and contain N-acetyl galactosamine polymers in addition to cellulose (West, 2003; Yin *et al.*, 2005).

Similar to *Dictyostelium* and in contrast to fungi, the vast majority of *Entamoeba* cyst wall glycoproteins are released by SDS (Van Dellen *et al.*, submitted; Frisardi *et al.*, 2000; West, 2003; Yin *et al.*, 2005). While some *Dictyostelium* cyst wall proteins have been shown to be cellulose-binding lectins, all of the proteins bound to the cyst wall of *E. invadens* have 6-Cys CBDs (Jacob lectins) or 8-Cys CBDs (Jessie 3 lectin and chitinase) (Frisardi *et al.*, 2000; Van Dellen *et al.*, 2002b; Van Dellen *et al.*, submitted). In the same way that *Giardia* cyst wall protein 2 is cleaved by a cysteine proteinase, Jacob lectins are cleaved by an endogenous cysteine proteinase at sites between chitin-binding domains (Touz *et al.*, 2002).

Like *Dictyostelium* spore coat proteins and insect peritrophins, cysteine-rich lectin domains of *E. invadens* cyst wall proteins are separated by serine- and threonine-rich domains that are heavily glycosylated (Frisardi *et al.*, 2000; West, 2003; Yin *et al.*, 2005; Van Dellen *et al.*, submitted). *S. cerevisiae* cyst wall proteins also have extensive serine- and threonine-rich domains that are heavily glycosylated (Yin *et al.*, 2005). These glycans likely protect proteins in cyst walls or fungal walls from exogenous proteases. While glycoproteins of the *E. invadens* cyst wall and *Dictyostelium* spore coat contain *O*-phosphodiester-linked glycans, *S. cerevisiae* wall glycoproteins contain *O*-glycans (Gemmill and Trimble, 1999; West *et al.*, 2005).

Like *S. cerevisiae*, *E. invadens* has enzymes in its wall that modify chitin (Yin *et al.*, 2005). Similar to chitinases of *S. cerevisiae* and bacteria, *E. invadens* chitinase has a CBD in addition to the catalytic domain (Kuranda and Robbins, 1991). It is likely that the CBD is present to localise chitinase to the cyst wall (*E. invadens*) or cell wall (*S. cerevisiae*). Finally, while *E. invadens* uses catecholamines as autocrines for encystation, *Dictyostelium* uses cAMP as an

autocrine for sporulation (Coppi *et al.*, 2002; Kriebel and Parent, 2004). An important goal of future research will be to translate what is known about the *E. invadens* cyst wall to that of *E. histolytica*.

10. EVIDENCE OF LATERAL GENE TRANSFER IN THE *E. hISTOLYTICA* GENOME

Lateral (or horizontal) gene transfer (LGT) plays a significant role in prokaryotic genome evolution, contributing up to ~20% of the content of a given genome (Doolittle *et al.*, 2003). LGT has therefore been an important means of acquiring new phenotypes, such as resistance to antibiotics and new physiological and metabolic capabilities, that may permit or facilitate adaptation to new ecological niches (Koonin *et al.*, 2001; Lawrence, 2005b; Ochman *et al.*, 2000). More recently, data from microbial eukaryote genomes suggest that LGT has also played a role in eukaryotic genome evolution, particularly among protists that eat bacteria (Andersson, 2005; Doolittle, 1998; Doolittle *et al.*, 2003; Lawrence, 2005a; Richards *et al.*, 2003). *Entamoeba histolytica* lives in the human gut, an environment that is rich in microorganisms and where LGT is thought to be common between bacteria (Shoemaker *et al.*, 2001). The *E. histolytica* genome thus provides a nice model for investigating prokaryote to eukaryote LGT. In the original genome description (Loftus *et al.*, 2005) 96 putative cases of LGT were identified using phylogenetic analyses of the *E. histolytica* proteome. These have now been reanalysed in the light of more recently published (August 2005) eukaryotic and prokaryotic genomes. This has allowed evaluation of how previous inferences were influenced by the sparse sampling of eukaryotic and prokaryotic genes and species available at the time of the original analysis. Sparse gene and species sampling is, and is likely to remain, a very serious problem for reconstructing global trees and inferring LGT (Andersson *et al.*, 2001; Richards *et al.*, 2003; Salzberg *et al.*, 2001). Thus, although ecologists differ in their claims for the extent of the unsampled microbial world, they all agree that those

species in culture, and the even smaller subset for which genome data exist, represent the smallest tip of a very large iceberg.

10.1 How Do The 96 LGT Cases Stand Up?

As before (Loftus *et al.*, 2005), Bayesian and maximum likelihood distance bootstrap phylogenetic analyses were used to identify putative LGT using the following *ad hoc* conservative criteria: Putative LGT was inferred where either no other eukaryote possessed the gene, or where the *E. histolytica* sequence was grouped with bacteria and separated from other eukaryotes by at least two strongly supported nodes (bootstrap support >70%, posterior probabilities >0.95). In cases where tree topologies were more weakly supported but still suggested a possible LGT, bootstrap partition tables were examined for partitions where the *E. histolytica* sequence clustered with another eukaryote. If no such partitions were found that gene was considered to be a putative LGT. Table 8 lists the results of the new analyses and also gives BlastP statistics for each sequence.

