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Identification of Differentially Expressed Genes in Virulent and Nonvirulent *Entamoeba* Species: Potential Implications for Amebic Pathogenesis†

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Entamoeba histolytica **is a protozoan parasite that causes colitis and liver abscesses. Several *Entamoeba* species and strains with differing levels of virulence have been identified. *E. histolytica* HM-1:IMSS is a virulent strain, *E. histolytica* Rahman is a nonvirulent strain, and *Entamoeba dispar* is a nonvirulent species. We used an *E. histolytica* DNA microarray consisting of 2,110 genes to assess the transcriptional differences between these species/strains with the goal of identifying genes whose expression correlated with a virulence phenotype. We found 415 genes expressed at lower levels in *E. dispar* and 32 genes with lower expression in *E. histolytica* Rahman than in *E. histolytica* HM-1:IMSS. Overall, 29 genes had decreased expression in both the nonvirulent species/strains than the virulent *E. histolytica* HM-1:IMSS. Interestingly, a number of genes with potential roles in stress response and virulence had decreased expression in either one or both nonvirulent *Entamoeba* species/strains. These included genes encoding Fe hydrogenase (9.m00419), peroxiredoxin (176.m00112), type A flavoprotein (6.m00467), lysozyme (6.m00454), sphingomyelinase C (29.m00231), and a hypothetical protein with homology to both a *Plasmodium* sporozoite threonine-asparagine-rich protein (STARP) and a streptococcal hemagglutinin (238.m00054). The function of these genes in *Entamoeba* and their specific roles in parasite virulence need to be determined. We also found that a number of the non-long-terminal-repeat retrotransposons (*EhLINE*s and *EhSINE*s), which have been shown to modulate gene expression and genomic evolution, had lower expression in the nonvirulent species/strains than in *E. histolytica* HM-1:IMSS. Our results, identifying expression profiles and patterns indicative of a virulence phenotype, may be useful in characterizing the transcriptional framework of virulence.**

The protozoan parasite *Entamoeba histolytica* causes 50 million cases of invasive disease and approximately 100,000 deaths each year (55). The most common manifestations of amebic infection are dysentery and liver abscess, but infections of the lung, heart, and brain also occur (28). Only ~10% of infections result in invasive disease, but the reasons behind this phenomenon remain largely unknown (27). The molecular mechanisms that regulate parasite invasion and pathogenesis are not well characterized.

Different species and strains of *Entamoeba* exhibit various levels of pathogenicity. *Entamoeba histolytica* and *Entamoeba dispar* are morphologically identical and highly similar species (their rRNAs are 98% identical), but they have vastly different virulence potentials in vivo (20). *E. histolytica* can cause invasive disease, whereas *E. dispar*, while able to colonize humans, appears to have no invasive potential in vivo. Key differences in a number of virulence determinants between the two species have been identified. Cysteine proteinases (CPs) are a family of cathepsin proteinases involved in the degradation of colonic mucin and extracellular matrix (47). Antisense inhibition of CPs in *E. histolytica* trophozoites results in reduced phagocytic activity (4), gut inflammation, and liver abscess formation (3). Two of the CP genes (*CP1* and *CP5*) are either missing or highly degenerate (11, 58), and another (*CP8*), although conserved, has significantly lower expression in *E. dispar* (12). Amebapores, which are pore-forming peptides, lyse target cells and ingested bacteria (33, 34). Silencing of amebapore A in *E. histolytica* results in decreased cytotoxicity against nucleated cells and erythrocytes, as well as decreased liver abscess formation in vivo (10). Although highly conserved orthologs of the amebapore family are present in *E. dispar* (88 to 95% identical), the most abundant amebapore (AP-A) is ~25-fold less active in *E. dispar* (42). KERP1, a lysine- and glutamic acid-rich protein postulated to be involved in attachment to host cells, was recently reported to be missing or divergent in *E. dispar* (49). The genetic element *EhSINE1*, a non-long-terminal-repeat (non-LTR) retrotransposon, and the *Ariel1* gene are also missing or divergent in *E. dispar* (56, 57).

