will prove useful in identifying genomic
differences linked to disease causation while that of *E. invadens* will be used to
study patterns of gene expression during encystation. Small-scale genome
surveys have been performed for two other species: *E. moshkovskii,* which is
primarily a free-living species although it occasionally infects humans, and *E. terrapinae*, a reptilian commensal species
(http://www.sanger.ac.uk/Projects/Comp_Entamoeba/).

2. GENOME STRUCTURE

2.1 The *E. histolytica* Genome Sequencing, Assembly and Annotation Process

The first choice to be made in the genome project was perhaps the easiest - the
identity of the strain to be used for sequencing. A significant majority of the
existing sequence data prior to the genome project was derived from one strain:
HM-1:IMSS. This culture was established in 1967 from a rectal biopsy of a
Mexican man with amoebic dysentery and axenised shortly thereafter. It has
been used widely for virulence, immunology, cell biology and biochemistry in
addition to genetic studies. In an attempt to minimise the effects of long-term
culture cryopreserved cells that had been frozen in the early 1970s were revived
and this uncloned culture used to generate the DNA for sequencing.

Before undertaking a genome scale analysis it is important to understand the
quality and provenance of the underlying data. The *E. histolytica* genome was
sequenced by whole genome shotgun approach with each center generating
roughly half of the reads. Several different DNA libraries containing inserts of
different sizes were produced using DNA that had been randomly sheared and
sequences were obtained from both ends of each cloned fragment. The Phusion
assembler (Mullikin and Ning, 2003) was used to assemble the 450,000 short
reads into larger contigs (contiguous sequences), resulting in 1819 genome
fragments that were approximately 12X deep, which means that each base has

1 been sequenced 12 times, on average. While the genome shotgun sequence

2 provides high coverage of each base it is inevitable that there will be

3 misassemblies and sequencing errors in the final consensus particularly towards

4 each end of the contigs. Another problem with draft sequence is that it contains

5 gaps, and while most of these will be small and will mostly contain repetitive

6 non-coding "junk" sequence, some of the gaps will probably contain genes.

7 This makes it impossible to be absolutely certain of the absence of particular

8 genes in *E. histolytica* and, in some cases, the presence or absence of particular

9 biological pathways. Due to the high repeat content and low GC content

10 (24.1%) of the *E. histolytica* genome, closure of the remaining gaps is likely to

11 be a lengthy process. Therefore it was decided to undertake and publish an

12 analysis of the genome draft following assembly of the shotgun reads.

13

14 Annotation of the protein coding regions of the genome was initially carried out

15 using two genefinders (GlimmerHMM (Majoros *et al.*, 2004) and Phat (Cawley

16 *et al.*, 2001)) previously used successfully on another low G+C genome, that of

17 *P. falciparum*. The software was re-trained specifically for analysis of the *E.*

18 *histolytica* genome. The training process involved preparing a set of 600

19 manually edited genes to be used as models with the subsequent genefinding

20 then being carried out on all of the assembled contigs to generate a 'complete'

21 gene set. Predicted gene functions were generated automatically by homology

22 searches using public protein and protein-domain databases, with subsequent

23 refinement of identifications being carried out by manual inspection. For

24 particular genes and gene families of special interest, members of the

25 *Entamoeba* scientific community were involved throughout this process as

26 expert curators with each individual assisting in the analysis and annotation of

27 their genes of interest. Therefore although the manual curation of the genome

28 has not been systematic, those areas of biology that are of primary interest to

29 the *Entamoeba* community have been annotated most thoroughly. The

30 publication of the genome by Loftus *et al* therefore represents a "first draft" of

31 the complete genome sequence and the level of annotation is similar to the

1   initial publications of other genomes such as *Drosophila* (Adams *et al.*, 2000;

2   Myers *et al.*, 2000) and human (Lander *et al.*, 2001).

3

4   **2.2 Karyotype and Chromosome Structure.**

5   The current *E. histolytica* genome assembly is approximately 23.7 million

6   basepairs (Mbp) in size (Table 1). This figure is not likely to be a very accurate

7   measure. In part this is due to misassembly of repetitive regions, which will

8   cause the genome to appear smaller, and in part because of the possibility of

9   aneuploidy in some regions of the genome, which would cause them to appear

10   more than once in the assembly. Overall, however, this size is not inconsistent

11   with data from pulse-field gels (Willhoeft and Tannich, 1999) and kinetic

12   experiments (Gelderman *et al.*, 1971a,b) making the *E. histolytica* genome

13   comparable in size (24 Mbp) to that of *Plasmodium falciparum* (23 Mbp)

14   (Gardner *et al.*, 2002)*, Trypanosoma brucei* (26 Mbp) (Berriman *et al.*, 2005),

15   and the free living amoeba *Dictyostelium discoideum* (34 Mbp) (Eichinger *et*

16   *al.*, 2005).

17

18   The current assembly does not represent complete chromosomes. Analysis of

19   pulse-field gels predicts 14 chromosomes ranging in sizes from 0.3 to 2.2 Mb

20   and possibly a ploidy of four (Willhoeft and Tannich, 1999). There is no current

21   information regarding the size and nature of the centromeres and there are no

22   contigs that appear to contain likely centromeric regions based on comparisons

23   with other organisms. A search for signature telomeric repeats within the data

24   indicates that these are either not present in the genome, not present in our

25   contigs, or are diverged enough to be unidentifiable. However, there is

26   circumstantial evidence that the chromosome ends may contain arrays of tRNA

27   genes (see 2.4 below).

28

29   **2.3 Ribosomal RNA Genes**

30   The organisation of the structural RNA genes in *E. histolytica* is unusual with

31   the rRNA genes carried exclusively on 24 kb circular episomes (Bhattacharya

1 *et al.*, 1998) that have two transcription units in an inverted repeat. These

2 episomes are believed to make up about 20% of the total cellular DNA; indeed,

3 roughly 15% of all of the sequencing reads generated in the genome project

4 were derived from this molecule with the exception of certain libraries where

5 attempts were made to exclude it. There are thought to be numerous other

6 circular DNA molecules of varying sizes present with unknown functions (Dhar

7 *et al.*, 1995; Lioutas *et al.*, 1995)but unfortunately they have not yet been

8 identified in the genome shotgun sequence data. The exact reasons for this are

9 unknown but the small size of the DNA may have prevented proper shearing

10 during the library construction process. These molecules represent an intriguing

11 unsolved aspect of the *E. histolytica* genome.

12

13 **2.4 tRNA Genes**

14 Perhaps the most unusual structural feature identified in the *E. histolytica*

15 genome is the unprecedented number and organisation of its tRNA genes (Clark

16 *et al.*, 2006a). Over 10% of the sequence reads contained tRNA genes and these

17 are (with a few exceptions) organised in linear arrays. The array organisation of

18 the tRNAs was immediately obvious in some cases from the presence of more

19 than one repeat unit in individual sequence reads and in other cases from their

20 presence in both reads from the two ends of the same clone. However because

21 of the near complete identity of the array units they were impossible to

22 assemble by the software used and therefore the size of the arrays cannot be

23 estimated accurately.

24

25 By manual assembly of tRNA gene-containing reads, 25 distinct arrays with

26 unit sizes ranging from under 500 bp to over 1750 bp were identified (Clark *et*

27 *al.*, 2006a). The arrayed genes are predicted to be functional because of the 42

28 acceptor types found in arrays none has been found elsewhere in the genome.

29 These array units encoded between one and five tRNAs and a few tRNA genes

30 are found in more than one unit. Three arrays also encode the 5S RNA and one

31 encodes what is thought to be a small nuclear RNA. Experimental quantitative

hybridisations suggest a copy number of between about 70 and 250 for various array units. In total it is estimated that there are about 4500 tRNA genes in the genome. The frequency of a particular tRNA isoacceptor appears to be independent of the codon usage in *E histolytica* protein-coding genes.

Between the genes in the array units are complex, non-coding, short tandem repeats ranging in size from 5 to over 36 bp. Some variation in short tandem repeat number is observed between copies of the same array unit but this variation is usually minor and not visible when inter-tRNA PCR amplification is performed. However, these regions often exhibit substantial variation when different isolates of *E. histolytica* are compared and this is the basis of a recently described genotyping method for this organism (Ali *et al.*, 2005).

There is indirect evidence to suggest that the tRNA arrays are present at the ends of chromosomes. Although allelic *E. histolytica* chromosomes often differ substantially in size in pulse-field gels, a central protein-encoding region appears to be conserved as DNA digested with rare cutting enzymes gives only a single band in Southern blots when most protein-coding genes are used as probes. In contrast, when some tRNA arrays are used as probes on such blots, the same number of bands is seen in digested and undigested DNA. It is therefore tempting to conclude that the tRNA genes are at the ends of the chromosomes and to speculate that these repeat units may perform a structural role. In *D. discoideum* it is thought that rDNA may function as a telomere in some cases (Eichinger *et al.*, 2005) and the tRNA arrays in *E. histolytica* may perform a similar role.

The chromosomal regions flanking the tRNA arrays are generally devoid of protein coding genes but often contain incomplete transposable elements (see next section) and other repetitive sequences (Clark *et al.*, 2006a). This is also consistent with a telomeric location.

1 **2.5 LINEs**

2 The *E. histolytica* genome is littered with transposable elements. There are two

3 major types autonomous LINEs (Long Interspersed Elements) of which there

4 are three subtypes (EhLINE 1, 2 and 3) and there are two types of SINEs (Short

5 Interspersed Elements) (Eh SINE1 and 2) (Table 2a). The classification of these

6 elements and their organisation has been reviewed recently (Bakre *et al.*, 2005).

7 Phylogenetic analysis of the EhLINEs places them in the R4 clade of non- Long

8 Terminal Repeat (LTR) elements, a mixed clade of elements that includes

9 members from nematodes, insects, and vertebrates (Van Dellen *et al.*, 2002a).

10 Analysis of the *E. histolytica* genome shows no evidence for the presence of

11 LTR retrotransposons and very few DNA transposons (of the *Mutator* family)

12 (Pritham *et al.*, 2005).

13

14 All copies of EhLINEs examined encode non-conservative amino acid changes,

15 frame shifts, and/or stop codons and no copy with a continuous open reading

16 frame (ORF) has yet been found. This suggests that the majority of these

17 elements are inactive. However, a large number of EhLINE1 copies do contain

18 long ORFs without mutations in the conserved protein motifs of the RT and EN

19 domains, suggesting that inactivity is quite recent. ESTs corresponding to

20 EhLINEs have been found suggesting that transcription of these elements still

21 occurs. Although most R4 elements insert in a site-specific manner, EhLINEs

22 do not show strict site-specificity and are widely dispersed in the genome. They

23 are quite frequently found close to protein-coding genes and inserted near T-

24 rich stretches (Bakre *et al.*, 2005).

25

26 All three EhLINE subtypes are of approximately equal size ranging from 4715

27 to 4811 bp in length. Individual members within an EhLINE family typically

28 share >85% identity, while between families they are <60% identical. By

29 aligning the available sequences, each EhLINE can be interpreted to encode a

30 single predicted ORF that spans almost the entire element (EhLINE1, 1589 aa;

31 EhLINE2, 1567 aa; EhLINE3, 1587 aa). However, a precise 5bp duplication at

nucleotide position 1442 in about 80% of the copies of EhLINE1 creates a stop

codon, dividing the single ORF in two. Similarly in 92% of EhLINE2 copies,

the single ORF contains a precise deletion of two nucleotides at position 1272,

resulting in two ORFs. Very few intact copies of EhLINE3 are found. The

location of the stop codon leading to two ORFs appears to be conserved since

in both EhLINE1 and EhLINE2 the size of ORF1 is about half that of ORF2

(Bakre *et al.*, 2005). Among the identifiable domains in the predicted proteins

are reverse transcriptase (RT) and a restriction enzyme-like endonuclease (EN).

The putative 5' and 3' untranslated regions are very short (3-44 bp).

EhLINEs 1 and 2 appear to be capable of mobilising partner SINEs (see next

section) for which abundant transcripts have been detected in *E. histolytica*.

Putative LINE/SINE partners can be assigned on the basis of conserved

sequences at the 3' -ends of certain pairs, which otherwise showed no sequence

similarity. The relevance of this assignment for the EhLINE1/SINE1 pair has

recently been demonstrated (Mandal *et al.*, 2004).

2.6 SINEs

The two EhSINEs are clearly related to the EhLINEs as they have a conserved

3' sequence. They are nonautonomous, non-LTR retrotransposons

(nonautonomous SINEs). The genetic elements encoding the abundant

polyadenylated but untranslatable transcripts found in *E. histolytica* cDNA

libraries (initially designated IE elements (Cruz-Reyes and Ackers, 1992;

Cruz-Reyes *et al.*, 1995) or *ehapt2* (Willhoeft *et al.*, 2002)) have now been

designated EhSINE1 (Van Dellen *et al.*, 2002a; Willhoeft *et al.*, 2002). BLAST

searching with representative examples of the first 44 EhSINE1s detected has

identified 90 full-length (= 99% complete) copies and at least a further 120

partial (= 50% of full length) copies in the genome. Length variation is

observed among EhSINE1s and is largely due to variable numbers of internal

26-27 bp repeats (Ackers, unpublished). The majority contain two internal

repeats and cluster closely around 546 bp in length.

A second *E. histolytica* SINE (EhSINE2) has recently been described (Van Dellen *et al.*, 2002a; Willhoeft *et al.*, 2002). Examination of the four published sequences again suggests the presence of variable numbers of short (20 bp) imperfect repeats. BLAST searching identified a total of 47 full-length (= 99%) and at least 60 partial copies in the genome. The 3'-end of EhSINE2 shows high similarity (76%) to the 3' end of EhLINE2.

A polyadenylated transcript designated UEE1 found commonly in cDNA libraries from *E. dispar* (Sharma *et al.*, 1999) is also a non-LTR retrotransposon. A single copy of a UEE1-like element has been identified in the *E. histolytica* genome and is here designated EhSINE3. There is no significant sequence identity between EhSINE3 and EhLINE3 but the 3' end of EhSINE3 is very similar to that of EhLINE1.

Analysis of an *E. histolytica* EST library identified over 500 significant hits to both EhSINE1 and EhSINE2. No convincing transcript from EhSINE3 could be identified although the nearly identical *E. dispar* UEE elements (EdSINE1; Shire and Ackers, submitted) are abundantly transcribed.

A very abundant polyadenylated transcript, *ehapt1*, was described by Willhoeft *et al.* (1999) in a cDNA library. However, only a small number of partial matches could be found in the current *E. histolytica* assembly and only 10-20 strong hits in the much larger *E. histolytica* EST library now available. *ehapt1* does not appear to be a SINE element and its nature is currently unclear. The lack of matches in the genome suggests either that it is encoded in regions missing from the current assembly or that it contains numerous introns.

2.7 Other Repeats

The *E. histolytica* genome contains a number of other repetitive elements whose functions are not always clear. There are over 75 genes encoding

leucine-rich tandem repeats (LRR) of the type found in BspA-like proteins of

the *Treponema pallidum* LRR (TpLRR) subfamily, which has a consensus

sequence of LxxIxIxxVxxIgxxAFxxCxx (Davis *et al.*, 2006). These proteins

generally have a surface location and may be involved in cell-cell interaction.

Genes encoding such proteins are found mainly in Bacteria and some Archaea;

so far they have been identified in only one other eukaryote, *Trichomonas*

vaginalis (Hirt *et al.*, 2002). An extensive description of the BspA-like proteins

of E. histolytica has recently been published (Davis *et al.*, 2006) and one of

them has been shown to be surface exposed (Davis *et al.*, 2006).

E. histolytica stress sensitive protein (Ehssp) 1 is a dispersed, polymorphic and

multicopy gene family (Satish *et al.*, 2003) and is present in ca. 300 copies per

haploid genome as determined by hybridisation (Table 2a). The average Ehssp1

ORF is 1 kb in length with a centrally-located acidic-basic region (ABR) that is

highly polymorphic. Unlike other such domains no clear repetitive motifs are

present. The protein has, on average, 21% acidic (aspartate and glutamate) and

17% basic (arginine and lysine) amino acids, most of which are located in the

ABR. The ABR varies in size from 5 to 104 amino acids among the various

copies. No size polymorphism is seen outside the central ABR domain. The

genes have an unusually long 5' untranslated region (UTR; 280 nucleotides).

Only one or a few copies of the gene are transcribed during normal growth, but

many are turned on under stress conditions. Homologues of this gene are

present in *E. dispar*, but there is very little size polymorphism in the *E. dispar*

gene family.

Eukaryotic genomes usually contain numerous microsatellite loci with repeat

sizes of 2-3 basepairs. With the exception of di- and tri-nucleotides made up

entirely of A+T such sequences are rare in the *E. histolytica* genome. In

contrast, two dispersed repeated sequences of unknown function occur far more

frequently than would be expected at random. Family 16 has a 42 base

consensus sequence and occurs approximately 38 times in the genome while

1 family 17 has a 27 base consensus sequence and occurs 35 time in the genome

2 (Table 2b). The significance of these sequences remains to be determined.

3

4 **2.8 Gene Number**

5 The current assembly predicts that the genome contains around 10,000 genes,

6 almost twice as many as seen in *P. falciparum* (Gardner *et al.*, 2002) or

7 *Saccharomyces cerevisiae* (Goffeau *et al.*, 1996) but closer to that of the free

8 living protist *Dictyostelium discoideum* (ca. 12,500; Eichinger *et al.*, 2005). It

9 should be remembered that this number will change as the assembly improves,

10 and is likely to decrease somewhat. Nevertheless, the comparatively large gene

11 number when compared to some other parasitic organisms reflects both the

12 relative complexity of *E. histolytica* and the presence of large gene families,

13 despite the loss of certain genes as a consequence of parasitism. Gene loss and

14 gain can both represent an adaptive response to life in the human host. Gene

15 loss is most evident in the reconstruction of metabolic pathways of *E.*

16 *histolytica*, which show a consistent pattern of loss of synthetic capacity as a

17 consequence of life in an environment rich in complex nutrient sources.

18 Similarly, analyses of expanded gene families with identifiable functions

19 indicate that many are directly associated with the ability to sense and adapt to

20 the environment within the human host and the ability to ingest and assimilate

21 the nutrients present. One consequence of these gene family expansions being

22 linked to phagocytosis of bacteria and other cells may be an association

23 between many of these gene families and pathogenicity.

24

25 **2.9 Gene Structure**

26 Most *E. histolytica* genes comprise only a single exon; however as many as

27 25% may be spliced and 6% contain two or more introns. Therefore mRNA

28 splicing is far less common than in the related protist *D. discoideum* or the

29 malaria parasite *P. falciparum*. The genome contains all of the essential

30 machinery for splicing (section 2.14) and a comparison of intron positions

31 suggests that *D. discoideum* and *E. histolytica* have both lost introns since their

shared common ancestor with *P. falciparum,* although many more have been lost in the *E. histolytica* lineage. A good example of this intron loss is the vacuolar ATP synthase subunit D gene (Figure 1). This protein is highly conserved but the number of introns in each gene varies. *P. falciparum* has 5 introns, *D. discoideum* has two and *E. histolytica* has one. The positions of three of the five *P. falciparum* introns are conserved in one of the other species which suggests that these three (at least) were present in the common ancestor and that intron loss has led to the lower number seen in *E. histolytica* today. This loss is consistent with reverse transcriptase mediated 3' intron loss (Roy and Gilbert, 2005) as the 5'-most introns are retained. It would appear that this process has been more active in the *E. histolytica* and *D. discoideum* lineages than in *P. falciparum*, possibly because *Plasmodium* lacks a reverse transcriptase.

2.10 Gene Size

Genes in *E. histolytica* are surprisingly short, not only due to the loss of introns but also in the predicted lengths of the proteins they code for. On average the predicted length of a protein in *E. histolytica* is 389 amino acids (aa) which is 129 aa and 372 aa shorter than in *D. discoideum* and *P. falciparum* respectively. In fact the protein length distribution is most similar to that of the microsporidian *Encephalitozoon cuniculi* (Figure 2) which has a very compact genome of 3Mb and less than 2000 genes. Direct comparison of orthologous genes between *E. histolytica* and its closest sequenced relative *D. discoideum* demonstrates this phenomenon quite well, with the majority of *E. histolytica* proteins being shorter than the *D. discoideum* counterpart (Hall, unpublished). Protein length is normally very well conserved among eukaryotes so the reason for protein shortening is unclear. It has been postulated that in bacteria reduced protein lengths reflects a reduced capacity for signaling (Zhang, 2000). This would not seem to be the case here as the number of genes identified as having a role in signaling suggests quite the opposite. An alternative theory is that as *E.*

1   *histolytica* has reduced organelles it is possible that its proteins contain fewer or

2   simpler targeting signals.

3

2.11 Protein Domain Content

5   The most common protein family (Pfam) domains of *E. histolytica* are shown in

6   Table 3. The domains that are unusually common in *E. histolytica* reflect some

7   of the more unusual aspects of the biology of this protist. For example, the Rab

8   and Rho families that are involved in signaling and vesicle trafficking are

9   among the most common domains in *E. histolytica* while in other species they

10   are not often among the top 50 families. This could well be due to the fact that

11   *E. histolytica* has a 'predatory' life style and these domains are intimately

12   involved in environmental sensing, endocytosis and delivery of lysosomes to

13   the phagosome. There are also a number of domains involved in actin

14   dynamics and cytoskeletal rearrangement that are not common in non-

15   phagocytic species, such as the gelsolin and SH3 domains. Myb domains are

16   the most common transcription regulatory domains in *E. histolytica*; this

17   domain is also common in plants where the proteins regulate many plant-

18   specific pathways (Ito, 2005). An important finding from an initial analysis

19   was the presence of unusual multidomain proteins, including five proteins

20   containing both RhoGEF and Arf-GAP domains, suggesting a mechanism for

21   direct communication between the regulators of vesicle budding and

22   cytoskeletal rearrangement. Over 80 receptor kinases were identified (section

23   7.2.2), each containing a kinase domain and a C rich extracellular domain.

24   These kinases fall into distinct classes depending on the presence of CXC or

25   CXXC repeats. There are also domains that are common in most other

26   sequenced genomes but rare or missing from *E. histolytica*. For example, most

27   mitochondrial carrier domain proteins are not needed in *E. histolytica* as it lacks

28   a normal mitochondrion (section 8).

29

2.12 Translation-Related Proteins

Two of the predicted tRNAs (Ile^{TAT} and Tyr) need to be spliced due to the presence of an intron. tRNA introns are distinct in structure from those in protein-coding genes and require a distinct splicing machinery. The expected enzymes required for this splicing are present as are a number of tRNA modification enzymes (including those for sythesising queuine and pseudouridine) and rRNA methylases that act on specific bases in their respective RNA molecules. The expected panel of tRNA synthetases necessary for aminoacylating the tRNAs is also present, with one or two gene copies for each type.

The majority of ribosomal protein genes are well-conserved in *E. histolytica* and only the gene for large subunit protein L41 could not be identified. The missing protein is only 25 amino acids in length, 17 of which are arginines or lysines, which would make it difficult to identify in this A+T-rich genome, but it is highly conserved, having been reported from Archaea to mammals. However, it also appears to be dispensable, as *S. cerevisiae* can grow relatively normally after deletion of both its copies (Yu and Warner, 2001). Nevertheless, deletion of L41 in *S. cerevisiae* reduces the level of 80S ribosomes, suggesting that it is involved in ribosomal subunit association, reduces peptidyl transferase activity, and increases translocation (Dresios *et al.*, 2003). In addition, L41 has been shown to interact with the beta subunit of protein kinase CKII and to stimulate phosphorylation of DNA topoisomerase II alpha by CKII (Lee *et al.*, 1997b). If this gene is truly absent from *E. histolytica* it may have important consequences for the cell.

No genes for mitochondrial ribosomal proteins were found. Their absence is not surprising since *E. histolytica* lacks typical mitochondria (see section 8 below).

In eukaryotic translation, elongation factor EF-1 is activated upon GTP binding and forms a ternary complex with aminoacyl tRNAs and ribosomes. EF-1 beta and delta subunits work as GDP-GTP exchange factors to cycle EF-1 alpha

between two forms while EF-1 gamma provides structural support for the formation of this multimeric complex. EF2 assists in the translocation of tRNAs on the mRNA by exactly one codon. *E. histolytica* has most of the expected factors except for EF-1 delta, a protein involved in exchanging GDP with GTP. This is also absent from *S. cerevisiae* and *P. falciparum*. It is likely that EF-1 beta carries out this activity. It is thought that the EF-1 complex can exist in two forms, EF-1-alpha/beta/gamma and EF-1-alpha/delta/gamma. In *E. histolytica*, probably only the former complex exists.

Eukaryotes typically have two polypeptide release factors, eRF1 and eRF3. Both of these factors have been found in *E. histolytica*.

2.13 Analysis of Cell Cycle Genes

Alternation of DNA duplication and chromosome segregation is a hallmark in the cell cycle of most eukaryotes. Carefully orchestrated processes coordinate an ensemble of cell cycle regulating 'checkpoint' proteins ensure that progeny cells receive an exact copy of the parental genetic material (Hartwell and Weinert, 1989). Unlike most eukaryotes, *Entamoeba histolytica* cells can reduplicate their genome several times before cell division occurs (Gangopadhyay *et al.*, 1997). Approximately 5-20% of the trophozoites (depending on the growth phase) of axenic culture are multi-nucleated. Additionally, DNA reduplication may occur without nuclear division so that single nuclei contain 1X -6X or more genome contents (Das and Lohia, 2002). Thus axenically cultured *E. histolytica* trophozoites display heterogeneity in their genome content suggesting that eukaryotic cell cycle checkpoints are either absent or altered in this organism. Around 200 genes have been identified in yeast that play a direct role in cell cycle progression.

2.13.1 DNA replication initiation and DNA duplication

The DNA replication licensing system is one of the crucial mechanisms that ensures the alternation of S-phase with mitosis in most cells (Tye, 1999).

1   Initiation of DNA replication involves binding of the replicative helicases to

2   DNA replication origins in late mitosis. Loading of the replicative helicase

3   Mcm2-7 proteins is preceded by formation of the pre-replicative complex (pre-

4   RC) and its subsequent activation. Formation of pre-RC requires the ordered

5   assembly of the origin recognition complex (ORC), Cdc6, Cdt1 and the Mcm2–

6   7 proteins. The pre-RC is activated by the protein kinase Cdc7p and its

7   regulatory subunit Dbf4 (Masai and Arai, 2002). Other factors that regulate the

8   transition from pre-RC to replication initiation are Mcm10p, Cdc45p, TopBP1,

9   RecQL4 and the GINS complex (Gregan *et al.*, 2003; Machida *et al.*, 2005;

10   Merchant *et al.*, 1997; Wohlschlegel *et al.*, 2002). Two other Mcm proteins –

11   Mcm8 and Mcm9 - have been identified in metazoan systems and are believed

12   to be part of the replicative helicase (Maiorano *et al.*, 2006). Replication origin

13   licensing is inactivated during S-phase but Mcm2–9p may function as a

14   helicase that unwinds DNA ahead of the replication fork during S-phase

15   (Maiorano *et al.*, 2006). Once S-phase has begun, the formation of new pre-RC

16   is kept in check by high CDK activity and by the activity of the protein geminin

17   (Bell and Dutta, 2002).

18

19   A detailed analysis of the *E. histolytica* genome shows that homologues of

20   several proteins required for DNA replication initiation are absent. These

21   include ORC (Origin Recognition Complex) 2-6, Cdt1, geminin, Cdc7/Dbf4

22   and Mcm10. A single gene encoding a homologue of the archaeal and human

23   Cdc6/Orc1p (Capaldi and Berger, 2004) was identified. This suggests that DNA

24   replication initiation in *E.histolytica* is likely similar to archaeal replication

25   initiation where a single Cdc6p/ORC1p replaces the hetero-hexameric ORC

26   complex (Kelman and Kelman, 2004). Several proteins described from

27   metazoa, such as Cdt1, geminin, Mcm8 and Mcm9, have not been found in

28   yeast. Surprisingly, Mcm8 and Mcm9 were identified in the *E.histolytica*

29   genome.

30

Of the four known checkpoint genes that regulate DNA replication in *S. cerevisiae* only Mec1 and Mrc1 have homologues in *E. histolytica*. *E. histolytica* homologues of several proteins involved in G1-S transitions are absent, such as Sic1, Chk1. The S-phase checkpoint genes p21, p27, p53 and retinoblastoma (RB) required for transition from G1 to S-phase in humans were absent in *E. histolytica*. Chk1 and Chk2 genes encode kinases that act downstream from the ATM and ATR kinases (intra-S phase checkpoint genes). The Chk1 homologue is absent but a Chk2 homologue has been identified in *E. histolytica* and partially characterised (Iwashita *et al.*, 2005).

2.13.2 Chromosome segregation and cell division

A large number of genes are known to regulate different events during the transition from G2-Mitosis - spindle formation checkpoint, chromosome segregation, mitosis, exit from mitosis, and cytokinesis - in *S.cerevisiae*. Many of the proteins required by yeast for kinetochore formation have no obvious homologues in *E. histolytica* suggesting that amoeba kinetochores may have an altered composition and structure. Proteins of the Anaphase Promoting Complex (APC) regulate transition from metaphase to anaphase. With the exception of APC11, none of the APC proteins could be identified in *E.histolytica*. In contrast two genes encoding CDC20 homologues, which are known to activate the APC complex, were identified in *E.histolytica* along with ubiquitin and related proteins (Wöstmann *et al.*, 1992), indicating that although most APC subunit homologues were absent the pathway of proteasomal degradation for regulation of cell cycle proteins may still be functional in *E.histolytica*. Effectors of the apoptotic pathway and meiosis were also largely absent.

2.13.3 CDKs and cyclins

The CDC28 gene encodes the single cyclin dependant kinase (CDK) in *S. cerevisiae* and regulates cell cycle progression by binding to different cyclins at the G1/S or G2/M boundaries (Reed, 1992; Surana *et al.*, 1991; Wittenberg *et*

1   *al.*, 1990). Similarly, *Schizosaccharomyces pombe* also encodes a single CDK

2   (*cdc2*) (Simanis and Nurse, 1986) . Mammals and plants can encode multiple

3   CDKs and an equally large number of cyclins (Morgan, 1995; Vandepoele *et*

4   *al.*, 2002). Association of different CDKs with specific cyclins regulates the

5   cell cycle in different developmental stages as well as in specific tissues. CDKs

6   belong to the serine/threonine family of kinases with a conserved PSTAIRE

7   domain where cyclins are believed to bind (Jeffrey *et al.*, 1995; Morgan, 1996)

8   although some mammalian and plant CDKs have been shown to have divergent

9   PSTAIRE motifs. This heterogeneity may or may not affect cyclin binding

10   (Poon *et al.*, 1997). The *E. histolytica* genome encodes at least 9 different

11   CDKs among which not even one has the conserved PSTAIRE motif. The

12   closest homologue of the CDC28/cdc2 gene, which shows only conservative

13   substitutions in the PSTAIRE motif (PVSTVRE), was cloned previously (Lohia

14   and Samuelson, 1993). The remaining 8 CDK homologues exhibit even greater

15   divergence in this motif. Eleven putative cyclin homologues with a high degree

16   of divergence have been found. Identifying their CDK/cyclin partner along with

17   their roles in the cell cycle is a major task that lies ahead. Some of the CDKs

18   may not function by associating with their functional cyclin partners but may

19   play a role in regulating global gene expression, either by activation from non-

20   cyclin proteins or by other mechanisms (Nebreda, 2006).

21

22   *E. histolytica* presents a novel situation where the eukaryotic paradigm of a

23   strictly alternating S-phase and mitosis is absent. Discrete G1, S and G2

24   populations of cells are not routinely found in axenic cultures. Instead cells in

25   S-phase show greater than 2x genome contents, suggesting that the G2 phase is

26   extremely short and irregular. This observation together with the absence of a

27   large number of checkpoint genes suggests that regulation of genome

28   partitioning and cell division in *E. histolytica* may be additionally dependant on

29   extracellular signals. *E. histolytica* must however contain regulatory

30   mechanisms to ensure that its genome is maintained and transmitted with

31   precision even in the absence of the expected checkpoint controls. The

1 discovery of these mechanisms will be crucial to our understanding of how the

2 *E. histolytica* cell divides.

3

2.14 Transcription

5 RNA polymerase II transcription in *E. histolytica* is known to be α-amanitin-

6 resistant (Lioutas and Tannich, 1995). The F homology block of the RNA

7 polymerase II large subunit has been identified as the putative α-amanitin

8 binding site. This block is highly divergent in the α-amanitin resistant

9 *Trichomonas vaginalis* RNA polymerase II (Quon *et al.*, 1996). The *E.*

10 *histolytica* RPB1 homologue also diverges from the consensus in this region

11 but, interestingly, it is also quite dissimilar to the *T. vaginalis* sequence.

12

13 The heptapeptide repeat (TSPTSPS) common to other eukaryotic RNA

14 polymerase II large subunit C terminal domains (CTD) is not present in the *E.*

15 *histolytica* protein. Indeed, the *E. histolytica* CTD is not similar to any other

16 RNA polymerase II domain in the current database. However, the CTD of the

17 *E. histolytica* enzyme does remain proline/serine-rich (these amino acids

18 constitute 40% of the CTD sequence). The *E. histolytica* CTD also retains the

19 potential to be highly phosphorylated: of the 24 serines, 6 threonines and 3

20 tyrosines within the CTD, 9 serines, 3 threonines and 1 tyrosine are predicted to

21 be within potential phosphorylation sites. It is therefore possible that, despite

22 its divergence, modification of the CTD by kinases and phosphatases could

23 modulate protein-protein interactions as is postulated to occur in other RNA

24 polymerases (Yeo *et al.*, 2003). In yeast, phosphorylation of the CTD regulates

25 association with the mediator protein (Davis *et al.*, 2002; Kang *et al.*, 2001;

26 Kornberg, 2001). The yeast mediator protein complex consists of 20 subunits.

27 However, perhaps due to the divergence of the CTD, only two of these proteins

28 have been identified in *E. histolytica* (Med7 and Med10). Homologues of the

29 Spt4 and Spt5 elongation factors, also thought to interact with the CTD, have

30 been identified.

