Published in final edited form as:
Cell Microbiol. 2009 January ; 11(1): 51–69. doi:10.1111/j.1462-5822.2008.01236.x.

Entamoeba histolytica modulates a complex repertoire of novel genes in response to oxidative and nitrosative stress: implications for amebic pathogenesis

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SUMMARY

Upon host infection, the protozoan parasite *Entamoeba histolytica* is confronted with reactive oxygen and nitrogen species (ROS and RNS) and must survive these stresses in order to cause invasive disease. We analyzed the parasite's response to oxidative and nitrosative stresses, probing the transcriptional changes of trophozoites of a pathogenic strain after a 60min exposure to H_2O_2 (1mM) or a NO donor (DPTA-NONOate, 200μM), using whole-genome DNA microarrays. Genes encoding ROS and RNS detoxification enzymes had high transcriptional levels under basal conditions and upon exposure to both stresses. On a whole genome level, there was significant modulation of gene expression by H_2O_2 (286 genes regulated) and DPTA-NONOate (1,036 genes regulated) with a significantly overlap of genes modulated under both conditions (164 genes). A number of transcriptionally regulated genes were in signaling/regulatory and repair/metabolic pathways. However, the majority of genes with altered transcription encode unknown proteins, suggesting as yet unraveled response pathways in *E. histolytica*. Trophozoites of a non-pathogenic *E. histolytica* strain had a significantly muted transcriptional response to H_2O_2 compared to the pathogenic strain hinting that differential response to oxidative stress may be one factor that contributes to the pathogenic potential of *E. histolytica*.

Keywords

parasite; virulence; microarray; gene expression; ROS; RNS

INTRODUCTION

Infection by *Entamoeba histolytica*, the causative agent of amoebiasis, is a global health problem, which affects 500 million people worldwide (Stanley, 2003). Most commonly, this pathogen causes hemorrhagic dysentery and liver abscesses. During tissue invasion, *E. histolytica* adapts to changing oxygen tensions as it goes from the anaerobic colonic lumen to an oxygen rich environment in the colonic tissue (Stanley, 2003). Additionally, the parasite must cope with cytotoxic reactive oxygen species (ROS) and reactive nitrogen

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species (RNS) that are produced and released by activated phagocytes that are attracted to the site of infection (Stanley, 2003; Bogdan *et al.*, 2000; MacMicking *et al.*, 1997). Therefore, a significant contribution to *E. histolytica*'s pathogenic potential is likely to be due to its ability to cope with oxidative and nitrosative stresses generated during tissue invasion.

The cellular components targeted by ROS and RNS include proteins (metal cofactors, thiolate side chains, tyrosine and methionine residues), nucleic acids and lipids (Halliwell and Gutteridge, 2007). Common defense strategies against oxidative and nitrosative stresses include detoxification enzymes and repair systems that enable cells to resist RNS and ROS (Vandenbroucke *et al.*, 2008; Justino *et al.*, 2005). Several microbial transcription factors and regulons, which are involved in the response to both oxidative and nitrosative stress as well as in the transition from anaerobic to aerobic metabolism, have redox sensitive active sites that are modified and/or damaged by both ROS and RNS. Not surprisingly, given the overlap in the types of damage caused by ROS and RNS, common mechanisms exist to deal with these stressors. These include the Crp-Fnr superfamily of transcriptional regulators, which respond both to nitrosative and oxidative stress (Korner *et al.*, 2003), as do other transcriptional regulators, e.g., in *Escherichia coli*: NsrR, OxyR, SoxRS, MetR, ferric uptake regulator and NorR, regulating a wide range of cellular processes (Spiro, 2006). A survey of the genomes of the parasitic protists *E. histolytica* (Loftus *et al.*, 2005), *Giardia lamblia* (Morrison *et al.*, 2007) and *Trichomonas vaginalis* (Carlton *et al.*, 2007) revealed the absence of homologues of any of the above mentioned transcriptional regulators. In contrast, genes coding for detoxification systems for ROS and RNS are present in the genomes of these anaerobic protists. Some of these genes may have been acquired by lateral gene transfer from prokaryotes (Andersson *et al.*, 2006; Andersson *et al.*, 2003). The *E. histolytica* genome has four genes encoding flavodiiron proteins (FDPs), enzymes endowed with oxygen and/or nitric oxide reductase activity which are widespread in prokaryotes (Kurtz, 2007; Saraiva *et al.*, 2004), and have been studied in the protozoa *T. vaginalis* (Sarti *et al.*, 2004) and *G. lamblia* (Di Matteo *et al.*, 2008). *E. histolytica*'s genome also contains genes encoding other enzymes involved in the detoxification of ROS, including peroxiredoxin, rubrerythrin, hybrid-cluster protein, and superoxide dismutase (SOD). Peroxiredoxin constitutes a major defense against oxidative stress as it is induced by a high-oxygen environment (Akbar *et al.*, 2004) and trichostatin A (Isakov *et al.*, 2008), and contributes to *E. histolytica*'s virulence (Sen *et al.*, 2007; Davis *et al.*, 2006). Although peroxiredoxin and SOD are ubiquitous in all domains of life, FDPs, rubrerythrin and hybrid-cluster proteins have thus far been identified only in prokaryotes and in these anaerobic protists.

Whole-genome expression profiling has been used to assess the effects of oxidative and nitrosative stress in diverse eukaryotes and prokaryotes (Vandenbroucke *et al.*, 2008; Thum and Bauersachs, 2007). A recent meta-analysis of microarray data performed to assess the common denominators in the oxidative stress response across different domains of life revealed that there are both strong species-specific responses, and common strategies for diverse organisms to cope with this challenge (Vandenbroucke *et al.*, 2008). Microarray technology has been used in *Entamoeba* to investigate a wide variety of biological questions, including virulence (Davis *et al.*, 2007; MacFarlane and Singh, 2006), host colonic and hepatic invasion (Santi-Rocca *et al.*, 2008; Gilchrist *et al.*, 2006) and development (Ehrenkaufer *et al.*, 2007). In order to determine the molecular mechanisms by which *E. histolytica* trophozoites respond when challenged with oxidative and nitrosative stresses, we used whole genome expression profiling using a short oligonucleotide microarray containing 9,435 of the annotated 9,938 amebic genes. Our results demonstrated a significant transcriptional response of *E. histolytica* HM-1:IMSS, a canonical virulent strain, to H_2O_2 (286 genes regulated), NO (1,036 genes regulated), and a significant overlap among the genes responsive to both conditions (164 genes). To further identify which components of

these response mechanisms may be correlated with *E. histolytica*'s virulence potential, the response to oxidative stress was assessed for a non-pathogenic strain, *E. histolytica* Rahman. In contrast to the observations for the virulent HM-1:IMSS strain, the Rahman strain had fewer transcriptional changes and the overall fold-changes for the regulated genes were significantly lower. Overall, our results provide insights into the molecular network regulating adaptation to oxidative and nitrosative stresses in *E. histolytica* and suggest that one important difference between virulent and non-virulent amebae is their ability to deal with the stresses encountered during host invasion.

RESULTS AND DISCUSSION

Sensitivity of *E. histolytica* trophozoites to oxidative and nitrosative stress

To determine the response of *E. histolytica* to nitrosative and oxidative stress, trophozoites from the HM-1:IMSS strain were exposed to dipropylenetriamine (DPTA)-NONOate (nitric oxide releaser) or hydrogen peroxide (H_2O_2). We analyzed the viability of *E. histolytica* trophozoites in varying concentrations of H_2O_2 and DPTA-NONOate in order to identify conditions in which *E. histolytica* trophozoites stressed but still ≥90% viable. A 1-hour exposure to 1 mM H_2O_2 or 200 μM DPTA-NONOate resulted in a significant fraction of the cells being stressed (as judged by rounded morphology) but only a few dead cells (≤5% and≤10%, respectively), based on Trypan blue staining (data not shown). We tested the sensitivity of both the virulent HM-1:IMSS and the non-virulent Rahman strains to 1 mM H_2O_2, and found a similar percentage of dead cells (data not shown). The two stress agents are differently released into the cultures: addition of hydrogen peroxide results in immediate exposure to the added concentration, whereas DPTA-NONOate is a slow releaser of nitric oxide. The tested concentration of H_2O_2 (1 mM) is within physiologically relevant concentrations found in the gastrointestinal lumen (Mayol *et al.*, 2006). At the chosen concentration (200 μM), DPTA-NONOate releases NO at a rate of ~25 nM $NO.s^{-1}$, which after 1 hour of exposure would accumulate to a maximum of ~82 μM of NO in the medium, a concentration above physiological concentrations (Halliwell and Gutteridge, 2007). It is however likely that the NO concentration experienced by *E. histolytica* under the tested conditions is lower than that released, due to the general reactivity of nitric oxide within biological milieu. Both NO and H_2O_2 may react with components of the TYI-S-33 medium, such as serum and cysteine, in which *E. histolytica* is cultured. However, no defined medium lacking serum or cysteine has been developed in which *E. histolytica* can be reliably grown (Diamond and Cunnick, 1991; Gillin and Diamond, 1980). Thus, the TYI-S-33 medium, in which parasite growth is well standardized and robust and in which many of the previous transcriptome analyses done previously have been performed, is the best option for our studies.

Expression analysis of *E. histolytica* strains challenged with oxidative or nitrosative stress

Affymetrix whole-genome microarrays were used to determine the global transcriptional changes in *E. histolytica* HM-1:IMSS trophozoites upon exposure to 1 mM hydrogen peroxide for 1 hour. Data from three independent H_2O_2-exposed cultures were compared with those from *E. histolytica* HM-1:IMSS trophozoites from standard axenic culture conditions. All sets of arrays displayed high correlation values (0.94–0.98) (Table 1). Genes with a ≥ 2-fold change and FDR < 0.05 were considered differentially expressed: 184 genes were up-regulated by H_2O_2 and 102 genes were down-regulated (Tables 2 and 3; Supplementary Table 2).

E. histolytica HM-1:IMSS trophozoites were also subjected to nitrosative stress by addition of the NO releaser DPTA-NONOate (200 μM, 1 hour) and resulting transcriptional changes assayed. Three arrays from independent parasite cultures challenged with DPTA-NONOate

were performed; all arrays displayed good correlation values (0.94–0.97 (Table 1)). Although *E. histolytica* displayed similar percentages of cell death in H_2O_2 compared to NO, a substantially greater number of genes were transcriptionally regulated by nitrosative stress compared to oxidative stress. Using the same statistical criteria applied above (≥2-fold change and FDR < 0.05), 443 genes were up-regulated and 593 genes down-regulated by DPTA-NONOate (Tables 4 and 5, and Supplementary Table 3). To confirm the array results, seven genes were selected for semi-quantitative RT-PCR analysis. In every case the data from the microarray analyses were confirmed (Figure 1).