Entamoeba histolytica Rahman, isolated from an asymptomatic individual, has reduced cytotoxic capabilities for epithelial cells in vitro, does not form liver abscesses in animal models, and is classically referred to as a nonvirulent *E. histolytica* strain (2, 23). A few genetic differences between *E. histolytica* HM-1:IMSS and *E. histolytica* Rahman have been identified. One observation, which has been genetically proven to be related to virulence, is the decreased level of the light subunit of the Gal/Gal-NAc lectin in *E. histolytica* Rahman compared to that in *E. histolytica* HM-1:IMSS (2). Additionally, the pro-

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TABLE 1. Primers used for generation of probes[a]

Locus	Primer
194.m00115 F	5′-ATGGGCAATAGACCCAACAC-3′
194.m00115 R	5′-TCCATAAATTTTTGCATGACCA-3′
2.m00567 F	5′-ATGAACGCTATTAAACCTAAAACAAT-3′
2.m00567 R	5′-CTATTATTTGTCTGAATTGTCTAAAGCTG-3′
2.m00567 putative promoter region F	5′-AAAAGCCATTGAAAATGGATG-3′
2.m00567 putative promoter region R	5′-TCCATCAACATCCAATACAATTG-3′
238.m00054 segment #1 F	5′-ATGGCTGAACAAATCAGAGTCTGACA-3′
238.m00054 segment #1 R	5′-CTAAAGTAATAATGCTGGAACATTTGGATGG-3′
238.m00054 segment #2 F	5′-GAATCACCGAATGTTATTGCATCTGG-3′
238.m00054 segment #2 R	5′-CTACTGTGAATCTTTAACACGGATGTTCGA-3′
238.m00054 segment #3 F	5′-TCGAACATCCGTGTTAAAGATTCACAG-3′
238.m00054 segment #3 R	5′-CTACATTGTCTTTCCTCCAATTTCATCTCC-3′
29.m00210 F	5′-ATGCAACAAGAGACAGTTGTTGG-3′
29.m00210 R	5′-CTATTATTTTTGTGCAAAGAATTTCTCT-3′
29.m00231 F	5′-CCCCTTCATCGACAAACAAT-3′
29.m00231 R	5′-TCGATGACGTCTTGATTTGG-3′
297.m00063 F	5′-TCAGCATGGATTTGATTGGA-3′
297.m00063 R	5′-CAGCAACACCTTTTTCAACG-3′
5.m00482 F	5′-TCTGGTGCTTTTGATGTTGC-3′
5.m00482 R	5′-CCACCGAAGGATCACACTCT-3′
51.m00161 F	5′-GTCAAAGAGCTGTTGCATGG-3′
51.m00161 R	5′-TTCTGCAACATTTCCTGGTG-3′
6.m00454 F	5′-GATTCTTCATTTGCCGTGCT-3′
6.m00454 R	5′-CCGAATGAAGCCCAATTATC-3′
6.m00467 F	5′-AATGGGCACAACCTATTGCT-3′
6.m00467 R	5′-TTTCCCCATTCAAAGCATGT-3′
9.m00419 F	5′-AACCACCAAAAATTCCACCA-3′
9.m00419 R	5′-AATTGGTGAACGGGCAGTAG-3′
EhActin F	5′-GCTGGTATGGGTCAAAAGGA-3′
EhActin R	5′-TTTCTGTGGACAATAGCTGGTC-3′
EhLINE1 ORF1 F	5′-GATCCTTTTCCAATGCAGGA-3′
EhLINE1 ORF1 R	5′-TGCTTTTCTCTTCGATTTCCA-3′
EhLINE1 ORF2 F	5′-TGAARATAGGGATTACTTCMGTGT-3′
EhLINE1 ORF2 R	5′-CCCATTAGACATGGTAAGTGGAA-3′
M13 F	5′-TGTAAAACGACGGCCAGT-3′
M13 R	5′-CAGGAAACAGCTATGACC-3′

[a] Probes were used for Northern blot analyses and sequencing particular loci in *E. histolytica* Rahman. T_m values ranged between 56 and 58°C.

teophosphoglycans coating the surface of *E. histolytica* Rahman have truncated glucan side chains compared to those of the virulent *E. histolytica* HM-1:IMSS (40). The relationship of this observation to amebic virulence is not clear; however, the authors hypothesized that the glycans may protect parasite adhesion molecules from proteolysis or that the proteophosphoglycans may regulate the ability of parasite surface molecules to interact with host cell receptors.

The effort to sequence the genome of *E. histolytica* HM-1:IMSS has unveiled a number of unusual aspects of amebic biology (36). However, the essential differences between the virulent and nonvirulent species/strains and the factor(s) that determines the invasive potential in *E. histolytica* remain elusive. A recent comparative genomic hybridization approach identified a number of genomic differences between *E. histolytica* and *E. dispar*, with 67 genes out of 1,640 studied (4%) identified as highly or significantly divergent (50). Fewer genetic differences were identified between *E. histolytica* HM-1:IMSS and *E. histolytica* Rahman, with 5 out of 1,817 (0.3%) genes identified as highly or significantly divergent. Whether these genomic differences contribute to the various virulence phenotypes remains to be determined. Previous studies have shown that *E. histolytica* HM-1:IMSS is able to lyse colonic cell monolayers without major changes in its transcriptional profile, indicating that many of the genes important in host cell damage may be constitutively expressed under tissue culture conditions (37).