31

1 The RNA polymerase core is composed of 12 putative subunits in *S. cerevisiae*

2 (Young, 1991), while *S. pombe* contains a subset of 10 of these proteins,

3 lacking the equivalents of subunits 4 and 9 (Yasui *et al.*, 1998). In *E.*

4 *histolytica* only 10 of the RNA polymerase subunits have been identified,

5 identifiable homologues of subunits 4 and 12 being absent. While the

6 homologue to subunit 9 was present it lacks the first of the two characteristic

7 zinc binding motifs of this protein and the DPTLPR motif in the C terminal

8 region. A similar sequence, DPTYPK, is however present and a homologue of

9 the TFIIE large subunit Tfa1, which is proposed to interact with this region of

10 the protein, has been identified (Hemming and Edwards, 2000; Van Mullem *et*

11 *al.*, 2002). The conserved N terminal portion (residues 1-52) of Rpb9 is

12 thought to interact with both Rpb1 and Rpb2 in *S. cerevisiae* (Hemming and

13 Edwards, 2000) and homologues of these have been identified.

14

15 The core promoter of *E. histolytica* has an unusual tripartite structure consisting

16 of the three conserved elements TATA, GAAC and INR (Purdy *et al.*, 1996;

17 Singh and Rogers, 1998; Singh *et al.*, 2002; Singh *et al.*, 1997). Singh and

18 Rogers (1998) have speculated that the GAAC motif may be the binding site of

19 a second or alternative *E. histolytica* DNA binding protein in the preinitiation

20 complex. It is therefore of interest that, in addition to the *E. histolytica* TATA-

21 binding protein (TBP), two other proteins contain the TATA -binding motif

22 (Hernandez *et al.*, 1997). TBP is a subunit of the TFIID general transcription

23 factor (GTF) which in other organisms is required for the recognition of the

24 core promoter. In light of the variation in the core promoter previously

25 mentioned, and the divergence in proteins that bind to the core promoter in

26 other parasitic protists, it is not surprising that only six of the 14 evolutionary

27 conserved subunits of TFIID, TBP Associated Factors (TAFs) 1, 5, 6, 10, 12

28 and 13 were identified. Homologues of some of the global regulatory subunits

29 of the Ccr4/Not complex, which interacts with TBP and TAFs 1 and 13, have

30 also been identified.

31

1 TAFs 5, 6, 10 and 12 are also components of the histone acetyltransferase

2 (HAT) complexes in other organisms as is SPT6 and 16 (Carrozza *et al.*, 2003).

3 While all known components of the HAT complexes have by no means been

4 identified or the role of the previously unknown bromodomain containing

5 proteins encoded in the *E. histolytica* genome, histone acetylation complexes

6 are known to be active in *E. histolytica* (Ramakrishnan *et al.*, 2004). Other

7 potential members of chromatin remodeling complexes of *E. histolytica* include

8 the TBP interacting helicase (RVB1 & 2) and the SNF2 subunit of the

9 SWI/SNF complex.

10

11 Homologues of some of the other GTFs (TFII E, F and H) but not the large or

12 small subunits were identified. In contrast to the difficulty identifying some of

13 the GTFs, the *E. histolytica* spliceosomes components U1, U2, U4/6, U5 and

14 the Prp19 complex have all been identified. In fact homologues of ten of the

15 fourteen "core" snRNP proteins, two of the U1 specific snRNPs, seven of the

16 ten U2 specific snRNPs, five of the six U5 specific snRNPs, three of the U4/6

17 specific snRNPs, and four of the nine subunits of the Prp19 complex have been

18 found. In fact *E. histolytica* has homologues of approximately 80% of the *S.*

19 *cerevisiae* splicing machinery (Jurica and Moore, 2003).

20

21 Like *G. intestinalis, E. histolytica* has short 5' untranslated regions on its

22 mRNAs. However, unlike those of *G. intestinalis, E. histolytica* mRNA has

23 been shown to be capped (Ramos *et al.*, 1997; Vanacová *et al.*, 2003).

24 Identification of homologues of the Ceg1 RNA guanylyltransferase - an

25 enzyme which adds an unmethylated GpppRNA cap to new transcripts - and of

26 Abd1 - which methylates the cap to form m7GpppRNA - gives new insight into

27 the probable cap structure in *E. histolytica* (Hausmann *et al.*, 2001; Pillutla *et*

28 *al.*, 1998).   It has been proposed that the capping enzymes interact with the

29 phosphorylated CTD of RNA polymerase (Schroeder *et al.*, 2000). The CTD of

30 *E. histolytica* large subunit is, as discussed earlier, not well conserved but

31 contains several probable phosphorylation sites.

1

2   mRNAs in *E. histolytica* are polyadenylated and the polyadenylation signal is

3   found within the short 3' untranslated region (Bruchhaus *et al.*, 1993; Li *et al.*,

4   2001). However only eight of the eighteen yeast Cleavage and Polyadenylation

5   Specificity Factor (CPSF) subunits are identifiable in *E. histolytica*.

6

7 **3. VIRULENCE FACTORS**

8 **3.1 Gal/GalNAc Lectin**

9   One of the hallmarks of *E. histolytica* pathogenicity is contact-dependent killing

10   of host cells. *E. histolytica* is capable of killing a variety of cells types including

11   human intestinal epithelium, erythrocytes, neutrophils, and lymphocytes

12   (Burchard and Bilke, 1992; Burchard *et al.*, 1992a; Burchard *et al.*, 1992b;

13   Guerrant *et al.*, 1981; Ravdin and Guerrant, 1981). Cytolysis occurs as a step-

14   wise process that begins with adherence to target cells via galactose/N-acetyl

15   D-galactosamine-inhibitable (Gal/GalNAc) lectin (Petri *et al.*, 1987; Ravdin

16   and Guerrant, 1982). Adherence via the Gal/GalNAc lectin is a requirement for

17   cell killing because in the presence of galactose or GalNAc targets cells are not

18   killed by the amoebae. Target cell death occurs within 5 to 15 minutes and is

19   often followed by phagocytosis. Inhibition of the Gal/GalNAc lectin with

20   galactose or specific antibody also blocks phagocytosis (Bailey *et al.*, 1990).

21   Resistance to lysis by the complement system is also mediated in part by the

22   Gal/GalNAc lectin. The lectin contains a CD59-like domain that likely helps

23   protect the trophozoites from complement; CD59 is a surface antigen of many

24   blood cells known to have this property (Braga *et al.*, 1992).

25

26   The Gal/GalNAc lectin is a membrane complex that includes heavy (Hgl) 170

27   kDa, and light (Lgl) 30-35 kDa subunits linked by disulphide bonds, and a non-

28   covalently associated intermediate (Igl) 150 kDa subunit (Cheng *et al.*, 2001;

29   Petri *et al.*, 1989). The structure and function of the Gal/GalNAc lectin has

30   recently been reviewed (Petri *et al.*, 2002). The heavy subunit is a type 1

31   transmembrane protein while the light and intermediate subunits have

1 glycosylphosphatidylinositol (GPI) anchors (Cheng *et al.*, 2001; McCoy *et al.*,

2 1993). Gal/GalNAc lectin subunits do not share any significant protein identity

3 or similarity to any other known proteins, though Hgl and Igl have some very

4 limited regions of similarity with known classes of proteins that will be

5 discussed below.

6

7 *3.1.1 The heavy (Hgl) subunit*

8 Based on pulse-field gel electrophoresis there are five loci in the genome with

9 similarity to the Hgl subunit. However, the current genome assembly only

10 identifies two complete genes, one of which corresponds to Hgl2 (Tannich *et*

11 *al.*, 1991b). The predicted proteins encoded by these loci are 92% identical. In

12 initial assemblies there were three other sequences with high similarity to the

13 Hgl subunit that were pseudogenes. These pseudogenes may account for the

14 additional loci detected by pulse-field gel electrophoresis. The large size of

15 these genes means that assembly problems may also be affecting our

16 interpretation.

17

18 Hgl subunit sequences can be divided in to domains based on amino acid

19 content and distribution (Figure 3). The amino-terminal domain of ca. 200

20 amino acids consist of 3.2% cysteine and 2.1% tryptophan residues. The next

21 domain, also ca. 200 amino acids, is completely devoid of these two amino

22 acids. The C-terminal domain of ca. 930 amino acids is cysteine-rich,

23 comprising 10.8 % cysteine. The number and spacing of all predicted

24 tryptophan and cysteine residues are 100% conserved in the two complete

25 genes. Although a portion of the C-terminal domain can be said to contain

26 cysteine-rich pseudo- repeats, there is no clear repetitive nature to the protein

27 (Tannich *et al.*, 1991b). The Hgl subunit has a single transmembrane domain

28 and a highly conserved 41 amino acid cytoplasmic domain. In addition to these

29 two *hgl* genes, the genome contains a newly identified divergent member of the

30 Hgl gene family (XP_650534). This ORF shares 43% similarity with the two

1   other Hgl isoforms, and is predicted to encode a protein with an almost

2   identical domain structure to that of Hgl described above.

3

4

5   *3.1.2 The light (Lgl) subunit*

6   The Lgl subunit is encoded by five genes (*lgl1-5*) that share 74-85% amino acid

7   identity. A sequence corresponding to Lgl2 is missing from the current genome

8   assembly. The light subunits range from 270 to 294 amino acids in length. Each

9   isoform has a 12 amino acid signal peptide, 5 conserved cysteine residues, and

10   a GPI-anchor addition site. Lgl1 has two potential glycosylation sites. Lgl2 has

11   one of these sites, Lgl3 has one different site, and Lgl4 and Lgl5 have none.

12

13   *3.1.3 The intermediate (Igl) subunit*

14   The Igl subunit was first identified by a monoclonal antibody that blocked

15   amoebic adherence to and cytotoxicity for mammalian cells (Cheng *et al.*,

16   1998). Co-purification of the Hgl, Lgl, and Igl suggests that these three subunits

17   form a complex (Cheng *et al.*, 1998; Cheng *et al.*, 2001). The Igl subunit also

18   has galactose-binding activity (Cheng *et al.*, 1998) and can serve as protective

19   antigen in vaccine trials (Cheng and Tachibana, 2001). There are two loci that

20   encode Igl subunits (Cheng *et al.*, 2001) and the predicted amino acid

21   sequences are 81% identical. The Igl subunit, like the Hgl subunit, does not

22   have any recognisable carbohydrate-binding domain.

23

24   *3.1.4 Conservation of Gal/GalNAc lectin subunits in other species of*

25   Entamoeba

26   There are clearly identifiable orthologues of the Hgl and Lgl subunits among

27   the limited sequences of *E. dispar, E. invadens, E. moshkovskii,* and *E.*

28   *terrapinae* available at present (Dodson *et al.*, 1997; Pillai *et al.*, 1997; Wang *et*

29   *al.*, 2003). Because these genomes are incomplete it is possible that as yet

30   unidentified family members will show greater similarity to the *E. histolytica*

31   sequences. Nevertheless, the Lgl subunit is quite conserved among the five

1   *Entamoeba* species. For instance, the *E. terrapinae* gene is 56% identical and

2   62 % similar to *E. histolytica* Lgl1 over a span of 201 amino acids. The Hgl

3   subunits are more diverse. The *E. dispar* Hgl orthologue is highly similar to the

4   *E. histolytica* subunit (86%) but the other species show more diversity,

5   including the region that corresponds to the CRD. However, the number and

6   positions of the cysteine residues are highly conserved, as is the sequence of the

7   cytoplasmic domain, showing only a few changes. It is difficult to put precise

8   numbers to these similarities because the complete sequences of Hgl subunits

9   from the other species are not present in the database. The character of the

10   conservation of the Hgl subunits suggests that the ligand specificity is different

11   for the Hgl subunits of each species but the signaling functions of the

12   cytoplasmic domains are similar, if not perhaps identical. Only *E. dispar* has an

13   identifiable Igl subunit.   The other three species clearly have paralogues of the

14   CXXC repeat family to which Igl belongs, but their similarity to Igl is mostly

15   restricted to the CXXC and CXC repeat motifs.

16

3.2 Cysteine endopeptidases

18   *Entamoeba histolytica* is characterised by its extraordinary capacity to invade

19   and destroy human tissues. The main lytic activity has been attributed to

20   cysteine endopeptidases. This class of enzymes, which is found in all

21   organisms, plays a major role in the pathogenicity of *E. histolytica* as

22   demonstrated in a large number of in vitro and in vivo studies (Ankri *et al.*,

23   1999; Gadasi and Kessler, 1983; Keene *et al.*, 1990; Li *et al.*, 1995; Luaces and

24   Barrett, 1988; Lushbaugh *et al.*, 1985; Reed *et al.*, 1989; Schulte and Scholze,

25   1989; Stanley *et al.*, 1995). Most striking are results from laboratory animal

26   infections showing that *E. histolytica* trophozoites with reduced cysteine

27   proteinase activity are greatly impaired in their ability to induce amoebic

28   disease (Ankri *et al.*, 1999; Stanley *et al.*, 1995). In addition, the discovery that

29   *E. histolytica* cysteine proteinases possess interleukin-1β convertase activity

30   suggests that these enzymes use a mechanism that is novel in microbial

31   pathogenicity (Zhang *et al.*, 2000).

1

2 Thiol-dependent proteolytic activity in *E. histolytica* was first attributed to a

3 neutral sulphydryl proteinase (McLaughlin and Faubert, 1977) and later to a

4 cytotoxic proteinase (Lushbaugh *et al.*, 1984). Other terms that have been used

5 to describe closely related or identical enzymes are cathepsin B (Lushbaugh *et*

6 *al.*, 1985), neutral proteinase (Keene *et al.*, 1990), histolysin (Luaces and

7 Barrett, 1988) (later changed to histolysain; Luaces *et al.*, 1992)), and

8 amoebapain (Scholze *et al.*, 1992). *E. histolytica* cysteine endopeptidases were

9 found to be secreted (Leippe *et al.*, 1995) and localised in lysosome-like

10 vesicles or at the surface of the cell (Garcia-Rivera *et al.*, 1999; Jacobs *et al.*,

11 1998). Molecular cloning has revealed a large number of cysteine

12 endopeptidase genes in the *E. histolytica* genome (Bruchhaus *et al.*, 2003;

13 Garcia-Rivera *et al.*, 1999; Reed *et al.*, 1993; Tannich *et al.*, 1991c; Tannich *et*

14 *al.*, 1992). Interestingly, most of these genes are not expressed during in vitro

15 cultivation (Bruchhaus *et al.*, 2003). As our current knowledge of *E. histolytica*

16 biology and pathogenicity is mostly based on analysis of cultured cells, the

17 function of most of the cysteine endopeptidases and their precise role in *E.*

18 *histolytica* virulence is largely unknown.

19

20 Homology searches using conserved active site regions revealed that the *E.*

21 *histolytica* genome contains at least 44 genes coding for cysteine

22 endopeptidases. Of these, the largest group is structurally related to the C1

23 papain superfamily (Table 4), whereas a few others are more similar to family

24 C2 (calpain-like cysteine proteinases), C19 (ubiquitinyl hydrolase), C54

25 (autophagin), and C65 (otubain), respectively (Table 5).

26

27 Phylogenetic analyses of the 36 C1-family members revealed that they

28 represent 3 distinct clades (A, B, C), consisting of 12, 11 and 13 members,

29 respectively. Clade A and B members correspond to the two previously

30 described subfamilies of *E. histolytica* cysteine proteinases, designated EhCP-A

31 and EhCP-B (Bruchhaus *et al.*, 2003). In contrast, clade C represents a new

1 group of *E. histolytica* cysteine endopeptidases that has not been described

2 before. EhCP-A and EhCP-B subfamily members are classical pre-pro enzymes

3 with an overall cathepsin L-like structure (Barrett 1998) as indicated by the

4 presence of an ERFNIN motif in the pro region of at least 21 of the 23 EhCP-A

5 and EhCP-B enzymes (Figure 4). Interestingly, biochemical studies with

6 purified EhCP-A indicated a cathepsin B-like substrate specificity (Scholze and

7 Schulte, 1988). This is likely due to the substitution of an alanine residue by

8 acidic or charged amino acids in the postulated S2 pocket, corresponding to

9 residue 205 of the papain sequence (Barrett 1998). As reported previously

10 (Bruchhaus *et al.*, 2003), the EhCP-A and EhCP-B subfamilies differ in the

11 length of the pro regions as well as of the catalytic domains, and have distinct

12 sequence motifs in the N-terminal regions of the mature enzymes (DWR vs.

13 PCNC). Moreover, none of the EhCP-A subfamily but 10 of the 11 EhCP-B

14 sequences contain hydrophobic stretches near or at the C-terminus, some of

15 which are predicted to constitute transmembrane helices (TMH) or GPI-

16 attachment moieties. This finding is consistent with previous reports on surface

17 localisation of *E. histolytica* cysteine proteinases but, so far, studies on the

18 cellular localisation of the various EhCP-B molecules have not been reported.

19

20 In contrast to the EhCP-A and EhCP-B subfamilies, primary structure

21 prediction indicates that EhCP-C members are not pre-pro enzymes, as they

22 lack hydrophobic signal sequences as well as identifiable pro regions. Instead,

23 they contain a hydrophobic region located 11 to 28 amino acids from the N-

24 terminus, which is predicted to form a TMH (Figure 4). Therefore, this new

25 group of molecules appears to be membrane associated via a signal anchor. All

26 EhCP-C enzymes have a conserved motif of the sequence $H/I(X)_6L/ICP$ in the

27 C-terminal half but they differ substantially in their pI, with values ranging

28 from 4.6 to 8.8. As there is no example of a structurally related cysteine

29 endopeptidase corresponding to the EhCP-C subfamily in other organisms, the

30 specific functions of this group of molecules remain completely unknown.

31

In addition to the large number of C1 superfamily members, the *E. histolytica* genome contains 2 genes encoding cysteine endopeptidases homologous to family C2 or calpain-like cysteine proteases (EhCALP1 and EhCALP2). Enzymes of this class contain several calcium-binding domains and have been shown to participate in a variety of cellular processes including remodeling of the cytoskeleton and membranes, signal transduction pathways and apoptosis.

Another 4 genes were identified coding for enzymes with homology to the peptidase family C54 also termed autophagins (EhAUTO1-4). The process of autophagy has been studied in human and yeast cells (Kirisako *et al.*, 2000; Marino *et al.*, 2003). Autophagy is a mechanism for the degradation of intracellular proteins and the removal of damaged organelles. During this process the cellular components become enclosed in double membranes and are subsequently degraded by lysosomal peptidases. Autophagins seem to be important for cytoplasm-to-vacuole targeting.

Two other genes encoding putative cysteine endopeptidases of *E. histolytica* show homology to the C19 and C65 families. These two groups of enzymes are known to be involved in ubiquitin degradation. Family C19 are ubiquitinyl hydrolases described as having ubiquitin-specific peptidase activity in humans. C65 or otubains are a group of enzymes with isopeptidase activity, which releases ubiquitin from polyubiquitin.

In summary, the *Entamoeba* genome contains a considerable number of endopeptidase genes. Elucidation of the precise role of each of the various enzymes will be a major challenge but may help us to understand the mechanism(s) of virulence and other unique properties of this protistan parasite.

3.3 Amoebapores and related proteins

In the lysosome-like granular vesicles of *E. histolytica* is found a family of small proteins, amoebapores, that are cytolytic towards human host cells,

1 display potent antibacterial activity, and cause ion channel formation in

2 artificial membranes (for a review see Leippe (1997)). Three amoebapore

3 isoforms have been isolated and biochemically characterised, and their primary

4 structure has been elucidated by molecular cloning of the genes encoding their

5 precursors (Leippe *et al.*, 1991; Leippe *et al.*, 1992; Leippe *et al.*, 1994b).

6 These membrane-permeabilising polypeptides are discharged by *E. histolytica*

7 into bacteria-containing phagosomes to combat growth of engulfed

8 microorganisms (Andrä *et al.*, 2003). Because of their potent cytolytic activity

9 against human cells in vitro (Berninghausen and Leippe, 1997; Leippe *et al.*,

10 1994a), amoebapores have been viewed as a crucial element of the machinery

11 use by the parasite to kill host cells. Trophozoites of *E. histolytica* lacking the

12 major isoform amoebapore A, whether through antisense inhibition of

13 translation (Bracha *et al.*, 1999) or epigenetic silencing of the gene (Bracha *et

14 al.*, 2003), became avirulent demonstrating that this protein plays a key role in

15 pathogenesis. Relatives of these protistan polypeptides are found in granules of

16 porcine and human cytotoxic lymphocytes where they are termed NK-lysin and

17 granulysin, respectively. All of these polypeptides are 70-80 amino acids in

18 length and are characterised by a compact alpha-helical, disulphide-bonded

19 structure known as the saposin-like fold. The structures of the amoebic and

20 mammalian polypeptides have been solved and compared (Anderson *et al.*,

21 2003; Hecht *et al.*, 2004; Leippe *et al.*, 2005; Liepinsh *et al.*, 1997). The

22 biological activities have also been measured in parallel (Bruhn *et al.*, 2003;

23 Gutsmann *et al.*, 2003) to evaluate the similarities and differences of these

24 effector molecules from organisms whose evolutionary paths diverged very

25 early. As they are active against both prokaryotic and eukaryotic target cells,

26 they may be viewed as broad-spectrum effector molecules.

27

28 In the genome of *E. histolytica*, 16 genes coding for putative saposin-like

29 proteins (SAPLIPs) were identified. All of these genes are transcribed by cells

30 growing in axenic culture (Winkelmann *et al.*, 2006). Like amoebapores, the

31 predicted proteins all contain one C-terminal SAPLIP domain and (with one

exception) a putative signal peptide (Table 6). As a transmembrane domain is not apparent in these proteins, it may well be that they are secretory products stored in the cytoplasmic vesicles and act synergistically with the amoebapores. However, only four of them have a similar size to amoebapores, the others being considerably larger (up to 1009 residues). At present, it is not clear whether these larger gene products represent precursor molecules that are processed further. None of the novel SAPLIPs contain the conserved unique histidine residue at the C-terminus that is a key residue for the pore-forming activity of amoebapores (Andrä and Leippe, 1994; Hecht *et al.*, 2004; Leippe *et al.*, 2005). Indeed, it has recently been shown that recombinant SAPLIP3 has no pore-forming or bactericidal activity, although it does cause membrane fusion in vitro (Winkelmann *et al.*, 2006). This is in agreement with the experimental evidence for only three pore-forming entities being present in trophozoite extracts. Therefore, it is most likely that the three amoebapores are the sole pore-forming molecules of the parasite. However, the lipid-interacting activity present in all SAPLIP proteins (Munford *et al.*, 1995) and a function that helps to kill bacterial prey may well characterise all members of the amoebapore/SAPLIP superfamily of this voraciously phagocytic cell.

3.4 Antioxidants

Entamoeba histolytica trophozoites usually reside and multiply within the human gut, which constitutes an anaerobic or microaerophilic environment. However, during tissue invasion, the amoebae are exposed to an increased oxygen pressure and have to eliminate toxic metabolites such as reactive oxygen or nitrogen species (ROS/RNS) produced by activated phagocytes during the respiratory burst. *E. histolytica* lacks a conventional respiratory electron transport chain that terminates in the reduction of O_2 to H_2O. However, *E. histolytica* does respire and tolerates up to 5% oxygen in the gas phase (Band and Cirrito, 1979; Mehlotra, 1996; Weinbach and Diamond, 1974). Thus, *E. histolytica* trophozoites must use different antioxidant enzymes for the removal of ROS, RNS and oxygen (Figure 5).

1

2 Among the enzymes in the first line of oxidative defence are superoxide

3 dismutases (SODs), which are metalloproteins that use copper/zinc (Cu/Zn),

4 manganese (Mn) or iron (Fe) as metal cofactors. SODs catalyse the dismutation

5 of superoxide radical anions to form H_2O_2 and O_2 (Fridovich, 1995). Analysis

6 of the *E. histolytica* genome revealed only a single gene coding for a FeSOD

7 and no sequences encoding MnSOD or Cu/ZnSOD. This reflects the situation

8 found in most protistan parasites and is consistent with biochemical studies

9 previously performed on *E. histolytica* lysates (Tannich *et al.*, 1991a).

10

11 *Entamoeba histolytica* lacks the tripeptide glutathione (Fahey *et al.*, 1984),

12 which constitutes the major low molecular weight thiol found in almost all

13 aerobic cells (Sies, 1999). Instead, *E. histolytica* uses cysteine as its principal

14 low molecular weight thiol (Ariyanayagam and Fairlamb, 1999; Fahey *et al.*,

15 1984; Nozaki *et al.*, 1999). As expected, coding sequences for enzymes that use

16 glutathione as a cofactor, such as glutathione-S-transferase, glutathione-

17 dependent peroxidase, glutathione reductase or glutaredoxin, are all absent from

18 the *E. histolytica* genome. In addition, genes encoding catalases and

19 peroxidases are also missing, as previously suggested (Sykes and Band, 1977;

20 Weinbach and Diamond, 1974).

21

22 Other genes were identified that code for proteins involved in detoxification of

23 H_2O_2, including one with homology to rubrerythrin. Rubrerythrin is a non-

24 haeme iron protein thought to be able to reduce H_2O_2 as part of an oxidative

25 stress protection system (Weinberg *et al.*, 2004). So far, the nature of its redox

26 partner is unknown in *E. histolytica* and it remains to be determined whether

27 protection against oxidative stress is indeed its main function. Another group of

28 H_2O_2-detoxifying proteins identified in *E. histolytica* are peroxiredoxins.

29 Peroxiredoxins are known from a wide variety of organisms. They are able to

30 reduce H_2O_2 as well as peroxynitrite with the use of electrons provided by

31 thiols. In addition to involvement in the detoxification of reactive oxygen

1 species peroxiredoxins seem to play a role in other processes such as signalling

2 and differentiation (Hofmann *et al.*, 2002; Rhee *et al.*, 2005; Wood *et al.*,

3 2003a,b). All peroxiredoxins contain a conserved cysteine residue that

4 undergoes a cycle of peroxide-dependent oxidation and thiol-dependent

5 reduction during the reaction. The whole protein family can be divided into

6 three classes based on the number and position of active site Cys residues (2-

7 Cys, atypical 2-Cys, and 1-Cys peroxiredoxins; Wood *et al.*, 2003a,b). In *E.*

8 *histolytica* five different genes coding for peroxiredoxins were identified (Prx1-

9 5). They all belong to the 2-Cys peroxiredoxin family. Four of them (Prx1-4)

10 share 98% sequence identity and have an unusual N-terminal Cys-rich repeat

11 (KECCKKECQEKECQEKECCC) of unknown function. In contrast, the fifth

12 peroxiredoxin (Prx5) lacks the cysteine-rich N-terminal extension and shares

13 only 30% identity with Prx1-4. Biochemical studies have shown that *E.*

14 *histolytica* peroxiredoxins are able to detoxify H_2O_2 and cumene hydroperoxide

15 (Bruchhaus *et al.*, 1997; Poole *et al.*, 1997). Moreover, up-regulation of

16 peroxiredoxin and FeSOD was associated with metronidazole resistance in

17 cultured *E. histolytica* trophozoites (Samarawickrema *et al.*, 1997; Wassmann

18 *et al.*, 1999).

19

20 Reactions catalysed by peroxiredoxins are dependent on the presence of

21 physiological thiols like thioredoxin (Rhee *et al.*, 2005; Wood *et al.*, 2003b).

22 Thioredoxins are small proteins involved in thiol-redox processes (Holmgren,

23 2000). They contain two redox-active site cysteine residues of the motif CXXC

24 (Watson *et al.*, 2004). Five genes coding for classical cytoplasmic thioredoxins

25 were identified in the *E. histolytica* genome (Trx1-5). These thioredoxins have

26 a length of 103-114 amino acids and share 25 – 47 % sequence identity. Trx1-3

27 have identical active site motifs of the sequence WCGPC, whereas the active

28 sites of Trx4 and Trx5 have the sequences SCPSC and WCKDC, respectively.

29 In addition, another five thioredoxin-related proteins were identified (Trx6-10).

30 All have a signal sequence of 15 to 19 amino acid residues and the active site

31 motif WCGHC, which is also known from the active site of protein disulphide

isomerases. However, in contrast to the latter group of enzymes, the *E. histolytica* thioredoxin-related molecules contain only one rather than two active-site motifs and only two of the proteins have an ER membrane retention signal (Freedman *et al.*, 2002). Thus it remains to be determined whether the thioredoxin-related molecules of *E. histolytica* do constitute protein disulphide isomerases or whether they undertake other functions within the cell.

Thioredoxins are kept in the reduced state by the enzyme thioredoxin reductase which catalyses the reduction of oxidised thioredoxin by NADPH using FAD and its redox-active disulphide (Nakamura, 2005). Two different genes with homology to thioredoxin reductases have been previously described from *E. histolytica* (thioredoxin reductase (TrxR) and NADPH:flavin oxidoreductase (p34)). They share about 87% sequence identity and both contain the two conserved sequence motifs forming the FAD and NAD(P)H binding domains. p34 was shown to catalyse the NADPH-dependent reduction of oxygen to H_2O_2 as well as of disulphides like DTNB and cystine (Bruchhaus *et al.*, 1998; Lo and Reeves, 1980). Therefore, in addition to disulphide reductase activity the enzyme has H_2O_2-forming NADPH oxidase activity. It was also shown that p34 can transfer reducing equivalents to peroxiredoxin, converting the protein from its non-active, oxidised form back into its active, reduced form (Bruchhaus *et al.*, 1997). However, it is unlikely that peroxiredoxin is directly reduced by p34 *in vivo*. It is more likely that *E. histolytica* contains a classical thioredoxin redox system consisting of thioredoxin reductase, thioredoxin and peroxiredoxin (Poole *et al.*, 1997).

In addition to genes coding for proteins with homology to thioredoxin reductase, four other gene families were identified that encode various flavoproteins. One of these families includes 4 members that have between 53% and 61% sequence identity to A-type flavoproteins (flavorubredoxin/flavodiiron). A-type flavoproteins belong to a large family of enzymes that are widespread among anaerobic and facultatively anaerobic

prokaryotes. In addition to bacteria, homologous genes are also found in the genomes of the pathogenic amitochondriate protistan parasites *Trichomonas vaginalis* and *Giardia intestinalis* (Andersson *et al.*, 2003; Sarti *et al.*, 2004). The A-type flavoproteins are made up of two independent structural modules. The N-terminal region forms a metallo-beta-lactamase-like domain, containing a non-haeme di-iron site, whereas the C-terminal region is a flavodoxin-like domain, containing one FMN moiety. These enzymes have significant nitric oxide reductase activity (Gomes *et al.*, 2002; Sarti *et al.*, 2004). For *Escherichia coli* it is known that the nitric oxide reductase (FIRd) receives electrons from a NADH:oxidoreductase (FIRd-red). Consistent with that situation, the *E. histolytica* genome contains a gene encoding an NADH oxidase with 25% sequence identity to several bacterial FIRd-reds.

The three other *E. histolytica* gene families with homology to iron-sulphur flavoproteins (families B-D) are characterised by the presence of a flavodoxin-like domain forming a typical FMN binding site. Family B and family C consist of three members each, which share sequence identity of 42% and 46%, respectively. Family D consists of two members, which share only 33% sequence identity. At present, the function of the various flavodoxin-like molecules remains to be determined and deserves to be investigated fully, particularly as to whether they do indeed have antioxidant capacity.

4. METABOLISM

Biochemical analysis of *E. histolytica* metabolism has a long history (Reeves, 1984), dating back to shortly after the development of culture media that allowed the generation of substantial numbers of axenic cells. The genome sequence has confirmed most of the predicted metabolic pathways shown biochemically to be present or absent in *E. histolytica* in the past. As with most parasites, secondary loss of biosynthetic pathways is a recurring theme. However, a few surprises have also been uncovered. Every single enzyme involved in metabolism cannot realistically be discussed in this review. In this

section the only the major energy generating and biosynthetic aspects of metabolism will be covered. Enzyme names, EC numbers and accession numbers are given in the the supplementary table for this section.

4.1 Energy Metabolism

4.1.1   Glycolysis

E. histolytica lacks a functional tricarboxylic acid (TCA) cycle and oxidative phosphorylation. It is not able to convert organic substrates such as glucose into H_2O and CO_2, but has to rely on the energy generated by various types of substrate level phosphorylation (Reeves, 1984). Glycolysis is the major pathway of ATP generation, but in addition the genome project has identified a number of genes that could result in more ATP generation through the catabolism of amino acids. These enzymes will be described further below. As *E. histolytica* lacks compartmentalised energy generation, it has been classified as a type I amitochondriate protist (Martin and Müller , 1998) in contrast to the type II amitochondriate protists containing hydrogenosomes, such as *Trichomonas vaginalis*. Nevertheless, it does contain a mitochondrial remnant, the mitosome (see section 8).

In *E. histolytica*, glycolysis appears to be localised in the cytosol. This is in contrast to trypanosomes where a major part is carried out in the glycosomes (Parsons, 2004) and the pathway is regarded as a potential target for chemotherapy (Opperdoes and Michels, 2001). The kinetic properties of recombinant *E. histolytica* glycolysis enzymes have recently been studied by Saavedra *et al.* (2005). Their analysis suggested that fructose-1,6-bisphosphate aldolase, phosphoglycerate mutase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate phosphate dikinase might be regulating the glycolytic flux.