Genes encoding known detoxification systems in *E. histolytica* trophozoites are not significantly modulated by oxidative or nitrosative stresses

In *E. histolytica* HM-1:IMSS strain growing under standard axenic culture conditions, the basal transcription levels of genes encoding the enzymatic ROS and RNS detoxification systems are generally high (Figure 2). Putative detoxification systems for reactive oxygen and nitrogen species, which were identified in the genome of *E. histolytica* are depicted in Scheme 1. Flavodiiron proteins (FDPs) have nitric oxide (NO) and/or molecular oxygen (O_2) reductase activities, although the molecular basis for substrate selectivity remains elusive (Vicente *et al.*, 2007). Out of the four copies of genes encoding FDPs, two (6.m00467 and 155.m00084) have much higher transcription levels than the other two homologues (65.m00171 and 146.m00121) (Figure 2A). The gene encoding superoxide dismutase (384.m00041, SOD), the sole known superoxide detoxifying enzyme in *E. histolytica*'s genome, displays high transcription levels in all conditions tested with no significant changes under either stress condition (Figure 2B). The same is observed for gene products involved in hydrogen peroxide detoxification, since the three distinct scavenging systems identified in its genome also display high and almost invariable expression levels: peroxiredoxin (176.m00112, Prx), rubrerythrin (131.m00144, Rbr), and hybrid-cluster protein (8.m00410, HCP) (Figure 2B). Although these genes do not display significant transcriptional changes upon H_2O_2 or NO exposure, basal expression levels of some of these genes do vary across *Entamoeba*: the FDP-encoding 6.m00467 gene, which is the most highly expressed FDP in *E. histolytica* HM-1:IMSS, has a lower expression in the non-virulent species, *E. dispar*, and the gene coding for peroxiredoxin, has reduced expression in both *E. histolytica* Rahman and *E. dispar* (Davis *et al.*, 2006; MacFarlane and Singh, 2006). Moreover, a proteomic analysis revealed that *E. histolytica* superoxide dismutase and peroxiredoxin have significantly higher expression levels in the virulent *E. histolytica* HM-1:IMSS strain as compared to the non-virulent Rahman strain (Davis *et al.*, 2006), indicating a possible contribution by these proteins to amebic virulence.

Although the majority of genes encoding the ROS and RNS stress detoxifying proteins were not significantly changed in parasites exposed to oxidative and nitrosative stresses, some transcriptional changes were noted. Three genes encoding iron-sulfur flavoproteins (35.m00253, 312.m00037 and 41.m00244) that are proposed to be related to the oxidative stress response, but whose function remains undetermined (Cruz and Ferry, 2006), were up-regulated by H_2O_2. Two homologous genes (187.m00073 and 646.m00021) encoding iron-sulfur flavoproteins were induced by nitrosative stress. Additionally, one of the four flavodiiron proteins (146.m00121) was up-regulated by NO (4.8-fold). Besides FDPs, no other known NO-detoxifying enzyme has thus far been identified in *E. histolytica*'s genome.

Aside from these limited changes, most of the genes in the ROS and RNS detoxification pathways were not transcriptionally regulated under a number of different conditions that model the host-pathogen interaction (parasites colonizing the mouse intestine) (Gilchrist *et al.*, 2006) or trophozoite to cyst stage conversion (Ehrenkaufer *et al.*, 2007). Thus, contrary to what has been observed in many prokaryotes and a few eukaryotes (Rodionov *et al.*, 2005; Paget and Buttner, 2003), the transcriptional response at the detoxification level

appears constitutive as *E. histolytica* trophozoites have a number of scavenging enzymes that are robustly expressed even under basal conditions.

Genes up-regulated in *E. histolytica* HM-1:IMSS in response to oxidative stress

Out of the 185 genes up-regulated by oxidative stress, 107 (58%) are annotated as encoding hypothetical proteins. The remaining genes code for proteins with roles in signaling/regulatory processes, metabolic/repair processes, energy metabolism, stress response, and transport (Figure 3). The categories comprising the largest numbers of up-regulated genes include the repair systems and signaling/regulatory pathways, which are detailed below.

Response to DNA damage—DNA is a known cellular target of oxidative stress (reviewed in (D'Autreaux and Toledano, 2007)) and 8% of the genes up-regulated by H_2O_2 in *E. histolytica* encode putative nucleic acids metabolism/repair proteins (Figure 3, Table 2). Two genes encoding deoxyuridine triphosphate nucleotidehydrolase (dUTPase) (194.m00123 and 375.m00057), considered to be essential for DNA integrity (Nguyen *et al.*, 2005), are among the genes most highly up-regulated by H_2O_2 (51 and 22-fold, respectively). A putative homologue of polynucleotide kinase-3-phosphatase (1.m00709, 4-fold) is involved in the repair of nicks and gaps in damaged DNA, including oxidatively generated DNA strand breaks (Blondal *et al.*, 2005; Betti *et al.*, 2001). A similar function in the repair of oxidative damage to DNA has been attributed to the large family of MutS repair proteins (Dzierzbicki *et al.*, 2004; Chang *et al.*, 2002; Khil and Camerini-Otero, 2002). A gene coding for a homologue of MutS DNA mismatch repair proteins in *E. histolytica* (115.m00144) was up-regulated by oxidative stress (2-fold). We also observed induction by hydrogen peroxide of a gene encoding a homologue of DEAD DEAH box helicase (27.m00240, 3-fold). The homologous gene from the pathogenic fungus *Candida albicans* is regulated by Cap1p, a transcription factor involved in oxidative stress tolerance (Wang *et al.*, 2006).

Response to protein and lipid damage—Hydrogen peroxide oxidatively damages proteins, mainly by reacting with thiol groups from cysteine side chains, and also with redox cofactors such as metal centers. Moreover, the hydroxyl radical generated in the reaction between H_2O_2 and free Fe^{2+} further reacts with amino acid residues, namely methionine (Halliwell and Gutteridge, 2007). In *E. histolytica* HM-1:IMSS challenged with H_2O_2, 4% of the up-regulated genes encode homologues of proteins involved in the repair or degradation of misfolded proteins (Figure 3 and Table 2). Genes encoding homologues of chaperone-like heat-shock proteins were up-regulated by oxidative stress, for example HSP40/DnaJ (21.m00247) which stimulates the ATPase activity of HSP70 chaperones (Qiu *et al.*, 2006) and HSP101 (64.m00187). An ubiquitin-conjugating enzyme (142.m00162), which may mark misfolded proteins for degradation, was also up-regulated. A homologue of peptidyl-prolyl cis-trans isomerase (75.m00189), a protein with a role in the repair of oxidatively damaged proteins from plants (Shapiguzov *et al.*, 2006) and mammalian cells (Hong *et al.*, 2002; Santos *et al.*, 2000) was also upregulated.

Oxidative damage exerted on lipids by H_2O_2 and other ROS may result in loss of membrane integrity (Halliwell and Gutteridge, 2007; Colles and Chisolm, 2000). Accordingly, some of the genes up-regulated by H_2O_2 have a role in lipid metabolism: phosphatidylcholine transport-like protein (99.m00180), and a phospholipid-transporting P-type ATPase (75.m00173), homologous to aminophospholipid translocases. Increased expression of the gene coding for glucosamine-6-phosphate N-acetyltransferase (34.m00243) suggests a requirement for cell wall repair and/or the assembly of cell wall proteins (Hurtado-Guerrero *et al.*, 2007). It has been reported that a mutant strain of *C. albicans* with a deletion in this gene displays decreased virulence (Mio *et al.*, 2000).

Signaling and regulatory pathways—The largest group of genes up-regulated by H_2O_2 (20%) is that encoding proteins that may be related to signaling pathways including protein kinases, phosphatases and acetyltransferases. *E. histolytica* possesses an intricate phosphorylation network involving a large number of kinases, which have roles in diverse cell processes (Anamika *et al.*, 2008), including effects on parasite virulence (Beck *et al.*, 2005; Batista Ede and de Souza, 2004). Other genes that may be involved in signal transduction mechanisms include those that encode GTPases and related proteins, such as the G protein regulator phosducin (407.m00055) (2.4-fold). The two genes coding for Rab GTPases, RabI1 (20.m00304, 3.6-fold) and RabM1 (103.m00161, 2.0-fold) (Saito-Nakano *et al.*, 2005), are homologues of Rab1B and Rab15, respectively involved in transport and endocytosis. A small GTPase CDC42 (encoded by 219.m00107, 11.3-fold) has a human homologue that regulates adherence and membrane permeability (Broman *et al.*, 2007) and more recently has been proposed to have a role in regulating protein ubiquitination (Shen *et al.*, 2008). In addition, ArfA3 (147.m00113) (Clark *et al.*, 2007), an ADP ribosylation factor (ARF) GTPase, was induced by H_2O_2 (2.6-fold). ARF GTPases participate in the regulation of organelle structure and vesicular trafficking, besides participating in diverse cellular functions, such as cytokinesis, endocytosis, phagocytosis and cell adhesion (D'Souza-Schorey and Chavrier, 2006). We also observed induction of a gene encoding a putative copine (333.m00053) (2.1-fold), whose homologue from *Dictyostelium discoideum* plays a role in cytokinesis and contractile vacuole function (Damer *et al.*, 2007). An AIG1 plant-like antibacterial protein (451.m00039) was induced (2.1-fold) and was also significantly up-regulated upon *E. histolytica* invasion of the mouse intestine (Gilchrist *et al.*, 2006). Two genes encoding BspA-like leucine rich proteins (1.m00705 and 310.m00070) were induced by H_2O_2 (6.6 and 2.4 fold, respectively). Homologues of these proteins from *Tannerella forsythia* have been reported to modulate the host response by interfering with interleukin-8 expression and ultimately contributing to invasion of the epithelial barrier (Onishi *et al.*, 2008; Inagaki *et al.*, 2006). In summary, *E. histolytica* responds to oxidative stress by inducing genes coding for a multitude of signaling/regulatory systems, with roles in diverse cellular functions.

Other pathways—Since *E. histolytica* redox homeostasis is sustained by free (homo)cysteine, in the absence of glutathione or any related metabolic enzymes, it is noteworthy that sulfur amino-acid metabolism enzymes are regulated by oxidative stress. The gene encoding the MGL1 isotype of methionine-γ-lyase (MGL1, 395.m00028), which in eukaryotes has thus far only been found in plants (Rebeille *et al.*, 2006), *E. histolytica* (Tokoro *et al.*, 2003) and *T. vaginalis* (Nozaki *et al.*, 2005) is up-regulated (3.6-fold) by H_2O_2. This enzyme catalyzes elimination reactions with a wide range of substrates, such as methionine, (homo)cysteine, and substituted (homo)serine homologues (Nozaki *et al.*, 2005). Moreover, the products of methionine degradation by MGL not only supply the energy metabolism, but may also be implicated in amebic pathogenicity since thiols and hydrogen sulfide can become toxic for the host cells by permeating the membrane barrier and interfering with host signaling systems (Nozaki *et al.*, 2005).

Genes down-regulated in *E. histolytica* HM-1:IMSS in response to oxidative stress

A total of 102 genes were down-regulated by H_2O_2 exposure; 52 (51%) of which encode hypothetical proteins (Table 3; Supplementary Table 2). As displayed in Figure 3, the majority of the *E. histolytica* HM-1:IMSS genes repressed by oxidative stress appear to be involved in the same general processes whose components were up-regulated, namely repair systems (mostly for nucleic acids), signaling pathways, and regulatory mechanisms. The numerous genes possibly involved in signaling and regulatory processes encode a number of putative GTPases, one of which (64.m00149) encodes a protein with RhoGEF and ArfGAP

domains and has a homologue proposed to be involved in *D. discoideum* development (Mondal *et al.*, 2007).