In order to identify the genes that are differentially expressed among the virulent and nonvirulent *Entamoeba* species/strains, we used a DNA microarray consisting of 2,110 unique genes to perform expression profiling of the virulent *E. histolytica* HM-1:IMSS and the nonvirulent *E. histolytica* Rahman and *E. dispar* SAW760. Using this technique, we have identified 415 genes that have lower expression in *E. dispar* SAW760 and 32 genes with lower expression in *E. histolytica* Rahman than in EH HM-1:IMSS. Interestingly, 29 genes showed decreased expression in both the nonvirulent species/strains *E. dispar* SAW760 and *E. histolytica* Rahman. These genes are of particular interest as their expression correlates with virulence, and it is interesting to speculate that they may play roles in amebic pathogenesis. Our work represents the first large-scale expression profiling of *Entamoeba* species/strains and opens the door to the investigation of genetic and expression differences which may relate to parasite virulence.

MATERIALS AND METHODS

Parasite culture. *E. histolytica* HM-1:IMSS was originally isolated from a patient with colitis in 1967 (21). *E. histolytica* Rahman was isolated from an asymptomatic cyst passer in 1972 (21). Both were obtained from ATCC (http://www.atcc.org/) and grown under axenic conditions in Trypticase-yeast extract-iron-serum

medium (TYI-S-33) with 15% adult bovine serum (Sigma), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) (Gibco BRL), and 1× Diamond's vitamins (Biosource International) in 15-ml glass culture tubes at 36.5°C as previously described (21). *E. dispar* SAW760 was isolated from an adult human male in England in 1979 (http://www.atcc.org/). RNA from *E. dispar* SAW760 and *E. histolytica* HM-1:IMSS grown axenically in liver digest-yeast extract-iron-serum medium (LYI-S-2) were kindly provided by C. Graham Clark (15). The strains were proven to be *E. histolytica* HM-1:IMSS and *E. histolytica* Rahman by PCR analysis of the rRNA episome and the serine-rich *Entamoeba histolytica* protein gene by using previously described methods (16, 52).

RNA isolation and Northern blot analysis. Amebae were harvested, and total RNA was extracted using TRIzol reagent (Invitrogen) and cleaned using the RNeasy cleanup kit (QIAGEN). Northern blot tests were performed using standard protocols (37). Briefly, 10 to 20 μg of total parasite RNA was separated by denaturing 1.2% agarose gel electrophoresis, transferred to membrane filters, and hybridized with radioactive probes using the ExpressHyb (Clontech, Palo Alto, CA) hybridization buffer. Probes were amplified by PCR from the appropriate clone or genomic DNA using M13F, M13R, or gene-specific primers, sequence verified, and labeled with [α-^{32}P]dATP with the Random Primed DNA labeling kit (Roche, Germany). Primers used in the study are shown in Table 1. Blots were exposed to film, subjected to autoradiography, scanned, and prepared for publication using Adobe Photoshop (version 7; San Jose, CA). Blots were stripped using 0.5% sodium dodecyl sulfate and reused for subsequent hybridizations per the manufacturer's suggestions. For a loading control, *EhActin* (locus 8.m00351) was PCR amplified from *E. histolytica* HM-1:IMSS genomic DNA and labeled as described previously (50).

Microarray hybridizations and data analysis. An 11,328-clone genomic DNA microarray was generated from *E. histolytica* HM-1:IMSS as described previously (50). Information from The Institute for Genomic Research (TIGR) gene annotation was used to identify clones that contained only one potential open reading frame (ORF) (defined as ≥98% identity over ≥200 bp). A total of 2,801 clones were in this category, representing 2,110 unique genes. Eight micrograms of total parasite RNA was prepared for array hybridizations as previously described (37). All experiments used at least two separate RNA samples, with each RNA sample isolated on different days. Microarrays were analyzed using the ScanAlyze program (Michael Eisen; http://rana.lbl.gov/EisenSoftware.htm) to determine the fluorescent intensities, and the data were then stored on the Stanford Microarray database (http://genome-www5.stanford.edu//). Data normalization and quality were assessed as previously described (37). Three arrays each were hybridized with RNA from *E. histolytica* HM-1:IMSS grown in TYI-S-33, *E. histolytica* HM-1:IMSS grown in LYI-S-2, *E. histolytica* Rahman grown in TYI-S-33, and axenic *E. dispar* SAW760 grown in LYI-S-2. In order to identify amebic genes that were differentially expressed between the different species/strains, the software Significance Analysis of Microarrays (SAM) version 1.21 (53) was used according to recommended procedures (http://www-stat.stanford.edu/~tibs/SAM/) as described previously (37). Two-class unpaired sample analysis was used on $\log_2$-transformed data from *E. histolytica* HM-1:IMSS and either *E. histolytica* Rahman or *E. dispar* SAW760 using the *K* nearest-neighbor imputer. The user-defined delta value was assigned by maximizing the number of significant genes while maintaining a false-discovery rate of ≤5%. Additional filtering was performed to include only those genes that are likely to be expressed under trophozoite conditions.