4.1.1 (a) Hexokinases

Glucose taken up by *E. histolytica* is phosphorylated by two hexokinase (EC 2.7.1.1) isoenzymes (Hxk1 and Hxk2). The two *E. dispar* isoenzymes are shifted towards a slightly more basic pI, which is the basis of the classical biochemical method for distinguishing *E. histolytica* from *E. dispar* by starch gel electrophoresis (Farri *et al.*, 1980). The pI differences among the two *E. histolytica* isoforms (Ortner *et al.*, 1995) and between the two species (Ortner *et al.*, 1997b) are the result of genetic differences that lead to different amino acid sequences and charge differences. Hxk1 phosphorylates glucose and mannose, while Hxk2 phosphorylates mainly glucose and is much less active with mannose as a substrate (Kroschewski *et al.*, 2000).

4.1.1 (b) Glucose-6-phosphate isomerase

Glucose 6-phosphate is converted to fructose 6-phosphate by glucose-6-phosphate isomerase (EC 5.3.1.9). The genome has 2 genes for this enzyme, which code for proteins that differ only by a single insertion/deletion of 7 amino acid residues. Glucose-6-phosphate isomerase is another of the enzymes for the classical differentiation of *Entamoeba* zymodemes by starch gel electrophoresis (Sargeaunt, 1987).

4.1.1 (c) Phosphofructokinases

The main phosphofructokinase activity in *E. histolytica* is PPi-dependent (EC 2.7.1.90; Reeves *et al.*, 1976). There is a single gene (Deng *et al.*, 1998) encoding this 60 kDa enzyme. The gene is a candidate for lateral transfer from bacteria (Loftus *et al.*, 2005) (see section 10). The enzyme is expressed at a tenfold higher level and displays about tenfold higher activity than a second phosphofructokinase of 48 kDa (XP_653373) (Chi *et al.*, 2001). The substrate specificity of the smaller enzyme is disputed. Whereas Bruchhaus *et al.* (1996) reported that this minor enzyme also used PPi as phosphate donor, Chi *et al.* (2001) found only an ATP-dependent activity. The 48 kDa and 60 kDa enzymes are highly divergent with less than 20% sequence identity. Interestingly, the specificity of the 60 kDa phosphofructokinase can be changed

from PPi to ATP by mutation of a single amino acid residue (Chi and Kemp,

2000). The authors concluded that ATP rather than PPi was the primordial high

energy compound. In the genome, there are two additional genes encoding

isoforms of the 48 kDa enzyme, which have not been studied at the protein

level.

4.1.1 (d)   Fructose-1,6-bisphosphate aldolase

Fructose 1,6-bisphosphate is cleaved to glyceraldehyde 3-phosphate and

dihydroxyacetone 3-phosphate by fructose-1,6-bisphosphate aldolase (EC

4.1.2.13). The enzyme, a Class II aldolase (Marsh and Lebherz, 1992) has been

cloned (XP_650373) and exhibits strong sequence similarity to eubacterial

aldolases (Sanchez *et al.*, 2002). A second gene (XP_655966) encodes a protein

differing from the first by a single deletion of 28 amino acids flanked by short

divergent stretches. These bacterial-type aldolases are also found in

Trichomonas vaginalis, *Giardia intestinalis* and other protists (Sanchez *et al.*,

2002). *E. histolytica* has no gene coding for a Class I aldolase like those found

in animals, which might make aldolase an interesting target for chemotherapy.

4.1.1 (e)   Triose-phosphate isomerase

Triose-phosphate isomerase (EC 5.3.1.1) converts dihydroxyacetone 3-

phosphate into glyceraldehyde 3-phosphate. The gene was previously cloned

(Landa *et al.*, 1997), and is highly similar to the annotated gene product. This

dimer-forming enzyme represents the first *E. histolytica* protein for which the

structure has been solved by X-ray crystallography (Rodriguez-Romero *et al.*,

2002).

4.1.1 (f)   Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) oxidises and

phosphorylates glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate in two

coupled reactions using NAD^+ as cofactor (Reeves, 1984). The genome project

revealed five putative genes, three of which encode the identical protein

1 sequence of 36.0 kDa and a predicted pI of 7.04. The fourth gene product,

2 XP_648981, differs from these three only by a 13 amino acid deletion, while

3 XP_650370 is a clearly distinct 34.8 kDa isoform with a lower predicted pI of

4 5.80. Interestingly, the isoforms XP_650356 and XP_650370 of different pI are

5 encoded within the same contig.

6

7 4.1.1 (g)   Phosphoglycerate kinase

8 Phosphoglycerate kinase has an unusual substrate (Reeves and South, 1974),

9 transferring the high energy phosphate group from 3-phosphoglyceroyl

10 phosphate to GDP leading to the formation of GTP (EC 2.7.2.10). There is one

11 candidate gene encoding a 45 kDa protein.

12

13 4.1.1 (h) Phosphoglycerate mutase

14 Phosphoglycerate mutase (Reeves, 1984) isomerises 3-phosphoglycerate to 2-

15 phosphoglycerate (EC 5.4.2.1). Five divergent putative genes for this enzyme

16 are found in the genome. Two gene products of 62 kDa were classified as 2,3-

17 bisphosphoglycerate-independent phosphoglycerate mutases (XP_649031 and

18 XP_654182); they differ only at their C-termini and display significant

19 similarity to bacterial phosphoglycerate mutases. The three other genes are very

20 divergent. XP_ 651808 was identified as a candidate for lateral gene transfer

21 (Loftus *et al.*, 2005) (see section 10). The remaining two gene products

22 XP_649053 and XP_657284 are related to genes found in both prokaryotes and

23 eukaryotes.

24

25 4.1.1 (i) Enolase (2-phosphoglycerate dehydratase)

26 Enolase (EC 4.2.1.11) converts 2-phosphoglycerate to phosphoenolpyruvate.

27 The gene has been cloned (Beanan and Bailey, 1995) and the protein

28 characterised (Hidalgo *et al.*, 1997) previously. The 47 kDa gene product is a

29 typical eukaryotic enolase (XP_649161). A carboxy-terminally truncated

30 incomplete ORF is also found.

31

4.1.1 (j) Pyruvate, orthophosphate dikinase and pyruvate kinase

In *E. histolytica*, both activities forming ATP and pyruvate from phosphoenolpyruvate have been found. The exergonic pyruvate kinase reaction uses ADP (Saavedra *et al.*, 2004), and the pyruvate, orthophosphate dikinase uses AMP and PPi in a slightly endergonic reaction (Varela-Gomez *et al.*, 2004). The dikinase activity is found in C4 plants where it is involved in phosphoenolpyruvate generation for gluconeogenesis. In *E. histolytica* it was discovered long before the pyruvate kinase (Reeves, 1968).

The cloning of pyruvate, orthophosphate dikinase (EC 2.7.9.1) was reported by two groups. The published sequences (Bruchhaus and Tannich, 1993; Saavedra Lira *et al.*, 1992) are highly similar or identical to XP_657332 and XP_654666. In addition there are two shorter related open reading frames.

In the genome 3 putative pyruvate kinase genes (EC 2.7.1.40) have been identified. The 3 are identical except for an amino-terminal deletion in XP_648240 and an internal deletion in XP_653635.

4.1.1 (k) Pyruvate:ferredoxin oxidoreductase (PFOR) and ferredoxin

PFOR (EC 1.2.7.1) is an enzyme of major importance to *E. histolytica*, as the parasite lacks NAD^+-dependent pyruvate dehydrogenase and pyruvate decarboxylase (Reeves, 1984). No evidence for the latter two genes was found in the genome, confirming the biochemical results. PFOR oxidatively decarboxylates pyruvate to acetyl-CoA. The electrons are transferred to ferredoxin which, in its reduced form, can activate and reduce metronidazole, the major anti-amoebic drug (Müller , 1986). The activated form of metronidazole can potentially react with a number of biomolecules and is able to cleave the parasite DNA. In human cells, metronidazole is not activated and is much less toxic. In *T. vaginalis*, down-regulation of PFOR is one mechanism of producing metronidazole resistance (Kulda, 1999); however PFOR expression appears unaltered in partially resistant *E. histolytica*

1 (Samarawickrema *et al.*, 1997; Wassmann *et al.*, 1999). All eukaryotic PFOR

2 genes, including that of *E. histolytica*, appear to have been acquired during an

3 ancient lateral gene transfer event from bacteria (Horner *et al.*, 1999; Rotte *et*

4 *al.*, 2001). There are two putative PFORs in the *E. histolytica* genome

5 displaying minor sequence differences.

6

7 The genome contains 7 ferredoxin genes in total with 5 quite divergent

8 sequences. All are related to eubacterial and archaeal ferredoxins (Nixon *et al.*,

9 2002). The gene pairs XP_655183 / XP_655182 and XP_654311 / XP_652694

10 are identical. The other three gene products represent more divergent open

11 reading frames. The deduced proteins have similar molecular masses, between

12 6.1 kDa and 8.8 kDa, and different predicted isoelectric points between 4.2 and

13 8.6.

14

15 4.1.1 (l) Acetyl-CoA synthetase (acetate thiokinase)

16 The normal fate of acetyl-CoA in mitochondriate organisms is entry into the

17 tricarboxylic acid cycle. However this pathway is absent from *E. histolytica*.

18 Instead, the cleavage energy of the thioester bond of acetyl-CoA can be used to

19 generate one ATP molecule. One of the known acetyl-CoA synthetases

20 generates ATP from ADP and Pi (EC 6.2.1.13). Such an enzyme has been

21 characterised by Reeves *et al.* (1977) and cloned (Field *et al.*, 2000), and

22 reported to be a 77 kDa protein. The common acetyl-CoA synthetase activity

23 that produces ATP from AMP and PPi (EC 6.2.1.1) appears to be absent in *E.*

24 *histolytica*.

25

26 4.1.1 (m) Aldehyde and alcohol dehydrogenases

27 The *E. histolytica* genome encodes a complex system of alcohol and/or

28 aldehyde dehydrogenases. In total, there are 25 predicted genes, 3 of which are

29 on the list of lateral gene transfer (LGT) candidates.

30

Alcohol dehydrogenase ADH1 was the first alcohol dehydrogenase to be characterised in *E. histolytica* (Reeves *et al.*, 1971) and is a NADPH-dependent enzyme (EC 1.1.1.2). The gene was previously cloned (Kumar *et al.*, 1992); in the genome three genes are almost identical to that sequence, while one (XP_652772) has 67% identity.

Fermentation in *E. histolytica* uses the bifunctional alcohol dehydrogenase / aldehyde dehydrogenase NADH-dependent enzyme ADH2, which belongs to the ADHE family and has both alcohol dehydrogenase and aldehyde dehydrogenase activities (Lo and Reeves, 1978). Under anaeobic conditions, reduction of the acetyl-CoA generated by PFOR to ethanol is one way to regenerate the NAD+ used by glyceraldehyde-3-phosphate dehydrogenase. ADH2 first reduces acetyl-CoA to an enzyme-bound hemiacetal which is then hydrolysed to acetaldehyde (EC 1.2.1.10) and further reduced to ethanol (EC 1.1.1.1). If the enzyme is also able to work in the reverse direction, *E. histolytica* would be able to generate acetyl-CoA and energy from ethanol in the presence of oxygen. This would explain older reports of ethanol stimulated oxygen uptake in *E. histolytica* (Weinbach and Diamond, 1974). The enzyme is closely related to AdhE from *E. coli* and other bacteria (Reid and Fewson, 1994) and there is strong support for its aquisition by LGT (Andersson *et al.*, 2006; Field *et al.*, 2000; Loftus *et al.*, 2005) (see section 10). Like its bacterial homologue, ADH2 appears to form helical rods that sediment with membrane fractions (Avila *et al.*, 2002). Two groups have previously cloned ADH2 (Bruchhaus and Tannich, 1994; Yang *et al.*, 1994), and in total the genome contains 5 full-length ADH2 genes and one that is truncated. All share between 98% and 100% sequence identity.

In total, there are 11 alcohol dehydrogenase ADH3 genes in the genome, two of which been reported previously (Kimura *et al.*, 1996; Rodriguez *et al.*, 1996). The recombinant enzyme characterised by Rodriguez *et al.* (1996) was NADPH-specific, like ADH1. There are five genes similar to these previously

reported sequences. The rest of the ADH3 sequences fall into two groups of 3 similar sequences. All 11 ADH3 sequences are between 44% and 100% identical on the amino acid level. XP_649823 was originally on the list of LGT candidates (Loftus *et al.*, 2005), and a similarity to ADH3 sequences of gram-negative bacteria had been noted before (Nixon *et al.*, 2002). However a related sequence is now known to exist in *T. vaginalis* also (see section 10).

The genome encodes three additional distinct alcohol dehydrogenases. XP_656535 is a putative Zn-containing enzyme, and is on the list of LGT candidates. XP_652753 has been annotated as a Fe-containing alcohol dehydrogenase and XP_652262 simply as putative alcohol dehydrogenase.

One NADPH-dependent aldehyde dehydrogenase encoding gene (ALDH1) is present and was reported previously (Zhang *et al.*, 1994).

4.1.2   *Energy storage: the glycogen metabolism*

E. histolytica uses glycogen as its major energy store. Glycogen is a polymer of alpha-1,4-linked glucose chains with alpha-1,6 branch points, which in *E. histolytica* has a compact structure as suggested by branch points every 5-6 glucose residues (Bakker-Grunwald *et al.*, 1995). The cytoplasm of trophozoites contains numerous glycogen granules which were first observed by electron microscopy (Rosenbaum and Wittner, 1970) and later characterised biochemically (Takeuchi *et al.*, 1977). A glycogen phosphorylase activity (EC 2.4.1.1), associated with the glycogen granules, generates glucose 1-phosphate from orthophosphate and the linear portion of various glucopolysaccharides (Werries and Thurn, 1989). The genome contains at least 6 putative full-length and truncated genes encoding glycogen phosphorylases, two of which were cloned by Wu and Müller (2003). These authors noted a marked sequence divergence in those regions of the enzymes involved in regulation by phosphorylation and concluded that classical regulation by phosphorylation may not occur.

1

2 Glycogen phosphorylase degrades the linear chains only down to the alpha-1,6

3 branch points. The remaining core molecule is called limit dextrin. Degradation

4 can proceed further with the help of a debranching enzyme that has been

5 purified (Werries *et al.*, 1990). It exhibits activities of both amylo-1,6-

6 glucosidase (EC 3.2.1.33) and 4-alpha-glucanotransferase (EC 2.4.1.25). The

7 genome contains two genes putatively encoding a full-length (XP_653608) and

8 a truncated glycogen debranching enzyme. The deduced molecular mass of the

9 large protein is 166 kDa which corresponds to the biochemical data (Werries *et*

10 *al.*, 1990).

11

12 Glucose 1-phosphate is isomerised to glucose 6-phosphate by

13 phosphoglucomutase (EC 5.4.2.2) before entering the glycolytic pathway. The

14 isoelectric points of the phosphoglucomutases from *E. histolytica* and *E. dispar*

15 differ which was exploited for differentiation of the two species by starch gel

16 electrophoresis (Sargeaunt *et al.*, 1978). The migration properties are

17 reproduced by recombinant enzymes and are the result of primary sequence

18 differences (Ortner *et al.*, 1997a). *E. histolytica* has one gene coding for this

19 important enzyme, and in addition there are two distantly related members of

20 the phosphoglucomutase / phosphomannomutase family.

21

22 Genes encoding the enzymes involved in glycogen biosynthesis in *E. histolytica*

23 have been identified: a glycogen synthase (EC 2.4.1.11) of 155 kDa and two

24 putative branching enzymes (EC 2.4.1.18). The glycogen precursor UDP-

25 glucose is generated from UTP and glucose 1-phosphate by UTP:glucose-1-

26 phosphate uridylyltransferase (EC 2.7.7.9). Two UTP-hexose-1-phosphate

27 uridyltransferases have been characterised biochemically, a larger glucose 1-

28 phosphate-specific enzyme of 45 kDa and a less specific enzyme of 40 kDa

29 reported to use both galactose 1-phosphate and glucose 1-phosphate (Lobelle-

30 Rich and Reeves, 1983). The genome contains one larger open reading frame

31 encoding a putative UTP:glucose-1-phosphate uridylyltransferase of 54.7 kDa

1 and two smaller ones encoding enzymes of 46.3 kDa with high similarity

2 identified as UTP:N-acetyl-glucosamine-1-phosphate uridyltransferases. These

3 enzymes are interesting in that they could possibly be involved in the activation

4 of N-acetyl-glucosamine 1-phosphate as a precursor of the chitin cyst wall.

5

6 *4.1.3 Catabolism of sugars other than glucose*

7 4.1.3 (a) Activation of fructose and galactose for glycolysis

8 Neither Hxk1 nor Hxk2 can use fructose or galactose as a substrate, but there

9 are 2 genes encoding bacterial-type enzymes that may do so, a 33 kDa

10 fructokinase, which is one of the candidates for lateral gene transfer to the *E.*

11 *histolytica* lineage (see section 10), and a 43 kDa galactokinase. The

12 fructokinase groups with bacterial fructose 6-kinases (EC 2.7.1.4), and the

13 galactokinase groups with galactose 1-kinases (EC 2.7.1.6). This substrate

14 specificity has been noted before (Reeves, 1984). Fructose 6-phosphate enters

15 as an intermediate of the glycolytic pathway (see 4.1.1 (c)). As described above

16 (4.1.2), galactose 1-phosphate can be activated to UDP-galactose (Lobelle-Rich

17 and Reeves, 1983), and then epimerised to UDP-glucose by UDP-glucose 4-

18 epimerase (EC 5.1.3.2) (Reeves, 1984). In the genome, a single candidate 38

19 kDa ORF for the latter enzyme has been identified. The UDP-bound glucose

20 can then be used either for the synthesis of glycogen or fed into the glycolysis

21 pathway via glucose 1-phosphate and glucose 6-phosphate. This efficient

22 pathway allows *E. histolytica* to grow on galactose instead of glucose (Reeves,

23 1984).

24

25 4.1.3 (b) Anomerisation of aldoses

26 The 1-position in the pyranose form of aldoses has a hydroxyl group that can be

27 in either the α- or β-configuration. These forms can be interconverted by means

28 of an aldose 1-epimerase (EC 5.1.3.3) an enzyme that has recently been

29 characterised (Villalobo *et al.*, 2005). There is a single gene encoding this

30 product.

31

1   4.1.3 (c) Activation of pentoses

2   Two gene candidates encoding pentose-activating enzymes have been identified

3   in the *E. histolytica* genome: a 35 kDa ribokinase (EC 2.7.1.15) and a 56 kDa

4   xylulokinase (EC2.7.1.17). The latter is another bacterial-type sequence

5   putatively acquired by lateral gene transfer.

6

7   4.1.3 (d) Interconversion of hexoses and pentoses

8   The pathway of interconversion between hexoses and pentoses in *E. histolytica*

9   was described many years ago (Reeves, 1984; Susskind *et al.*, 1982). A

10 transketolase (EC 2.2.1.1) converts fructose 6-phosphate and glyceraldehyde 3-

11 phosphate into xylulose 5-phosphate and erythrose 4-phosphate. Erythrose 4-

12 phosphate and dihydroxyacetone phosphate are condensed by the glycolytic

13 enzyme fructose-1,6-bisphosphate aldolase to sedoheptulose 1,7-bisphosphate,

14 an extended substrate specificity of the aldolase. Phosphofructokinase then is

15 able to remove a phosphate group forming diphosphate and sedoheptulose 7-

16 phosphate. This molecule and glyceraldehyde 3-phosphate are then converted

17 by transketolase to the pentoses ribose 5-phosphate and xylulose 5-phosphate.

18 A transaldolase activity is absent (Reeves, 1984) consistent with there being no

19 such gene in the genome. In contrast, 7 gene products were identified as likely

20 transketolases: three highly similar proteins of 73 kDa and four truncated

21 versions.

22

23 **4.2 Amino acid catabolism**

24 *4.2.1 General features*

25 As discussed above, glycolysis under anaerobic conditions can use only part of

26 the energy contained in glucose for ATP generation. *E. histolytica* is capable

27 not only of taking up amino acids (Reeves, 1984), but also using them for the

28 generation of energy, as suggested by Zuo and Coombs (1995). The genome

29 has revealed a number of unusual genes, often with bacterial affinities, coding

30 for enzymes of amino acid catabolism (Anderson and Loftus, 2005).

31

1 In many cases, the degradation of amino acids starts with a transamination

2 reaction (EC 2.6.1. -) generating a 2-ketoacid. The *E. histolytica* genome has

3 five ORFs identified as aminotransferases. These ORFs are distinct from each

4 other with the exception of XP_655090 and XP_655099, which differ only by

5 one insertion and are LGT candidates. So far there is no enzymological data on

6 this group of enzymes, so their substrate specificities in *E. histolytica* are

7 unknown.

8

9 Both amino acid degradation and glycolysis have 2-ketoacids as intermediates.

10 Pyruvate is one common intermediate, as amino acid degradation can produce

11 either pyruvate or other 2-ketoacids. PFOR (see 4.1.1 (k)) is known to have a

12 relaxed specificity, and in addition to pyruvate it can oxidatively decarboxylate

13 2-ketobutanoate, oxaloacetate, and 2-ketoglutarate (Samarawickrema *et al.*,

14 1997). The reaction generates CoA-thioesters with the potential of producing

15 one ATP per molecule.

16

17 The amino acids asparagine, aspartate, serine, alanine, tryptophan, cysteine,

18 threonine, methionine, glutamine, and glutamate can all be transformed into one

19 of these 2-ketoacids in one or very few steps. This underlines the major

20 importance of the PFOR in the energy metabolism of *E. histolytica*. The

21 enzyme is indispensable, and as it always generates reduced ferredoxin it will

22 always activate metronidazole. Consequently, it would be very difficult for *E.*

23 *histolytica* to become resistant to metronidazole.

24

25 *4.2.1 Aspartate and asparagine*

26 *E. histolytica* takes up asparagine and aspartate in the presence or absence of

27 glucose (Zuo and Coombs, 1995). Four putative asparaginases (EC 3.5.1.1) are

28 found in the genome. Three are identical and share only 48% amino acid

29 identity with the fourth (XP_656586). Asparaginase mediates the formation of

30 aspartate from aspargine by releasing ammonia. The predicted sequences

31 appear to possess a signal sequence, as suggested by TargetP program

1 (www.cbs.dtu.dk/services/TargetP/), which is reminiscent of a periplasmic

2 isotype (EcA, type II) (Swain *et al.*, 1993) that is up-regulated under anaerobic

3 and carbon-restricted conditions (Cedar and Schwartz, 1967).

4

5 Aspartate can be converted to fumarate and ammonia by aspartate ammonia-

6 lyase (aspartase, EC 4.3.1.1). Addition of a water molecule by fumarase (EC

7 4.2.1.2) produces malate. The genome encodes a putative fumarase that is

8 related to bacterial Class I fumarases. The aspartase is a member of the bacterial

9 Class II fumarase / aspartase protein family (Woods *et al.*, 1988), and also on

10 the list of LGT candidates.

11

12 Aspartate is also decomposed into oxaloacetate and ammonia by aspartate

13 aminotransferase, with the concomitant production of glutamate from 2-

14 oxoglutarate. Oxaloacetate is then converted into malate via malate

15 dehydrogenase (EC 1.1.1.37) and, since *E. histolytica* lacks both a functional

16 TCA cycle and phosphoenolpyruvate carboxykinase, the malate generated can

17 be oxidatively decarboxylated to pyruvate by malic enzyme (EC 1.1.1.39). Both

18 of these enzymes are present in *E. histolytica*. Two very similar genes have

19 been identified as encoding malic enzyme and are LGT candidates.

20

21 *4.2.2 Serine, threonine*

22 Serine and threonine are also taken up by *E. histolytica* in the presence and

23 absence of glucose (Zuo and Coombs, 1995). Serine can be deaminated by the

24 pyridoxal phosphate-dependent serine dehydratase (L-serine ammonia-lyase,

25 EC 4.3.1.17) to pyruvate and ammonia. The enzyme was characterised by

26 Takeuchi *et al.* (1979) who showed that addition of serine to the culture

27 medium stimulated oxygen consumption. In an analogous reaction, threonine

28 dehydratase (threonine ammonia-lyase, EC 4.3.1.19) breaks down threonine to

29 2-oxobutanoate. Both ketoacids can then be oxidised by PFOR to acetyl-CoA

30 or proprionyl-CoA. Both catabolic reactions can be carried out by the same

31 enzyme, as has been shown in yeast for example (Ramos and Wiame, 1982). In

the *E. histolytica* genome annotation, four gene products have been annotated

as threonine dehydratases, but none as serine dehydratase. XP_650405 and

XP_652480 are identical while XP_655614 and XP_657171 share 95% and

37% identity with the others, respectively. The exact substrate specificities of

these 4 putative serine / threonine dehydratases have not been reported.

Degradation of serine via the non-phosphorylated serine pathway, by the

sequential reactions of L-serine: pyruvate aminotransferase (EC 2.6.1.51), D-

glycerate dehydrogenase (EC 1.1.1.29), and D-glycerate kinase (EC 2.7.1.31)

(Snell, 1986) results in the glycolytic intermediate 3-phosphoglycerate. The

genome encodes several putative aminotransferases (see section 4.2.1), but it is

not yet known if serine is among their substrates. An unusual bacterial-type

NADPH-dependent D-glycerate dehydrogenase was characterised by Ali *et al.*

(2003) and there are 2 genes encoding D-glycerate dehydrogenases, one of

which (XP_648124) is among the weaker LGT candidates (see section 10). The

genome also contains 2 genes encoding identical glycerate kinases. The enzyme

has recently been characterised by Ali and Nozaki (unpublished).

4.2.3 Methionine, homocysteine and cysteine

Methionine γ-lyase (EC 4.4.1.11) decomposes methionine to methanethiol

(mercaptomethane), ammonia, and 2-oxobutanoate. In *E. histolytica*, two

methionine γ–lyases, EhMGL1 and EhMGL2, of similar molecular weights

have been characterised (Tokoro *et al.*, 2003). These two isoenzymes show

marked differences in substrate specificity, isoelectric point, enzymological and

biochemical parameters (Tokoro *et al.*, 2003). Both enzymes can also act on

other amino acids. In addition to degrading methionine, both EhMGL1 (pI

6.01) and EhMGL2 (pI 6.63) can convert homocysteine to hydrogen sulphide,

ammonia and 2-oxobutanoate. EhMGL2 also decomposes cysteine to hydrogen

sulphide, ammonia, and pyruvate, whereas EhMGL1 is only weakly active

against cysteine. Decomposition of homocysteine by methionine γ-lyase is

essential since this parasite lacks the other known enzymes capable of

destroying this toxic amino acid. In the genome, three open reading frames correspond to EhMGL1 and one to EhMGL2. So far, the only eukaryotes known to possess methionine γ–lyases are *E. histolytica* and *T. vaginalis* (Lockwood and Coombs, 1991). As the enzymes are absent from the human host and important for the generation of metabolic energy, they could be targets for chemotherapy (Coombs and Mottram, 2001; Tokoro *et al.*, 2003).

In addition to serving as a source of metabolic energy, another important role of methionine is as a donor of methyl groups via S-adenosylmethionine synthetase (synonymous with methionine adenosyltransferase, EC 2.5.1.6). Seven gene candidates were identified, four full-length and three truncated. The S-adenosylhomoserine left after the transfer of the activated methyl group can be hydrolysed by S-adenosylhomocysteine hydrolase (EC 3.3.1.1) giving adenosine and homocysteine. Two candidate genes with identical sequences and one truncated form are present.

However, *E. histolytica* lacks the remaining enzymes for the reverse transsulphuration pathway (forming cysteine from methionine) (Nozaki *et al.*, 2005), i.e. cystathionine β-synthase and cystathionine γ-lyase. In addition, *E. histolytica* lacks all enzymes involved in the forward transsulphuration (forming methionine from cysteine) including cobalamin-dependent methionine synthase (EC 2.1.1.13) or cobalamin-independent methionine synthase (EC 2.1.1.14), which suggests that *E. histolytica* is capable of neither converting homocysteine to cystathionine nor recycling homocysteine to methionine.

E. histolytica lacks the methylthioadenosine cycle enzymes except for two, 5'-methylthioadenosine/S-adenosyl homocysteine nucleosidase (EC 3.2.2.9) and aspartate aminotransferase (AT, EC 2.6.1.1). The significance of these two enzymes in *E. histolytica* is unknown.

4.2.4 Arginine

In *G. intestinalis* and *T. vaginalis* the arginine deiminase (EC 3.5.3.6) pathway is important for energy generation (Knodler *et al.*, 1994; Linstead and Cranshaw, 1983; Schofield and Edwards, 1994), generating one ATP molecule from the breakdown of arginine to ornithine. In contrast, no arginine deiminase gene or dihydrolase pathway was detected in the *E. histolytica* genome.

In *E. histolytica*, arginine can either be degraded by arginase (EC 3.5.3.1) via ornithine or arginine decarboxylase (EC 4.1.1.19) via agmatine. The arginine decarboxylase reaction uses up protons and may be involved in the acid resistance needed for the passage of cysts through the human stomach Anderson and Loftus, 2005). Another function suggested for arginine degradation was that it depletes arginine as a substrate for human macrophages, preventing NO synthesis and amoebicidal activity (Elnekave *et al.*, 2003). Both enzymes could also be important for the generation of the polyamine putrescine (see 4.3 below). The genome contains a single gene encoding a 96 kDa polypeptide annotated as ornithine/arginine/lysine decarboxylase, the substrate specificity of which has not yet been examined on the recombinant protein level. There is a single gene encoding a putative 33 kDa arginase.

4.2.5 Glutamate, glutamine

In aerobic organisms, the 2-oxoglutarate generated from glutamate in a transaminase reaction enters the citric acid cycle for further catabolism. In *E. histolytica*, which also contains transaminases, 2-oxoglutarate can be oxidised by PFOR to give succinyl-CoA from which one molecule of ATP can be generated.

Several other gene products of *E. histolytica* could act on glutamine and glutamate. The genome lacks a glutaminase (EC 3.5.1.2) to carry out the simple hydrolysis of glutamine. Instead there is a putative glucosamine-fructose-6-phosphate aminotransferase (EC 2.6.1.16), which uses the energy in the amide

group of glutamine to generate glucosamine 6-phosphate from fructose 6-phosphate. This product may be used for cyst wall biosynthesis.

4.2.6 Tryptophan

Tryptophan can be degraded to indole, pyruvate, and ammonia by the PLP-dependent enzyme tryptophanase (EC 4.1.99.1), for which one candidate gene exists. To date, tryptophanase has only been found in bacteria, never in eukaryotes, and it is also on the list of LGT candidates.

4.2.7 Alanine: a possible special case

Alanine could potentially be transformed into pyruvate by alanine aminotransferase (synonymous with alanine:pyruvate transaminase, EC 2.6.1.2). However, *E. histolytica* is reported to excrete alanine (Zuo and Coombs, 1995) suggesting that this enzyme is not used under the culture conditions tested. Conceivably, the purpose of the excretion process may be to carry excess nitrogen out of the cell in the absence of a functional urea cycle.

4.2.8 Catabolism of other amino acids

Most of the enzymes for branched-chain amino acid metabolism are missing in *E. histolytica* but leucine, isoleucine, and valine could be transformed to 2-oxoisocaproate, 2-oxo-3-methylvalerate, and 2-oxovalerate, respectively, by a putative branched-chain amino acid aminotransferase (EC 2.6.1.42), one of the aminotransferases mentioned above (section 4.2). This could produce ammonia or transfer the amino group to 2-oxoglutarate to form glutamate. Subsequent oxidative decarboxylation to give the respective CoA-derivatives could be envisaged but so far no gene candidates for the necessary dehydrogenases have been identified.

One gene encodes a putative histidine ammonia-lyase (EC 4.3.1.3), which is responsible for the decomposition of histidine into urocanate and ammonia. Other than the formation of ammonia, the significance of this enzyme is not

clear since the downstream enzymes involved in histidine catabolism from urocanate to glutamate were not found.

Currently, there is little information regarding the fate of the amino acids glycine, proline, phenylalanine, tyrosine, and lysine in *E. histolytica*. No genes for the catabolic enzymes necessary were detected except for a LGT candidate bacterial-type 96 kDa broad-specificity ornithine/arginine/lysine decarboxylase that may be acting on lysine.

4.3 Polyamine Metabolism

The absence of *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50), which converts *S*-adenosyl methionine into decarboxylated *S*-adenosyl methionine, spermidine synthase (EC 2.5.1.16), and spermine synthase (EC 2.5.1.22) suggests a complete lack of polyamine metabolism in this parasite (Anderson and Loftus, 2005). However, as mentioned above, *E. histolytica* possesses genes encoding arginase and arginine decarboxylase. Both could be involved in the production of putrescine via agmatine and agmatinase (EC 3.5.3.11) or via ornithine and ornithine decarboxylase (EC 4.1.1.17). The high putrescine concentration in trophozoites demonstrated by NMR spectroscopy (9.5 mM) (Bakker-Grunwald *et al.*, 1995) reinforces the physiological significance of putrescine. However, the fate of putrescine is unknown as neither spermine nor spermidine has been demonstrated in *E. histolytica*.