Genes up-regulated in *E. histolytica* HM-1:IMSS in response to nitrosative stress

From the 443 genes up-regulated by nitrosative stress, 248 (56%) are annotated as encoding unknown hypothetical proteins. Similar to what was observed with oxidative stress, the largest groups of genes induced by nitrosative stress are those related with signaling / regulatory processes and repair systems for nucleic acids, proteins and lipids.

Response to DNA damage—Upon exposure to NO, 33 up-regulated genes (7%) encode proteins involved in the metabolism and/or repair of nucleic acids (Figure 3). This reflects the direct damage exerted by reactive nitrogen species on nucleic acids, either NO directly or NO-derived species, such as peroxynitrite (Halliwell and Gutteridge, 2007). In *Salmonella enterica*, NO impairs DNA replication and arrests growth (Schapiro *et al.*, 2003). Two genes encoding dUTPase (194.m00123 and 375.m00057) are among the genes most highly up-regulated by NO (192 and 82-fold, respectively), and are essential enzymes for DNA integrity (Nguyen *et al.*, 2005). A homologue of MutS DNA mismatch repair proteins (115.m00144), was also up-regulated by nitrosative stress (3.3-fold). We observed induction of genes encoding a panoply of DNA repair enzymes, such as DNA excision repair protein (117.m00190) (4.0-fold), Rad3 DNA repair helicase (197.m00081) (2.1-fold), and Rad50 (102.m00081) (2.0-fold).

Response to protein and lipid damage—NO and its derived species can be harmful to proteins involved in all cellular processes, both by reacting with specific amino acids and/or with redox active cofactors, mostly metal centers. The reaction of RNS with amino acids results in S-nitrosylation or nitration of the corresponding side chains. NO and other RNS can also bind transiently or permanently to protein metal cofactors and either inhibit or definitely inactivate its function. The resulting modifications can be damaging to the overall folding and structural arrangements of proteins, which was confirmed by the significant number of *E. histolytica* genes with these functions that were up-regulated by NO exposure (5% of all upregulated genes by NO) (Figure 3). The vast majority of these genes encode either heat-shock proteins, HSP20 (134.m00124) (12.3-fold), three homologues of HSP40 (12.m00313, 28.m00338 and 28.m00311) (5.6, 3.8 and 3.3-fold, respectively), two of HSP70 (301.m00039 and 584.m00019) (21.1 and 2.3-fold), and three of HSP101 (81.m00150, 64.m00178 and 64.m00187) (20.5, 8.8 and 6.1-fold, respectively), or ubiquitin-conjugating proteins, which are all involved in degradation / repair of misfolded proteins and often responsive to many types of cellular stress.

Other major targets of NO and RNS reactivity are lipids. While this interaction may damage lipids, NO and RNS may also act as antioxidants, reacting with lipid radicals generated by oxidative stress, thus blocking and terminating harmful lipid radical chain reactions (Halliwell and Gutteridge, 2007). The low polarity of NO allows it to freely diffuse through membranes. Damage to lipids can not only affect metabolic processes, but most importantly may result in cell membrane permeability. The effect of nitrosative stress on lipids in *E. histolytica* is directly observable by the number of up-regulated genes encoding proteins related to lipid metabolism (3% of all induced genes): a PCTP-like protein (99.m00180) (2.8-fold), a myotubularin lipid phosphatase (35.m00216) (2.6-fold), and a phospholipid-transporting P-type ATPase (75.m00173) (2.6-fold). In addition to affecting the membranous environment, NO reacts directly with membrane proteins, such as ion channels and transport proteins, thus disturbing ion homeostasis. Two genes encoding putative importins (310.m00066 and 1.m00747) were induced by nitrosative stress (4.0 and 2.1-fold, respectively). The nuclear transport pathway mediated by importin in human cells has

recently been shown to be impaired by nitrosative stress (Qu *et al.*, 2007). A putative sodium/proton antiporter (152.m00122) was up-regulated by NO (3.5-fold). Nitric oxide has been shown to inhibit Na^+/H^+ exchange activity, mediated by the cyclic GMP signal transduction pathway (Gill *et al.*, 2002). Genes encoding homologues of amino acid transporters (2.m00499, 46.m00238 and 82.m00146) were also induced by nitrosative stress.

Signaling and regulatory processes induced by nitrosative stress—The largest groups of regulated genes (18%) are those involved in signaling and regulation of cellular processes (Figure 3). Part of this group overlaps with genes found to be up-regulated by oxidative stress. From the 78 genes in this group, at least 21 encode putative protein kinases, 7 code for protein phosphatases, and 5 encode acetyltransferases. A significant number of GTPases (4 Rab-type, 3 Rho family, 1 Rap Ran GTPase activating protein, and 2 Rab GTPase activating proteins) and zinc finger proteins were also up-regulated by nitrosative stress. Reversible inhibition of DNA-binding zinc containing proteins by nitrosative stress has been thought to be part of NO-related DNA replication inhibition in pathogenic bacteria (Schapiro *et al.*, 2003).

Other pathways—A putative FAD-binding NADH oxidoreductase (328.m00064) was induced (2.4-fold) by NO. Homologues of this enzyme are primary electron carriers in electron transfer chains with NO-detoxifying activity (Saraiva *et al.*, 2004). A nitroreductase (13.m00321) was induced upon NO exposure (3.9-fold). Nitroreductases are flavoproteins that catalyze the reduction of nitro groups in a wide range of substrates and are associated with resistance to the anti-parasitic antibiotic metronidazole (Mendz and Megraud, 2002). The gene that codes for methionine-γ-lyase (MGL1, 395.m00028) was also up-regulated by nitrosative stress (4.4-fold). The products of methionine degradation by MGL may permeate the host cells membrane barrier and interfere with signaling systems (Nozaki *et al.*, 2005). Interestingly, three genes encoding putative cysteine proteases were induced by NO (24.m00271, 180.m00101, and 97.m00133) (2.0, 2.1, and 2.1-fold, respectively). Cysteine proteases are an important virulence factor for *E. histolytica*, and are involved in cytotoxicity and colonic invasion (Stanley, 2003).

Genes downregulated by nitrosative stress

A surprisingly large number of genes had decreased expression upon exposure to DPTA-NONOate: 592 genes of which 366 (62%) are annotated as unknown hypothetical proteins (Table 4; Supplementary Table 3). The profile of down-regulated genes and their putative functions is shown in Figure 3. There is a marked repression of genes involved in the metabolism of nucleic acids (6%), repair and degradation of misfolded proteins (2%) and lipids (2%), and in transport (2%). The largest category of genes is that involved in cell signaling and regulatory processes (21%).

A significant down-regulation of genes encoding proteins involved in RNA metabolism was observed. Genes encoding RNA polymerase subunits were repressed by nitrosative stress (64.m00185, 73.m00160, 59.m00197, 51.m00170, and 406.m00050) (4.0, 2.1, 3.2, 2.5, and 3.2-fold, respectively), suggesting that the overall transcription may be slowed-down. Exposure to nitric oxide led to repression of genes coding for putative proteins related to ubiquitination (three cullin homologues, one ubiquitin and one ubiquitin-conjugating enzyme) and ribosome-related proteins. We also observed down-regulation of genes encoding proteins involved in (glyco)lipid metabolism and glycosylation, such as phosphatidylinositol-glycan biosynthesis class C protein (80.m00142) (3.4-fold), N-acetylglucosaminyl transferase (32.m00239) (3.3-fold) and N-acetylglucosaminyl-phosphatidylinositol de-N-acetylase (52.m00150) (2.5-fold). These genes are likely to participate in the biosynthesis of glycosylphosphatidylinositol (GPI) anchors, which have

been proposed to be involved in the regulation of cell growth, endocytosis and the adhesion to target cells by *E. histolytica* (Vats *et al.*, 2005). The bulk of genes repressed by NO that encode putative transport proteins comprise those coding for importin subunits.

From the extended list of NO repressed genes encoding putative signaling/regulatory proteins, 21 code for protein kinases and 23 for GTPases (9 Rab-type, 6 Ras-type and 8 Rho-type). There is also down-regulation of 10 genes coding for zinc finger proteins, 6 for WD repeat proteins and 10 for leucine rich proteins. Significantly, exposure to NO resulted in repression of gene 43.m00187 (2.4-fold). This gene encodes a key regulatory protein, phosphatidylinositol (PI)-3,4,5-trisphosphate 3-phosphatase (PTEN), which controls diverse cellular processes as the antagonist of PI 3-kinase (constituting the PTEN/PIK signaling pathway). *E. histolytica* PI 3-kinase inhibition has been shown to impair proliferation, encystation and autophagy (Picazarri *et al.*, 2008).

Common transcriptional responses of *E. histolytica* HM-1:IMSS to oxidative and nitrosative stress

We observed a substantial overlap in genes transcriptionally regulated byoxidative and nitrosative stress with 102 genes up-regulated under both stress conditions including those genes that are the most induced under each of those conditions (Figure 4) (Supplementary Table 5). Of these 102 genes, 63 encode unknown hypothetical proteins. The remaining ones are distributed according to the function profiles observed for the individual stresses (Figure 3). The observation of commonly induced repair systems-encoding genes by both stress types reflects the fact that nucleic acids and proteins are among the cell components that suffer similar damaging reactions upon exposure to both ROS and RNS (Halliwell and Gutteridge, 2007). The two genes showing the highest induction folds by both stress types are deoxyuridine 5-triphosphate nucleotidohydrolase (dUTPase, 194.m00123 and 375.m00057). As mentioned above, this enzyme is involved in nucleotide metabolism and contributes to DNA integrity and has been previously assessed as a potential target for anti-parasitic drugs (Nguyen *et al.*, 2005). Two genes (1.m00709 and 115.m00144) are homologues of a polynucleotide kinase-3-phosphatase and a MutS DNA mismatch repair protein, respectively, and are both involved in the repair of oxidatively damaged DNA (Dzierzbicki *et al.*, 2004; Betti *et al.*, 2001). Both stresses induced genes encoding systems involved in the degradation and repair of misfolded proteins such as heat-shock proteins HSP101 and DnaJ/HSP40 (64.m00187 and 21.m00247) and an ubiquitin-conjugating enzyme (142.m00162).

We observed induction under oxidative and nitrosative stresses of genes involved in lipid metabolism, transport and glycosylation. Two genes encoding proteins involved in lipid metabolism were phospholipid-transporting P-type ATPase (75.m00173) and phosphatidylcholine transfer protein (99.m00180). An aminophospholipid translocase (75.m00173), upon nitrosative inhibition in apoptotic cells, leads to an accumulation of extra-cellular phosphatidylserine that marks cells for macrophage engulfment (Tyurina *et al.*, 2007). *E. histolytica* has been reported to recognize externalized phosphatidylserine on the surface of host cells and target these cells for phagocytosis (Boettner *et al.*, 2005; Huston *et al.*, 2003). Genes encoding putative transport proteins (mainly ion transporters) were induced both by oxidative and nitrosative stress, consistent with the observation that nitrosative and oxidative stress disturb ion homeostasis (Beausejour *et al.*, 2007; Orsenigo *et al.*, 2007). A dTDP-D-glucose 4,6-dehydratase (116.m00108) was up-regulated by both types of stress; this enzyme is related to glycosylation in pathogenic bacteria (Allard *et al.*, 2001). Also induced by both stresses was a glucosamine-6-phosphate N-acetyltransferase (34.m00243) whose homologous gene in *C. albicans* has a role in virulence (Mio *et al.*, 2000).