A total of 41 LGT remain as strongly supported as before based upon the original criteria. For the remaining 55 tree topologies, support for recent LGT into the *Entamoeba* lineage is not as strong as before. For 27 of these 55 trees, two strongly supported nodes separating *E. histolytica* from other eukaryotes has been reduced to only one well-supported node. However, close scrutiny of the bootstrap partition tables for these trees revealed that, as before, there are no trees in which *E. histolytica* is found together with another eukaryote. Thus, LGT still remains the strongest hypothesis to explain 68 (70%) of the original 96 tree topologies. In a further 14 cases, the position of *E. histolytica* among prokaryotes and eukaryotes was not well supported. The taxonomic sampling of eukaryotes in these trees is very patchy and the trees do not depict consensus eukaryotic relationships. Thus, although the trees do not fulfill the conservative criteria for LGT they also do not provide strong support for the alternative

hypothesis, that the *E. histolytica* genes were vertically inherited from a common ancestor shared with all other eukaryotes.

In nine trees *E. histolytica* either clustered with a single newly published eukaryotic sequence, or such a relationship could not be ruled out. In six of these nine trees *E. histolytica* and *Trichomonas vaginalis* grouped together, and two trees grouped *E. histolytica* with the diatom *Thalassiosira* (for example see Figure 12). Such trees are also not easy to explain within the current consensus for eukaryotic relationships (Baldauf, 2003). Similar topologies have been previously reported for other eukaryotes (Andersson, 2005). The explanations advanced to explain the absence of the gene in other eukaryotes include 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 *Entamoeba* as well transcriptional response to heat shock, collagen and calcium exposure, and tissue invasion (Debnath *et al.*, 2004; Gilchrist *et al.*, 2006; MacFarlane and Singh, 2006; Weber *et al.*, 2006). 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. In comparing virulent and avirulent *Entamoeba* species and strains, MacFarlane and Singh (2006) confirmed that a number of the known virulence determinants have decreased expression in the non-virulent *Entamoeba*. Based on the hypothesis that potential virulence determinants will be more highly expressed in virulent strains compared to non-virulent strains, the authors identified 29 genes that have decreased expression in both the attenuated *E. histolytica* (Rahman) and the avirulent *E. dispar* (SAW760). The majority of these genes are annotated as hypothetical and whether these genes represent novel virulence genes will require genetic analysis of their functions. A large family of transmembrane receptor kinases has been identified in *E. histolytica* and Beck *et al.* (2005) found that a number of these are differentially expressed under *in vitro* trophozoite culture conditions. 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 was demonstrated by Weber *et al.* (2006), who interestingly identified lectin gene family members being differentially regulated during heat shock conditions.

The most definitive data to date is by Gilchrist *et al.* (2006), who developed a whole genome short oligonucleotide microarray (based on the Affymetrix platform) and used it to profile the transcriptional changes that occur as the parasite colonises and invades the host colon. Using a mouse model of colitis,

in which the microscopic features replicate human disease and substantial pathology can be seen, the authors assayed the transcriptional response of parasites soon after colonisation (1 day after injection into the caecum) and in a long-term (29 days) disease state. Overall, 326 genes were modulated at day 1 after infection, 109 at 29 days after infection, and 88 at both time points. A number of the well-characterised "virulence determinants" in *E. histolytica* were highly expressed under all conditions tested and not transcriptionally modulated, although some members of the cysteine proteinase gene family were highly regulated during tissue invasion. A summary of the genes and gene families that have been identified as being transcriptionally active under the conditions mentioned above are listed in Table 9.

The above studies used expression data to identify interesting genes and pathways potentially involved in amoebic pathogenesis. In another approach, Shah *et al.* (2005) used comparative genomic hybridisations to identify a number of interesting genomic characteristics in *Entamoeba*. The genome project revealed that a large number of *E. histolytica* genes are in multi-copy or members of highly similar gene families. Due to the repetitive nature of the genome there was some 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 (~14%) of the genome and validated the genome assembly. Additionally, Shah et al (2005) demonstrated genome-wide genetic diversity among strains of *E. histolytica* including the observation that an avirulent *E. histolytica* strain had a unique genetic pattern indicating 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*. The CGH analysis of *E. histolytica* and *E. dispar* revealed a significant amount of differences 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 characterisation of the parasite transcriptome from invasive hepatic disease as well as during 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

Although the genome of *E. histolytica* is not yet complete it has already revealed much about the biology of the parasite. There appear to be forces acting to compact the genome, leading to a reduction in the coding region and intron length of genes, and resulting in the loss of numerous metabolic pathways. However, there are also opposing evolutionary forces as many gene families have expanded. This applies particularly to genes involved in signaling and trafficking that allow the parasite to sense and respond to its environment, a necessary adaptation for a predatory protist. Unfortunately, it is difficult at present to understand the genome structure on a macro scale due to the fragmented nature of the current assembly. In other parasites, genome structure has been vital to unraveling important biological processes, such as antigenic variation in *T. brucei* and identification of rifin genes in *P. falciparum*. Until the *E. histolytica* genome is complete we will not know what else remains to be uncovered. Efforts are already underway to complete the genome by first

generating a HAPPY map (Dear and Cook, 1993). Over 2000 markers are being designed at approximately 25 kb intervals across all contigs. Using PCR, co-segregation analysis allows the identification of contigs that are physically linked in the genome. This will allow the ordering and orientation of the contigs and will facilitate gap closure. Shotgun genome sequencing projects of *E. invadens* and *E. dispar* are underway (Loftus and Hall, 2005). At present the *E. invadens* genome appears to assemble with fewer problems than were encountered with that of *E. histolytica*. It is anticipated that an essentially complete *E. invadens* genome sequence will be obtained, enabling extensive comparative analyses to be made, and facilitating the study of pathogenicity, host interaction and the evolutionary forces acting on the genome.

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