There is controversy regarding the presence or absence of trypanothione, a spermidine-containing thiol, in *E. histolytica*. Trypanothione is a major thiol in trypanosomes and leishmania (Fairlamb and Cerami, 1992) and contains two molecules of glutathione joined by a spermidine linker. The first reports detected the presence of trypanothione in *E. histolytica* (Ondarza *et al.*, 1997) but were contradicted soon after (Ariyanayagam and Fairlamb, 1999). More recently another study reaffirmed its presence (Ondarza *et al.*, 2005). However, the gene encoding trypanothione reductase reported from *E. histolytica* strain

HK-9 (AF503571) has no homologue in the genome of HM-1:IMSS. Although this matter has not been resolved, there is general agreement that the major thiol in *E. histolytica* is cysteine (Fahey *et al.*, 1984).

The *E. histolytica* genome encodes a 46 kDa ornithine decarboxylase with similarity to both plant and vertebrate enzymes and there is also the 96 kDa ornithine/arginine/lysine decarboxylase (see section 4.2.4). Only the former enzyme has been characterised at the biochemical level (Arteaga-Nieto *et al.*, 2002) and has been shown to be insensitive to difluoromethylornithine (DFMO), as is *E. histolytica* (Gillin *et al.*, 1984).

The conversion of arginine into putrescine via agmatine, in a reaction initiated by arginine decarboxylase, is generally present in bacteria and plants. Although arginine decarboxylase is present in *E. histolytica*, agmatinase (EC 3.5.3.11), which further catalyses conversion of agmatine to putrescine and urea, appears absent. However, one gene identified as a 33 kDa arginase also shares 21% sequence identity with human mitochondrial agmatinase and therefore its substrates need to be examined on the biochemical level to see whether the enzyme can act on arginine, agmatine, or both. At present, the role of arginine decarboxylase in *E. histolytica* is not clear, although as mentioned above this enzyme may also be involved in acid resistance in *E. histolytica.*

4.4 Biosynthesis of Amino Acids

4.4.1 Cysteine and serine

One of the areas in which reduction of metabolism is most evident is in amino acid biosynthesis. Biosynthetic pathways for most amino acids other than serine and cysteine (Ali *et al.*, 2003; Ali *et al.*, 2004a; Nozaki *et al.*, 1998a; Nozaki *et al.*, 1999) have been lost in *E. histolytica*. Similarly, *P. falciparum*, which predominantly acquires amino acids from host haemoglobins, lacks biosynthesis of most amino acids (Gardner *et al.*, 2002). Intracellular concentrations of some amino acids (glutamate, leucine, valine, and proline, in

1   descending order of abundance) are very high in *E. histolytica* ranging from 6-

2   21 mM (Bakker-Grunwald *et al.*, 1995). In particular, the glutamate and proline

3   concentrations are much higher in the cells than in the growth medium (21 and

4   7.3 mM vs. 5.9 and 1.8 mM, respectively). Glutamate accounts for over one

5   third of the total amino acid pool (Bakker-Grunwald *et al.*, 1995), and is likely

6   to play a central role in homeostasis not only of amino acids but of energy

7   metabolism in general. Thus, it is likely that these amino acids are actively

8   taken up by as-yet unidentified amino acid transporters.

9

10   Retention of the serine and cysteine biosynthetic pathways when the others

11   have been lost is likely related to the physiological importance of cysteine,

12   which is the major intracellular thiol of this parasite. The cysteine biosynthetic

13   pathway consists of two major steps, catalysed by serine acetyltransferase (EC

14   2.3.1.30), which produces *O*-acetylserine from serine and acetyl-coenzyme A,

15   and cysteine synthase (EC 2.5.1.47), which subsequently transfers an alanyl

16   moiety from *O*-acetylserine to sulphide to produce cysteine. *E. histolytica*

17   possesses three genes each for cysteine synthase and serine acetyltransferase.

18   Cysteine synthase 1 and 2 were considered to be allelic isotypes (Nozaki *et al.*,

19   1998b), while cysteine synthase 3 appears to be distinct, with only 83% identity

20   to cysteine synthase 1 and 2. In contrast, all three serine acetyltransferase genes

21   seem to be distinct, showing only 48-73% identity (Ali and Nozaki,

22   unpublished). It was previously shown that cysteine synthase 1/2 and serine

23   acetyltransferase 1 are unique in that (a) they do not form a heterocomplex, in

24   contrast to other organisms (Bogdanova and Hell, 1997; Droux *et al.*, 1998) and

25   (b) serine acetyltransferase 1 is sensitive to allosteric inhibition by both L-

26   cysteine and L-cystine (Nozaki *et al.*, 1999). Since all variants of these two

27   enzymes lack organelle-targeting sequences, the significance of the multiple

28   isotypes is unknown. It is important to determine subcellular distribution and

29   specific functions of these isotypes to understand the significance of the

30   redundancy. As this pathway is absent in humans, it is a rational target for

31   development of new chemotherapeutic drugs against amoebiasis.

1

2   Serine is synthesised de novo utilising the glycolytic intermediate 3-

3   phosphoglycerate, in a pathway that includes three sequential reactions

4   catalysed by D-phosphoglycerate dehydrogenase (EC 1.1.1.95), phospho-L-

5   serine aminotransferase (EC 2.6.1.52), and *O*-phospho L-serine phosphatase

6   (EC 3.1.3.3). Although the final enzyme has not yet been enzymologically and

7   functionally analysed, the first two enzymes have been characterised (Ali and

8   Nozaki, 2006; Ali *et al.*, 2004a).

9

10   *4.4.2 Interconversion of glutamate-glutamine and aspartate-asparagine*

11   The single step interconversions of glutamate and glutamine, catalysed by

12   glutamate synthase (EC 1.4.1.13) and glutamine synthetase (EC 6.3.1.2), and of

13   aspartate and asparagine by asparagine synthase (EC 6.3.5.4) are found in *E.*

14   *histolytica*. There are two isotypes of glutamine synthetase with 47% amino

15   acid identity and 5 candidate genes. NADPH-dependent glutamate synthase

16   (EC 1.4.1.13) catalyses the formation of two glutamates from glutamine and 2-

17   oxo-glutarate in bacteria, yeast, and plants, and together with glutamine

18   synthetase is involved in ammonia fixation under ammonia-restricted

19   conditions. NADPH-dependent glutamate synthase is normally composed of

20   two large and two small subunits (Petoukhov *et al.*, 2003). Although three

21   genes encoding the small subunit are present, the large subunit appears to be

22   absent in *E. histolytica*. These putative NADPH-dependent glutamate synthase

23   small subunits share 80% amino acid identity and show 44% amino acid

24   identity to homologues from the Archaea. The similarity to archaeal-type

25   glutamate synthase (Nesbo *et al.*, 2001) suggests that the *E. histolytica* small

26   subunits may function as a glutamate synthase without the large subunit, as

27   shown for gltA from the archaean *Pyrococcus* (Jongsareejit *et al.*, 1997).

28

29   The two enzymes that catalyse interconversion between aspartate and

30   asparagine, aspartate ammonia ligase (EC 6.3.1.1) and asparaginase (EC

31   3.5.1.1; see 4.2.1), are present in *E. histolytica*. Two types of aspartate

ammonia ligases, AsnA and AsnB, are known from other organisms: the former utilises only ammonia, while the latter uses both ammonia and glutamine as amide donors in a reverse reaction. Mammals possess only AsnA, whereas prokaryotes have both AsnA and AsnB (Boehlein *et al.*, 1996; Nakamura *et al.*, 1981). Interestingly, *E. histolytica* possesses only the AsnB homologue. Thus, the amoebic enzyme is likely involved in the formation of glutamate from glutamine, in addition to asparagine formation from aspartate.

4.4.3 Synthesis of glutamate and aspartate

Glutamate can be formed from 2-oxo-glutarate and ammonia in a reversible reaction catalysed by glutamate dehydrogenase (EC 1.4.1.2), which is present in *E. histolytica*. It is known that this enzyme plays a dominant role in ammonia fixation under ammonia-non-restricted conditions as this reaction consumes no ATP. In addition, glutamate dehydrogenase is also involved in gluconeogenesis from glutamate.

Aspartate ammonia lyase (synonymous with aspartase, EC 4.3.1.1), which decomposes aspartate into fumarate and ammonia in a reversible reaction, is also present in *E. histolytica* (see 4.2.1 above).

4.5 Lipid Metabolism

For *E. histolytica*, the lack of oxidative phosphorylation means that the high energy content of lipids such as fatty acids cannot be exploited. Therefore lipids such as phospholipids and cholesterol are primarily membrane components in *E. histolytica* (Das *et al.*, 2002; Sawyer *et al.*, 1967). Although these components are acquired mainly from their food or from the human host, *E. histolytica* does have some capability for biosynthesis, as well as extending and remodeling lipids, and for attaching lipids to proteins.

4.5.1 Lipid biosynthetic capabilities

4.5.1 (a) Polyisoprene biosynthesis and protein prenylation

1 Cholesterol is an important membrane constituent generated from C_5 isoprene

2 precursors. *E. histolytica* trophozoites in axenic culture need cholesterol in their

3 growth medium (Reeves, 1984), and it is likely that they acquire it from their

4 human host. Reeves (1984) even cites several studies which show that

5 hypercholesteremia in the host increases the damage inflicted by amoebic

6 infection. *E. histolytica* lacks several enzymes for the classical sterol

7 biosynthesis pathway (Schroepfer, 1981). The first stage of sterol biosynthesis

8 is the formation of isopentenyl- or dimethylallyl diphosphate. In the *E.*

9 *histolytica* genome no candidate genes for the generation of these intermediates

10 were found, neither for the mevalonate pathway nor for the mevalonate-

11 independent methylerythritol 4-phosphate (MEP) pathway that operates in

12 bacteria and plants (Hunter *et al.*, 2003; Rohmer *et al.*, 1993). In a later step

13 towards cholesterol sythesis, two molecules of C_{15} farnesyl diphosphate are

14 dimerised to give C_{30} presqualene diphosphate (EC 2.5.1.21). This enzyme

15 activity and those catalysing the subsequent steps also appear to be absent. The

16 genome data thus support the long standing conclusion that cholesterol

17 biosynthesis is absent from *E. histolytica*.

18

19 Unexpectedly, the *E. histolytica* genome appears to encode enzymes involved

20 in the intermediate stages of cholesterol biosynthesis from C_5 isopentenyl

21 diphosphate to C_{15} farnesyl diphosphate. The latter compound, and the larger

22 C_{20} compound geranylgeranyl diphosphate, may serve as precursors for the

23 hydrophobic modification of GTP-binding proteins allowing them to bind to

24 membranes (Grunler *et al.*, 1994). Protein prenylation is a ubiquitous process. It

25 is important in human cell biology, health and disease (McTaggart, 2006), but it

26 is also essential for parasites such that protein farnesylation has been proposed

27 as a potential novel target for anti-parasitic chemotherapy (Maurer-Stroh *et al.*,

28 2003) including anti-*E. histolytica* chemotherapy (Ghosh *et al.*, 2004).

29

30 The first enzyme in this pathway is the isopentenyl-diphosphate delta-isomerase

31 which catalyses the conversion of isopentenyl diphosphate to dimethylallyl

1   diphosphate (EC 5.3.3.2). There is a single gene encoding this enzyme that is of

2   presumed bacterial origin and is on the list of LGT candidates. The two

3   isomeric C_5 isoprenyl diphosphates undergo condensation to C_{10} geranyl

4   diphosphate, catalysed by geranyl-diphosphate synthase (EC 2.5.1.1). Farnesyl-

5   diphosphate synthase (EC 2.5.1.10) then adds another C_5 unit to give C_{15}

6   farnesyl diphosphate. Finally geranylgeranyl-diphosphate synthase (EC

7   2.5.1.29) adds another C_5 prenyl unit to give C_{20} geranylgeranyl diphosphate.

8   The genome contains five putative prenyl transferase genes, which all have

9   been annotated as geranylgeranyl-diphosphate synthases. Their sequences are

10   highly similar, with the exception that the open reading frames are disrupted in

11   two of them (XP_650479 and XP_655958). These prenyl transferases appear to

12   be of bacterial origin as well, and XP_650913 is on the list of LGT candidates.

13   When searching for geranyl-diphosphate synthase or farnesyl-diphosphate

14   synthase in the *E. histolytica* genome, the closest matches are for the same

15   genes, so that the substrate specificity of these enzymes is unclear and needs to

16   be examined biochemically.

17

18   The *E. histolytica* genome contains one sequence each for the alpha and beta

19   chains of protein farnesyltransferase (EC 2.5.1.58), which were previously

20   cloned and characterised as recombinant proteins (Kumagai *et al.*, 2004).

21

22   In addition to the protein farnesyltransferase, a protein

23   geranylgeranyltransferase I (EC 2.5.1.59) beta chain has recently been cloned

24   and expressed together with the protein farnesyltransferase alpha chain

25   (Makioka *et al.*, 2006). The heterodimeric molecule had protein

26   geranylgeranyltransferase activity of unusually broad substrate specificity. The

27   alpha and beta chains of the protein (Rab-) geranylgeranyltransferase II (EC

28   2.5.1.60) have also been cloned, as cDNAs (Kumagai *et al.* unpublished

29   results).

30

The *E. histolytica* genome encodes candidate enzymes for the modification of prenylated proteins. There are two highly divergent proteins both identified as CAAX prenyl proteases (EC 3.4.24.84). CAAX is the carboxy-terminus of the substrate protein, in which C is the prenylated cysteine residue, A is an aliphatic amino acid and X is the terminal residue. The proteases cleave after the modified cysteine. After the processing step, a prenylcysteine carboxyl methyltransferase (EC 2.1.1.100) methylates the carboxy-terminal residue; there are two divergent candidate genes for this enzyme.

Taken together, the *E. histolytica* genome contains all the necessary genes to encode the pathway from isopentenyl diphosphate to a processed farnesylated or geranylgeranylated protein. The source of the starting material, isopentenyl diphosphate, remains unknown at this time, but there may be a previously unknown pathway for its synthesis or *E. histolytica* may be able to aquire it from its environment.

4.5.1 (b) Fatty acid biosynthesis

E. histolytica encodes an unusual 138 kDa acetyl-CoA carboxylase with two bacterial-type carboxylase domains, an acetyl-CoA carboxylase and a pyruvate carboxylase. Since no biotin carboxylase domain is found in the *E. histolytica* genome, it was proposed that the enzyme removes a carboxyl group from oxaloacetate and transfers it to acetyl-CoA, forming malonyl-CoA and pyruvate (Jordan *et al.*, 2003; Loftus *et al.*, 2005). This fusion protein has not been identified in any organisms other than *Giardia* and *Entamoeba*.

In the classical pathway of fatty acid biosynthesis, starting from acetyl-CoA sequential two-carbon units are added from malonyl-CoA. In each round of extension, the beta-keto group is reduced in three steps before a new two-carbon unit is added. The whole pathway is carried out in a large fatty acid synthase complex, where the growing chain is linked to an acyl carrier protein. *E. histolytica* lacks this classical pathway. There are, however, plant

homologues of fatty acid chain elongases such as *Arabidopsis thaliana* KCS1

(Todd *et al.*, 1999). There are eight putative fatty acid elongases in the *E.*

histolytica genome, and all are very similar to each other. These enzymes could

be involved in elongation of fatty acids taken up from the host or food sources,

but their function and substrate specificity are unknown at this time.

4.5.2 Phospholipid metabolism

Phospholipids amount to 60-70% of the total lipids in *E. histolytica* (Sawyer *et*

al., 1967). So far little information is available at the biochemical level on how

phospholipids are synthesised, acquired or remodelled. The genome project has

revealed a number of genes indicating that the phospholipid metabolism could

be more complex than expected.

4.5.2 (a) Phospholipid biosynthesis

In order to produce phospholipids one has to generate the important

intermediate phosphadidate (1,2-diacylglycerol 3-phosphate) by

phosphorylation and acylation of glycerol. *E. histolytica* contains one gene for a

glycerol kinase (EC 2.7.1.30). The second step would be the transfer of the acyl

group to glycerol-3-phosphate by glycerol-3-phosphate O-acyltransferase (EC

2.3.1.15), but no candidate gene for this enzyme has been found in the genome.

There are, however, two potential 1-acylglycerol-3-phosphate O-

acyltransferases (EC 2.3.1.51) that could attach the second acyl group. After the

attachment of the acyl groups, and in preparation for the attachment of the

activated aminoalcohols, the phosphate is removed by phosphadidate

phosphatase (EC 3.1.3.4), for which there is one gene, resulting in a

diacylglycerol.

The activation of ethanolamine (EC 2.7.1.82) or choline (EC 2.7.1.32) for

attachment to the phosphadidate starts with phosphorylation. There are two

genes identified as choline/ethanolamine kinases that share 37% amino acid

identity. Next, ethanolamine phosphate and choline phosphate are converted to

1   CDP-ethanolamine (EC 2.7.7.14) and CDP-choline (EC 2.7.7.15), respectively.

2   The genome encodes two enzymes sharing 57% sequence identity that are

3   identified as ethanolamine-phosphate cytidylyltransferases. The substrate

4   specificity of these enzymes needs to be examined on the biochemical level.

5   Finally the activated ethanolamine or choline is attached to diacylglycerol by

6   the enzymes ethanolaminephosphotransferase (EC 2.7.8.1) or diacylglycerol

7   cholinephosphotransferase (EC 2.7.8.2) producing phosphatidylethanolamine or

8   phosphatidylcholine, respectively. For these activities a total of 8 possible

9   genes are found that share varying degrees of sequence similarity.

10

11   In *E. histolytica,* an alternative pathway of phospholipid biosynthesis could

12   involve the biosynthesis of phosphatidylserine. In this pathway, the

13   phosphatidate itself is activated by CTP in a reaction catalysed by

14   phosphatidate cytidylyltransferase (EC 2.7.7.41) resulting in CDP-

15   diacylglycerol. Three genes have been identified. Phosphatidylserine synthase

16   then catalyses the reaction of CDP-diacylglycerol with serine to give

17   phosphatidylserine (EC 2.7.8.8); one gene has been found.

18

19   Some organisms can form phosphatidylethanolamine from phosphatidylserine

20   using a decarboxylase, but such an enzyme appears to be absent from the *E.*

21   *histolytica* genome. There are, however, several candidate methyltransferases of

22   yet unknown substrate specificity, which might be able to generate

23   phosphatidylcholine from phosphatidylethanolamine.

24

25   Taken together, large portions of the pathways needed to generate the most

26   important phospholipids can be assembled from genes tentatively identified to

27   date in the *E. histolytica* genome. The first acylation of glycerol 3-phosphate to

28   lysophosphatidate remains an important gap. As *E. histolytica* could potentially

29   aquire all the necessary phospholipids from the host, the functional relevance of

30   the described biosynthetic pathways may not be high.

31

1 Finally, two additional interesting enzymes present in *E. histolytica* should be

2 mentioned. The first was previously characterised using cDNA sequences and

3 recombinant proteins as L-myo-inositol 1-phosphate synthase (EC 5.5.1.4;

4 Lohia *et al.*, 1999). This enzyme catalyses the complicated isomerisation of

5 glucose 6-phosphate to L-myo-inositol 1-phosphate. Inositol is found in

6 phosphatidylinositol and in glycosylphosphatidylinositol- (GPI-) anchors of

7 some membrane proteins, as well as playing a major role in signal transduction

8 via the secondary messenger 1,4,5-inositol trisphosphate. There are three myo-

9 inositol 1-phosphate synthase genes, all highly similar to each other and to the

10 previously sequenced cDNA.

11

12 The second is phospholipid-cholesterol acyltransferase (EC 2.3.1.43), which

13 transfers an acyl group from phospholipids such as phosphatidylcholine to

14 cholesterol giving a cholesterol ester. The genome contains 7 genes for this

15 enzyme. So far nothing is known about the importance of cholesterol esters for

16 *E. histolytica.*

17

18 4.5.2 (b) Phospholipid degradation

19 Phospholipids are degraded by phospholipases. Whereas phospholipases A1

20 (EC 3.1.1.32) and A2 (EC 3.1.1.4) cleave the acyl residues in the 1 or 2 position

21 of the glycerol core, phospholipases C (EC 3.1.4.3) and D (EC 3.1.4.4) cleave

22 at the phosphate, phospholipase C on the glycerol side, and phospholipase D on

23 the aminoalcohol side. In *E. histolytica* phospholipase A activity has been

24 implicated in virulence (Ravdin *et al.*, 1985) as it liberates toxic fatty acids and

25 lysophospholipids (Said-Fernandez and Lopez-Revilla, 1988). Phospholipases

26 A have been found in two forms, a membrane-bound Ca-dependent form active

27 at alkaline pH and a soluble Ca-independent form active at acid pH (Long-Krug

28 *et al.*, 1985; Vargas-Villarreal *et al.*, 1998). The genome encodes 11 potential

29 phospholipases A with predicted pI values between 4.8 and 8.8 and various

30 degrees of sequence similarity. In addition the *E. histolytica* genome encodes 3

31 potential phospholipases D.

Finally, there are 2 highly similar genes for phospholipases C, but these are homologous to phosphatidylinositol-specific phospholipases C (EC 3.1.4.11) and most likely do not cleave phosphatidylinositol or phosphatidylcholine but GPI-anchors instead. So far there are no studies using individual recombinant phospholipases, and it is not yet known how much these enzymes may contribute to the virulence of *E. histolytica*.

4.6 Coenzyme A Biosynthesis and Pantothenate Metabolism

Analysis of the genome revealed a complete lack of known folate-dependent enzymes and folate transporters, suggesting this cofactor is not utilised by *E. histolytica*. This is at odds with a study on the nutritional requirements of *E. histolytica* in which folate was found to be essential for growth (Diamond and Cunnick, 1991). More experimental research will be needed to resolve this discrepancy. Most organisms require folate as a cofactor for several reactions of amino acid metabolism and for synthesis of thymidylate, a component of DNA. The microsporidian *Encephalitozoon cuniculi*, which possesses the smallest known eukaryotic genome, still contains a folate transporter and several folate-dependent enzymes (Katinka *et al.*, 2001). In eukaryotes possessing mitochondria or chloroplasts, folate is required for the formylation of methionine on the initiator tRNA used for organelle protein synthesis. Although *E. histolytica* possesses a mitochondrion-derived organelle, the mitosome, there is no organellar genome (Leon-Avila and Tovar, 2004) and so no need for organellar protein synthesis. The most important metabolic consequences of the loss of folate metabolism for *E. histolytica* are therefore the absence of thymidylate synthesis and methionine recycling, although it remains possible that *E. histolytica* possesses folate-independent enzymes carrying out these steps.

Phosphopantothenoyl-cysteine decarboxylase (EC 4.1.1.36) and phosphopantothenoyl-cysteine synthetase (EC 6.3.2.5, synonymous with

phosphopantothenate-cysteine ligase) exist as a fusion protein in *E. histolytica,* as in Bacteria and Archaea. The amino- and carboxyl-terminal domain possesses decarboxylase and synthetase activity, respectively (Kupke, 2002; Kupke, 2004; Kupke *et al.*, 2000; Strauss *et al.*, 2001). The role of this enzyme in coenzyme A biosynthesis is not well understood in *E. histolytica* as the other necessary enzymes are absent.

4.7 Nucleic Acid Metabolism

Like many protistan parasites, *E. histolytica* lacks *de novo* purine synthesis (Reeves, 1984). The genome reveals that nucleic acid metabolism of *E. histolytica* is similar to that of the other lumenal parasites *G. intestinalis* and *T. vaginalis* in lacking pyrimidine synthesis and thymidylate synthase (Aldritt *et al.*, 1985; Wang and Cheng, 1984). In addition *E. histolytica* appears to lack ribonucleotide reductase, a characteristic shared with *G. intestinalis* (Baum *et al.*, 1989). Ribonucleotide reductase was found, however, in genomic sequences of the species *E. invadens* and *E. moshkovskii*, indicating that the enzyme was lost or replaced relatively recently. Among eukaryotes, the loss of these areas of nucleic acid metabolism is otherwise rare. The enzymes were likely lost during adaptation to living in an organic nutrient rich environment.

4.8 Missing Pieces

Several important enzymes and pathways could not be found within the genome and their presumed sequence divergence from known enzymes and pathways labels them as possible drug targets once they are identified. Phosphopyruvate carboxylase, which reversibly converts phosphoenolpyruvate to oxaloacetate, is a central enzyme of carbon metabolism in *E. histolytica* (Reeves, 1970), but could not be identified. Isoprenyl-PP synthesis and aminoethylphosphonate synthesis are also likely to be present but no candidate genes could be identified.

4.9 Transporters

A total of 174 transporters were identified within the genome, a number

intermediate between the 62 transporters of *P. falciparum* and the 286

transporters of *S. cerevisiae* (membranetransport.org). *E. histolytica* has a

number of ion transporters similar to those of yeast, but fewer identifiable

nutrient and organellar transporters. *Plasmodium* and *Entamoeba* both have

reduced metabolisms and take up many complex nutrients. The higher number

of transporters in *Entamoeba* suggests that they may be more substrate specific

than the *Plasmodium* transporters or that they may have a higher level of

redundancy.

Since glucose transport activity has been experimentally characterised in *E.*

histolytica and glucose is thought to be the major energy source, it was

surprising to find no homologues of known hexose transporters in the genome.

Most hexose transporters belong to the sugar porter subfamily of the major

facilitator superfamily (TC 2.A.1.1), members of which are found in

prokaryotes, animals, fungi, plants, and other protists, including *D. discoideum*,

but no proteins of this family were found in the *E. histolytica* genome. A group

of candidate monosaccharide transporters found within the genome is related to

the glucose/ribose porter family from prokaryotes (TC 2.A.7.5). These

transporters consist of two related domains, and the *Entamoeba* proteins appear

to have the N-terminal and C-terminal domains switched relative to the

bacterial proteins. Functional characterisation of transporter-encoding genes

will be necessary for a more complete picture.

5. THE CYTOSKELETON

The eukaryotic cytoskeleton is composed of three main elements: actin

microfilaments, tubulin-based microtubules and intermediate filaments. Despite

the fact that *E. histolytica* is very motile and performs phagocytosis very

efficiently, its cytoskeletal components are rather simple. No genes encoding

homologues of intermediate filament network proteins, including keratins,

desmin and vimentin, have been identified in *E. histolytica*, providing further

evidence that these particular cytoskeletal components are rather poorly conserved in evolution. In contrast, microfilament and microtubule components have been readily identified.

5.1 Actin and Microfilaments

Genome information suggests that *E. histolytica* has a greater dependence than other protists on an actin-rich cytoskeletal network. Microfilament proteins are represented by actin and several actin-binding proteins, although there are notable differences with respect to analogous proteins in other eukaryotes. There are eight actin genes in the *E. histolytica* genome, in addition to six others that encode divergent actins. Three divergent actins surprisingly contain an extra N-terminal domain with as yet unknown functional characteristics. Examples of hybrid actins are rather scarce and have been found as ubiquitin fusions (Archibald *et al.*, 2003). The functional significance of these *E. histolytica* hybrid actins is as yet unknown.

Under physiological salt concentrations, monomeric actin assembles into polymers of F-actin, thus building microfilaments. Actin assembles and disassembles in an extremely dynamic and highly controlled process which is dependent on many different actin-binding proteins (Winder and Ayscough, 2005). The *E. histolytica* genome encodes homologues of actin-binding proteins involved in the severing, bundling, cross-linking and capping of filamentous actin. The number and variety of actin-binding proteins support the view that the actin-rich cytoskeleton is very dynamic in *E. histolytica*.

Since the spontaneous polymerisation of actin monomers is inhibited by the action of sequestering proteins such as thymosin $\beta4$ and profilin, efficient actin polymerisation requires the intervention of an actin polymerisation-promoting factor. The best described promoting factors are the Arp2/3 complex and the formin protein family.

1   The Arp2/3 complex is composed of two actin-related proteins (Arp2 and Arp3,

2   which act as a template for new actin filaments) and works in conjunction with

3   five additional subunits: ARPC1 -to 5 (Vartiainen and Machesky, 2004). All

4   subunits have been clearly identified in the *E. histolytica* genome, and among

5   these the Arp2 and Arp 3 subunits are the best conserved. The Arp2/3

6   complex's ability to nucleate new actin filaments is stimulated by its interaction

7   with nucleation promoting factors such as the Wiskott-Aldrich Syndrome

8   protein (WASP) or the suppressor of cAMP-receptor (SCAR) factor.

9   Surprisingly, no proteins with homology to WASP/SCAR components were

10   found in the genome, suggesting that actin nucleation depends on the activity of

11   other, as yet unidentified proteins.

12

13   In contrast, *E. histolytica* possesses six genes coding for formins, which have

14   emerged as potent regulators of actin dynamics in eukaryotic cells through their

15   ability to increase actin filament assembly (Higgs and Peterson, 2005). Formins

16   control rearrangements of the actin cytoskeleton, especially in the context of

17   cytokinesis and cell polarisation. Members of this family have been found to

18   interact with Rho-GTPases, profilin and other actin-associated proteins. The

19   precise nature of this polymerisation-accelerating activity differs from one

20   formin to another: some nucleate filaments *de novo*, some require profilin for

21   effective nucleation, while yet others seem to use filament severing as their

22   basic mechanism. However, the Formin Homology 2 Domain (FH2, comprising

23   roughly 400 amino acids) is central to formin activity (Otomo *et al.*, 2005; Xu

24   *et al.*, 2004). Actin nucleation by formins is thought to occur by stabilisation of

25   an unfavourable nucleation intermediate, possibly through FH2 domains

26   binding to monomers in the same manner that they bind to barbed ends (an

27   activity influenced by profilin). The formin homologues from *E. histolytica* all

28   contain an FH2 domain, suggesting that they are potential actin nucleation

29   factors.

30

1 Once nucleated, actin filaments are able to grow rapidly by addition of

2 monomers at their barbed ends. Filaments are regulated by several mechanisms

3 (Winder and Ayscough, 2005). Filament length is controlled by capping

4 proteins: barbed end cappers (such as capping protein and gelsolin) block

5 addition of new monomers and thus act to decrease the overall length of the

6 filament. In addition, gelsolin severs actin filaments, thereby rapidly increasing

7 actin dynamics. Actin filaments appear to be significantly shorter in *E.*

8 *histolytica* when compared with those from fibroblasts and stress fibres are not

9 formed in this amoeba. Although *E. histolytica* actin has been shown not to

10 bind DNase I (Meza *et al.*, 1983), the inferred amino acid sequence indicates

11 conservation of all the residues likely to participate in this binding event -

12 suggesting that post-translational modifications of actin monomers may prevent

13 DNAse I-actin binding. It remains to be determined whether such modifications

14 of actin participate in the regulation of actin polymerisation. The genome

15 encodes multiple genes associated with filament capping and severing, as well

16 as candidates for proteins that cross-link actin filaments and thus organise them

17 into a supramolecular network. The organisation of actin into networks and

18 higher-order structures is crucial for both cell shape and function. These

19 structures can be responsible for overall cell shape and related processes, such

20 as bundle formation through α-actinin activity, for example. The arrangement

21 of actin filaments into cross-linked arrays is also mediated by proteins with

22 multiple actin-binding domains, which allows a more perpendicular

23 arrangement of actin filaments. Examples of this type of protein are the large,

24 flexible filamin dimer (Vargas *et al.*, 1996) and the spectrin tetramer. Genome

25 analysis has now identified many candidate genes for actin-binding proteins in

26 *E. histolytica*, and additional protein partners of this versatile family

27 responsible for cytoskeleton regulation are likely to emerge from curation of the

28 sequence and cellular studies of cell motility and phagocytosis in this parasite.

29

5.2 Tubulins and microtubules

E. histolytica has a lower dependence on a tubulin-based cytoskeleton than most other eukaryotic cells. Protein homologues of the basic (α, β and γ) tubulins are present, although other tubulins more characteristic of organisms with basal bodies and flagella (e.g.: ϵ- and δ-tubulins) are absent from *E. histolytica* (Dutcher, 2001). Nine different tubulins (grouped into multigene families) exist in most eukaryotic cells. Microtubules (MTs) composed of α- and β- tubulin are intranuclear in *E. histolytica* (Vayssie *et al.*, 2004), and this raises the question of how such structures are modulated within the nucleus, given that MT dynamics require MT nucleation-based renewal at the minus end and MT capping at the plus end. Proteins involved in MT nucleation act in concert with γ-tubulin (which is also intra-nuclear in *E. histolytica*), and this parasite possesses at least one homologue to the Spc98 factor, a component of the MT-nucleating Tub4p-γ tubulin complex. In contrast, no homologues of EB1, CLIP-170, APC (all involved in MT capping) or centrins (which operate at the MT organising centre) have yet been identified, suggesting that other factors (or mechanical constraints within the MT) may be required in blocking MT growth. *E. histolytica* does encode candidate proteins involved in MT severing or chromosome segregation. All these proteins are good candidates for experimental analysis of the mechanisms of intranuclear MT localisation and turnover, as well as of the trafficking of tubulins between the cytoplasm and nucleus.

There is little information available on the precise organisation of microtubules and F-actin cytoskeleton during *E. histolytica* motility. In many eukaryotic cells, F-actin-microtubule interactions can be observed in lamellipodia at all stages. Interestingly, microtubules preferentially grow along actin bundles in filopodia, suggesting that a physical link between the structures exists (Leung *et al.*, 2002). Multifunctional MT-associated proteins (MAPs, like MAP1B, MAP2 and plakins) are promising candidates for acting as such links, either via dimerisation of MAPs with single microtubule and actin binding sites or by

direct bridging of the two cytoskeletons (for example via plakins, which contain binding sites for both microtubules and actin within a single molecule). Plakin homologues have not been identified in the *E. histolytica* genome but a MAP is present. Furthermore, proteins with domains that can bind to actin (and potentially to MT) have been described in *E. histolytica* - the ABP-120 gelation factor, for example (Vargas *et al.*, 1996).