It is worth noting that all four protein families that respond to oxidative stress in all eukaryotic kingdoms (Vandenbroucke *et al.*, 2008) (heat-shock proteins, ubiquitin-conjugating enzymes, kinases and small GTPases) have homologues which were transcriptionally regulated in *E. histolytica* by both oxidative and nitrosative stress: HSP101 (64.m00187), an ubiquitin-conjugating enzyme (142.m00162), three protein kinases (199.m00096, 46.m00221 and 275.m00123) and one Rab family GTPase (20.m00304).

In previous sections we emphasized the induction of a gene encoding methionine-γ-lyase (MGL) (395.m00028) by both stress types, since its methionine degradation products may contribute to the permeation and disruption of the host epithelial barrier by *E. histolytica*. MGL is an attractive drug target which is being actively pursued (Sato *et al.*, 2008; Ali and Nozaki, 2007).

The majority of the genes most significantly repressed by each individual stress were down-regulated under both stresses (Supplementary Table 5). Sixty-two genes were down-regulated by both stresses, 35 (56%) of which encode unknown hypothetical proteins. The remaining genes encode putative proteins mostly involved in signaling and regulatory mechanisms, and also include a few genes encoding nucleic acid metabolism proteins. No genes encoding repair systems for misfolded proteins or lipids were commonly down-regulated under both stresses, although both stresses did regulate genes in these pathways. In summary, the overlap between genes responsive to oxidative and nitrosative stress by *E. histolytica* is significant and shows common strategies to overcome the cytotoxicity of reactive oxygen and nitrogen species.

We also analyzed the overlap between the transcriptional responses of *E. histolytica* to oxidative or nitrosative stress and other conditions. As expected, some genes regulated by H_2O_2 were also regulated by cyst conversion indicating that these genes respond to multiple stress conditions (Ehrenkaufer *et al.*, 2007; Hackney *et al.*, 2007; Weber *et al.*, 2006). A significant fraction of the genes repressed by H_2O_2 (47%) were also repressed in response to heat-shock (Hackney *et al.*, 2007; Weber *et al.*, 2006). Of the 443 genes up-regulated by DPTA-NONOate, 50 were also induced by heat-shock, some of which encode putative repair systems for damaged nucleic acids and proteins, 25 were also up-regulated in cysts, but only a few were induced upon colonization of the mouse intestine (Gilchrist *et al.*, 2006). Almost half of the 592 genes down-regulated by nitrosative stress were also down-regulated by heat-shock, a much smaller fraction (10%) was down-regulated in cysts, and 12 genes were down-regulated during an *in vivo* mouse colitis model (Table 5 and Supplementary Table 3). The limited overlap between genes regulated by oxidative and nitrosative stress and the changes seen during colonization of the mouse colon and hepatic invasion deserve further comment (Santi-Rocca *et al.*, 2008; Gilchrist *et al.*, 2006). Whether this represents technical issues (differences in time points of colonic animal model, colonic model represents more colonization than invasion, or differences in the arrays used for the liver invasion model compared to these studies) or biological differences (animal models are more complex and difficult to compare directly to studies utilizing in vitro methods) is not currently clear. However, since the genes and pathways transcriptionally modulated by oxidative or nitrosative stress overlap significantly with changes seen in multiple other systems using the same stress conditions, the approach with the *in vitro* model of stress exposure is identifying conserved mechanisms of stress response between *Entamoeba* and other systems.

Differential response to oxidative stress may contribute to the decreased virulence phenotype of the *E. histolytica* Rahman strain

In order to determine whether virulent and non-virulent amebic strains had differing responses to oxidative stress, we characterized the transcriptional changes of the non-

pathogenic *E. histolytica* Rahman strain to hydrogen peroxide. It has previously been demonstrated that the non-virulent *E. histolytica* Rahman is more susceptible to hydrogen peroxide than the pathogenic *E. histolytica* HM-1:IMSS and that higher levels of peroxiredoxin in the virulent strain contribute to *E. histolytica's* virulence (Davis *et al.*, 2006) Three arrays using RNA from *E. histolytica* Rahman exposed to 1 mM H_2O_2 for one hour were performed and displayed good correlation values (0.97–0.99) (Table 1). Expression was compared to array data from *E. histolytica* Rahman under standard axenic culture conditions (Ehrenkaufer *et al.*, 2007). Using the same fold-change criteria described in the Materials and Methods (≥2-fold change and FDR<0.5), a total of 153 genes were up-regulated by H_2O_2 in *E. histolytica* Rahman and 65 genes were down-regulated (Tables 6 and 7; Supplementary Table 4).

Overall, *E. histolytica* Rahman had a decreased repertoire of transcriptional changes in response to oxidative stress - both in terms of the numbers of genes regulated but also in the magnitude of their regulation (Figure 5 and Tables 6 and 7; Supplementary Table 4). Of the genes up-regulated in *E. histolytica* Rahman, only 36 (24%) were also up-regulated in the *E. histolytica* HM-1:IMSS strain under the same conditions (Figure 5). Furthermore, only 20% of the genes induced by oxidative stress in HM-1:IMSS strain also up-regulated in Rahman. Importantly, even for the genes that overlapped in their expression patterns between *E. histolytica* HM-1:IMSS and Rahman, the extent of up-regulation was much higher in the pathogenic strain *E. histolytica* HM-1:IMSS (Supplementary Table 6). Given the degree of genetic identity between *E. histolytica* HM-1:IMSS and *E. histolytica* Rahman (Shah *et al.*, 2005), the limited similarity of the response to the same stress condition was unexpected. Indeed, previous comparisons of the two strains under standard culture conditions have identified a limited number of genes with lower expression in Rahman compared to the HM-1:IMSS strain (Davis *et al.*, 2006; MacFarlane and Singh, 2006). The more robust transcriptional response of the virulent strain may contribute to its decreased sensitivity to oxidative stress. Furthermore, the differential response may signify that other genes exclusively up-regulated in the pathogenic *E. histolytica* HM-1:IMSS strain possibly contribute to this strain's virulence potential. These include highly induced genes such as those encoding deoxyuridine 5-triphosphate nucleotidohydrolase (194.m00123 and 375.m00057), aminophospholipid translocase (75.m00173), dTDP-D-glucose 4,6-dehydratase (116.m00108) and methionine-γ-lyase (395.m00028). These genes are highly up-regulated by both oxidative and nitrosative stress in the HM-1:IMSS strain but are not regulated at all in the Rahman strain. Moreover, some of these genes have roles in the pathogenicity of other organisms and have been tested as potential novel drug targets (Sato *et al.*, 2008; Ali and Nozaki, 2007; Nguyen *et al.*, 2005).

Of the genes induced in *E. histolytica* Rahman by oxidative stress, 93 (61%) encode unknown hypothetical proteins. Some genes with known functions in response to oxidative stress were regulated in the Rahman strain. One such is a UDP-glucose 4-epimerase (226.m00073) whose homologue from *C. albicans* contributes to morphology and cell-wall integrity and gene silencing results in fungi that are more susceptible to H_2O_2-derived oxidative stress (Singh *et al.*, 2007). The remaining genes displayed a different profile from those of the *E. histolytica* HM-1:IMSS strain challenged with oxidative stress (Figure 3). The number of genes encoding nucleic acids metabolism / repair proteins that were induced by oxidative stress in *E. histolytica* Rahman was much lower than the number of such genes up-regulated by oxidative stress in the pathogenic HM-1:IMSS strain (Figure 3). Notably, no induction of ubiquitin-conjugating enzymes was observed, contrary to what was observed in *E. histolytica* HM-1:IMSS.

Overall it appears that the Rahman strain may lack the transcriptional regulatory mechanisms for coping with oxidative damage. During tissue invasion trophozoites are

exposed to an oxygen rich environment and the Rahman strain's limited ability to cope to oxidative stress may contribute to its avirulent phenotype.

CONCLUSIONS

Upon invasion of the host intestinal epithelium, *E. histolytica* trophozoites are confronted with varying oxygen tensions and cytotoxic reactive oxygen and nitrogen species. The transcriptional changes in *E. histolytica* HM-1:IMSS upon exposure to oxidative and nitrosative stress were extensive both in the numbers of regulated genes as well as the fold-changes of these genes. A significant fraction of the genes modulated by both stresses code for unknown proteins, which may constitute response mechanisms yet to be unraveled. Among the genes regulated by H_2O_2 exposure, we identified genes encoding members of four protein families proposed to compose the core of oxidative stress response in eukaryotes: heat-shock proteins, ubiquitin-conjugating enzymes (misfolded protein degradation and repair), protein kinases, and small GTPases (signaling and regulation). Strikingly, genes coding for members of these four families were also induced by nitrosative stress and a significant fraction of these genes responded to both stress types. The common responses thus reflect the overlapping regulatory mechanisms to both stresses by *E. histolytica*.

Following the premise that an important component of *E. histolytica*'s pathogenic potential is related to its resistance to ROS and RNS cytotoxicity, we identified a number of genes responsive to either or both stresses, which may contribute to this organism's virulence. Furthermore, we demonstrated that the non-pathogenic *E. histolytica* Rahman strain had a marked difference in response to oxidative stress. The differential transcriptional regulation observed for both strains upon exposure to the same oxidative stress conditions suggests that Rahman may experience a higher degree of oxidative damage and these changes could contribute to a decreased virulence phenotype of the Rahman strain.

Overall, our work demonstrates that (i) response to oxidative and nitrosative stress is modulated by a large and complex network of genes in *E. histolytica*, (ii) a number of known virulence genes are regulated by these stresses, (iii) the decreased virulence phenotype of the non-pathogenic *E. histolytica* Rahman may be in part due to its limited response to oxidative stress, and (iv) some genes responding to these stress pathways may represent important novel drug targets.

EXPERIMENTAL PROCEDURES

Entamoeba histolytica strains and culture methods

E. histolytica HM-1:IMSS (pathogenic, ATCC 30459) and *E. histolytica* Rahman (non-pathogenic, ATCC 30886) (Dvorak *et al.*, 2003; Ankri *et al.*, 1999) were obtained from ATCC, strain identity confirmed by PCR and RFLP of known genomic loci (Clark and Diamond, 1993) and grown axenically in TYI-S-33 medium at 36.5°C, as previously described (Diamond *et al.*, 1978).

Sensitivity of *E. histolytica* trophozoites to oxidative and nitrosative stress

To determine the sensitivity of *E. histolytica* trophozoites to NO and oxidative stress, parasites from the HM-1:IMSS strain were seeded into 48-well plates (2×10^4 cells per well), each well filled with growth medium and individually sealed with parafilm. After 16–18 h, cells in mid-log phase (50–70% confluent) were exposed to dipropylenetriamine (DPTA)-NONOate (100 μM to 1 mM, nitric oxide releaser with a half-time 180 min^{-1} at 37°C (Nittler *et al.*, 2005)), or hydrogen peroxide (H_2O_2, 100 μM to 5 mM) for 1 to 8h. At regular intervals the percentage of rounded up parasites and the number of cells that stained

with Trypan blue were determined. For microarray experiments, *E. histolytica* trophozoites were grown in capped 16-ml Falcon glass tubes to mid-log phase, the medium changed and culture tubes capped. In the case of the nitrosative stress assays, the tube cap included a rubber seal that could be punctured without significantly compromising the anaerobic conditions within the culture. Two hours after replacing the medium, DPTA and H_2O_2 were added, cultures incubated for 60 min, observed to assess the cell morphology, chilled for 5 min and parasites harvested (spun at 1,000 g, 4°C, 5 min). The supernatant was removed, the cells re-suspended in trypan blue and the percentage of dead cells assessed.

Isolation of RNA and microarray hybridization

RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's protocol, purified with a Qiagen® RNeasy kit, and microarray hybridization performed at the Stanford University Protein and Nucleic Acids facility (http://cmgm.stanford.edu/pan/) using previously published protocols (Ehrenkaufer *et al.*, 2007). A custom generated Affymetrix platform microarray described in (Gilchrist *et al.*, 2006), with probe sets that represents 9,435 open reading frames was used for all studies. A fraction of probe sets are predicted to cross-hybridize with multiple genes and are annotated as follows: probe sets labeled (_at) represent a single gene; probe sets labeled (_x_at) have at least one probe that may cross-hybridize with another gene(s); probe sets labeled (_s_at) are those in which all the probes for a given gene cross-hybridize with another gene(s). Probes for intergenic noncoding regions were excluded from all analyses. The arrays were scanned after hybridization and the probe intensities were calculated using Affymetrix GCOS software (http://www.affymetrix.com/products/software/specific/gcos.affx).

Microarray data normalization and analysis

Analysis was performed as in (Ehrenkaufer *et al.*, 2007). A minimum of three arrays were used for each condition. Standard correlation coefficients were calculated in Genespring. Normalized expression values for each probe set were obtained from raw probe intensities in R 2.2.0 downloaded from the BioConductor project (http://www.bioconductor.org), using robust multi-array averaging with correction for oligosequence (gcRMA) (Wu *et al.*, 2004). To identify differentially expressed genes, we used local pooled error testing along with Benjamini-Hochberg multiple test correction (Benjamini and Hochberg, 1995). In addition, fold-change was calculated in Genespring GX (http://www.chem.agilent.com/scripts/pds.asp?lpage=27881). The threshold for a probe set to be considered differentially expressed was set at a 2-fold change with a false discovery rate (FDR) of < 0.05. Differentially expressed genes that were not annotated as hypothetical proteins were grouped according to their putative functions.

Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from *E. histolytica* HM-1:IMSS and Rahman trophozoites was isolated using Trizol reagent (Invitrogen) and further purified with a Qiagen RNeasy kit. cDNA was synthesized from total RNA (2.5μg) with the Universal riboclone cDNA system (Promega), following the manufacturer's instructions. The cDNA samples were quantified in a Nanodrop spectrophotometer (NanoDrop Technologies, LLC) and PCR reactions performed with 100ng of cDNA (15 cycles at 55°C plus 15 cycles at 50°C for every gene, except for gene 879.m00008, which was amplified with 15 cycles at 50°C plus 15 cycles at 57°C). The PCR products were fractionated on 2% agarose gels and analyzed with a Kodak Digital Science electrophoresis documentation and analysis system 120. The primers used are listed in Supplementary Table 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge all members of the Singh and Teixeira laboratories for helpful discussions. This work is supported by FCT grant SFRH/BPD/26895/2006 for JBV, NIH grants AI-068899 and AI-069382 for GME, FCT project grant POCI/SAU-IMI/56088/2004 to LMS, FCT project grant PTDC/BIA-PRO/67267/2006 to MT, and a grant from the NIAID (AI-053724) to US. We wish to thank Lígia S. Nobre (ITQB, Portugal) for the support with the semi-quantitative RT-PCRs. All array data is available at the GEO database website at NCBI (Accession number GSE11811).

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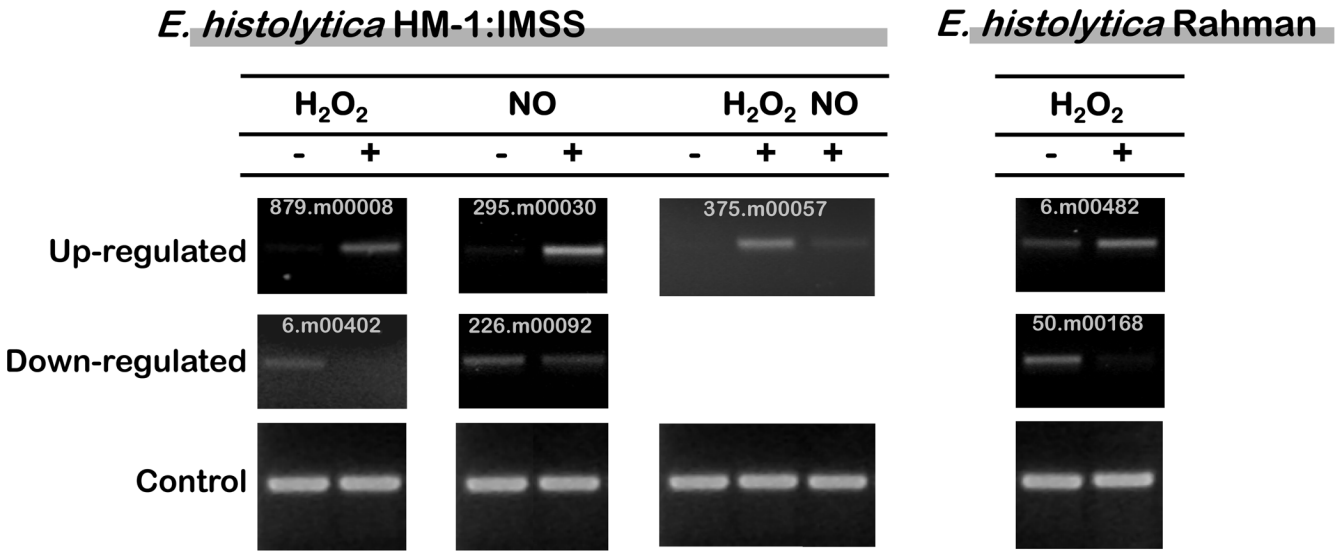


Figure 1.
Semi-quantitative RT-PCR analysis of selected genes, for validation of array results. Up - Genes induced by exposure to the corresponding stress; Down Genes repressed by exposure to the corresponding stress.

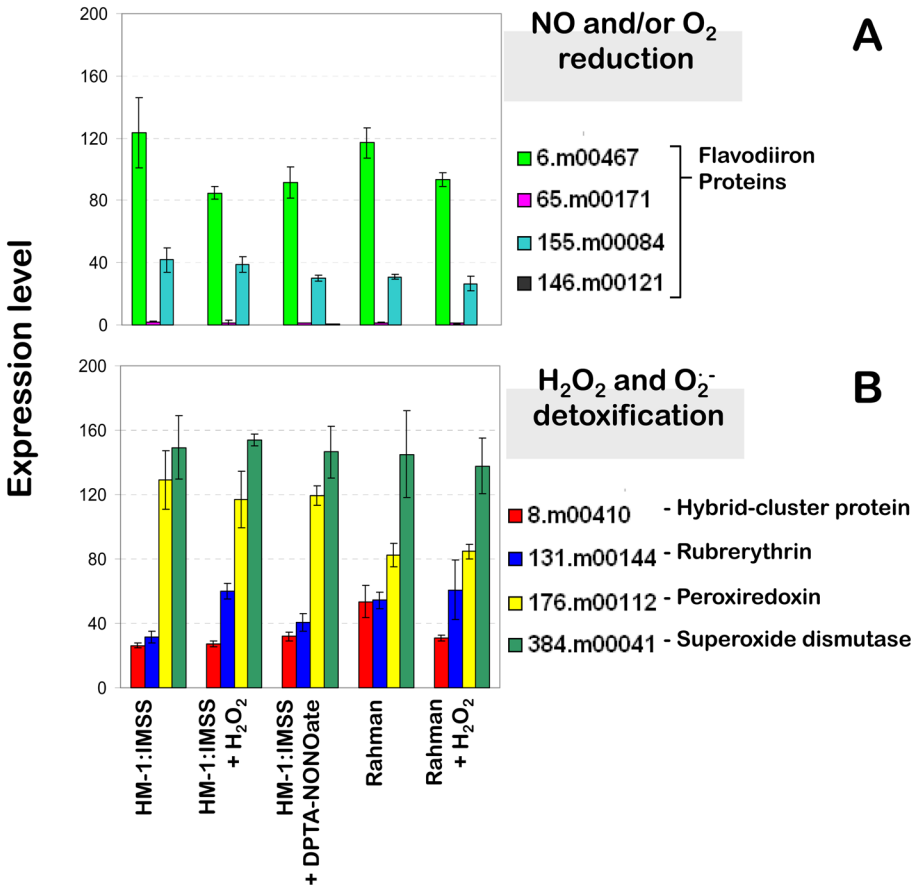


Figure 2.

Expression levels of genes encoding identified detoxification pathways for reactive oxygen and nitrogen species (top and middle panels) and iron-sulfur center assembly systems (bottom panel) for unchallenged cultures of *E. histolytica* strains HM-1:IMSS (pathogenic) and Rahman (non-pathogenic), and for cultures challenged with hydrogen peroxide and the nitric oxide releaser DPTA-NONOate.

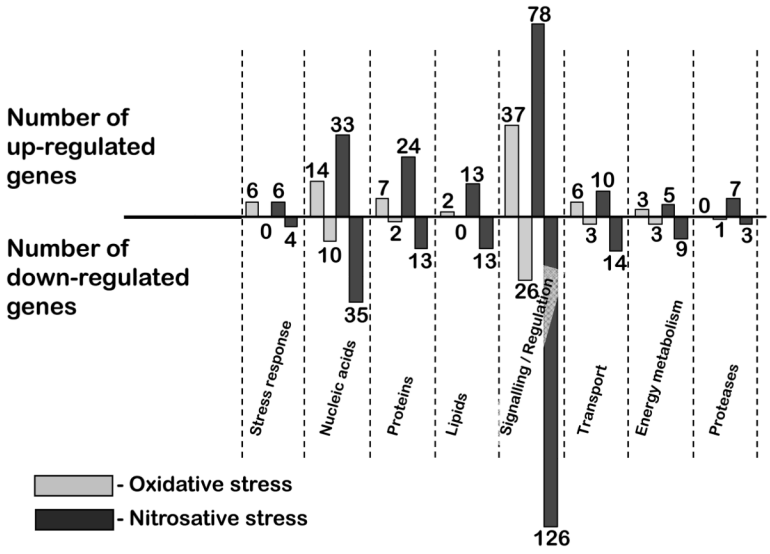


Figure 3.