5.3 Molecular motors

The distribution of intracellular factors and vesicles is performed using three sets of molecular transporters: myosin along microfilaments and kinesin and dynein along MTs. Although *E. histolytica* is a highly motile cell, stress fibres and cytoplasmic MTs have never been observed. The fluidity of the parasite's cytoplasm may be related to features of its molecular motors some of which are very surprising. The myosin family of actin filament-based molecular motors consists of at least 20 structurally and functionally distinct classes. The human genome contains nearly 40 myosin genes, representing 12 of these classes. Remarkably, *E. histolytica* is the first reported instance of a eukaryote with only one unconventional myosin. This myosin heavy chain (myosin IB) belongs to the type I myosin family, of which 12 are present in the *Dictyostelium* genome (Eichinger *et al.*, 2005).

All members of the myosin family share a common structure composed of three modules: the head, neck and tail domains. The N-terminal region harbours the motor unit, which uses ATP to power movement along the actin filaments. By interacting with specific proteins and 'cargoes', the tail is responsible for the myosin's specific function and location. In particular, the presence of an SH3 domain in the tail region is important for linking these myosin I molecules with the endocytic machinery and the Arp2/3 complex. Protistan class I myosins are able to recruit the Arp2/3 complex towards the CARMIL adapter protein and Acan125. These homologous adapters consist of multiple, leucine-rich repeat sequences and bear two carboxyl-terminal polyproline motifs that are ligands

for the myosin I SH3 domains. CARMIL has been shown to bind the Arp2/3 complex via an acidic motif similar to those found in WASP. In view of the fact that *E. histolytica* does not have WASP homologues, the discovery of a CARMIL homologue through proteomic analysis of *E. histolytica* phagosomes (Marion et al, 2005) provides an important clue for understanding actin nucleation in *E. histolytica*. Interestingly, myosin IB in *E. histolytica* plays a structural role in the actin network, due to its ability to cross-link filaments (Marion *et al.*, 2004). The cytoskeletal structuring activity of myosin IB regulates the gelation state of cell cytoplasm and the dynamics of cortical F-actin during phagocytosis.

The most studied myosin has been the conventional or class II myosin. This double-headed molecule is composed of two heavy chains and two pairs of essential and regulatory light chains. The heavy chain tail consists of an α-helical, coiled coil protein able to form a parallel dimer that in turn can self-associate into bipolar, thick filaments. This enables myosin II to operate in huge filament arrays, which drive high speed motility. In addition to myosin IB, *E. histolytica* also has a conventional myosin II heavy chain (very closely related to its homologue in *Dictyostelium*) which has been reported to be involved in crucial phases of parasite motility, surface receptor capping and phagocytosis (Arhets *et al.*, 1998). *E. histolytica*'s sole isoform shapes the actin network and maintains cytoskeletal integrity. Candidate genes for the regulatory and essential light chain activities were also found, and these possess the EF hand domains necessary for Ca^{2+} binding.

Directional transport along the MTs depends on dynein and kinesin, both MT-associated motor proteins which convert the chemical energy from ATP hydrolysis into movement. These motors are unidirectional and move towards either the MT plus- or minus- ends (Mallik and Gross, 2004). Kinesins and dyneins have been implicated in a wide range of functions - principally intracellular organelle transport during interphase and spindle function during

mitosis and meiosis. Members of the dynein family are minus-end directed, although this remains to be confirmed for a few uncharacterised, vertebrate, cytoplasmic dynein heavy chains. It has not yet been reliably established that the *E. histolytica* genome contains a dynein heavy chain gene, although a dynein light chain gene is present: improvements in gene assembly should provide us with more information on this high molecular mass protein.

Kinesins are microtubule-dependent molecular motors that play important roles in intracellular transport and cell division. Even though the motor domain is found within the N-terminus in most kinesins (N-type), it is located within the middle or C-terminal domains in some members of the family (M-type and C-type kinesins, respectively) (Asbury, 2005). The position of the motor domain dictates the polarity of the movement of kinesin along the MT: whereas N- and M-type kinesins are plus-end directed, the C-type kinesins are minus-end directed. Humans possess 31 different kinesins and trypanosomes have more than 40. The *E. histolytica* genome sequence predicts only six kinesin-encoding genes (four N-type, two C-type and no M-type homologues have been found). One of the N-kinesins also contains a domain homologous to the HOOK protein required for the correct positioning of microtubular structures within the cell (Walenta *et al.*, 2001). Bearing in mind that *E. histolytica* MTs are intranuclear, the study of kinesin function and trafficking should help us elucidate what is likely to be a very interesting MT functional mechanism.

6. VESICULAR TRAFFIC

The requirement for nutritional uptake from the extracellular milieu in the host intestine imposes a heavy reliance on endocytic and phagocytic activities in *Entamoeba* (Espinosa-Cantellano and Martínez-Palomo, 2000). Proliferating trophozoites secrete a number of peptides and proteins including cysteine proteases (Que and Reed, 2000) and amoebapores (Leippe, 1999) required for bacterial cell killing and degradation as well as being implicated in virulence (Petri, 2002). During encystation, the cells also secrete substrates used for the

formation of the cyst wall (Eichinger, 1997). Electron micrographic studies have revealed a complex membrane organisation. The trophozoites contain numerous vesicles and vacuoles varying in size and shape (Clark *et al.*, 2000; Mazzuco *et al.*, 1997). Intracellular transport of both endocytosed and synthesised molecules between compartments is regulated by the elaborate orchestration of vesicle formation, transport, docking and fusion to the target compartment (Bonifacino and Glick, 2004; Kirchhausen, 2000).

6.1 Complexity of Vesicle Trafficking

Among a number of molecules and structures involved in vesicular trafficking, three types of coated vesicles, named coatomer protein (COP) I, COPII, and clathrin-coated vesicles are the best characterised (Bonifacino and Glick, 2004; Kirchhausen, 2000). COPI vesicles primarily mediate transport from the Golgi to the endoplasmic reticulum (ER) and between the Golgi cisternae, while COPII vesicles are involved in the transport from the ER to the *cis*-Golgi The clathrin-dependent pathway has a few independent routes: from the plasma membrane to endosomes, from the Golgi to endosomes, and from endosomes to the Golgi. It has been well established that certain subfamilies of Ras-like small GTPases, widely conserved among eukaryotes, regulate both the formation of transport vesicles and their docking and fusion to the target organelles. The ADP-ribosylation factor (Arf) and secretion-associated Ras-related protein (Sar) families of GTPases regulate the formation of COPI and COPII vesicles (Memon, 2004), respectively. In contrast, the Rab family of GTPases (Novick and Zerial, 1997) is involved in the targeting and fusion of vesicles to the acceptor organelles together with the tethering machinery SNARE (a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) (Chen and Scheller, 2001). Since individual coat proteins, small GTPases, SNAREs, and their associated proteins show distinct intracellular distributions in both unicellular and multicellular organisms, they are believed to play a critical role in the determination of membrane trafficking specificity (Chen and Scheller, 2001; Munro, 2004; Novick and Zerial, 1997). It is generally believed that the

1   total number of proteins involved in the membrane traffic reflects the

2   complexity and multiplicity of its organism. The total number of the putative

3   amoebic genes encoding Arf/Sar, Rab, SNARE, and coat proteins together with

4   those from *S. cerevisiae, C. elegans, D. melanogaster, H. sapiens,* and *A.*

5   *thaliana*, is shown in Table 7. *E. histolytica* reveals complexity similar to yeast,

6   fly, and worm in case of Sar/Arf and SNAREs, while the number of genes

7   encoding three coat proteins (COPI, COPII, and Adapter Proteins (APs)) was

8   higher in *E. histolytica* than these organisms and comparable to that in

9   mammals and plants. In contrast, the number of Rab proteins in *E. histolytica* is

10   exceptionally high, exceeding that in mammals and plants.

11

12   **6.2 Proteins Involved in Vesicle Formation**

13   *6.2.1 COPII-coated vesicles and Sar1 GTPase*

14   COPII components were originally discovered in yeast using genetic and

15   biochemical approaches (reviewed in Bonifacino and Glick (2004)). COPII

16   vesicles mediate the transport from the ER to the Golgi and consists of three

17   major cytosolic components and a total of five essential proteins: the Sec23p-

18   Sec24p complex, the Sec13p-Sec31p complex, and the small GTPase Sar1p

19   (Barlowe *et al.*, 1994). Sar1p and Sec23p-Sec24p complex are involved in the

20   formation of the membrane-proximal layer of the coat, while Sec13p-Sec31p

21   complex mediates the formation of the second membrane-distal layer (Shaywitz

22   *et al.*, 1997). These proteins are well conserved among various organisms

23   (Table 7). *E. histolytica* encodes one each of Sar1, Sec13 and Sec31, two of

24   Sec23, and five proteins corresponding to Sec24 (Table 7). The yeast and

25   human genomes also encode multiple Sec24 isotypes (3 and 4, respectively).

26   Although Sec24 isotypes have been shown to be responsible for the selection of

27   transmembrane cargo proteins in yeast (Peng *et al.*, 2000; Roberg *et al.*, 1999),

28   the significance of the Sec24 redundancy in *E. histolytica* is not clear.

29   Additional regulatory proteins participate in COPII assembly in yeast, including

30   Sec16p, a putative scaffold protein (Espenshade *et al.*, 1995), and Sec12p, a

1 guanine nucleotide exchange factor (GEF) for Sar1p (Barlowe and Schekman,

2 1993). Homologues of Sec12p and Sec16p appear to be absent in *E. histolytica.*

3 The p24 protein is a non-essential component of vesicle formation (Springer *et*

4 *al.*, 2000) and in yeast it functions as a cargo adaptor through binding to Sec23p

5 (Kaiser, 2000; Schimmoller *et al.*, 1995). *E. histolytica* encodes four p24

6 proteins, fewer than in yeast and humans which have eight. GTPase-activating

7 protein (GAP) Sec23p is also present in *E. histolytica*; this activates the

8 intrinsic GTPase activity of Sar1p after the formation of COPII vesicle, and

9 inactivates the function of Sar1p (Yoshihisa *et al.*, 1993), resulting in the

10 uncoating of COPII vesicles.

11

12 *6.2.2 COPI-coated vesicles and Arf GTPases*

13 COPI-coated vesicles, which mediate transport from the Golgi to the ER and

14 between the Golgi cisternae (Kirchhausen, 2000), consist of seven proteins (α,

15 β, β', γ, δ, ϵ, and ζ-COP) (Hara-Kuge *et al.*, 1994). The number of proteins

16 making up the COPI coat, and thus the complexity of COPI components, varies

17 among organisms (Table 7). While human possesses two isotypes of γ-COP and

18 ζ-COP, yeast has a single gene for each. In humans, the two isotypes of γ-COP

19 and ζ-COP form three different COPI complexes ($\gamma1/\zeta1$, $\gamma1/\zeta2$, and $\gamma2/\zeta1$),

20 which have different intracellular distributions (Wegmann *et al.*, 2004). This

21 implies that COPI-coated vesicles are also involved in functions other than

22 Golgi-to-ER transport (Whitney *et al.*, 1995). In *E. histolytica,* the COPI

23 complex appears more heterogeneous: *E. histolytica* encodes two isotypes each

24 of γ-COP, δ-COP, and α-COP and three isotypes of β-COP. In contrast, *E.*

25 *histolytica* lacks ϵ-COP, which is known to stabilise α-COP (Duden *et al.*,

26 1998). It has been shown in yeast that all genes encoding components of COPI

27 coat except for Sec28p, the yeast ϵ-COP homologue, are essential for growth

28 (Duden *et al.*, 1998).

29

Recruitment of COPI to the Golgi membrane requires the association of a GTP-bound GTPase called Arf (Donaldson *et al.*, 1992; Kahn *et al.*, 2006). Arf was initially identified due to its ability to stimulate the ADP-ribosyltransferase activity of cholera toxin A (Kahn and Gilman, 1984). To recruit the COPI coat, Arfs are activated by a Sec7 domain-containing protein, Arf-GEF, which is a target of a fungal metabolite brefeldin A (Helms and Rothman, 1992; Sata *et al.*, 1998). Among Arf family proteins, Arf1 is involved in the formation of COPI-coated vesicles in the retrograde transport from the Golgi to ER, and is also involved in the assembly of clathrin-AP1 (see next section) on the *trans*-Golgi network (TGN) (Stamnes and Rothman, 1993), clathrin-AP3 on endosomes (Ooi *et al.*, 1998), and the recruitment of AP-4 to the TGN (Boehm *et al.*, 2001). The specific roles of Arfs3-5 are less clear, although Arf4 and Arf5 show *in vitro* activities similar to Arf1. Functional cooperativity of Arfs in the vesicular formation has also been demonstrated recently. At least two of four human Arf isotypes (Arf1, Arf3-5) are essential for a retrograde pathway from the Golgi to the ER, in the secretory pathway from the Golgi to the TGN, and in the recycling from endosomes to the plasma membrane (Volpicelli-Daley *et al.*, 2005). In contrast to these Arfs, Arf6 regulates the assembly of actin filaments and is involved in endocytosis on the plasma membrane (Radhakrishna and Donaldson, 1997).

GTPases that share significant similarity to Arf, but do not either activate cholera toxin A or rescue *S. cerevisiae Arf* mutants are known as Arls (Arf-like GTPases) (Lee *et al.*, 1997a). Arl1 is involved in endosome-to-Golgi trafficking (Lu *et al.*, 2001; Lu *et al.*, 2004). Other Arls (Arls 2-11) and Arf-related proteins (Arp or ArfRP 1-2) have been localised to the cytosol, nucleus, cytoskeleton and mitochondria (Burd *et al.*, 2004; Pasqualato *et al.*, 2002). The number of Arf, Arl, and Arf-related proteins varies among organisms (Table 7). Among 27 members identified in humans, only about a half dozen Arf/Arl/Arp proteins, including Arf1-6 and Arl1 (Wennerberg *et al.*, 2005), have been

1   shown to function in membrane traffic (Lu *et al.*, 2001). The localisation and

2   function of the remaining Arf/Arl/Arp remained unclear.

3

4   *E. histolytica* encodes ten Arf/Arl proteins (Table 7). Only two *E. histolytica*

5   Arfs (A1 and A2) have a high percentage identity to human Arfs 1, 3, 5, and 6

6   and yeast Arfs 1-3 (57-76% identity), while the remaining eight Arf/Arl fall

7   into three groups (A4-6, B1-3, and C) and are equally divergent from one

8   another and from other organisms. Both the intracellular distributions and the

9   specific steps in vesicular trafficking mediated by these *Entamoeba* Arf/Arl

10   proteins are unknown. It is worth noting that five of these Arfs lack a conserved

11   glycine residue at the second amino acid position of the amino terminus; this

12   glycine is known to be myristylated and essential for membrane association in

13   other organisms (Randazzo *et al.*, 1995). *Eh*ArfA4 also lacks one of the

14   conserved GTP-binding consensus regions (Box2). Similar deletion of GTP-

15   binding domains has also been observed in proteins belonging to the Rab

16   family (see section 6.3.1).

17

18   *6.2.3 Clathrin-coated vesicle and its adaptor proteins*

19   Clathrin-coated vesicles and pits, as demonstrated by electron microscopy, are

20   often indicative of clathrin-mediated endocytosis. However, there is no clear

21   ultrastructural evidence for their occurrence in *Entamoeba* (Chavez-Munguia *et*

22   *al.*, 2000). Interestingly, heavy- but not light-chain clathrin is encoded in the

23   genome. Since a majority of proteins, including adaptor proteins (APs,

24   Adaptins), known to be involved in the assembly of clathrin-coated vesicles are

25   encoded in *E. histolytica*, the fundamental mechanisms and components of

26   clathrin-mediated endocytosis are probably present in this organism, but are

27   likely to be divergent from other eukaryotes. AP is a cytosolic heterotetramer

28   that mainly mediates the integration of membrane proteins into clathrin-coated

29   vesicles in the secretory and endocytic pathways (Boehm and Bonifacino, 2001;

30   Kirchhausen, 2000). AP is composed of two large, one medium, and one small

1   subunit (Keen, 1987). Four major types of AP complexes (AP1-4) have been

2   identified (Boehm and Bonifacino, 2001; Nakatsu and Ohno, 2003). AP-2

3   (consisting of α, $\beta2$, $\sigma2$, and $\mu2$) mediates endocytosis from the plasma

4   membrane (Conner and Schmid, 2003; Motley *et al.*, 2003), while AP-1

5   (γ, $\beta1$, $\sigma1$, and $\mu1A$) (Meyer *et al.*, 2000), AP-3 (δ, $\beta3A$, $\sigma3$, and $\mu3A$) (Le

6   Borgne *et al.*, 2001; Vowels and Payne, 1998), and AP-4 (ϵ, $\beta4$, $\sigma4$, and $\mu4$)

7   (Aguilar *et al.*, 2001), play a role in the Golgi-endosome, endosomal-lysosomal,

8   or the Golgi/lysosome sorting pathway, respectively. AP-4, which is present

9   only in mammals and plants (Boehm and Bonifacino, 2001), was also identified

10   in non-clathrin-coated vesicles mediating the transport from TGN to the plasma

11   membrane or endosomes (Hirst *et al.*, 1999). A few isotypes of AP-1 and AP-3,

12   e.g., AP-1B (γ, $\beta1$, $\sigma1$, and $\mu1B$) and AP-3B (δ, $\beta3B$, $\sigma3$, and $\mu3B$), showed

13   tissue specific expression (Faundez *et al.*, 1998; Folsch *et al.*, 1999). *E.*

14   *histolytica* encodes ten large subunits (α, β, γ, δ, and ϵ), four medium

15   subunits (one each of $\mu1$ and $\mu2$, and two $\mu3$), and four small subunits ($\epsilon1-\epsilon4$).

16   This suggests that *E. histolytica* produces four types of AP complex, as in

17   humans and plants.

18

19   **6.3 Proteins Involved in Vesicle Fusion**

20   *6.3.1 Rab GTPases*

21   The docking and fusion of transport vesicles to a specific target compartment

22   requires the appropriate Rab protein. Specific interaction of a Rab with its

23   effector molecules in conjunction with the interaction between SNAREs plays a

24   central role in vesicle fusion (Zerial and McBride, 2001). In general, the

25   complexity of the Rab gene family correlates with the degree of

26   multicellularity. For example, *S. pombe, S. cerevisiae, C. elegans*, *D.*

27   *melanogaster*, and *H. sapiens* consist of one, one, ca. 10^3, 10^9, and 10^{13} cells,

28   and have 7, 11, 29, 29, and 60 Rab genes, respectively (Pereira-Leal and

29   Seabra, 2001). It has been also shown that in multicellular organisms, Rab

30   proteins are expressed in a highly coordinated (i.e. tissue-, organ-, or

developmental stage-specific) fashion (Seabra *et al.*, 2002; Zerial and McBride, 2001). *E. histolytica* possesses an extremely high number of Rab genes - 91 (Figure 6). Among its 91 Rabs only 22, including *Eh*Rab1, *Eh*Rab2, *Eh*Rab5, *Eh*Rab7, *Eh*Rab8, *Eh*Rab11, *Eh*Rab21, and their isotypes showed >40% identity to Rabs from other organisms. The 69 remaining *E. histolytica* Rab proteins showed only moderate similarity (<40% identity) and represent unique, presumably *Entamoeba*-specific, Rab proteins. Approximately one third of Rab proteins form 15 subfamilies, including Rab1, Rab2, Rab7, Rab8, Rab11, and RabC-P, each of which contains up to 9 isoforms. Interestingly, approximately 70% of *E. histolytica* Rab genes contain one or more introns (Saito-Nakano *et al.*, 2005). SNARE genes are also intron-rich whereas the Sar/Arf GTPase and the three coat protein genes have a low frequency of introns. The high frequency of introns in the Rab and SNARE gene families may indicate the presence of post-transcriptional regulation of these genes.

Although Rab proteins generally possess a CXC or CC at the carboxyl terminus, twenty-five *E. histolytica* Rabs have an atypical carboxyl terminus, such as CXXX, XCXX, XXCX, XXXC, or no cysteine at all. The enzyme(s) involved in the lipid modification of these unusual Rab proteins remain poorly understood (see 4.5.1 (a)). It is also worth noting that >20 *E. histolytica* Rab lack or contain only a degenerate form of the consensus sequence for structural elements such as the GTP-binding regions and the Switch I and II regions, implicated in the binding to GEF, GAP, effectors, or guanine nucleotides (Saito-Nakano *et al.*, 2005). These non-conventional *Eh*Rabs are not pseudogenes since at least some of the genes are known to be expressed as mRNA (Saito-Nakano *et al.*, 2001). It has been shown that neither *Eh*Rab5 nor *Eh*Rab7A rescued the corresponding yeast mutant (Saito-Nakano *et al.*, 2004). Therefore, many, if not all, *E. histolytica* Rabs may have lost functional interchangeability with their homologues in other organisms despite the relatively high percentage of sequence identities. Classification and annotation

1   of the *E. histolytica* Rab proteins has been previously described (Saito-Nakano

2   *et al.*, 2005).

3

4   One of the peculiarities of *E. histolytica* Rab proteins was demonstrated by the

5   unprecedented function of *Eh*Rab7A, which plays an important role in the

6   transport of cysteine proteases via interaction with the retromer complex. The

7   *E. histolytica* retromer complex consists of three components, Vps26, Vps29

8   and Vps35, rather than the 4-5 found in yeast and mammals (Nakada-Tsukui *et*

9   *al.*, 2005). Homologues of Vps5, Vps17, and sorting nexins are not encoded in

10   the genome. It has been suggested that the *Eh*Rab7A-retromer interaction,

11   mediated by direct binding of *Eh*Rab7A to a unique carboxyl-terminal region of

12   Vps26, regulates intracellular trafficking of cysteine proteases, and possibly

13   other hydrolases as well, by modulating the recycling of a putative cysteine

14   protease receptor from lysosomes and phagosomes to the Golgi or post-Golgi

15   compartment (Nakada-Tsukui *et al.*, 2005).

16

17   *6.3.2 SNARE and their accessory proteins*

18   The final step in membrane trafficking is the fusion of a transport vesicle with

19   its target membrane, which is mediated by the SNARE family of proteins.

20   SNAREs are integral membrane proteins that are present on both donor and

21   acceptor membranes and form a stable complex to tether the two membranes. It

22   is believed that the formation of a SNARE complex pulls the vesicle and target

23   membrane together and provides the energy to drive fusion of the lipid bilayers

24   (Chen and Scheller, 2001; Chen *et al.*, 1999). In a prototypical model, a

25   SNARE complex, which consists of four helices, is formed at each fusion site

26   (Hanson *et al.*, 1997; Poirier *et al.*, 1998). For instance, the fusion of synaptic

27   vesicles with the presynaptic nerve terminus is mediated by the formation of a

28   complex comprising one helix each from syntaxin 1A (Qa-SNARE, also termed

29   target-SNARE (t-SNARE)) and VAMP2 (R-SNARE, vesicular SNARE (v-

30   SNARE)) and two helices from SNAP-25 (Qb- and Qc-SNARE).

The complexity of SNAREs has remained largely unchanged in yeast, fly, and worm, but has increased remarkably in mammals and plants (Table 7) indicating that although expansion of SNARE repertoires occurs, a set of core SNAREs is sufficient to mediate vesicular fusion of most pathways in multicellular organisms. *E. histolytica* encodes 28 putative SNAREs, 18 Q-SNAREs and 10 R-SNAREs, which is comparable to the complexity to humans and plants. A notable peculiarity of SNAREs in *E. histolytica* is the lack of a group of proteins possessing two helixes (Qb and Qc SNAREs) such as SNAP-25. Thus, the prototype model of membrane tethering by a combination of four helixes (from Qa, R, and Qb/Qc) does not appear to be possible in this organism.

A group of proteins that interact directly with the syntaxin subfamily, including the prototypical member yeast Sec1p and mammalian Munc-18, are essential cytosolic proteins peripherally associated with membranes (Toonen and Verhage, 2003). They are presumed to be chaperones, putting syntaxins into the conformations required for interaction with other SNAREs (Dulubova *et al.*, 1999; Yang *et al.*, 2000). Sec1/Munc-18 proteins are also conserved in *E. histolytica* (there are 5 Sec1 genes). Two additional important components involved in the recycling of fusion machinery, *N*-ethylmaleimide sensitive factor (NSF) (Beckers *et al.*, 1989) and soluble NSF attachment protein (SNAP) (Clary *et al.*, 1990; Mayer *et al.*, 1996) are also found in *E. histolytica*.

Other proteins involved in vesicle fusion are the saposin-like proteins mentioned earlier (Section 3.3). The membrane-fusogenic activity of the *E. histolytica* SAPLIPs may play a role in vesicle fusion (Winkelmann *et al.*, 2006) but how they interface with the Rab/SNARE processes remains to be determined.

6.4 Comparisons and Implications

While the fundamental machinery of vesicular trafficking is conserved in *E. histolytica*, the high activity of the endocytic and biosynthetic transport pathway in this organism appears to have resulted in the dramatic expansion of the Rab gene repertoire. The diversity and complexity of Rab proteins present in *E. histolytica* likely reflect the vigorous dynamism of membrane transport and the reliance on Rab proteins for the specificity of vesicular trafficking. The high degree of Rab complexity observed in *E. histolytica* (91) has no precedent in other organisms, although the incomplete genome of *Trichomonas vaginalis* appears to encode 65 Rabs (Lal *et al.*, 2005) while *Dictyostelium* encodes 50 (Eichinger *et al.*, 2005). Rab proteins have been extensively studied in *Trypanosoma brucei* and the recent completion of *T. brucei, T. cruzi* and *Leishmania major* genomes led to identification of all Rab genes in these haemoflagellates (Ackers *et al.*, 2005; Berriman *et al.*, 2005; Quevillon *et al.*, 2003). Among the 16 Rab present in *T. brucei*, there are only three Rab proteins (RabX1-X3) that appear to be unique to kinetoplastids. *T. brucei* possesses 11 Rab proteins homologous to those in humans, suggesting significant conservation of the Rab-dependent core endomembrane systems in kinetoplastids. *Plasmodium falciparum* possesses only 11 Rab genes all of which are considered orthologues of yeast and mammalian Rabs, although Rab5a, 5b, and 6 revealed unique features (Quevillon *et al.*, 2003). Interestingly, some of these Rabs are expressed in a stage-dependent manner (Quevillon *et al.*, 2003). The comparatively small number of Rabs in these protists reinforces the tremendous diversity and complexity of Rabs seen in *E. histolytica* (Table 7).

In marked contrast to the complexity of Rab proteins in *E. histolytica*, the number of SNARE proteins, the other major components of vesicular fusion, is comparable to that in yeast. The apparent disparity in the number of Rab and SNARE proteins suggests one of three possibilities: 1) *Eh*Rab proteins share a single SNARE complex as an interacting partner (Huber *et al.*, 1993; Rowe *et*

1   *al.*, 2001; Torii *et al.*, 2004), 2) a majority of *Eh*Rabs do not require SNARE

2   proteins for membrane fusion (Demarque *et al.*, 2002), 3) some *Eh*Rabs are

3   primarily involved in cellular functions other than membrane fusion, like Arl

4   GTPases (Burd *et al.*, 2004; Pasqualato *et al.*, 2002). Genome-wide surveys of

5   SNAREs in other protists are not available. The three major types of coatomer

6   protein, which are conserved in *E. histolytica*, are also conserved in

7   kinetoplastids (Berriman *et al.*, 2005). However, in contrast to *E. histolytica, T.*

8   *brucei* does not possess multiple isotypes of COPI and II components except

9   for Sec24, which has two isotypes. *T. cruzi* encodes all four AP complexes

10   while *L. major* and *T. brucei* lack AP-4 or AP-2, respectively, which suggests

11   that the repertoire of AP complexes in kinetoplastids is variable and species-

12   specific. Although low similarity of the *E. histolytica* components to either

13   yeast or mammalian orthologues make unequivocal assignment of *Entamoeba*

14   AP complexes challenging, tentative assignments have been made. It is likely

15   that *E. histolytica* encodes four kinds of AP complex corresponding to APs 1-4.

16

6.5 Glycosylation and Protein Folding.

18   *6.5.1 Asparagine-linked glycan precursors.*

19   Mammals, plants, *Dictyostelium*, and most fungi synthesise asparagine-linked

20   glycans (N-glycans) by means of a common 14-sugar precursor dolichol-PP-

21   $Glc_3Man_9GlcNAc_2$ (Figures 7 and 8) (Helenius and Aebi, 2004). This lipid-

22   linked precursor is made by at least fourteen glycosyltransferases, which are

23   present in the cytosolic aspect or lumen of the ER. The reducing end of the

24   glycan contains two N-acetylglucosamines, while nine mannoses are present on

25   three distinct arms. Three glucoses are added to the left arm, which is the same

26   arm that is involved in the quality control (QC) of protein folding (see next

27   section) (Trombetta and Parodi, 2003).

28

29   *Entamoeba* is missing luminal glucosylating and mannosylating enzymes and

30   so makes the truncated, 7-sugar N-glycan precursor dolichol-PP-$Man_5GlcNAc_2$

31   (Figures 7 and 8) (Samuelson *et al.*, 2005). Five mannoses on this N-glycan

1   include the left arm, which is involved in the quality control of protein folding.

2   In contrast, *Entamoeba* is missing the middle and the right arms, which are

3   involved in N-glycan associated QC of protein degradation (see next section).

4   Because *Dictyostelium*, which is phylogenetically related to *Entamoeba*, makes

5   a complete 14-sugar N-glycan precursor, it is likely that *Entamoeba* has lost

6   sets of glycosyltransferases in the ER lumen (Samuelson *et al.*, 2005).

7   Similarly, secondary loss of glycosyltransferases best explains the diversity of

8   N-glycan precursors in fungi, which contain 0-14 sugars, and apicomplexa,

9   which contain 2-10 sugars (Samuelson *et al.*, 2005).

10

11   The 14-sugar N-glycan precursor of mammals, plants, *Dictyostelium*, and most

12   fungi is transferred to the nascent peptide by an oligosaccharyltransferase

13   (OST), which is composed of a catalytic peptide and 6-7 non-catalytic peptides

14   (Kelleher and Gilmore, 2006). In contrast, the *Entamoeba* OST contains a

15   catalytic peptide and just three non-catalytic peptides, while other protists (e.g.

16   *Giardia* and *Trypanosoma*) have an OST with a single catalytic peptide. This

17   reduced complexity does not likely affect the site of N-glycan addition to the

18   nascent peptides, which is NxS or NxT (the so-called sequon) (Kornfeld and

19   Kornfeld, 1985).

20

21   *6.5.2 N-glycans and quality control of protein folding.*

22   Protein folding in the lumen of the ER is a complex process that involves N-

23   glycan-dependent and N-glycan-independent QC systems (Helenius and Aebi,

24   2004; Trombetta and Parodi, 2003). *Entamoeba* has four of five systems

25   present in higher eukaryotes for protein folding (Figure 9).

26   1) *Entamoeba* has the minimum component parts for N-glycan-dependent QC

27   of protein folding (Helenius and Aebi, 2004; Trombetta and Parodi, 2003;

28   Banerjee, Robbins, and Samuelson, unpublished data). These include a UDP-

29   glucose-dependent glucosyltransferase (UGGT), which adds a single glucose to

30   the left arm of the N-glycans of misfolded proteins and so form

31    $GlcMan_5GlcNAc_2$ (Figure 7). The glucosylated N-glycan is then bound and

1 refolded by the lectin calreticulin (CRT), which is a chaperone that works with

2 a protein disulfide isomerase (PDI) to make and break disulfide bonds. A

3 glucosidase (Gls2) removes glucose from the well-folded protein, which is

4 transferred to the Golgi by a mannose-binding lectin (ERGIC-53). The

5 *Entamoeba* system is similar to that of mammals and fungi, which add glucose

6 to the $Man_9GlcNAc_2$ precursor to make $GlcMan_9GlcNAc_2$ (Figure 7).

7 Mammals have a second glucosidase to remove glucose from the

8 $Glc_3Man_9GlcNAc_2$ precursor (Figure 7).

9 2) *Entamoeba* has N-glycan-independent QC of protein folding within the

10 lumen of the ER, which includes the chaperones Hsp70 and Hsp90 (also

11 known as BiP and Grp94, respectively) (Figure 9) (Helenius and Aebi, 2004;

12 Trombetta and Parodi, 2003; Banerjee, Cui, Robbins, and Samuelson,

13 unpublished data). Also involved in this QC system are PDIs; DnaJ proteins

14 that increase the ATPase activity of Hsp70 and Hsp90; and peptidyl-prolyl cis-

15 trans isomerases (PPIases). This N-glycan-independent QC system for protein

16 folding is present in all eukaryotes (Banerjee, Cui, Robbins, and Samuelson,

17 unpublished data).

18 3) *Entamoeba* and all other eukaryotes have an N-glycan-independent system

19 for ER-associated degradation (ERAD) of misfolded proteins (Figure 9) (Hirsch

20 *et al.*, 2004; Banerjee, Cui, Robbins, and Samuelson, unpublished data). This

21 system is composed of proteins (Sec61 and Der1) that dislocate misfolded

22 proteins from the ER lumen to the cytosol. There a complex of proteins

23 (Cdc48, Npl4, and Ufd1) ubiquinate misfolded proteins, which are then

24 degraded in the proteasome. In contrast, *Entamoeba* and the vast majority of

25 eukaryotes are missing an N-glycan-dependent system of ERAD of misfolded

26 proteins (Helenius and Aebi, 2004; Trombetta and Parodi, 2003; Banerjee, Cui,

27 Robbins, and Samuelson, unpublished data). In this system, the middle arm of

28 $Man_9GlcNAc_2$ is trimmed to $Man_8GlcNAc_2$, which is recognised by a unique

29 mannose-binding lectin (EDEM) before dislocation into the cytosol for

30 degradation (Figure 9).