Transcriptional profiles of *E. histolytica* HM-1:IMSS exposed to oxidative and nitrosative stress. Genes were grouped according to putative functions inferred from the respective annotations.

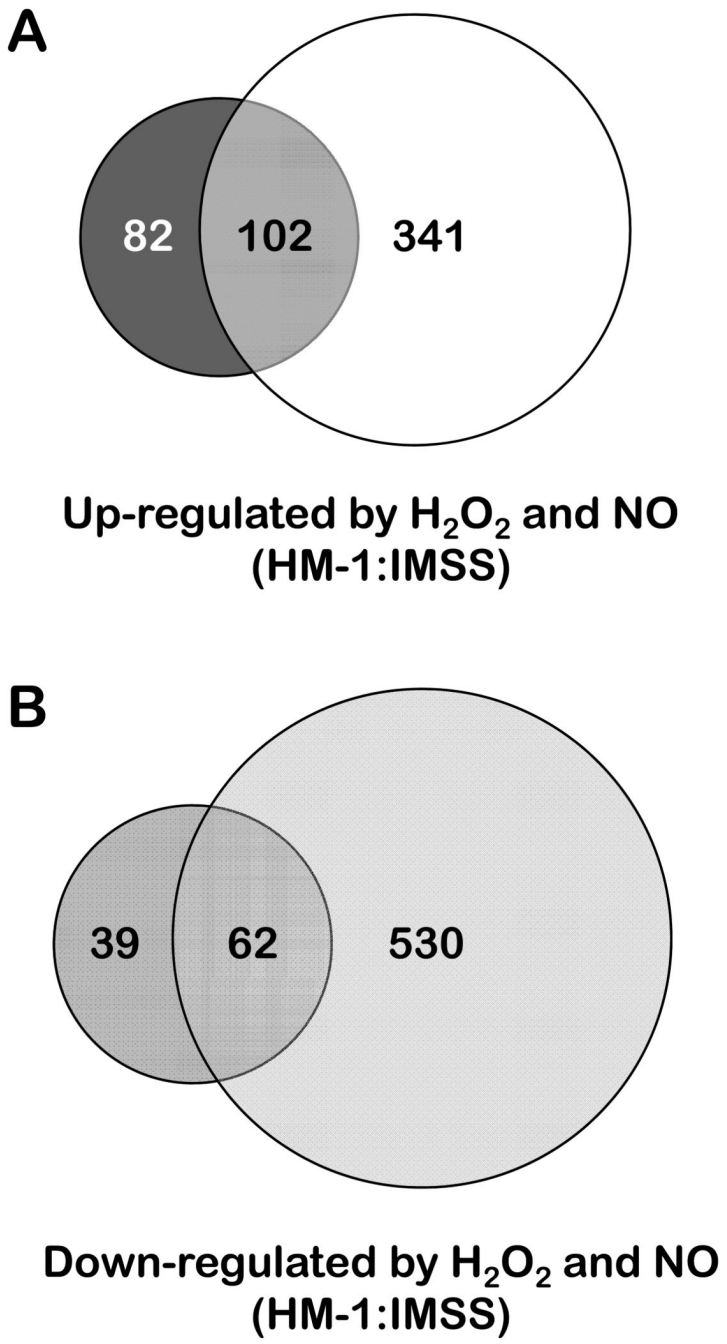


Figure 4.
Venn diagrams depicting the overlap between the transcriptional changes observed upon exposing *E. histolytica* HM-1:IMSS to oxidative and nitrosative stress.

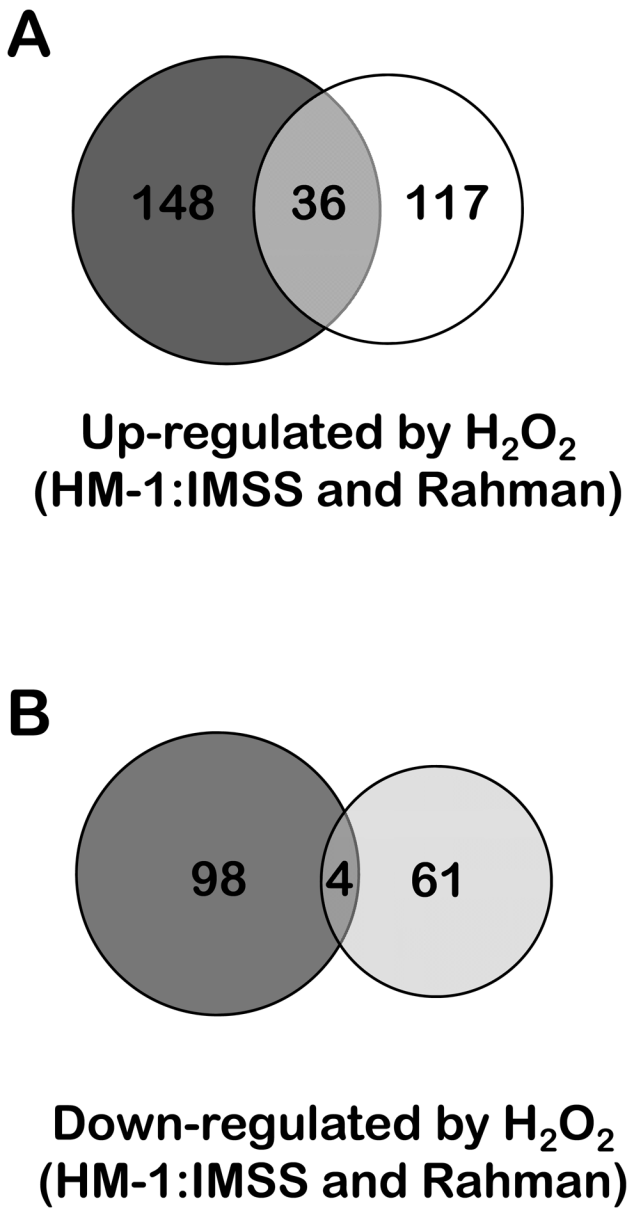
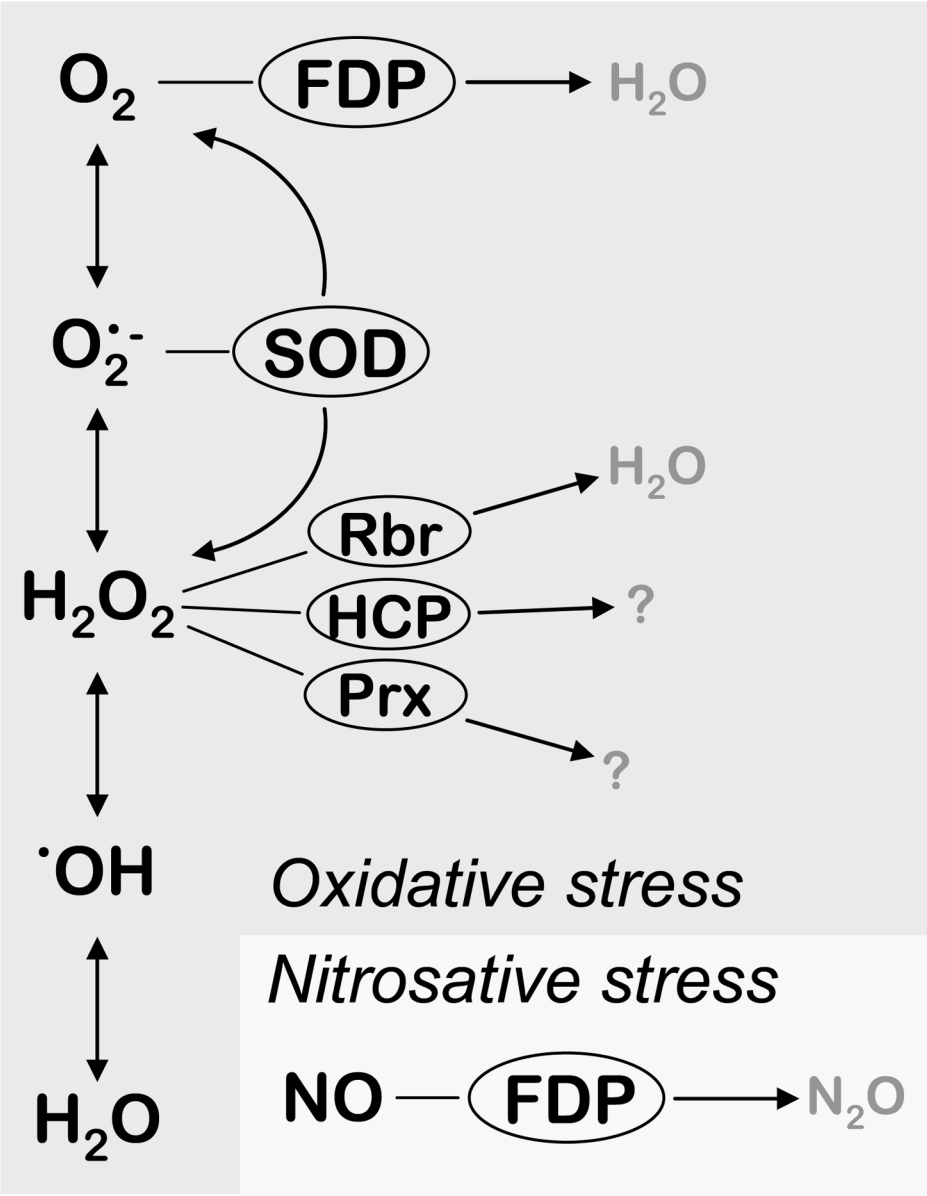


Figure 5.
Venn diagrams depicting the overlap between the transcriptional changes observed upon exposing a pathogenic and non-pathogenic *E. histolytica* strain to oxidative stress.



Scheme 1.

Putative detoxification pathways for reactive oxygen and nitrogen species identified in the genome of *E. histolytica* HM-1:IMSS. Reactive oxygen species depicted as the sequential one-electron reduced intermediates of oxygen reduction. FDP – flavodiiron proteins, oxygen and/or nitric oxide reductases; SOD – superoxide dismutase; Rbr – rubrerythrin, hydrogen peroxide reductase; HCP – hybrid-cluster protein; Prx – peroxiredoxin.

Table 1A

Correlations between individual DNA microarrays for *E. histolytica* HM-1:IMSS and Rahman under each experimental condition.

	HM-1:IMSS		
	1	**2**	**3**
1		0.97	0.95
2			0.94
3			
	HM-1:IMSS + H_2O_2		
	1	**2**	**3**
1		0.97	0.97
2			0.98
3			
	HM-1:IMSS + DPTA-NONOate		
	1	**2**	**3**
1		0.96	0.95
2			0.97
3			
	Rahman		
	1	**2**	**3**
1		0.99	0.97
2			0.98
3			
	Rahman + H_2O_2		
	1	**2**	**3**
1		0.99	0.98
2			0.99
3			

Table 1B

Averaged correlations between DNA microarrays for each experimental condition of *E. histolytica* HM-1:IMSS.

Array condition (# of arrays)	***HM-1:IMSS (3)***	***HM-1+H_2O_2 (3)***	***HM-1+DPTA (3)***
HM-1:IMSS (3)		0.98	0.96
HM-1 + H_2O_2 (3)			0.98
HM-1 + DPTA (3)			

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Table 2

Genes up-regulated by hydrogen peroxide in *Entamoeba histolytica* HM-1:IMSS. The probe ID, accession number, description, baseline expression level, fold-change, p-value, and regulation under DPTA or other conditions are shown. The 30 most highly regulated genes are listed. HS (heat shock) (adapted from Hackney et al); Cysts (*E. histolytica* cysts) (adapted from Ehrenkaufer et al).

Probe ID	Accession number	Description	Baseline expression level	Fold-change	p-value	Regulated under DPTA	Regulated under other conditions
879.m00008_at	XM_642785	hypothetical protein	0.07	189.6	0.000	-	-
194.m00102_s_at	XM_645780	hypothetical protein	0.06	127.1	0.001	+	HS
654.m00032_x_at	XM_642891	hypothetical protein	1.39	114.9	0.023	+	-
363.m00049_x_at	XM_643869	hypothetical protein	0.17	89.05	0.015	+	HS
256.m00084_x_at	XM_644865	hypothetical protein	0.12	86.64	0.014	+	HS
256.m00083_x_at	XM_644864	hypothetical protein	1.22	82.85	0.014	+	-
692.m00024_s_at	XM_642872	hypothetical protein	0.05	76.14	0.000	+	-
248.m00060_s_at	XM_644979	hypothetical protein	0.05	71.78	0.000	+	-
266.m00066_s_at	XM_644755	hypothetical protein	0.13	53.25	0.002	+	-
194.m00123_x_at	XM_645776	dUTP diphosphatase, putative	0.11	50.95	0.011	+	-
397.m00055_s_at	XM_643659	hypothetical protein	0.18	42.56	0.020	+	-
654.m00031_s_at	XM_642894	hypothetical protein	0.06	41.05	0.000	+	HS
397.m00062_x_at	XM_643664	hypothetical protein	1.35	37.88	0.034	+	-
692.m00026_s_at	XM_642873	hypothetical protein	0.06	35.78	0.001	+	-
711.m00021_x_at	XM_642861	hypothetical protein	0.10	33.68	0.018	+	HS
375.m00057_x_at	XM_643790	deoxyuridine 5 -triphosphate nucleotidohydrolase	0.05	21.75	0.001	+	-
397.m00054_x_at	XM_643658	hypothetical protein	0.06	15.74	0.001	+	-
344.m00044_at	XM_644000	hypothetical protein	0.24	15.1	0.025	+	-
245.m00039_x_at	XM_645025	hypothetical protein	0.47	14.39	0.026	+	Cysts
219.m00107_at	XM_645351	cell division control protein 42, putative	0.10	11.34	0.003	-	HS
267.m00069_at	XM_644746	hypothetical protein	0.10	11.2	0.034	-	-
358.m00033_at	XM_643905	hypothetical protein	0.64	11.05	0.012	+	Cysts
194.m00103_at	XM_645781	hypothetical protein	0.07	10.44	0.005	+	-
8.m00393_at	XM_651687	late competence protein, putative	0.25	8.85	0.027	-	HS + Cysts
356.m00029_s_at	XM_643911	hypothetical protein	0.18	7.68	0.021	+	HS
20.m00272_at	XM_650868	conserved hypothetical protein	0.13	6.83	0.046	+	-

Probe ID	Accession number	Description	Baseline expression level	Fold-change	p-value	Regulated under DPTA	Regulated under other conditions
36.m00211_at	XM_650002	hypothetical protein	0.09	6.78	0.009	+	Cysts
35.m00253_at	XM_650038	iron-sulfur flavoprotein, putative	2.16	6.7	0.006	-	Cysts
312.m00037_at	XM_644279	iron-sulfur flavoprotein, putative	0.99	6.62	0.005	-	HS + Cysts
1.m00705_at	XM_652401	BspA-like leucine rich repeat protein, putative	0.27	6.56	0.004	-	HS

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Table 3

Genes down-regulated by hydrogen peroxide in *Entamoeba histolytica* HM-1:IMSS. The probe ID, accession number, description, baseline expression level, fold-change, p-value, and regulation under DPTA or other conditions are shown. The 30 most highly regulated genes are listed. HS (heat shock) (adapted from Hackney et al); Colitis (ameba from a mouse model of amebic colitis) (adapted from Gilchrist et al); Trophs (*E. histolytica* trophozoites) (adapted from Ehrenkaufer et al).

Probe ID	Accession number	Description	Baseline expression level	Fold-change	p-value	Regulated under DPTA	Regulated under other conditions?
172.m00078_at	XM_646199	hypothetical protein	0.86	−9.09	0.000	+	HS
537.m00017_x_at	XM_643063	AIG1 family protein, putative	4.58	−7.41	0.039	+	-
2.m00528_at	XM_652349	hypothetical protein	0.35	−6.8	0.019	+	HS
3.m00563_at	XM_652186	hypothetical protein	6.39	−5.75	0.002	+	-
86.m00158_s_at	XM_648244	hypothetical protein	0.46	−5.59	0.039	+	HS
226.m00092_at	XM_645246	Rab family GTPase	0.26	−4.83	0.000	+	-
233.m00105_at	XM_645153	hypothetical protein	6.57	−4.41	0.004	+	-
9.m00372_x_at	XM_651612	hypothetical protein	0.32	−4.18	0.004	+	HS
87.m00165_at	XM_648195	hypothetical protein	0.21	−4.13	0.009	+	-
87.m00154_at	XM_648212	formate nitrite transporter family protein, putative	0.78	−4.02	0.001	-	Colitis
31.m00209_x_at	XM_650257	conserved hypothetical protein	0.25	−3.79	0.019	+	-
66.m00150_at	XM_648887	high mobility group protein, putative	0.39	−3.77	0.006	+	HS
343.m00064_x_at	XM_644015	WD repeat protein	0.21	−3.76	0.039	+	-
125.m00091_at	XM_647175	cyclin, putative	0.71	−3.69	0.006	+	HS
286.m00057_at	XM_644512	mitotic inducer phosphatase, putative	0.21	−3.31	0.000	+	HS
37.m00216_at	XM_649962	hypothetical protein	0.79	−3.26	0.018	+	-
56.m00175_at	XM_649238	elongation factor 1 beta, putative	12.26	−3.15	0.038	-	-
46.m00250_at	XM_649627	hypothetical protein	3.53	−3.07	0.005	-	-
19.m00301_at	XM_650878	hypothetical protein	2.32	−3.03	0.015	+	HS
408.m00045_s_at	XM_643583	putative GTPase	2.43	−3.02	0.006	+	HS
80.m00159_at	XM_648429	hypothetical protein	7.76	−2.99	0.004	+	-
1.m00606_s_at	XM_652473	hypothetical protein	9.86	−2.91	0.006	+	HS
40.m00246_s_at	XM_649855	rRNA biogenesis protein, putative	4.83	−2.89	0.041	+	HS
95.m00147_at	XM_647953	PfkB family carbohydrate kinase, putative	6.85	−2.89	0.005	-	HS
51.m00159_s_at	XM_649424	hypothetical protein	0.30	−2.88	0.031	+	-

Probe ID	Accession number	Description	Baseline expression level	Fold-change	p-value	Regulated under DPTA	Regulated under other conditions?
221.m00089_s_at	XM_645315	hypothetical protein	3.74	−2.85	0.027	+	-
127.m00147_at	XM_647143	predicted protein	1.33	−2.82	0.019	+	HS
4.m00641_at	XM_652060	tRNA-specific adenosine deaminase, putative	13.13	−2.8	0.040	+	-
34.m00273_at	XM_650116	Rab family GTPase	4.84	−2.77	0.048	-	Trophs
13.m00334_at	XM_651296	conserved hypothetical protein	2.85	−2.67	0.027	+	Trophs

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Table 4

Genes up-regulated by DPTA-NONOate in *Entamoeba histolytica* HM-1:IMSS. The probe ID, accession number, description, baseline expression level, fold-change, p-value, and regulation under other conditions are shown. The 30 most highly regulated genes are listed. HS (heat shock) (adapted from Hackney et al); Colitis (ameba from a mouse model of amebic colitis) (adapted from Gilchrist et al); Cysts (*E. histolytica* cysts) (adapted from Ehrenkaufer et al).

Probe ID	Accession number	Description	Baseline expression level	Fold-change	p-value	Regulated under other conditions?
194.m00102_s_at	XM_645780	hypothetical protein	0.06	380.2	0.009	HS
363.m00049_x_at	XM_643869	hypothetical protein	0.17	305.5	0.002	-
256.m00084_x_at	XM_644865	hypothetical protein	0.12	252.8	0.002	-
248.m00060_s_at	XM_644979	hypothetical protein	0.05	240.9	0.006	-
692.m00024_s_at	XM_642872	hypothetical protein	0.05	219.5	0.003	-
194.m00123_x_at	XM_645776	dUTP diphosphatase, putative	0.11	191.5	0.003	-
266.m00066_s_at	XM_644755	hypothetical protein	0.13	176.2	0.004	-
654.m00032_x_at	XM_642891	hypothetical protein	1.39	162.9	0.015	-
654.m00031_s_at	XM_642894	hypothetical protein	0.06	130.4	0.008	-
256.m00083_x_at	XM_644864	hypothetical protein	1.22	123.6	0.006	-
397.m00055_s_at	XM_643659	hypothetical protein	0.18	119.3	0.004	-
692.m00026_s_at	XM_642873	hypothetical protein	0.06	107.7	0.010	-
375.m00057_x_at	XM_643790	deoxyuridine 5 -triphosphate nucleotidohydrolase	0.05	82.54	0.020	-
711.m00021_x_at	XM_642861	hypothetical protein	0.10	81.62	0.006	-
397.m00062_x_at	XM_643664	hypothetical protein	1.35	58.47	0.019	-
397.m00054_x_at	XM_643658	hypothetical protein	0.06	57.66	0.020	-
36.m00211_at	XM_650002	hypothetical protein	0.09	49.28	0.005	-
245.m00039_x_at	XM_645025	hypothetical protein	0.47	22.34	0.014	-
301.m00039_s_at	XM_644356	heat shock protein 70, putative	0.30	21.14	0.003	-
81.m00150_s_at	XM_648418	heat shock protein 101, putative	1.54	20.48	0.001	-
450.m00030_at	XM_643384	hypothetical protein	0.20	19.96	0.027	Colitis
356.m00029_s_at	XM_643911	hypothetical protein	0.18	16.2	0.008	-
7.m00453_s_at	XM_651737	hypothetical protein	0.30	14.84	0.001	-
344.m00044_at	XM_644000	hypothetical protein	0.24	13.53	0.033	Cysts
295.m00030_at	XM_644416	conserved hypothetical protein	0.16	13.28	0.048	-
134.m00124_at	XM_646949	heat shock protein, Hsp20 family, putative	1.44	12.29	0.016	-

Probe ID	Accession number	Description	Baseline expression level	Fold-change	p-value	Regulated under other conditions?
33.m00209_x_at	XM_650174	hypothetical protein	0.10	12.1	0.004	-
141.m00082_at	XM_646820	protein kinase, putative	0.54	12.09	0.027	-
20.m00272_x_at	XM_650868	conserved hypothetical protein	0.17	9.86	0.043	-
796.m00013_s_at	XM_642828	conserved hypothetical protein	0.23	9.69	0.015	-

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Table 5

Genes down-regulated by DPTA-NONOate in *Entamoeba histolytica* HM-1:IMSS. The probe ID, accession number, description, baseline expression level, fold-change, p-value, and regulation under other conditions are shown. The 30 most highly regulated genes are listed. HS (heat shock) (adapted from Hackney et al); Colitis (ameba from a mouse model of amebic colitis) (adapted from Gilchrist et al); Trophs (*E. histolytica* trophozoites) (adapted from Ehrenkaufer et al).