1 4) *Entamoeba* has a transmembrane kinase (Ire1), which recognises misfolded

2 proteins in the lumen of the ER and triggers the unfolded protein response

3 (Figure 9) (Patil and Walter, 2001; Banerjee, Cui, Robbins, and Samuelson,

4 unpublished data; and see section 7.2.2). The amoebic unfolded protein

5 response is likely to be different from those of mammals and fungi, because

6 *Entamoeba* is missing an important downstream target, which is a transcription

7 factor called Hac1.

8

9 *6.5.3 Unique N-glycans.*

10 Mammals make complex N-glycans in the Golgi by trimming back the

11 precursor to $Man_3GlcNAc_2$ and then adding N-acrtyl glucosamine, galactose,

12 sialic acid, and fucose (Figure 8) (Hubbard and Ivatt, 1981). In each case, the

13 activated sugars (UDP-GlcNAc, UDP-Gal, CMP-sialic acid, and GDP-fucose)

14 are transferred from the cytosol to the lumen of the Golgi by a specific

15 nucleotide-sugar transporter (NST) (Hirschberg *et al.*, 1998). In turn, each

16 activated sugar is added to the N-glycans by a specific glycosyltransferase.

17 *Entamoeba* N-glycans are remarkable for two properties. First, the most

18 abundant N-glycan is unprocessed $Man_5GlcNAc_2$ (Figure 7) (Magnelli, Ratner,

19 Robbins, and Samuelson, unpublished data). This N-glycan is recognised by

20 the mannose-binding lectin Concanavalin A, which caps glycoproteins on the

21 *Entamoeba* surface (Silva *et al.*, 1975). Unprocessed $Man_5GlcNAc_2$ is also

22 recognised by the anti-retroviral lectin cyanovirin, which binds $Man_9GlcNAc_2$

23 on the surface of gp120 (Adams *et al.*, 2004; Magnelli, Ratner, Robbins, and

24 Samuelson, unpublished data). This result suggests the possibility that the anti-

25 retroviral lectin may be active against numerous protists.

26 Second, complex N-glycans of *Entamoeba*, which are built upon the same

27 $Man_3GlcNAc_2$ core as higher eukaryotes, contain just two additional sugars

28 (galactose and glucose) (Figure 9, D and H) (Magnelli, Ratner, Robbins, and

29 Samuelson, unpublished data). Galactose is added first to both arms of

30 $Man_3GlcNAc_2$, and then glucose is added to galactose. To make these complex

31 N-glycans, *Entamoeba* has NSTs for glucose (UDP-Glc) and galactose (UDP-

1 Gal) (Bredeston *et al.*, 2005). Glucose is also transferred to N-glycans during

2 the QC of protein folding in the ER, while both galactose and glucose are

3 transferred to proteophosphoglycans (PPGs) (see next section) (Moody-Haupt

4 *et al.*, 2000). Because the complex N-glycans of *Entamoeba* are unique, it is

5 possible that they may be targets of anti-amoebic antibodies.

6

7 *6.5.4 O-glycans and GPI anchors.*

8 The surface of *E. histolytica* trophozoites is rich in glycoconjugates as shown

9 by the ability of many lectins and carbohydrate specific antibodies to recognise

10 the cell surface (Srivastava *et al.*, 1995; Zhang *et al.*, 2002).

11 Proteophosphoglycans (PPG) constitute the major glycoconjugate of the *E.*

12 *histolytica* cell surface. PPG is anchored to the cell surface through a GPI

13 moiety (Bhattacharya *et al.*, 1992). The structure of the PPG GPI has been

14 tentatively determined (Moody-Haupt *et al.*, 2000). In most eukaryotes,

15 phosphatidylinositol (PI) is glycosidically linked to the reducing end of de-

16 acetylated glucosamine followed by three mannoses which are in turn attached

17 to the ethanolamine that links the protein to the GPI. However, the GPI anchor

18 of *E. histolytica* PPG was found to have a unique backbone that is not observed

19 in other eukaryotes, namely Gal-Man-Man-GlcN-myoinositol. The

20 intermediate and light subunits of the *E. histolytica* Gal/GalNAc lectin, among

21 other cell surface molecules, are anchored to the cell surface through GPI

22 anchors. Though the structure of the GPI anchors is not known, they are

23 thought to be functionally important (Ramakrishnan *et al.*, 2000). In humans,

24 23 genes are known to participate in the biosynthesis of GPI anchors. However,

25 only 15 of these were identified in *E. histolytica* (Vats *et al.*, 2005).

26 Interestingly, all the catalytic subunits were identified in *E. histolytica*, the

27 missing genes encoding the accessory subunits suggesting that the biosynthetic

28 pathway may not be significantly different from that in other eukaryotes. The

29 presence of the pathway was also confirmed by detecting the biochemical

30 activities of the first two enzymes - N-acetyl glucosamine transferase and

31 deacetylase. In addition, antisense inhibition of the deacetylase blocked GPI

anchor biosynthesis and reduced virulence of the parasite (Vats *et al.*, 2005). A novel GIPL (glycosylated inositol phospholipid) was also identified in *E. histolytica* (Vishwakarma *et al.*, 2006). Structural studies indicate that a galactose residue is attached to glucosamine as the terminal sugar instead of mannose. This suggests that *E. histolytica* is capable of synthesising unusual GPI-containing glycoconjugates not observed in other organisms.

In PPG, glycans are attached to a peptide backbone by an *O*-Phosphodiester-linkage (O-P glycans). The *E. histolytica* O-P-glycans have galactose at the reducing end followed by a chain of glucoses. *Entamoeba invadens* also has O-P-glycans on its cyst wall proteins but the reducing sugar is a deoxysugar rather than galactose (Van Dellen *et al.*, 2006b). While *Dictyostelium* also has O-P-glycans on glycoproteins in its spore wall, glycoproteins with O-P-glycans are absent from the vast majority of animals and plants (West, 2003).

6.5.5 Significance.

The unique glycans of *Entamoeba* lead to three important evolutionary inferences. First, much of the diversity of eukaryotic N-glycans is due to secondary loss of enzymes that make the 14-sugar lipid-linked precursor, which was present in the common ancestor to extant eukaryotes. Despite the truncated N-glycan precursor, *Entamoeba* has conserved the relatively complex N-glycan-dependent QC system for protein folding. Third, the unique N-glycans and O-P-linked glycans are based upon a novel set of glycosyltransferases, which are present in *Entamoeba* and remain to be characterised molecularly.

7. PROTEINS INVOLVED IN SIGNALLING
7.1 Phosphatases

The combined actions of protein kinases and phosphatases regulate many cellular activities through reversible phosphorylation of proteins. These activities include such basic functions as growth, motility, and metabolism. Although it was once assumed that kinases played the major regulatory role, it is now clear that phosphatases can also be critical participants in some cellular

1   events (Li and Dixon, 2000). There are few publications on the role of

2   phosphatases in *E. histolytica,* however, several investigators have established a

3   role for phosphatases in proliferation, and growth. Chaudhuri *et al.* (1999)

4   observed that there was an increase in phospho-tyrosine levels in serum starved,

5   growth inhibited, *E. histolytica* cultures. Upon the additional serum and

6   subsequent growth simulation, an increase in tyrosine phosphatase activity

7   occurred. These investigators also demonstrated that genistein, a tyrosine

8   kinase inhibitor, had no effect on growth, while the addition of sodium

9   orthovanadate, a phosphatase inhibitor, produced a major decrease in cell

10   proliferation. Membrane-bound and secreted acid phosphatase activities have

11   been detected in *E. histolytica* (Aguirre-Garcia *et al.*, 1997; Anaya-Ruiz *et al.*,

12   1997). The secreted acid phosphatase activity is absent from *E. dispar*

13   (Talamas-Rohana *et al.*, 1999). This secreted acid phosphatase was found to

14   have phosphotyrosine hydrolase activity, and caused cell rounding and

15   detachment of HeLa cells (Anaya-Ruiz *et al.*, 2003), suggesting that

16   phosphatase activity contributes to the virulence of the organism.

17

18   There are four families of phosphatases (Stark, 1996). Members of the PPP

19   (protein phosphatase P) family are serine/threonine phosphatases, and include

20   PP1, PP2A, and PP2B (calcineurin-like) classes. The PPM (protein

21   phosphatase M) family phosphatases also dephosphorylate serine/threonine

22   residues but are unrelated to the PPP family proteins. A third family consists of

23   protein tyrosine phosphatases (PTP) and dual phosphatases. Low molecular

24   weight phosphatases make up the fourth family. In eukaryotic cells, greater than

25   99% of protein phosphorylation is on serine or threonine residues (Chinkers,

26   2001). Human cells have about 500 serine/threonine phosphatases and 100

27   tyrosine phosphatases (Hooft van Huijsduijnen, 1998; Hunter, 1995).

28   *Saccharomyces cerevisiae* has 31 identified or putative protein phosphatases

29   (Stark, 1996). *E. histolytica* has over 100 putative protein phosphatases. Only a

30   few of these phosphatases have potential transmembrane domains. Some *E.*

31   *histolytica* phosphatases have varying numbers of leucine-rich-repeats (LRR).

1 The LRR domain is thought to be a site for protein:protein interactions (Hsiung

2 *et al.*, 2001; Kobe and Deisenhofer, 1994). LRR domains have been found in a

3 few kinases, but had not been identified in any phosphatases until recently (Gao

4 *et al.*, 2005).

5

6 *7.1.1 Serine/Threonine Protein Phosphatases*

7 Members of the PPP family of protein phosphatases are closely related

8 metalloenzymes, and complex with regulatory subunits. In contrast, PPM

9 family members are generally monomeric, ranging 42-61 kDa in size. By Blast

10 analysis, the serine/threonine protein phosphatases of *E. histolytica* are most

11 closely related to PPP phosphatases PP2A, PP2B, and PPM phosphatase PP2C.

12

13 7.1.1 (a) PP2A and PP2B (Calcineurin-like) serine/threonine phosphatases

14 PP2A phosphatases are trimeric enzymes consisting of catalytic, regulatory, and

15 variable subunits (Wera and Hemmings, 1995). Calcineurin is a calcium-

16 dependent protein serine/threonine phosphatase (Rusnak and Mertz, 2000).

17 Orthologues of calcineurin are widespread, from yeast to mammalian cells.

18 Calcineurin is a heterodimeric complex with catalytic (CaNA) and regulatory

19 (CaNB) subunits. CaNA ranges in size from 58-64 kDa. Its conserved domain

20 structure includes a catalytic domain, a CaNB-binding domain, a calmodulin

21 binding domain, and an autoinhibitory (AI) domain. The binding of CaNB and

22 calmodulin activates CaNA. CaNB subunit is 19 kDa, contains 4 EF hand

23 calcium binding motifs and has similarity to calmodulin. The binding of

24 calmodulin releases the autoinhibitory domain and results in activation of the

25 phosphatase. Deletion of the AI domain results in a constitutively active

26 protein. Calcineurin is specifically inhibited by cyclosporin A and FK506.

27 Cyclosporin A and FK506 first bind to specific proteins, cyclophilin A and

28 FK506BP, respectively, then bind to CaNA at the CaNB binding site.

29 Cyclophilin A has been identified in *E. histolytica* and treatment with

30 cyclosporin A decreases growth and viability (Carrero *et al.*, 2000; Carrero *et*

31 *al.*, 2004; Ostoa-Saloma *et al.*, 2000).

1

2   The *E. histolytica* genome has 51 PP2A and calcineurin-like protein

3   phosphatases. The Pfam motif that classifies proteins as PPP phosphatases is

4   Metallophos (PF00149, calcineurin-like phosphoesterase). This motif is also

5   found in a large number of proteins involved in phosphorylation, including

6   DNA polymerase, exonucleases and other phosphatases. The genome

7   annotation identifies three loci as CaNA orthologues. However, due to the

8   similarity among this family of phosphatases, it is difficult to tell by sequence

9   analyses alone those that are calcium-dependent. Identification of CaNA will

10 have to be confirmed experimentally.

11

12 Two of the PPM phosphatases contain a TPR domain (PF00515). TPR is

13 thought to be involved in protein:protein interactions (Das *et al.*, 1998).

14 Activities that have been ascribed to TPR include regulatory roles, lipid binding

15 and auto-inhibition.

16

17 7.1.1 (b) PP2C phosphatases

18 PP2C phosphatases are also widespread and are often involved in

19 terminating/attenuating phosphorylation during the cell cycle or in response to

20 environmental stresses such as osmotic and heat shock (Kennelly, 2001).

21 Thirty-five genes were identified as PP2C phosphatases. These proteins can be

22 divided into three broad categories: 1) PP2C domain only-small (235-381

23 amino acids), 2) PP2C domain only-large (608-959 amino acids), and 3) PP2C

24 with LRR domains.

25

26 *7.1.2 Tyrosine phosphatases (PTP)*

27 Tyrosine phosphorylation-dephosphorylation is a key regulatory mechanism for

28 many aspects of cell biology, and development (Li and Dixon, 2000). PTPs are

29 a large class of enzymes that have catalytic domains of ~300 amino acids.

30 Forty of these residues are highly conserved (Hooft van Huijsduijnen, 1998).

31 PTPs can be divided into membrane (receptor) and non-membrane (soluble)

1 PTPs (Li and Dixon, 2000). The soluble PTP group includes those that contain

2 conserved SH2, PEST, Ezrin, PDZ, or CH2 domains. Two other classes of

3 PTPs are the low molecular weight and dual phosphatases. *Saccharomyces*

4 *cerevisiae* lacks classic PTPs but does contain dual phosphatases, such as the

5 MAP kinase kinases.

6

7 *E. histolytica* has only four potential PTPs none of which are receptor PTPs,

8 (*i.e.* PTPs with recognisable transmembrane spanning regions). Two of the

9 PTPs (XM_650778, XM_645883) are 350 and 342 amino acids in length and

10 share 48% identity. Neither of these phosphatases has any other recognisable

11 conserved domain. Non-receptor type 1 PTPs are the closest match to these

12 proteins (Li and Dixon, 2000). Membrane and secreted forms of a PTP that

13 cross-react with anti human PTP1B have been reported in *E. histolytica*

14 (Aguirre-García *et al.*, 2003; Talamas-Rohana *et al.*, 1999). Both forms have an

15 apparent molecular weight of 55 kDa and disrupt host actin stress fibers.

16 However, since none of the putative PTPs identified by the genome project

17 appear to encode secreted or membrane forms it is unlikely that these loci

18 represent these previously reported PTP1B cross-reacting proteins.

19

20 A third PTP contains a protein tyrosine phosphatase like protein (PTPLA)

21 domain (PF04387). The PTPLA domain is related to the catalytic domains of

22 tyrosine kinases, but it has an arginine for proline substitution at the active site

23 (Uwanogho *et al.*, 1999). It is not yet clear whether this family of proteins

24 actually has phosphatase activity or serves some other regulatory role.

25

26 An orthologue of a low molecular weight PTP has also been identified. Low

27 molecular weight protein tyrosine phosphatases have been found in bacteria,

28 yeast, and mammalian cells (Ramponi and Stefani, 1997). They are not similar

29 to other PTPs except in the conserved catalytic domain.

1

2 *7.1.3 Dual-specificity protein phosphatases*

3 Dual specificity PTPs (DSP) can hydrolyse both tyrosine and serine/threonine

4 residues, though they hydrolyse phosphorylated tyrosine substrates 40-500 fold

5 faster (Zhang and Van Etten, 1991). In other organisms, DSPs are found

6 mostly in the nucleus and have roles in cell cycle control, nuclear

7 dephosphorylation and inactivation of MAP kinase.

8

9 The *E. histolytica* genome has 23 sequences related to DSPs. They fall into

10 three main subclasses: those with the DSP domain only, those with DSP plus a

11 variable number (1-5) of LRRs, and those with the Rhodanese homology

12 domain (RHOD; IPR001763). Rhodanese is a sulphurtransferase involved in

13 cyanide detoxification. Its active site, RHOD, is also found in the catalytic site

14 of the dual specificity phosphatase CDC25 (Bordo and Bork, 2002).

15

16 *7.1.4 Leucine Rich Repeats (LRRs)*

17 LRRs are tandem arrays of 20-29 amino acid, leucine-rich motifs. LRRs have

18 been found in a number of proteins with varied functions including enzyme

19 inhibition, regulation of gene expression, morphology and cytoskeleton

20 formation (Kobe and Deisenhofer, 1994). LRRs are thought to provide versatile

21 sites for protein:protein interaction and have been found linked to a variety of

22 secondary domains. Most LRRs form curved horseshoe-shaped structures with

23 "a parallel beta sheet on the concave side and mostly helical elements on the

24 convex side" (IPR001611).

25

26 The LRR_1 Pfam is the second most abundant Pfam domain found in the *E.*

27 *histolytica* genome (Table 3). The LRR motifs in *E. histolytica* most closely

28 resemble the LRR found in BspA (section 2.7; Davis *et al.*, 2006). Several *E.*

29 *histolytica* proteins that contain LRRs are associated with other recognised

30 domains. These include the protein phosphatases PP2C and DSP, as well as

31 protein kinase (PK), F-box (PF00646), gelsolin/villin headpiece (IPR007122),

DNA J (IPR001623), Band 41 (B41;IPR000299), WD-40 (IPR001680), and
Zinc binding (IPR000967) domains. The association of LRRs with
phosphatases is unusual. One published example is the phosphatase that
dephosphorylates the kinase Akt (Gao *et al.*, 2005). Fungal adenylate cyclases
have both LRR and PP2C-like domains but this is not a wide spread feature of
adenylate cyclases in other species (Mallet *et al.*, 2000; Yamawaki-Kataoka *et al.*, 1989). The LRR may be a site for interaction with phosphorylated residues
in *E. histolytica*. This speculation is supported by the example of the Grr1
protein of yeast, which contains an F-box and a LRR (Hsiung *et al.*, 2001).
Grr1 is involved in ubiquitin-dependent proteolysis. The LRR domain of Grr1
binds to phosphorylated targets in the proteosome complex. Another example
is the fission yeast phosphatase regulatory subunit, Sds22, which also has LRRs
(MacKelvie *et al.*, 1995). The LRR containing phosphatases of *E. histolytica*
may represent fusions of regulatory and catalytic subunits.

7.2 Kinases

7.2.1. Cytosolic kinases.
Eukaryotic protein kinases are a superfamily of enzymes, which are important
for signal transduction and cell-cycle regulation. Six families of
Serine/Threonine kinases (STKs), which include AGC, Ste, CK1, CaMK,
CMGC, and TKL (tyrosine kinase-like), have conserved aspartic acid and
lysine amino acids in their active sites and phosphorylate serine or threonine on
target proteins (Hanks and Hunter, 1995). Tyrosine kinases (TK), which lack
active site lysine, phosphorylate tyrosine on target proteins. Phosphorylated
tyrosine is in turn recognised by Src-homology 2 (SH2) domains that are
present on some kinases and other proteins. All seven families of protein
kinases are present in metazoa and in *D. discoideum*, while plants lack TK, and
S. cerevisiae lacks both TK and TKL.

Over 150 predicted *E. histolytica* cytosolic kinases, those that lack signal
peptides and trans-membrane helices, can be identified, including

representatives of each of the seven groups of kinases (AGC, CAMK, CK1, CMGC, STE, TKL, and TK) (Loftus *et al.*, 2005; Cui and Samuelson, unpublished data). Two predicted *E. histolytica* TKs, which group with human TKs in phylogenetic trees, contain an AAR peptide in the active site and a Kelch domain at the C-terminus (Gu and Gu, 2003). Four cytosolic protein kinases contain C-terminal SH2 domains, which bind phosphorylated tyrosine residues. Phosphotyrosine has been identified in *E. histolytica* using specific antibodies (Hernandez-Ramirez *et al.*, 2000). The thirty-five predicted cytosolic *E. histolytica* TKLs include some that contain Leu-rich repeats (LRR) and ankyrin repeats at their N-termini. In contrast, the vast majority of *Entamoeba* cytosolic kinases lack accessory domains.

7.2.2. Receptor-kinases.

Five distinct families of eukaryotic proteins have an N-terminal ectoplasmic domain, a single transmembrane helix, and a C-terminal cytoplasmic kinase domain (Blume-Jensen and Hunter, 2001). Ire-1 transmembrane kinases, which are present in *S. cerevisiae*, plants, and metazoa, detect unfolded proteins in the lumen of the ER and help splice a transcription factor mRNA by means of a unique C-terminal ribonuclease (Patil and Walter, 2001). Receptor tyrosine kinases (RTKs), which include growth hormone and epidermal growth factor (EGF) receptors, are restricted to metazoa and have a diverse set of N-terminal ectoplasmic domains and a conserved C-terminal cytosolic TK (Schlessinger, 2000). Receptor serine/threonine kinases (RSK) of metazoa and receptor-like kinases (RLKs) of plants each contain a C-terminal TKL domain (Massague *et al.*, 2000; McCarty and Chory, 2000; Shiu and Bleecker, 2001). Phylogenetic analyses suggest that plant RLKs, animal RSKs, and animal RTKs each form monophyletic groups, and that plant RLKs closely resemble cytosolic TKLs of animals called Pelle or IRAK (Shiu and Bleecker, 2001).

E. histolytica contains >80 novel receptor RSKs, each of which has a N-terminal signal sequence, a conserved ectoplasmic domain, a single

transmembrane helix (TMH), and a cytosolic kinase domain (Beck *et al.*, 2005). The largest group of *E. histolytica* RSKs has a CXXC-rich ectoplasmic domain with 6 to 31 internal repeats that each contains 4 to 6 cysteine residues (Figure 10). Very similar CXXC-rich domains are present in the ectoplasmic domain intermediate subunit of the Gal/GalNAc lectin (section 3.1.3). CXXC-rich domains are also present in hypothetical secreted proteins of *E. histolytica*, while cysteine-rich domains are also present in the heavy subunit of the Gal/GalNAc lectin and at the cytosolic aspect of some cysteine proteases (Figure 10).

Ectoplasmic domains of other large families of *Entamoeba* RSKs have one or two 6-Cys domains at the N-terminus and four 6-Cys domains proximal to the plasma membrane. There are no plasma membrane proteins or secreted proteins with similar domains. A minority of RSKs do not contain Cys-rich ectoplasmic domains. Numerous *Entamoeba* RSKs are expressed at the same time, but the specific ligands for the *Entamoeba* RSKs have not been identified (Beck *et al.*, 2005).

As discussed in the section on protein folding (6.5.2), *Entamoeba* has an Ire1 transmembrane kinase, which recognises misfolded proteins in the lumen of the ER and triggers the unfolded protein response (Figure 8).

7.2.3 Significance

While most protists lack TK, TKL, receptor-kinases, and Ire1 *E. histolytica* has all four. It is very likely that the *E. histolytica* receptor-kinases, which are extensively duplicated, will have important roles in pathogensis (Beck *et al.*, 2005; Okada *et al.*, 2005). Similarly, trimeric G-proteins and the associated adenyl-cyclases likely have important roles in cyst formation and virulence (Coppi *et al.*, 2002; Frederick and Eichinger, 2004).

7.3 Calcium Binding Proteins

Ca^{2+} signaling plays a crucial role in the pathogenesis of many protozoan parasites, including *E. histolytica* (Ravdin *et al.*, 1985). Many of the calcium-mediated processes are carried out with the help of calcium binding proteins (CaBPs). CaBPs have been identified and characterised in almost all eukaryotic systems. Some of these, such as calmodulin (CaM) and troponin C, have been studied extensively. A number of CaBPs have also been identified in *E. histolytica*. Among these are two related EF-hand containing proteins, grainin 1 and granin 2, which are likely to be localised in intracellular granules (Nickel *et al.*, 2000). Another protein, URE3-BP, was shown to have a transcription regulatory function (Gilchrist *et al.*, 2001). The CaM-dependent secretion of collagenases from electron dense granules has been demonstrated using *E. histolytica* lysate. However, there is as yet no direct molecular evidence for the presence of CaM in *E. histolytica* (Muñoz *et al.*, 1991). The CaM-like protein EhCaBP1 has four canonical EF-hand Ca^{2+} binding domains but no functional similarity to CaM (Yadava *et al.*, 1997). Inducible expression of EhCaBP1 antisense RNA demonstrated this protein's role in actin-mediated processes (Sahoo *et al.*, 2004).

Analysis of the whole genome revealed presence of 27 CaBPs with multiple EF-hand calcium binding domains (Bhattacharya *et al.*, 2006). Many of these proteins are architecturally very similar but functionally distinct from CaM. Moreover, functional diversity was also observed among closely related CaBPs, such as EhCaBP1 and EhCaBP2 (79% identical at the amino acid level; Chakrabarty *et al.*, 2004). Analysis of partial EST and proteomic databases combined with Northern blots and RT-PCR shows that at least one third of these genes are expressed in trophozoites, suggesting that many if not all of the 27 are functional genes (Bhattacharya *et al.*, 2006).

What are the roles of these proteins in the context of *E. histolytica* biology? At present the function of only two EhCaBPs are known, EhCaBP1 and URE3-BP. The rest of the proteins are likely to be Ca^{2+} sensors involved in a number of

different signal transduction pathways. After binding Ca^{2+} these may undergo conformational changes and the bound form then activates downstream target proteins. It is not clear why *E. histolytica* would need so many Ca^{2+} sensors when many other organisms do not. It is likely that with Ca^{2+} being involved in many functions, some of which are localised in different cellular locations, the various CaBPs may participate in different functions that are spatially and temporally separated.

8. THE MITOSOME

One of the expectations for the *E. histolytica* genome project was that it would identify the function of the mitochondrial remnant known as the mitosome (Tovar *et al.*, 1999) or crypton (Mai *et al.*, 1999). Under the microscope mitosomes are ovoid structures smaller than 0.5 μm in diameter (Leon-Avila and Tovar, 2004). While it is now clear that no mitochondrial genome still persists, from both genome sequencing and cellular localisation data (Leon-Avila and Tovar, 2004), the protein complement of the organelle is still somewhat obscure. The number of identifiable mitosomal proteins remains very small and does not provide great insight into the organelle's function. Genes encoding mitochondrial-type chaperonins (cpn60, hsp10 and mt-hsp70) have been identified and appear to be synthesised with amino-terminal signal sequences. The importation machinery has been shown to be conserved with that in true mitochondria (Mai *et al.*, 1999; Tovar *et al.*, 1999) but none of the proteins involved in mitosomal protein import have been identified with certainty.

Other genes encoding putative mitosomal proteins include pyridine nucleotide transhydrogenase (which moves reducing equivalents between NAD and NADP, and acts as a proton pump (Clark and Roger, 1995); only an incomplete gene is present in the assembly), and ADP/ATP transporter (Chan *et al.*, 2005), a P-glycoprotein-like protein (Pgp6), and a mitochondrial type thioredoxin, although the latter two are identified based largely on their amino terminal

1 extensions. The only enzymatic pathway that is normally mitochondrial in

2 location is iron-sulphur cluster synthesis. Genes encoding homologues of both

3 IscS/NifS and IscU/NifU proteins are present, but uniquely among eukaryotes

4 the *E. histolytica* homologues are not of mitochondrial origin, having been

5 acquired by distinct lateral gene transfer from an ε-proteobacterium (Ali *et al.*,

6 2004b; van der Giezen *et al.*, 2004). The location of these proteins appears to be

7 cytoplasmic as determined by immunofluorescence, using antibodies against

8 both the native proteins as well as detection of epitope-tagged proteins in

9 transformed *E. histolytica* (Ali and Nozaki, unpublished). The same pathway

10 has been localised to mitosomes in *Giardia* and is also retained in all other

11 organisms with remnant mitochondria. Given the apparently unique non-

12 compartmentalised nature of iron-sulphur cluster synthesis in *E. histolytica* the

13 location of the proteins needs to be confirmed by immuno-electron-microscopy;

14 such experiments are currently underway (Ali and Nozaki, unpublished). The

15 function of the *E. histolytica* mitosome therefore remains an enigma.

16

17 ## 9. ENCYSTATION

18 The infectious stage of *Entamoeba histolytica,* and also that most often used for

19 diagnosis, is the quadrinucleate cyst. Because it is not possible to encyst *E.*

20 *histolytica* in axenic culture, *Entamoeba invadens*, which is a reptilian parasite,

21 has been used as a model organism for encystation (Eichinger, 2001; Wang *et*

22 *al.*, 2003). The *E. invadens* cyst wall is composed of three parts: deacetylated

23 chitin (also known as chitosan), lectins that bind chitin (e.g. Jacob and Jessie)

24 or cyst wall glycoproteins (e.g. plasma membrane Gal/GalNAc lectin), and

25 enzymes that modify chitin or cyst wall proteins (e.g. chitin deacetylase,

26 chitinase, and cysteine proteases) (Figure 11).

27

28 ### 9.1 Chitin synthases

29 Chitin fibrils, which are homopolymers of β-1,4-linked N-acetyl glucosamine

30 (GlcNAc), are synthesised by chitin synthases. Chitin synthases share common

31 ancestry with cellulose synthases and hyaluronan synthase. They are

transmembrane proteins with a catalytic domain in the cytosol (Bulawa, 1993), where UDP-GlcNAc is made into a homopolymer and is threaded through the transmembrane domains into the extracellular space. In *Saccharomyces cerevisiae*, four accessory peptides, encoded by the Chs4-7 genes, are necessary for the function of its chitin synthases (Trilla *et al.*, 1999). Remarkably, the *E. histolytica* chitin synthase 2 (EhChs2) complements a *S. cerevisiae* chs1/chs3 mutant and the function of EhChs2 is independent of the four accessory peptides (Van Dellen *et al.*, 2006a). This result suggests the possibility that chimaeras of *E. histolytica* and *S. cerevisiae* chitin synthases may be used to map domains in the *S. cerevisiae* chitin synthase that interact with the accessory peptides.

9.2 Chitin Deacetylases

Chitin fibrils in the cyst wall are modified by deacetylases and chitinases (see section 9.3). There are two *E. invadens* chitin deacetylases, which convert chitin to chitosan (Das *et al.*, 2006). Chitosan is a mixture of N-acetyl glucosamine and glucosamine and so has a positive charge. It is also present in spore walls of *S. cerevisiae* and in lateral walls of *Mucor* (Kafetzopoulos *et al.*, 1993; Mishra *et al.*, 1997). It is likely that the positive charge of chitosan fibrils contributes to the binding of cyst wall proteins, all of which are acidic (de la Vega *et al.*, 1997; Frisardi *et al.*, 2000; Van Dellen *et al.*, 2002b). Monosaccharide analyses of the *E. invadens* cyst walls following treatment with SDS to remove proteins strongly suggest that chitosan is the only sugar homopolymer present (Das *et al.*, 2006).

9.3 Chitinases

Entamoeba species encode numerous chitinases with a conserved type 18 glycohydrolase domain (de la Vega *et al.*, 1997). Recombinant *Entamoeba* chitinases have both endo- and exo -chitinase activities. Two other domains are important in *Entamoeba* chitinases: 1) At the N-terminus is a unique 8-Cys chitin-binding domain (CBD), which is also present as a single domain in *E.*

1   *histolytica* Jessie lectins (Figure 11) (Van Dellen *et al.*, 2002b). Chitinase and

2   Jessie-3 lectin bind to the *E. invadens* cyst wall by means of this 8-Cys CBD

3   (Van Dellen *et al.*, submitted). This *E. histolytica* chitinase CBD has the same

4   function as CBDs in chitinases of fungi, nematodes, insects, and bacteria, but

5   has no sequence similarity (i.e. it has arisen by convergent evolution) (Shen and

6   Jacobs-Lorena, 1999). 2) Between the CBD and chitinase domains of

7   *Entamoeba* species are low complexity sequences that contain heptapeptide

8   repeats (Ghosh *et al.*, 2000). These polymorphic repeats may be used to

9   distinguish isolates of *E. histolytica* within the same population and may be

10   able to discriminate among isolates from New and Old World (Haghighi *et al.*,

11   2003). These polymorphic repeats, which are rich in serine and resemble

12   mucin-like domains in other glycoproteins, may also be the sites for addition of

13   *O*-phosphodiester linked sugars (see section 6.5.4).

14

15   **9.4 Jacob lectins**

16   Chitin fibrils in the cyst wall of *E. invadens* are cross-linked by Jacob lectins,

17   which contain 3 to 5 unique 6-Cys CBDs (Frisardi *et al.*, 2000). *E. invadens*

18   has at least nine genes encoding Jacob lectins, and the mRNA levels from each

19   gene increase during encystation (Van Dellen *et al.*, submitted). In addition, at

20   least six Jacob lectin proteins are present in *E. invadens* cyst walls (Van Dellen

21   *et al.*, submitted). Between the CBDs, Jacob lectins have low complexity

22   sequences that are rich in serine as in the case of chitinase [5]. Jacob lectins are

23   post-translationally modified in two ways. First, they are cleaved by cysteine

24   proteinases at conserved sites in the serine- and threonine-rich spacers between

25   CBDs. Second, they have *O*-phosphodiester-linked sugars added to serine and

26   threonine residues. *O*-phosphodiester-linked glycans are also present in

27   proteophosphoglycans (PPGs) on the surface of *E. histolytica* trophozoites

28   (Moody-Haupt *et al.*, 2000).