Probe ID	Accession number	Description	Baseline expression level	Fold-change	p-value	Regulated under other conditions?
4.m00678_s_at	XM_652013	hypothetical protein	41.94	−49.02	0.002	-
172.m00078_at	XM_646199	hypothetical protein	0.86	−14.43	0.001	HS
99.m00179_at	XM_647858	inositol polyphosphate kinase, putative	4.53	−11.48	0.001	-
341.m00039_s_at	XM_644035	hypothetical protein	2.41	−10.59	0.015	-
229.m00063_at	XM_645215	hypothetical protein	5.04	−9.35	0.019	-
221.m00089_s_at	XM_645315	hypothetical protein	3.74	−9.01	0.002	-
13.m00349_at	XM_651311	protein kinase, putative	2.17	−8.85	0.004	-
65.m00147_at	XM_648922	hypothetical protein	2.93	−8.47	0.003	Trophs + HS
255.m00049_at	XM_644881	conserved hypothetical protein	1.25	−8.2	0.022	-
93.m00151_at	XM_648019	WD repeat protein	1.99	−8.2	0.004	-
22.m00263_at	XM_650739	hypothetical protein	1.42	−7.94	0.019	-
74.m00199_at	XM_648611	hypothetical protein	2.53	−7.87	0.001	-
232.m00071_at	XM_645176	hypothetical protein	1.15	−7.46	0.004	-
4.m00607_at	XM_652082	protein kinase, putative	3.19	−7.46	0.002	HS
408.m00044_at	XM_643584	hypothetical protein	0.95	−7.46	0.043	-
86.m00158_s_at	XM_648244	DEAD DEAH box helicase, putative	0.46	−7.35	0.044	HS
32.m00207_at	XM_650196	zinc finger protein, putative	12.62	−6.94	0.009	-
32.m00202_s_at	XM_650191	hypothetical protein	0.72	−6.9	0.008	-
4.m00640_at	XM_652059	conserved hypothetical protein	3.07	−6.9	0.000	-
113.m00152_at	XM_647500	BspA-like leucine rich repeat protein, putative	1.09	−6.85	0.015	Trophs
289.m00071_s_at	XM_644481	putative GTPase	7.01	−6.29	0.000	-
82.m00139_s_at	XM_648379	hypothetical protein	0.59	−6.21	0.005	-
408.m00045_s_at	XM_643583	hypothetical protein	2.43	−6.17	0.007	HS
2.m00528_at	XM_652349	protein phosphatise, putative	0.35	−5.99	0.028	HS
201.m00110_at	XM_645662	hypothetical protein	1.35	−5.92	0.006	HS
12.m00322_at	XM_651338	hypothetical protein	3.67	−5.81	0.030	HS

Probe ID	Accession number	Description	Baseline expression level	Fold-change	p-value	Regulated under other conditions?
48.m00216_s_at	XM_649527	hypothetical protein	3.05	−5.75	0.019	Colitis
98.m00148_s_at	XM_647890	hypothetical protein	1.02	−5.52	0.001	-
66.m00150_at	XM_648887	hypothetical protein	0.39	−5.49	0.000	HS
816.m00009_at	XM_642817	hypothetical protein	4.41	−5.38	0.049	HS

Table 6

Genes up-regulated by hydrogen peroxide in *Entamoeba histolytica* Rahman. The probe ID, accession number, description, baseline expression level, fold-change and p-value are shown. The 30 most highly regulated genes are listed. If the gene is regulated and its fold-change in HM-1:IMSS are listed.

Probe ID	Accession number	Description	Baseline expression level	Fold-change	p-value	Regulated in HM1:1MSS	Fold-change in HM1:IMSS
301.m00039_s_at	XM_644356	heat shock protein 70, putative	0.30	7.37	0.005	-	
205.m00100_s_at	XM_645582	hypothetical protein	8.19	6.9	0.013	-	
134.m00124_at	XM_646949	heat shock protein, Hsp20 family, putative	1.44	6.62	0.006	-	
606.m00014_s_at	XM_642953	hypothetical protein	0.28	6.59	0.043	-	
64.m00187_s_at	XM_648976	heat shock protein 101, putative	36.55	6.53	0.015	+	2.42
8.m00393_at	XM_651687	late competence protein, putative	0.25	6.48	0.010	+	8.85
264.m00070_x_at	XM_644777	hypothetical protein	0.05	6.31	0.030	-	
181.m00068_s_at	XM_646040	hsp101-related protein	18.45	6.1	0.009	-	
562.m00023_at	XM_643023	protein kinase, putative	1.18	5.74	0.022	-	
256.m00083_x_at	XM_644864	hypothetical protein	1.22	5.72	0.050	+	82.85
188.m00103_at	XM_645894	hypothetical protein	0.48	5.68	0.038	-	
451.m00037_s_at	XM_643383	hypothetical protein	0.07	5.59	0.016	-	
81.m00150_s_at	XM_648418	heat shock protein 101, putative	1.54	5.51	0.001	-	
110.m00118_x_at	XM_647568	Rho family GTPase	0.11	5.47	0.030	-	
64.m00178_s_at	XM_648975	heat shock protein 101, putative	3.41	5.24	0.011	-	
654.m00032_x_at	XM_642891	hypothetical protein	1.39	5.01	0.047	+	114.90
42.m00175_at	XM_649778	hypothetical protein	0.96	4.92	0.018	-	
363.m00049_x_at	XM_643869	hypothetical protein	0.17	4.9	0.030	+	89.05
344.m00045_at	XM_644001	hypothetical protein	0.60	4.82	0.008	+	6.21
442.m00023_at	XM_643432	hypothetical protein	0.69	4.75	0.015	+	4.49
50.m00195_s_at	XM_649449	hypothetical protein	0.05	4.73	0.018	-	
30.m00249_at	XM_650277	1-O-acylceramide synthase, putative	0.40	4.63	0.026	-	
39.m00253_at	XM_649881	CXXC-rich protein	8.57	4.56	0.009	-	
168.m00119_s_at		pseudogene, N- acetylmuraminidase	0.35	4.55	0.029	-	
167.m00116_x_at	XM_646306	hypothetical protein	0.08	4.48	0.014	-	
458.m00058_at	XM_643333	hypothetical protein	0.14	4.45	0.017	-	
6.m00424_at	XM_651810	hypothetical protein	0.22	4.26	0.012	+	3.00

Probe ID	Accession number	Description	Baseline expression level	Fold-change	p-value	Regulated in HM1:1MSS	Fold-change in HM1:IMSS
70.m00178_s_at	XM_648752	hypothetical protein	0.24	3.96	0.027	-	
245.m00039_x_at	XM_645025	hypothetical protein	0.47	3.84	0.040	+	14.39
7.m00453_s_at	XM_651737	hypothetical protein	0.30	3.7	0.021	+	3.46

Cell Microbiol. Author manuscript; available in PMC 2012 August 13.

Table 7

Genes down-regulated by hydrogen peroxide in *Entamoeba histolytica* Rahman. The probe ID, accession number, description, baseline expression level, fold-change and p-value are shown. The 30 most highly regulated genes are listed. If the gene is regulated and its fold-change in HM-1:IMSS are listed.

Probe ID	Accession number	Description	Baseline expression level	Fold-change	p-value	Regulated in HM1:1MSS	Fold-change in HM1:IMSS
50.m00168_at	XM_649471	hypothetical protein	107.85	−30.49	0.012	-	
233.m00105_at	XM_645153	hypothetical protein	6.57	−4.13	0.005	+	−4.41
214.m00072_at	XM_645429	hypothetical protein	1.02	−3.69	0.015	-	
249.m00072_at	XM_644965	hypothetical protein	0.24	−3.5	0.042	-	
459.m00030_at	XM_643329	hypothetical protein	74.08	−3.4	0.034	-	
25.m00245_at	XM_650545	conserved hypothetical protein	0.35	−3.19	0.006	-	
67.m00091_x_at	XM_648866	protein kinase, putative	6.08	−3.1	0.046	-	
113.m00152_at	XM_647500	hypothetical protein	1.09	−2.92	0.002	-	
224.m00085_at	XM_645268	cullin, putative	0.44	−2.82	0.033	-	
41.m00219_s_at	XM_649804	ABC transporter, putative	47.84	−2.74	0.006	-	
380.m00029_at	XM_643755	hypothetical protein	0.09	−2.72	0.019	-	
54.m00183_at	XM_649345	hypothetical protein	28.38	−2.65	0.025	+	−2.30
129.m00151_at	XM_647089	hypothetical protein	0.08	−2.56	0.015	-	
264.m00067_at	XM_644788	hypothetical protein	2.21	−2.56	0.034	-	
234.m00047_at	XM_645144	hypothetical protein	1.04	−2.51	0.007	-	
232.m00071_at	XM_645176	hypothetical protein	1.15	−2.49	0.022	-	
41.m00243_s_at	XM_649788	hypothetical protein	5.24	−2.46	0.000	-	
310.m00064_at	XM_644295	RNA polymerase I largest subunit, putative	26.67	−2.43	0.009	-	
103.m00165_x_at	XM_647745	hypothetical protein	0.14	−2.4	0.034	-	
291.m00043_at	XM_644448	leucine rich repeat protein	0.78	−2.38	0.041	-	
26.m00293_at	XM_650512	poly(A) polymerase, putative	1.35	−2.37	0.029	-	
247.m00075_at	XM_644998	LIM domain protein	1.74	−2.33	0.007	-	
2.m00522_at	XM_652343	U6 snRNA-associated Sm-like protein, putative	0.63	−2.32	0.008	-	
9.m00420_at	XM_651594	carbonic anhydrase, putative	2.67	−2.32	0.031	-	
129.m00135_at	XM_647073	PH domain protein	4.92	−2.31	0.002	-	
34.m00252_at	XM_650085	conserved hypothetical protein	0.29	−2.3	0.030	-	
25.m00242_at	XM_650542	hypothetical protein	7.15	−2.29	0.011	-	

Probe ID	Accession number	Description	Baseline expression level	Fold-change	p-value	Regulated in HM1:1MSS	Fold-change in HM1:IMSS
277.m00057_at	XM_644616	actobindin homolog, putative	126.03	−2.28	0.007	-	
108.m00108_at	XM_647607	hypothetical protein	0.50	−2.26	0.019	-	
59.m00197_x_at	XM_649134	DNA-directed RNA polymerase subunitN, putative	7.02	−2.25	0.023	-	