29

30   **9.5 Gal/GalNAc lectins**

1   The Gal/GalNAc lectins present on the surface of *E. histolytica* trophozoites

2   have been described above (section 3.1) and in the literature (Mann *et al.*, 1991;

3   Petri *et al.*, 2002). Their possible role in encystation is suggested by two

4   independent experiments. First, the signal for encystation likely depends in part

5   on aggregation of *E. invadens*, which is inhibited by exogenous galactose

6   (Coppi and Eichinger, 1999). Aggregated *E. invadens* secrete catecholamines,

7   which in an autocrine manner stimulate amoebae to encyst (Coppi *et al.*, 2002).

8   Second, in the presence of excess galactose, *E. invadens* forms wall-less cysts

9   that contain four nuclei and makes Jacob lectins and chitinase (Frisardi *et al.*,

10 2000). Because *E. invadens* trophozoites have a Gal/GalNAc lectin on their

11 surface that is capable of binding sugars on Jacob lectin, and because Jacob

12 lectins have no carboxy-terminal transmembrane helix or GPI-anchor, it is

13 likely that the cyst wall is bound to the plasma membrane by the Gal/GalNAc

14 lectin.

15

16 **9.6 Summary and Comparisons**

17 Similar to the cyst wall of *Giardia*, the cyst wall of *E. invadens* is a single

18 homogeneous layer and contains a single homopolymer, chitosan (Figure 11)

19 (Frisardi *et al.*, 2000; Gerwig *et al.*, 2002; Shen and Jacobs-Lorena, 1999). In

20 contrast, *S. cerevisiae* spore walls have multiple layers and contain β-1,3-

21 glucans in addition to chitin, while *Dictyostelium* walls have multiple layers

22 and contain N-acetyl galactosamine polymers in addition to cellulose (West,

23 2003; Yin *et al.*, 2005).

24

25 Similar to *Dictyostelium* and in contrast to fungi, the vast majority of

26 *Entamoeba* cyst wall glycoproteins are released by SDS (Van Dellen *et al.*,

27 submitted; Frisardi *et al.*, 2000; West, 2003; Yin *et al.*, 2005). While some

28 *Dictyostelium* cyst wall proteins have been shown to be cellulose-binding

29 lectins, all of the proteins bound to the cyst wall of *E. invadens* have 6-Cys

30 CBDs (Jacob lectins) or 8-Cys CBDs (Jessie 3 lectin and chitinase) (Frisardi *et*

31 *al.*, 2000; Van Dellen *et al.*, 2002b; Van Dellen *et al.*, submitted). In the same

1 way that *Giardia* cyst wall protein 2 is cleaved by a cysteine proteinase, Jacob

2 lectins are cleaved by an endogenous cysteine proteinase at sites between

3 chitin-binding domains (Touz *et al.*, 2002).

4

5 Like *Dictyostelium* spore coat proteins and insect peritrophins, cysteine-rich

6 lectin domains of *E. invadens* cyst wall proteins are separated by serine- and

7 threonine-rich domains that are heavily glycosylated (Frisardi *et al.*, 2000;

8 West, 2003; Yin *et al.*, 2005; Van Dellen *et al.*, submitted). *S. cerevisiae* cyst

9 wall proteins also have extensive serine- and threonine-rich domains that are

10 heavily glycosylated (Yin *et al.*, 2005). These glycans likely protect proteins in

11 cyst walls or fungal walls from exogenous proteases. While glycoproteins of

12 the *E. invadens* cyst wall and *Dictyostelium* spore coat contain *O*-

13 phosphodiester-linked glycans, *S. cerevisiae* wall glycoproteins contain *O*-

14 glycans (Gemmill and Trimble, 1999; West *et al.*, 2005).

15

16 Like *S. cerevisiae*, *E. invadens* has enzymes in its wall that modify chitin (Yin

17 *et al.*, 2005). Similar to chitinases of *S. cerevisiae* and bacteria, *E. invadens*

18 chitinase has a CBD in addition to the catalytic domain (Kuranda and Robbins,

19 1991). It is likely that the CBD is present to localise chitinase to the cyst wall

20 (*E. invadens*) or cell wall (*S. cerevisiae*). Finally, while *E. invadens* uses

21 catecholamines as autocrines for encystation, *Dictyostelium* uses cAMP as an

22 autocrine for sporulation (Coppi *et al.*, 2002; Kriebel and Parent, 2004). An

23 important goal of future research will be to translate what is known about the *E.*

24 *invadens* cyst wall to that of *E. histolytica*.

25

26 **10. EVIDENCE OF LATERAL GENE TRANSFER IN THE *E.***

27 ***hISTOLYTICA* GENOME**

28 Lateral (or horizontal) gene transfer (LGT) plays a significant role in

29 prokaryotic genome evolution, contributing up to ~20% of the content of a

30 given genome (Doolittle *et al.*, 2003). LGT has therefore been an important

31 means of acquiring new phenotypes, such as resistance to antibiotics and new

1   physiological and metabolic capabilities, that may permit or facilitate

2   adaptation to new ecological niches (Koonin *et al.*, 2001; Lawrence, 2005b;

3   Ochman *et al.*, 2000). More recently, data from microbial eukaryote genomes

4   suggest that LGT has also played a role in eukaryotic genome evolution,

5   particularly among protists that eat bacteria (Andersson, 2005; Doolittle, 1998;

6   Doolittle *et al.*, 2003; Lawrence, 2005a; Richards *et al.*, 2003). *Entamoeba*

7   *histolytica* lives in the human gut, an environment that is rich in

8   microorganisms and where LGT is thought to be common between bacteria

9   (Shoemaker *et al.*, 2001). The *E. histolytica* genome thus provides a nice

10   model for investigating prokaryote to eukaryote LGT. In the original genome

11   description (Loftus *et al.*, 2005) 96 putative cases of LGT were identified using

12   phylogenetic analyses of the *E. histolytica* proteome. These have now been

13   reanalysed in the light of more recently published (August 2005) eukaryotic and

14   prokaryotic genomes. This has allowed evaluation of how previous inferences

15   were influenced by the sparse sampling of eukaryotic and prokaryotic genes

16   and species available at the time of the original analysis. Sparse gene and

17   species sampling is, and is likely to remain, a very serious problem for

18   reconstructing global trees and inferring LGT (Andersson *et al.*, 2001; Richards

19   *et al.*, 2003; Salzberg *et al.*, 2001). Thus, although ecologists differ in their

20   claims for the extent of the unsampled microbial world, they all agree that those

21   species in culture, and the even smaller subset for which genome data exist,

22   represent the smallest tip of a very large iceberg.

23

24   **10.1 How Do The 96 LGT Cases Stand Up?**

25   As before (Loftus *et al.*, 2005), Bayesian and maximum likelihood distance

26   bootstrap phylogenetic analyses were used to identify putative LGT using the

27   following *ad hoc* conservative criteria: Putative LGT was inferred where either

28   no other eukaryote possessed the gene, or where the *E. histolytica* sequence was

29   grouped with bacteria and separated from other eukaryotes by at least two

30   strongly supported nodes (bootstrap support >70%, posterior probabilities

31   >0.95). In cases where tree topologies were more weakly supported but still

1   suggested a possible LGT, bootstrap partition tables were examined for

2   partitions where the *E. histolytica* sequence clustered with another eukaryote.

3   If no such partitions were found that gene was considered to be a putative LGT.

4   Table 8 lists the results of the new analyses and also gives BlastP statistics for

5   each sequence.

6

7   A total of 41 LGT remain as strongly supported as before based upon the

8   original criteria. For the remaining 55 tree topologies, support for recent LGT

9   into the *Entamoeba* lineage is not as strong as before. For 27 of these 55 trees,

10 two strongly supported nodes separating *E. histolytica* from other eukaryotes

11 has been reduced to only one well-supported node. However, close scrutiny of

12 the bootstrap partition tables for these trees revealed that, as before, there are no

13 trees in which *E. histolytica* is found together with another eukaryote. Thus,

14 LGT still remains the strongest hypothesis to explain 68 (70%) of the original

15 96 tree topologies. In a further 14 cases, the position of *E. histolytica* among

16 prokaryotes and eukaryotes was not well supported. The taxonomic sampling of

17 eukaryotes in these trees is very patchy and the trees do not depict consensus

18 eukaryotic relationships. Thus, although the trees do not fulfill the conservative

19 criteria for LGT they also do not provide strong support for the alternative

20 hypothesis, that the *E. histolytica* genes were vertically inherited from a

21 common ancestor shared with all other eukaryotes.

22

23 In nine trees *E. histolytica* either clustered with a single newly published

24 eukaryotic sequence, or such a relationship could not be ruled out. In six of

25 these nine trees *E. histolytica* and *Trichomonas vaginalis* grouped together, and

26 two trees grouped *E. histolytica* with the diatom *Thalassiosira* (for example see

27 Figure 12). Such trees are also not easy to explain within the current consensus

28 for eukaryotic relationships (Baldauf, 2003). Similar topologies have been

29 previously reported for other eukaryotes (Andersson, 2005). The explanations

30 advanced to explain the absence of the gene in other eukaryotes include

31 massive gene loss from multiple eukaryotic lineages, or LGT between the

eukaryotic lineages concerned. *Entamoeba* species can ingest both eukaryotes and prokaryotes and it has been suggested that LGT between eukaryotes, subsequent to one lineage acquiring the gene from a prokaryote, could explain such peculiar tree topologies and sparse distribution (Andersson, 2005). The fact that six of the nine cases recover a relationship between *Entamoeba* and *Trichomonas*, whose relatives often share the same niche, is consistent with this idea. In prokaryotes, recent large-scale analyses support the hypothesis that species from the same environment may share a set of niche specific genes (Beiko *et al.*, 2005; Mira *et al.*, 2004).

For five trees, the *E. histolytica* gene now appears to be present in eukaryotes from a different taxonomic group and the analysis cannot exclude a common origin for all eukaryotic sequences. Thus, for about 5% of the original 96 cases the simplest explanation is no longer LGT, but vertical inheritance from a common ancestor shared with other eukaryotes.

10.2 Where Do The Genes Come From?

As before, certain prokaryotic groups are favoured as the potential donors of LGT genes in the *E. histolytica* genome (Loftus *et al.*, 2005). In 15 well-resolved trees *E. histolytica* is recovered next to a member of the Bacteroidetes/Chlorobii group. Bacteroidetes/Chlorobii are abundant members of the intestinal microflora (Shoemaker *et al.*, 2001) providing plenty of opportunity for LGT to occur. Members of the Bacteroidetes/Chlorobii and Fusobacterium (one tree) groups are all obligate anaerobes. This bias is consistent with the idea that prokaryotic and eukaryotic cohabitants of the same anaerobic niche are sharing genes (Andersson *et al.*, 2001; Beiko *et al.*, 2005; Lawrence, 2005a). For example, Figure 13 shows an intriguing example where the *T. vaginalis* gene clusters with members of the Bacteroidetes/Chlorobii and *E. histolytica* clusters with *Fusobacterium.*

10.3 What Kinds of Gene Are Being Transferred?

Most of the 68 laterally transferred genes that can be assigned to a functional category encode enzymes involved in metabolism (Figure 14). This is consistent with the complexity hypothesis, which posits that LGT of genes involved in processing a single substrate are more likely to be transferred than those genes encoding proteins that interact with many other cellular components, such as ribosomal proteins for example (Jain *et al.*, 1999). Mapping the LGT enzymes on the *E. histolytica* metabolic pathway (Loftus *et al.*, 2005) indicates that LGT has affected some important pathways, including iron-sulphur cluster biosynthesis, amino acid metabolism, and nucleotide metabolism. Since only eight of the 68 LGT have obvious homologues in the human genome, the proteins are potentially specific to the parasite and may thus be worth exploring as potential drug targets. The rest of the LGT cases involve hypothetical or unclassified proteins.

11. MICROARRAY ANALYSIS

Microarray-based analyses can be utilised in conjunction with genome sequencing to assign functional roles to annotated genes and to clarify genomic architecture. A number of groups have utilised DNA microarrays in *E. histolytica* (made from random genomic DNA fragments or long or short oligonucleotides based on annotated genes) to successfully study transcriptional differences between virulent and avirulent *E. histolytica* as well transcriptional responses to heat shock, collagen and calcium exposure, tissue invasion, and cyst development (Debnath *et al.*, 2004; Gilchrist *et al.*, 2006; MacFarlane and Singh, 2006; Weber *et al.*, 2006; Davis *et al.*, 2007; Ehrenkaufer *et al.*, 2007). Additionally, using a genomic DNA microarray, comparative genomic hybridisations (CGH) between strains and species of *Entamoeba* have been performed (Shah *et al.*, 2005).

Some interesting aspects of amoebic biology have been uncovered using DNA microarray based expression profiling. To investigate the hypothesis that virulence determinants will be more highly expressed in virulent strains, the

transcriptomes of virulent and avirulent *Entamoeba* species and strains have been studied. It has been confirmed that a number of known virulence determinants have decreased expression in avirulent *Entamoeba* (MacFarlane and Singh, 2006; Davis *et al.*, 2007). A genomic DNA microarray composed of 2,110 genes identified 29 genes with decreased expression in both an attenuated *E. histolytica* strain (Rahman) and the avirulent *E. dispar* (strain SAW760) (MacFarlane and Singh, 2006), while an oligonucleotide microarray composed of 6,242 genes identified 152 genes with a higher level of expression in the virulent *E. histolytica* HM-1:IMSS than in the attenuated Rahman strain (Davis *et al.*, 2007). A majority of these genes are annotated as hypothetical and whether these genes encode novel virulence factors will require genetic analysis of their functions. A peroxiredoxin gene identified as having decreased expression in *E. histolytica* Rahman has been shown to be a virulence factor (Davis *et al.*, 2006), indicating that these comparisons between virulent and avirulent strains are likely to be a fruitful avenue of investigation.

In other microarray based studies, the large family of transmembrane receptor kinases identified in *E. histolytica* has been found to be differentially expressed under *in vitro* trophozoite culture conditions (Beck *et al.*, 2005). One can easily envision that these kinases may have roles in signaling, allowing the parasite to adapt to its ever changing environmental milieu. A substantial transcriptional response to heat shock has been demonstrated (Weber *et al.,* 2006), and interestingly lectin gene family members were identified as being differentially regulated under heat shock conditions.

The most comprehensive microarray data to date used a whole genome short oligonucleotide microarray (based on the Affymetrix platform) to profile the transcriptional changes that occur as the parasite colonises and invades the host colon (Gilchrist *et al.*, 2006). Using a mouse model of colitis, in which the microscopic features replicate human disease and substantial pathology can be seen, the transcriptional response of parasites was assayed soon after

1 colonisation (1 day after injection into the caecum) and in a long-term (29 days)

2 disease state. Overall, 326 genes were modulated at day 1 after infection, 109

3 at 29 days after infection, and 88 at both time points. A number of the well-

4 characterised "virulence determinants" in *E. histolytica* were highly expressed

5 under all conditions tested and not transcriptionally modulated, although some

6 members of the cysteine proteinase gene family were highly regulated during

7 tissue invasion. A summary of the genes and gene families that have been

8 identified as being transcriptionally active under the conditions mentioned

9 above are listed in Table 9.

10

11 The life cycle of *E. histolytica* involves transition between the trophozoite

12 stage, responsible for colonisation as well as invasive disease and the cyst,

13 responsible for infection transmission. Despite its central role, little is known

14 about cyst development in *E. histolytica,* largely due to our inability to generate

15 *E. histolytica* cysts in axenic culture. Using a whole genome microarray and

16 xenic cultures of recently isolated *E. histolytica* strains that contained

17 spontaneously produced cysts, a cyst transcriptome was developed that

18 identified 1,439 developmentally regulated genes (672 cyst-specific and 767

19 trophozoite-specific genes; Ehrenkaufer *et al.*, 2007). This first large-scale

20 insight into encystation indicates that ca. 15% of *E. histolytica* genes are

21 transcriptionally controlled in this developmental pathway. Among the genes

22 identified were a number of stage-specific cysteine proteinases, transmembrane

23 kinases, transcriptional regulators, and other potential initiators of the

24 developmental cascade. Future characterisation of these genes and pathways

25 will provide important insights into developmental processes in this parasite.

26

27 The above microarray studies used expression data to identify interesting genes

28 and pathways potentially involved in amoebic pathogenesis or development. In

29 another application of microarrays, comparative genomic hybridisations (CGH)

30 identified a number of interesting genomic characteristics of *Entamoeba* (Shah

31 *et al.,* 2005). The *E. histolytica* genome project revealed that a large number of

genes are multi-copy or members of highly similar gene families. Due to the
repetitive nature of the genome there has been difficulty with genome assembly
and thus the large number of gene duplications could have represented an
assembly artifact. The data from CGH confirmed the high copy number of a
significant portion (ca. 14%) of the genome and validated the genome
assembly. Additionally, genome-wide genetic diversity was demonstrated
among strains of *E. histolytica* (Shah *et al.*, 2005) including the observation that
the attenuated *E. histolytica* strain Rahman had a unique genetic pattern
suggesting the possibility that a genomic signature may correlate with invasive
potential. Since genome sequencing for different *E. histolytica* strains,
including clinical isolates, is unlikely the promise of CGH to study genetic
diversity and identify genotype-phenotype associations is substantial.

E. dispar, the closely related but avirulent species, had been identified early on
as having some genetic divergence from the virulent *E. histolytica*. CGH
analysis of *E. histolytica* and *E. dispar* revealed a significant amount of
difference between the two species. Whether the genetic drift in these genes is
responsible for the non-invasive phenotype of *E. dispar* is not known, but the
work has highlighted a number of genes for further functional analyses.

Taken together the DNA microarray analyses of *Entamoeba* have been useful to
begin to dissect the genome of this parasite and provide functional context to
the genes identified in the genome sequencing effort. Future directions will
include analysis of the parasite transcriptome in invasive hepatic disease as well
as further characterisation of the developmental conversion to the cyst form.
Those data may be useful in the development of novel diagnostic and
therapeutic options. Additionally, genetic approaches can now be applied to
definitively assign a role for these genes in amoebic biology and pathogenesis.

12. FUTURE PROSPECTS FOR THE *E. HISTOLYTICA* GENOME

1   Although the genome of *E. histolytica* is not yet complete it has already

2   revealed much about the biology of the parasite. There appear to be forces

3   acting to compact the genome, leading to a reduction in the coding region and

4   intron length of genes, and resulting in the loss of numerous metabolic

5   pathways. However, there are also opposing evolutionary forces as many gene

6   families have expanded. This applies particularly to genes involved in signaling

7   and trafficking that allow the parasite to sense and respond to its environment, a

8   necessary adaptation for a predatory protist. Unfortunately, it is difficult at

9   present to understand the genome structure on a macro scale due to the

10   fragmented nature of the current assembly. In other parasites, genome structure

11   has been vital to unraveling important biological processes, such as antigenic

12   variation in *T. brucei* and identification of rifin genes in *P. falciparum.* Until

13   the *E. histolytica* genome is complete we will not know what else remains to be

14   uncovered. Efforts are already underway to complete the genome by first

15   generating a HAPPY map (Dear and Cook, 1993). Over 2000 markers are being

16   designed at approximately 25 kb intervals across all contigs. Using PCR, co-

17   segregation analysis allows the identification of contigs that are physically

18   linked in the genome. This will allow the ordering and orientation of the contigs

19   and will facilitate gap closure. Shotgun genome sequencing projects of *E.*

20   *invadens* and *E. dispar* are underway (Loftus and Hall, 2005). At present the *E.*

21   *invadens* genome appears to assemble with fewer problems than were

22   encountered with that of *E. histolytica.* It is anticipated that an essentially

23   complete *E. invadens* genome sequence will be obtained, enabling extensive

24   comparative analyses to be made, and facilitating the study of pathogenicity,

25   host interaction and the evolutionary forces acting on the genome.

26

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1

1   *Table 1.* **Genome summary statistics for selected single celled**

2   **organisms with sequenced genomes**.

3

	Entamoeba histolytica	*Plasmodium falciparum*	*Dictyostelium discoideum*	*Saccharomyces cerevisiae*	*Encephalitozoon cuniculi*
Genome Size (Mb)	**23.7**	22.8	33.8	12.5	2.5
G+C content (%)	**24.1**	19.4	22.5	38	45.5
Gene number	**9,938**	5268	12,500	5538	1,997
Av. gene size (bp)	**1167**	2,534	1,756	1428	1077
% coding DNA	**49.2**	52.6	ND	70.5	ND
Av. protein size (aa)	**389**	761	518	475	359
Av. intergenic dist. (kb)	**0.8**	1.7	0.8	0.6	0.1
Gene density (kb per gene)	**1.9**	4.3	2.5	2.2kb	1.1
% Genes with introns	**25.2**	54	69	5	<1
Av. intron size (bp)	**102.1**	179	146	ND	-
Av. number of intron s/gene	**1.5**	2.6	1.9	1	1

4

1 *Table 2.* **Summary properties of the repeated DNAs**.

2 (a)

Type	Size (kb)	Estimated copy no. from genome sequence (Ref)	Estimated copy no. per haploid genome from hybridisation (Ref)	Transcript size (kb)(Ref)
EhLINE1	4.8	142 (1) 409; 49 full-length(2)	140 (3)	No full-length transcript (4)
EhLINE2	4.72	79 (1) 290; 56 full-length (2)	Not Determined	Not Determined
EhLINE3	4.81	12 (1) 52; 3 full-length(2)	Not Determined	Not Determined
EhSINE1	0.5-0.6	219 (1) 272; 81 full-length(2) 214; >90 full-length (3)	500	0.7 (6)
EhSINE2	0.65	120 (1) 117; 62 full-length(2) 122; ~50 full-length (3)	Not Determined	0.75 (7)
EhSINE3	0.58	1 (1,2)	Not Determined	Not detected (3)
Tr	0.7	1 per rDNA episome (5)	Not Determined	0.7 (5)
BspA-like	0.96	77 (8)	190 (3)	Not detected(3) [a]
Ehssp1	0.9-1.1	Not Determined	306 (9)	1.5 (9)

3

4 (b)

Family 16	GTAATGAATATAYAACTAAGAATTTCATTTAAAATGRATATG
Family 17	CAACAAATAAATRGKTTCAATAAAATA

5

6 (a) References for data: (1): (Van Dellen *et al.*, 2002a), (2): (Bakre *et al.*,

7 2005), (3): This analysis, (4) Bakre and Bhattacharya, unpublished

8 observations; (5): (Burch *et al.*, 1991), (6): (Cruz-Reyes *et al.*, 1995), (7): Shire

9 and Ackers, submitted, (8): (Davis *et al.*, 2006), (9): (Satish *et al.*, 2003). [a] -

10 although no transcript was detected the protein has been demonstrated on the

11 cell surface and in Western blots using antibodies (Davis *et al.*, 2006).

1   (b) Consensus sequences of Family 16 and 17 repeats. Standard abbreviation

2   for degenerate sequence positions are used: R= purine, Y= pyrimidine, K= G or

3   T.

1   *Table 3*. **Number and ranking of Pfam domains across different genomes**.

		EH		EC		PF		SC		AT		CE		DD	
		#	Rank	#	Rank	#	Rank	#	Rank	#	Rank	#	Rank	#	Rank
WD40	WD domain, G-beta repeat	249	1	139	1	287	2	414	1	1137	3	694	1	719	2
LRR_1	Leucine Rich Repeat	131	2	40	2	55	12	43	17	3793	2	494	5	372	4
Pkinase	Protein kinase domain	95	3	27	5	78	8	116	2	839	4	405	8	225	7
HEAT	HEAT repeat	70	4	13	15	44	17	114	3	220	17	162	26	108	12
efhand	EF hand	58	5	7	28	80	7	29	25	422	8	213	20	153	9
RRM_1	RNA recognition motif.	57	6	30	3	95	6	86	6	375	10	223	19	134	10
Ras	Ras family	46	7	9	22	13	44	25	28	78	68	66	76	126	11
TPR_1	Tetratricopeptide repeat	42	8	23	7	48	15	103	4	334	12	180	22	168	8
Ank	Ankyrin repeat	34	9	6	34	55	12	61	9	431	6	629	2	446	3
PUF	Pumilio-family RNA binding repeat	33	10	8	23	15	34	51	13	142	32	75	68	34	62
RhoGAP	RhoGAP domain	27	11	2	118	1	520	11	80	9	559	31	138	45	39
Myb_DNA-binding	Myb-like DNA-binding domain	22	12	15	12	10	62	21	34	424	7	30	141	55	26
RhoGEF	RhoGEF domain	22	12	1	230	0	1215	3	366	0	2581	34	130	47	37
Helicase_C	Helicase conserved C-terminal domain	20	14	28	4	64	11	74	8	150	31	98	49	84	20
DEAD	DEAD/DEAH box helicase	20	14	22	9	49	14	59	10	103	50	76	67	48	35
PH	PH domain	19	16	1	230	5	123	25	28	22	255	77	63	94	16
Metallophos	Calcineurin-like phosphoesterase	19	16	6	34	16	32	21	34	66	83	78	62	31	67
Gelsolin	Gelsolin repeat	18	18	2	118	2	295	4	255	33	169	12	323	29	68
LIM	LIM domain	17	19	0	703	0	1215	8	116	16	341	103	47	56	25
CH	Calponin homology (CH) domain	16	20	4	54	1	520	7	137	26	211	57	87	49	33
Filamin	Filamin/ABP280 repeat	16	20	0	703	1	520	0	1842	2	1450	55	91	10	203

18   Columns labeled "#" give the total number of occurrences of a particular domain. Columns labeled "Rank" give the ranking of the domain where

19   the most common domain is ranked 1. The organisms shown are *Entamoeba histolytica* (EH), *Encephalitozoon cuniculi* (EC), *Plasmodium*

20   *falciparum* (PF), *Arabidopsis thaliana* (AT), *Saccharomyces cerevisae* (SC), *Dictyostelium discoideum* (DD)..

Table 4. **Family C1-like cysteine endopeptidases of *E. histolytica*.**

Protein Name	Previous designation	Accession No.	Protein length Total (pre,pro,mature)	Active site residues	Conserved motifs	Remarks
EhCP-A1	EhCP1	XP_650156	315 (13,80,222)	QCHN	ERFNIN, DWR	
EhCP-A2	EhCP2	XP_650642	315 (13,80,222)	QCHN	ERFNIN, DWR	
EhCP-A3	EhCP3	XP_653254	308 (13,79,216)	QCHN	ERFNIN, DWR	
EhCP-A4	EhCP4	XP_656602	311 (20,73,218)	QCHN	ERFNIN, DWR	
EhCP-A5	EhCP5	XP_650937	318 (20,72,225)	QCHN	ERFNIN, DWR, RGD	Degenerate in *E. dispar*
EhCP-A6	EhCP6	XP_657364	320 (17,79,224)	QCHN	ERFNIN, DWR	
EhCP-A7	EhCP8	XP_648996	315 (13,80,222)	QCHN	ERFNIN, DWR	
EhCP-A8	EhCP9	XP_657446	317 (15,82,220)	QCHN	ERFNIN, DWR	
EhCP-A9	EhCP10	XP_655675	297 (17,90,190)	QCHN	ERFNIN, DWR	
EhCP-A10	EhCP17	XP_651147	420 (18,148,254)	QCHN	ERFNIN, DWR	
EhCP-A11	EhCP19	XP_651690	324 (17,79,228)	QC IN [a]	ERFNIN, DWR	
EhCP-A12	new	XP_653823	317 (14,83,220)	(d)	ERFNIN, DWR	
EhCP-B1	EhCP7	XP_651581	426 (15,106,305)	QCHN	ERFNIN, PCNC	hydrophobic C-terminus
EhCP-B2	EhCP11	AAO03568	431 (15,106,310)	QCHS [a]	ERFNIN, PCNC	GPI cleavage site
EhCP-B3	EhCP12	XP_656747	474 (16,107,351)	QCHN	ERFNIN, PCNC	TMH:444-466 aa
EhCP-B4	EhCP13	XP_648501	379 (16,105,258)	QCHN	ERFNIN, PCNC	TMH or GPI cleavage site
EhCP-B5	EhCP14	XP_652671	434 (12,108,314)	QCHN	ERFNIN, PCNC	GPI cleavage site
EhCP-B6	EhCP15	XP_652465	300 (14,55,231)	QCHN	PCNC	hydrophobic C-terminus
EhCP-B7	EhCP16	XP_650400	650 (18,144,488)	QCHN	ERFNIN, PCNC	hydrophobic C-terminus, Cys-rich profile
EhCP-B8	EhCP18	XP_651049	473 (15,105,353)	QCHN	ERFNIN, PCNC, RGD	GPI cleavage site
EhCP-B9	EhCP112	XP_652993	446 (19,112,315)	QCHN	ERFNIN, PCNC, RGD	hydrophobic C-terminus, Cys-rich profile
EhCP-B10	new	XP_648306	372 (b)	QCHN	ERFNIN, PCNC, RGD	hydrophobic C-terminus
EhCP-B11	new	XP_648013	133 (b)	Q ? ? ?	PCNC	
EhCP-C1	new	XP_654453	586 (c)	QCIN [a]	HS(X)$_6$ICP	TMH:12-34
EhCP-C2	new	XP_656632	567 (c)	QCHN	HS(X)$_6$ICP	TMH:27-49
EhCP-C3	new	XP_655128	572 (c)	QCHN	HS(X)$_6$LCP	TMH:17-39
EhCP-C4	new	XP_655800	502 (c)	QCHN	LT(X)$_6$LCP	
EhCP-C5	new	XP_654800	557 (c)	QCHN	IS(X)$_6$ICP	TMH:20-42
EhCP-C6	new	XP_651553	557 (c)	QCHD [a]	HS(X)$_6$LCA	TMH:14-36
EhCP-C7	new	XP_657273	595 (c)	QCHN	IS(X)$_6$LCP	TMH:19-41
EhCP-C8	new	XP_655479	627 (c)	QCHN	IS(X)$_6$ICP	TMH:29-51

1	EhCP-C9	new	XP_655011	518 (c)	(d)	HS(X)$_6$ICP	TMH:12-34
2	EhCP-C10	new	XP_654829	530 (c)	QCHN	IS(X)$_6$ICP	TMH:15-37
3	EhCP-C11	new	XP_648083	526 (c)	(d)	HS(X)$_6$ICP	TMH:20-42
4	EhCP-C12	new	XP_650829	473 (c)	(d)	MS(X)$_6$LCG	TMH:26-48 & 449-471
5	EhCP-C13	new	XP_656556	564 (c)	QCHN	VS(X)$_6$RCG	TMH:21-43
6							

7   a - active sites that lack the canonical motif QCHN; b - incomplete sequence; c - cleavage sites to be determined; d - not conserved

Table 5. **Family C2-, C19-, C54-, and C65-like Cysteine endopeptidases of *E. histolytica***

Name	Homology	Family	ProteinID	Protein length	Active site
EhCALP1	Calpain-like	C2	XP_649922	591 aa	not cons.
EhCALP2	Calpain-like	C2	XP_657312	473 aa	QCHN
EhUBHY	Ubiquitin Hydrolase-like	C19	XP_657356	444 aa	NDTN
EhAUTO1	Autophagin-like	C54	XP_651386	325 aa	YCHS
EhAUTO2	Autophagin-like	C54	XP_653798	364 aa	YCHD
EhAUTO3	Autophagin-like	C54	XP_652043	364 aa	YCHD
EhAUTO4	Autophagin-like	C54	XP_656724	348 aa	YCHD
EhOTU	Otubain-like	C65	XP_654013	259 aa	DCH

Table 6. **Attributes of the identified SAPLIPs of *E. histolytica***

Name	Size, aa			SAPLIP domain		Identical to annotated protein	SAPLIP domain can be found within this sequence[g]	Similar to (aa sequence identity, %)[f]	Homologous proteins in other organisms (aa sequence identity; %)
	entire	signal peptide (predicted)[a]	proform / mature[c]	position aa residues	similarity[e] name, Acc. no.				
Amoebapore A	98	21[b]	77	22 -98	SAPOSIN B, IPR 008139	amoebapore A precursor ACCESSION XP_653265		Amoebapore A Acc. AAA29111 (100%)	Disparpore A Acc. AAA18632 *E.dispar* (94%)
SAPLIP 1	92	15	77	16 - 92	SAPOSIN B, IPR 008139	saposin-like protein ACCESSION XP_655836		Amoebapore A Acc. AAA29111 (64%)	Disparpore A Acc. AAA18632 *E.dispar* (68%)
Amoebapore B	96	19[b]	77	20 - 96	SAPOSIN B, IPR 008139	Pore-forming peptide amoebapore B precursor (EH-APP) ACCESSION Q24824		Amoebapore B Acc.CAA54226 (100%)	Disparpore B Acc.AAF04195 *E.dispar* (90%)
Amoebapore C	101	24[b]	77	25 - 101	SAPOSIN B, IPR 008139	amoebapore C ACCESSION XP_656029		Amoebapore C Acc. CAA54225 (100%)	Disparpore C Acc.AAF04196 *E.dispar* (88%)
SAPLIP 2	153	15	138	71 - 153	SAPOSIN B, IPR 008139	hypothetical protein ACCESSION XP_656037		-	-
SAPLIP 3	94	16	78	18 - 94	SAPOSIN B, IPR 008139	hypothetical protein ACCESSION XP_656682		Amoebapore A Acc. AAA29111 (30%)	Invapore X Acc. AAP80381 *E.invadens* (67%)
SAPLIP 4	96	17	79	18 - 96	SAPOSIN B, IPR 008139	hypothetical protein ACCESSION XP_652159 AND hypothetical protein ACCESSION XP_652303		Amoebapore C Acc.CAA54225 (27%)	Disparpore C Acc.AAF04196 *E.dispar* (30%)
SAPLIP 5	1026	18	1008	946 - 1026	SAPOSIN B, IPR 008139	chromosome partition protein ACCESSION XP_655789		-	-
SAPLIP 6	92	15	77	14 - 92	SAPOSIN B, IPR 008139	hypothetical protein ACCESSION XP_655820		-	-
SAPLIP 7	926	17	909	855 - 926	SAPOSIN B, IPR 008139	conserved hypothetical protein ACCESSION XP_656441		-	-
SAPLIP 8	980	15	965	902 - 980	SAPOSIN B, IPR 008139	hypothetical protein ACCESSION XP_656913		-	-
SAPLIP 9	140	15	125	61 - 140	SAPOSIN B, IPR 008139	hypothetical protein ACCESSION XP_650376		-	-
SAPLIP 10	657	16	641	577 - 657	SAPOSIN B, IPR 008139	-	Genomic survey sequence ACCESSION AZ687176	-	-
SAPLIP 11	693	17	676	615 - 693[d]	-	-	Genomic survey sequence ACCESSION AZ692153	-	-
SAPLIP 12	873	16	857	793 - 873	SAPOSIN B, IPR 008139	hypothetical protein ACCESSION XP_652721		-	-
SAPLIP 13	1009	no signal peptide predicted	1009	931 - 1005	SAPOSIN B, IPR 008139	hypothetical protein ACCESSION XP_655089		-	-
SAPLIP 14	915	17	898	834 - 915	SAPOSIN B, IPR 008139	-	Genomic survey sequence ACCESSION AZ690015	-	-

SAPLIPs were named according to the similarity of their SAPLIP domain to amoebapore A:

[a] by the programme SignalP and manually corrected if predicted cleavage site is within the SAPLIP domain

[b] verified by experimental data

[c] with the exception of amoebapores it is not possible to decide whether proteins are further processed

1   [d] identified manually

2   [e] extracted from InterPro databases

3   [f] if no similarity is reported, there is none outside of the SAPLIP domain

4   [g] sequences only found in GSS section of GenBank with given identifier

1   *Table 7.* **The number of genes encoding representative proteins involved in vesicular trafficking in *E. histolytica*.**

Protein	*E. histolytica*	*S. cerevisiae*	*C. elegans*	*D. melanogaster*	*H. sapiens*	*A. thaliana*	References
Sar1	1	1	1	1	2	4	1,2
COPII	9	6	5	4	9	12	1
Arf	10	6	11	11	25	17	3
COPI	11	7	7	7	9	9	1
AP-1		5	7	5	8	9	
AP-2		4	5	5	5	6	
AP-3		4	4	4	7	4	
AP-4		0	0	0	4	4	
AP total	18	13	16	14	24	23	1
Rab	91	11	29	26	60	57	1,4
Qa	8	7	9	7	12	18	
Qb	10(b+c)	6	7	5	9	11	
Qc		8	4	5	8	8	
R	10	5	6	5	9	14	
SNARE total	28	24	23	20	35	54	1,5,6
NSF	1	1	1	2	1	1	1,7
SNAP	1	1	1	3	1	3	
Sec1	5	4	6	5	7	6	8

2   References (1), Bock *et al.* (2001); (2), Wennerberg *et al.* (2005); (3), Pasqualato *et al.* (2002); (4), Pereira-Leal and Seabra (2001); (5), Burri

3   and Lithgow (2004); (6), Uemura *et al.* (2004); (7), Sanderfoot *et al.* (2000); (8) Boehm *et al.* (2001).

1   *Table 8.* Reassessment of the 96 candidate LGT cases identified in the original genome publication.

Acc.[a] RefSeq	Acc.[a]	EhL[b]	Top Prokaryotic blast hit	PL[c]	%ID[d]	Top Eukayotic blast hit	EL[c]	%ID[d]	PE-score[e]	EE-score[f]	P/E Ratio[g]
41 LGT cases that remain strongly supported according to our criteria											
EAL43201	XP_648590.1	487	*Treponema denticola*	507	57	*Trichomonas vaginalis*	398	43	1.00E-167	5.00E-88	2.00E-80
EAL43619	XP_649008.1	621	*Vibrio vulnificus*	673	41	*Saccharomyces cerevisiae*	664	40	1.00E-132	1.00E-125	1.00E-07
EAL43678	XP_649067.1	538	*Fusobacterium nucleatum*	562	47	*Trichomonas vaginalis*	477	34	1.00E-135	2.00E-60	5.00E-76
EAL43850	XP_649240.1	880	*Mannheimia succiniciproducens*	898	63	*Mastigamoeba balamuthi*	882	45	0	0	N/A
EAL44182	XP_649570.1	260	*Bacteroides thetaiotaomicron*	273	34	*Yarrowia lipolytica*	298	29	2.00E-35	4.00E-10	5.00E-26
EAL44226	XP_649612.1	262	*Bacteroides thetaiotaomicron*	267	28	*Tetrahymena thermophila*	1476	30	2.00E-25	0.11	1.82E-24
EAL44778	XP_650165.1	188	*Bacteroides thetaiotaomicron*	188	43	*Neurospora crassa*	546	34	8.00E-41	1.8	4.44E-41
EAL45076	XP_650453.1	358	*Bacteroides fragilis*	362	46	*Trichomonas vaginalis*	562	22	1.00E-87	0.24	4.17E-87
EAL45145	XP_650531.1	825	*Staphylococcus aureus*	1036	30	*Trichomonas vaginalis*	2468	20	3.00E-59	0.016	1.88E-57
EAL45220	XP_650606.1	479	*Clostridium tetani*	471	45	*Arabidopsis thaliana*	581	31	1.00E-114	1.00E-54	1.00E-60
EAL44744	XP_650131.1	160	*Bacteroides fragilis*	424	41	*Yarrowia lipolytica*	169	31	3.00E-24	7.00E-11	4.29E-14
EAL46110	XP_651498.1	157	*Bacteroides fragilis*	166	49	*Arabidopsis thaliana*	627	35	5.00E-35	3.2	1.56E-35
EAL45378	XP_650765.1	311	*Haloarcula marismortui*	299	43	*Leishmania major*	411	43	3.00E-54	1.00E-32	3.00E-22
EAL45618	XP_651004.1	159	*Bacteroides thetaiotaomicron*	157	46	*Plasmodium vivax*	1275	33	2.00E-28	0.69	2.90E-28
EAL46311	XP_651697.1	248	*Synechococcus elongates*	270	36	*Trichomonas vaginalis*	3075	18	1.00E-30	0.38	2.63E-30
EAL46679	XP_652065.1	218	*Methanosarcina mazei*	230	37	*Candida glabrata*	461	24	8.00E-31	0.079	1.01E-29
EAL46975	XP_652361.1	370	*Bordetella bronchiseptica*	368	46	*Cryptococcus neoformans*	372	40	8.00E-83	3.00E-71	2.67E-12
EAL47525	XP_652912.1	380	*Clostridium perfringens*	296	23	*Plasmodium falciparum*	390	34	2.00E-13	1.3	1.54E-13
EAL47905	XP_653291.1	227	*Clostridium perfringens*	259	33	*Tetrahymena thermophila*	1425	24	4.00E-19	0.32	1.25E-18
EAL48587	XP_653973.1	425	*Desulfovibrio vulgaris*	442	60	*Yarrowia lipolytica*	572	37	1.00E-149	9.00E-57	1.11E-93
EAL48979	XP_654365.1	732	*Thermotoga neapolitana*	740	40	*Cryptococcus neoformans*	735	28	1.00E-135	3.00E-64	3.33E-72
EAL49084	XP_654474.1	350	*Methanococcus jannaschii*	241	29	*Anopheles gambiae*	784	40	1.00E-24	5.00E-06	2.00E-19
EAL49209	XP_654596.1	247	*Bacteroides fragilis*	243	38	*Thalassiosira pseudonana*	269	22	7.00E-43	0.0002	3.50E-39
EAL49277	XP_654665.1	737	*Bacteroides thetaiotaomicron*	781	31	*Cryptococcus neoformans*	935	24	1.00E-111	6.00E-44	1.67E-68
EAL49613	XP_654999.1	168	*Sulfolobus solfataricus*	237	34	*Tetrahymena thermophila*	487	38	1.00E-16	6.00E-06	1.67E-11
EAL49813	XP_655200.1	186	*Escherichia coli*	200	31	*P. brasiliensis*	257	26	2.00E-13	0.47	4.26E-13
EAL49869	XP_655257.1	390	*Campylobacter jejuni*	407	56	*Ashbya gossypii*	490	39	1.00E-124	8.00E-73	1.25E-52
EAL50263	XP_655646.1	390	*Porphyromonas gingivalis*	408	48	*Yarrowia lipolytica*	428	38	1.00E-98	3.00E-60	3.33E-39
EAL50440	XP_655826.1	344	*Bacillus anthracis*	491	54	*Rhizopus oryzae*	510	40	1.00E-101	2.00E-67	5.00E-35
EAL50508	XP_655888.1	348	*Wolinella succinogenes*	340	55	*Mus musculus*	168	40	1.00E-106	2.00E-18	5.00E-89
EAL50603	XP_655988.1	567	*Bacteroides thetaiotaomicron*	622	45	*Trichomonas vaginalis*	632	39	1.00E-141	2.00E-99	5.00E-43
EAL50801	XP_656185.1	499	*Bacteroides thetaiotaomicron*	513	52	*Trichomonas vaginalis*	514	28	1.00E-145	3.00E-40	3.33E-106
EAL50992	XP_656375.1	140	*Archaeoglobus fulgidus*	184	40	*Trichomonas vaginalis*	195	46	1.00E-27	0.018	5.56E-26

EAL50997	XP_656380.1 656	*Bacteroides thetaiotaomicron*	718	53	*Cryptococcus neoformans*	770	32	0	2.00E-69	0.00E+00
EAL51149	XP_656535.1 343	*Bacteroides fragilis*	359	43	*Pichia ofunaensis*	378	34	8.00E-84	1.00E-53	8.00E-31
EAL51236	XP_656622.1 259	*Symbiobacterium thermophilum*	274	45	*Oryza sativa*	315	21	3.00E-51	0.003	1.00E-48
EAL51348	XP_656749.1 171	*Methanopyrus kandleri*	204	37	*Tetrahymena thermophila*	2872	22	3.00E-21	0.1	3.00E-20
EAL51525	XP_656903.1 316	*Bacteroides thetaiotaomicron*	300	29	*Candida boidinii*	314	32	8.00E-27	0.0007	1.14E-23
EAL51565	XP_656946.1 415	*Clostridium perfringens*	900	43	*Trichomonas vaginalis*	897	40	1.00E-89	5.00E-81	2.00E-09
EAL51925	XP_657304.1 448	*T. tengcongensis*	481	43	*Giardia lamblia*	937	33	3.00E-96	2.00E-60	1.50E-36
EAL52001	XP_657387.1 303	*Oceanobacillus iheyensis*	306	27				2.00E-15	0.00E+00	

27 LGT cases that are more weakly supported than before according to our criteria

EAL45152	XP_650539.1 122	*Shewanella oneidensis*	132	34	*Trypanosoma bruzeii*	385	24	5.00E-10	6.6	7.58E-11
EAL43347	XP_648734.1 848	*Burkholderia pseudomallei*	779	38	*Plasmodium falciparum*	2463	32	1.00E-136	4.00E-44	2.50E-93
EAL44257	XP_649643.1 407	*Clostridium acetobutylicum*	406	25	*Homo sapiens*	468	24	6.00E-23	1.00E-14	6.00E-09
EAL45586	XP_650972.1 460	*Clostridium tetani*	476	47	*Xenopus laevis*	513	38	1.00E-116	5.00E-84	2.00E-33
EAL46313	XP_651699.1 118	*Prochlorococcus marinus*	163	42	*Hordeum vulgare*	223	22	2.00E-21	1.4	1.43E-21
EAL46399	XP_651785.1 218	*Clostridium perfringens*	235	65	*Trypanosoma bruzeii*	295	52	3.00E-73	9.00E-54	3.33E-20
EAL46421	XP_651808.1 205	*Clostridium acetobutylicum*	230	40	*Arabidopsis thaliana*	241	33	7.00E-34	6.00E-12	1.17E-22
EAL46701	XP_652087.1 294	*Bacteroides fragilis*	308	45	*Thalassiosira pseudonana*	348	27	4.00E-63	1.00E-14	4.00E-49
EAL46757	XP_652143.1 95	*Lactococcus lactis*	103	31	*Tetrahymena thermophila*	112	32	3.00E-09	1.00E-07	3.00E-02
EAL46858	XP_652245.1 192	*Pseudomonas aeruginosa*	195	41	*Caenorhabditis briggsae*	229	40	6.00E-36	2.00E-17	3.00E-19
EAL47026	XP_652397.1 164	*Bacillus subtilis*	181	30	*Trichomonas vaginalis*	182	26	3.00E-10	2.00E-08	1.50E-02
EAL47464	XP_652839.1 504	*Treponema denticola*	509	39	*Piromyces sp.*	555	27	5.00E-88	2.00E-30	2.50E-58
EAL47648	XP_653034.1 259	*Methanosarcina mazei*	272	36	*Arabidopsis thaliana*	345	25	2.00E-39	3.00E-11	6.67E-29
EAL47787	XP_653173.1 546	*Spirochaeta thermophila*	571	56	*Solanum tuberosum*	552	46	1.00E-175	1.00E-135	1.00E-40
EAL48186	XP_653572.1 232	*Bacillus cereus*	279	34	*Thalassiosira pseudonana*	271	32	2.00E-10	2.00E-08	1.00E-02
EAL49309	XP_654698.1 358	*Methanosarcina mazei*	379	42	*Leishmania major*	373	31	5.00E-77	9.00E-44	5.56E-34
EAL48568	XP_653954.1 113	*Chlamydia pneumoniae*	271	38	*Debaryomyces hansenii*	699	38	5.00E-14	7.00E-16	7.14E+01
EAL48767	XP_654156.1 165	*Bacteroides fragilis*	177	40	*Trichomonas vaginalis*	189	28	7.00E-28	2.00E-05	3.50E-23
EAL48783	XP_654172.1 217	*Pseudomonas putida*	225	46	*Giardia lamblia*	239	35	2.00E-43	7.00E-24	2.86E-20
EAL49703	XP_655090.1 396	*Clostridium acetobutylicum*	398	34	*Tetrahymena thermophila*	445	29	4.00E-64	3.00E-44	1.33E-20
EAL49996	XP_655383.1 358	*Bacteroides thetaiotaomicron*	368	60	*Brachydanio rerio*	367	43	1.00E-121	5.00E-76	2.00E-46
EAL50325	XP_655711.1 447	*Clostridium tetani*	448	30	*Trichomonas vaginalis*	871	29	4.00E-46	1.00E-37	4.00E-09
EAL50521	XP_655905.1 285	*Streptococcus agalactiae*	323	29	*Leishmania major*	452	24	2.00E-22	3.00E-06	6.67E-17
EAL50620	XP_656005.1 261	*Wolinella succinogenes*	655	27	*Trichomonas vaginalis*	261	28	6.00E-21	1.00E-06	6.00E-15
EAL50838	XP_656225.1 299	*Anabaena* sp.	287	27	*Trichomonas vaginalis*	336	29	4.00E-15	0.0009	4.44E-12
EAL50986	XP_656369.1 219	*Bacteroides thetaiotaomicron*	240	31	*Xenopus laevis*	309	29	2.00E-20	1.00E-12	2.00E-08
EAL52121	XP_657511.1 220	*T. tengcongensis*	222	36	*Caenorhabditis elegans*	255	26	1.00E-30	1.00E-07	1.00E-23

14 cases where increased sampling has weakened that case for LGT

EAL42539	XP_647925.1 213	*Bacteroides thetaiotaomicron*	319	47	*Entodinium caudatum*	411	43	3.00E-53	1.00E-32	3.00E-21
EAL42738	XP_648124.1 313	*Campylobacter jejuni*	324	40	*Trichomonas vaginalis*	313	36	1.00E-63	4.00E-42	2.50E-22
EAL44270	XP_649657.1 179	*Methanococcus maripaludis*	193	37	*Anopheles gambiae*	186	21	2.00E-27	2.00E-09	1.00E-18

		EhL		PL	%ID		EL	%ID			
EAL44593	XP_649979.1	220	*Vibrio vulnificus*	244	24	*Trichomonas vaginalis*	238	21	0.0002	2.6	7.69E-05
EAL45320	XP_650707.1	154	*Geobacillus kaustophilus*	183	53	*Thalassiosira pseudonana*	182	43	8.00E-38	2.00E-32	4.00E-06
EAL45332	XP_650718.1	392	*Methanosarcina acetivorans*	420	48	*Trichomonas vaginalis*	396	47	8.00E-99	2.00E-93	4.00E-06
EAL45528	XP_650913.1	349	*Sulfolobus acidocaldarius*	343	28	*Cyanophora paradoxa*	313	27	1.00E-24	5.00E-17	2.00E-08
EAL45907	XP_651293.1	380	*Streptomyces coelicolor*	603	32	*Dictyostelium discoideum*	457	30	2.00E-39	2.00E-35	1.00E-04
EAL46026	XP_651412.1	176	*Bacteroides fragilis*	184	51	*Tetrahymena thermophila*	323	32	2.00E-44	8.00E-08	2.50E-37
EAL46116	XP_651488.1	662	*Bacillus clausii*	684	48	*Solanum tuberosum*	761	48	0	1.00E-172	0.00E+00
EAL46656	XP_652044.1	419	*Dictyoglomus thermophilum*	579	30	*S. pombe*	493	41	2.00E-35	2.00E-19	1.00E-16
EAL50605	XP_655990.1	392	*Thermotoga maritima*	417	38	*Cryptococcus neoformans*	445	30	2.00E-69	1.00E-33	2.00E-36
EAL51270	XP_656656.1	251	*Porphyromonas gingivalis*	261	50	*Anopheles gambiae*	272	39	6.00E-53	1.00E-35	6.00E-18
EAL52102	XP_657492.1	345	*Bacteroides thetaiotaomicron*	358	54	*Thalassiosira pseudonana*	354	47	1.00E-105	7.00E-86	1.43E-20
Nine cases where *Entamoeba* is now recovered with a recently sequenced gene from another microbi al eukaryote											
EAL44213	XP_649600.1	710	*Bdellovibrio bacteriovorus*	698	37	*Trichomonas vaginalis*	713	35	1.00E-127	1.00E-127	1.00E+00
EAL44435	XP_649823.1	250	*Bacteroides fragilis*	395	40	*Trichomonas vaginalis*	395	33	1.00E-43	3.00E-35	3.33E-09
EAL44766	XP_650152.1	401	*Porphyromonas gingivalis*	419	36	*Trichomonas vaginalis*	445	32	3.00E-65	1.00E-51	3.00E-14
EAL47785	XP_653171.1	234	*Bacillus anthracis*	242	32	*Trichomonas vaginalis*	256	39	2.00E-30	3.00E-33	6.67E+02
EAL47859	XP_653246.1	337	*Clostridium acetobutylicum*	322	50	*C. reinhardtii*	352	44	9.00E-74	0	N/A
EAL49158	XP_654544.1	397	*T. tengcongensis*	412	49	*Trichomonas vaginalis*	416	46	1.00E-100	4.00E-99	2.50E-02
EAL49488	XP_654874.1	320	*Geobacter sulfurreducens*	336	34	*Leishmania major*	357	31	1.00E-38	4.00E-30	2.50E-09
EAL49791	XP_655177.1	164	*Oceanobacillus iheyensis*	177	42	*Thalassiosira pseudonana*	96	38	8.00E-30	6.00E-09	1.33E-21
EAL50404	XP_655790.1	718	*T. tengcongensis*	717	37	*Trichomonas vaginalis*	721	34	1.00E-139	1.00E-118	1.00E-21
Five cases where vertical inheritance is now the simplest explanation for the new tree											
EAL44346	XP_649732.1	314	*Oceanobacillus iheyensis*	239	47	*Dictyostelium discoideum*	278	65	1.00E-52	3.00E-95	3.33E+42
EAL45466	XP_650849.1	209	*Agrobacterium tumefaciens*	254	31	*Thalassiosira pseudonana*	227	35	3.00E-23	1.00E-27	3.00E+04
EAL45548	XP_650934.1	259	*Bacillus cereus (strain ZK)*	233	29	*Candida glabrata*	270	30	7.00E-06	5.00E-05	1.40E-01
EAL45595	XP_650981.1	284	*Pyrobaculum aerophilum*	293	27	*Ashbya gossypii*	343	27	1.00E-23	7.00E-16	1.43E-08
EAL50185	XP_655571.1	186	*Aeropyrum pernix*	192	31	*Thalassiosira pseudonana*	149	30	4.00E-13	5.00E-06	8.00E-08

All 96 trees reanalysed here can be downloaded (in pdf format) from the following web site: http://www.ncl.ac.uk/microbial_eukaryotes/

[a]GenBank accession numbers and RefSeq accession numbers, respectively, for the 96 original candidates LGT identified by phylogenetic analysis (Loftus *et al.*, 2005)

[b]EhL, the length of the *E. histolytica* protein

[c]PL/EL, the protein length of the prokaryotic or eukaryotic top BlastP hit, respectively

[d]%ID, the percent identity between the *E. histolytica* protein and the top prokaryotic or eukaryotic protein in BlastP alignments (in respective columns)

1   [e]PE-score, the e-score of the top, prokaryotic hit

2   [f]EE-score, the e-score of the top eukaryotic hit

3   [g]P/E Ratio, the e-score ratio between the top prokaryotic hit and top eukaryotic hit

4   Abbreviated taxon names (to fit the columns):

5   *Chlamydomonas reinhardtii: C. reinhardtii; Paracoccidioides brasiliensis: P. brasiliensis*; *Schizosaccharomyces pombe: S. pombe*;

6   *Thermoanaerobacter tengcongensis: T. tengcongensis*

7

Table 9. **Examples of microarray-detected transcriptional changes in some gene families and the conditions tested**

Gene family	Total number of genes in gene family	Number of genes transcriptionally regulated under condition tested	
		Heat shock[a] (1,131 genes on array)	Host colonisation and invasion[b] (9,435 genes on array)
Cysteine proteinases	29[c]	2 upregulated (CPs 6, 4); 7 down-regulated (CPs 1, 2, 3, 8, 13, 17,	21 genes on array; 4 up-regulated (CPs 1, 9, 4, 6);1 down-regulated (CP8)
Lectin (Heavy, Light, and Intermediate subunits)	12	1 up-regulated (Hgl-2); 5 down-regulated (Lgl-1 and 3, Igl 1 and 2, Hgl-3)	No change in heavy or intermediate subunits; Light subunit lgl2 and lgl3 down-regulated)
Amoebapore	3	1 down-regulated (amoebapore C)	No substantial changes
Transmembrane receptor kinases	>80	NA	6 up-regulated (TMKs 69, 53, 95, 105, 63, 56) 2 down-regulated (TMKs 03 and 17)
AIG-1 (similar to plant antibacterial proteins)	15	NA	5 up-regulated at day 1; 6 down-regulated at day 29 (all non-overlapping)

[a] Adapted from Weber *et al.* (2006); [b] Adapted from Gilchrist *et al.* (2006);

[c] Number of cysteine proteinase gene families in genome annotation at time studies were performed

1   Figure Legends

2

3   *Figure 1.*

4   **Positions of introns in the vacuolar ATPase subunit D gene in *P. falciparum, D.***

5   ***discoideum*, and *E. histolytica***

6

7   *Figure 2.* **Comparison of protein sizes in *E. histolytica* and *D. discoideum*.**

8   a: The graph shows the distribution of predicted amino acid length across sequenced

9   genomes from single celled eukaryotes*: D. discoideum* (DD) *Encephalitozoon*

10 *cuniculi* (EC), *P. falciparum* (PF), *E. histolytica* (EH), and *S. cerevisiae* (SC). *E.*

11 *histolytica* and *E. cuniculi* have a distribution that is skewed toward smaller proteins

12 relative to the other species.

13 b: The histogram displays the degree of size change of genes in *E. histolytica* relative

14 to *D. discoideum* when comparing orthologous genes identified by reciprocal best

15 blast hits. The black bars show genes that are smaller in *E. histolytica* where as the

16 grey bars are smaller in *D. discoideum.*

17

18 *Figure 3.* **Domain diagram of the Hgl subunit of the Gal/GalNAc lectin.** **CW**-

19 Cysteine-Tryptophan region; **CF**- Cysteine free region; **C-Rich**- Cysteine rich region.

20 The black vertical box near the carboxy-terminus of the protein represents the single

21 transmembrane domain. The horizontal black bars above the diagram indicate the

22 location of a carbohydrate recognition domain (**CRD**), the region with similarity to

23 the hepatic growth factor receptor, **c-Met**, and the region that has similarity to the

24 **CD59**, the membrane inhibitor of the complement membrane attack complex. The

25 numbers in parentheses indicate the location of these regions in the Hgl1 isoform

26 (Mann *et al.*, 1991), where the methionine of the immature protein is residue 1.

27

28 *Figure 4.* **Structural domains of the 3 different types of family C1-like cysteine**

29 **endopeptidases EhCP-A, EhCP-B and EhCP-C.** Shown are the location and length

30 of domains specific for each the 3 types as well as the conserved active site and

31 cysteine residue

32

33 *Figure 5.* **Predicted antioxidant system of *Entamoeba histolytica*.** A. Superoxide

34 radical anions are detoxified by an iron-containing superoxide dismutase (FeSOD).

1   Molecular oxygen is reduced to hydrogen peroxide by a NADPH:flavin

2   oxidoreductase (thioredoxin reductase, p34). Hydrogen peroxide is converted to water

3   by rubrerythrin (Rbr). The nature of its redox partner is unknown. Hydrogen peroxide

4   can also be converted to water via a classical thioredoxin redox system consisting of

5   thioredoxin reductase (TrxR, p34), thioredoxin (Trx) and peroxiredoxin (Prx). B.

6   Nitric oxide is reduced by an A-type flavoprotein (FprA) to nitrous oxide and water.

7   For this reaction FprA receives electrons from NADH oxidase (Far).

8

9   *Figure 6.* **A phylogenetic tree of Rab proteins from *Entamoeba histolytica,***

10   **human, and yeast.** The number on the nodes represent the bootstrap proportions (%)

11   of 1000 pseudo samples; only bootstrap proportions >30% are shown. *E. histolytica*

12   Rab proteins are indicated in bold. Tentative subfamilies that revealed significant

13   similarity (>40% identity) to their human or yeast counterpart are shaded dark, while

14   *Entamoeba*-specific subfamilies have light shading. The scale bar indicates 0.1

15   substitutions at each amino acid position. *: *Eh*Rab proteins that lack the conserved

16   effector region, switch regions, or GTP-binding boxes. **: *Eh*Rab proteins that

17   possess a non-conventional carboxyl-terminus or lack carboxyl-terminal cysteines.

18   ***: Rab proteins that were not classified as isotypes based on <40% identity to other

19   members of the subfamily. References on tree: (1), Temesvari *et al.* (1999); (2),

20   Rodríguez *et al.* (2000); (3), Saito-Nakano *et al.* (2001); (4), Juarez *et al.* (2001); (5),

21   Saito-Nakano *et al.* (2004); and (6), Okada *et al.* (2005).

22

23   *Figure 7.* **Synthesis of N-glycan precursors by *S. cerevisiae* (A) and *E. histolytica***

24   **(B).** The N-glycan precursor of *S. cerevisiae* contains 14 sugars ($Glc_3Man_9GlcNAc_2$),

25   each of which is added by a specific enzyme. The *E. histolytica* N-glycan precursor

26   contains just seven sugars ($Man_5GlcNAc_2$), as the protist is missing enzymes that add

27   mannose and glucose in the lumen of the ER. The figure is redrawn from Figure 1 of

28   Samuelson *et al.* (2005). Glc = Glucose; GlcNAc = N-acetyl glucosamine; Man =

29   Mannose.

30

31   *Figure 8.* **Selected N-glycans of mammals (A-E) and *Entamoeba* (F-H).** Precursors

32   transferred to nascent peptide (A and F). Glycosylated products involved in N-

33   glycan-associated QC of protein folding (B and G). Mannosidase product involved in

N-glycan-associated protein degradation (mammals only) (C). Trimmed product that is building block for complex N-glycans (mammals and *Entamoeba*) (D). Complex N-glycans made in the Golgi (E and H). Glc = Glucose; GlcNAc = N-acetyl glucosamine; Man = Mannose; Gal = Galactose; Fuc = Fucose.

Figure 9. **Model of quality control of protein folding in *Entamoeba*.** 1. N-glycan-dependent QC of protein folding. 2. N-glycan-independent QC of protein folding. 3. N-glycan-independent ERAD. 4. Ire1 and unfolded protein response (see text for details).

Figure 10. **Structure of cysteine-rich plasma membrane proteins of *E. histolytica*.** These proteins include the various subunits of the Gal/GalNAc lectin, a cysteine protease, and numerous receptor kinases. Ire1, which is involved in the unfolded protein response, is also a receptor kianse but has no Cys-rich domain.

Figure 11. **Model for the *Entamoeba* cyst wall derived primarily from experiments with *E. invadens*.** A. The cyst wall consists of chitosan fibrils, which are made by chitin synthase and chitin deacetylase. Wall proteins include Jacob lectins with tandem arrays of 6-Cys chitin-binding domains (CBDs), as well as chitinase and Jessie lectins that have a single 8-Cys CBD. The Gal/GalNAc lectin in the plasma membrane binds sugars on the Jacob and Jessie lectins. B. Structures of representative lectins illustrated in A.

Figure 12. **Phylogenetic relationships of *E. histolytica* glutamine synthase**. The gene encoding glutamine synthase (EC 6.3.1.2) is now shared by *E. histolytica* and the diatom *Thalassiosira*. This gene is mainly restricted to prokaryotic genomes (eukaryotes are highlighted by arrows). *T. vaginalis* also contains a homologue but in this case it clusters weakly with *Fusobacterium*. The scale bar represents 10% of inferred sequence divergence. Both the GenBank and RefSeq accession numbers are given for the *E. histolytica* entry.

Figure 13. **Phylogenetic relationships of *E. histolytica* tryptophanase.** This tree suggests that the *E. histolytica* gene encoding a tryptophanase was acquired by LGT from a relative of the anaerobic bacterium *Fusobacterium*. In contrast, the *T.*

1   *vaginalis* gene appears to have a separate origin with a LGT from a relative of the

2   anaerobic *Bacteroides* group. The scale bar represents 10% of inferred sequence

3   divergence. Both the GenBank and RefSeq accession numbers are given for the *E.*

4   *histolytica* entry. The EC number is also shown.

5

6   *Figure 14.* **Pie chart of functional categories for the 68 strongest LGT cases.** The

7   cases are those discussed in the text and listed in Table 8. Most entries encode

8   metabolic enzymes (KEGG annotation).

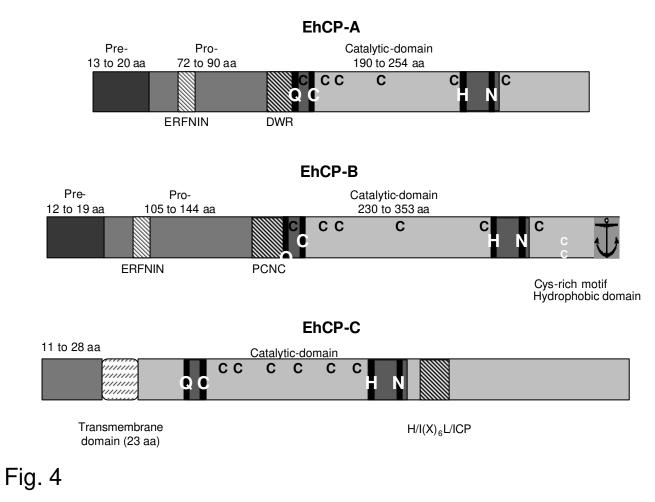Fig. 1

1

a

Protein Size Distribution

b

Degree of reduction in length of proteins in *E. histolytica* compared to *D. discoideum*

Fig. 2

1
2

1

CD59 (779-870)
cMet (918-955)
CRD (911-1016)

NH₂- CW CF C-Rich -COOH

Fig. 3

2

EhCP-A

Pre-
13 to 20 aa

Pro-
72 to 90 aa

Catalytic-domain
190 to 254 aa

C C C   C   C   C

Q C   H N

ERFNIN

DWR

EhCP-B

Pre-
12 to 19 aa

Pro-
105 to 144 aa

Catalytic-domain
230 to 353 aa

C   C C   C   C   C

C   H N

Q

ERFNIN

PCNC

Cys-rich motif
Hydrophobic domain

EhCP-C

11 to 28 aa

Catalytic-domain

C C   C C   C C

Q C   H N

Transmembrane
domain (23 aa)

$H/I(X)_6L/ICP$

Fig. 4

1

Fig. 5

1

Fig. 6

1
2

A. *Saccharomyces*:

Glc$_3$Man$_9$GlcN

B. *Entamoeba*:

Dol-PP   GlcNAc   Man   Glc
peptide

1

Fig. 7

Fig. 8

Fig. 9

Fig. 10

1

A. *Entamoeba* cyst wall

Jacob lectins

Jessie lectin

Chitosan fibrils

Chitinase

Chitin Synthase

Gal/GalNAc lectins

Chitin deacetylase

6-cys lectin domain

8-Cys lectin domain

Enzyme domain

B. *Entamoeba* cyst wall-associated lectins

Eh Gal/GalNAc lectin

| Not Cys-rich | Cys-rich lectin domain |

Signal

Ei Jacob-1 lectin

6-Cys   6-Cys   6-Cys   6-Cys   6-Cys

Cleavage sites   CBDs

Eh Jacob-2 lectin

6-Cys   6-Cys   Low complexity   6-Cys

Eh chitinase

8-Cys CBD   Catalytic domain

heptapeptide repeats

Fig. 11

1
2

Fig. 12

Fig. 13

Fig. 14