PCR and sequence analysis. Five genetic loci that displayed decreased expression in *E. dispar* SAW760 and *E. histolytica* Rahman were sequenced and analyzed. Primers were designed based on the *E. histolytica* HM-1:IMSS sequence and used to generate PCR products from *E. histolytica* Rahman genomic DNA. The PCR products (or several cloned PCR products) were sequenced using an ABI PRISM 310 genetic analyzer (PE Applied Biosystems). The primers used are listed in Table 1. The melting temperature for the primers ranged from 56 to 58°C.

BLAST analysis against the *E. dispar* sequence database. Sequences of the genes of interest were downloaded from the GeneDB website (http://www.genedb.org/) and compared by BLASTN analysis to both the TIGR (http://www.tigr.org/tdb/e2k1/eha1/) and Sanger Institute (http://www.sanger.ac.uk/Projects/E_histolytica/) *E. dispar* databases (sequence data available as of March 2005), and the top hit for each locus was used in subsequent analyses. Since the current *E. dispar* sequence data are limited to twofold-genome coverage, we wanted to ascertain the likelihood of identifying orthologs in the *E. dispar* database. *E. dispar* genes encoding RabB (GenBank accession number AY882575), GEF2 (AY561277), Jacob (AF401985), peroxiredoxin (AB026184), cysteine synthase 2 (AB028632), cysteine synthase 1 (AB028631), pore forming protein (AF082529), GalNac lectin *hgl* (U73710.1), and GalNac lectin *lgl*1 (U85823.1) were analyzed by BLASTN analysis to see whether their full-length sequences could be identified in the current *E. dispar* databases. For these genes, a match in the *E. dispar* database could be identified with ≥90% identity over ≥50% of the locus. Thus, we used these criteria (≥90% identity over ≥50% of the locus size) as our cutoff to designate an *E. histolytica* gene as one that is highly conserved in *E. dispar*. For BLAST analyses involving *EhLINEs* and *EhSINEs*, we used the consensus sequence defined by Bakre et al. (5; A. Bakre and S. Bhattacharya, personal communication).

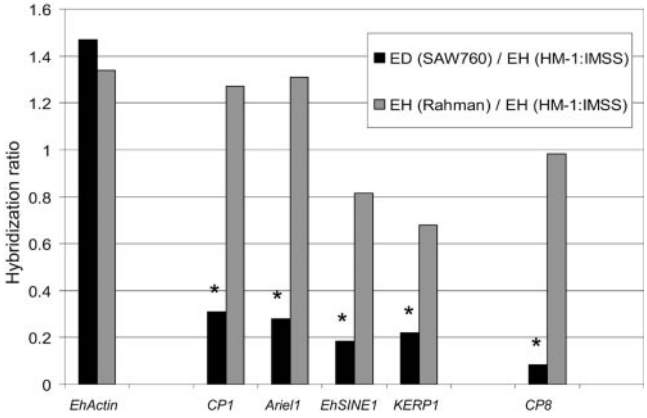


FIG. 1. The hybridization ratios of *E. dispar* SAW760 and *E. histolytica* Rahman to *E. histolytica* HM-1:IMSS are shown for *EhActin*, *CP1*, *Ariel1*, *EhSINE1*, *KERP1*, and *CP8*. Actin has similar expression levels in all *Entamoeba* species/strains studied. *CP1*, *Ariel1*, *EhSINE1*, and *KERP1*, all previously shown to be missing at a genomic level in *E. dispar* SAW760, showed significantly less expression in *E. dispar* SAW760 than in *E. histolytica* HM-1:IMSS. *CP8*, present in *E. dispar* SAW760 but known to have less expression, shows significantly less hybridization for *E. dispar* SAW760 than *E. histolytica* HM-1:IMSS. *E. histolytica* Rahman had expression levels equivalent to those of *E. histolytica* HM-1:IMSS for all genes shown. Genes with significantly decreased (*P* value of <0.05) expression levels compared to those of *E. histolytica* HM-1:IMSS are denoted with an asterisk. ED, *E. dispar*; EH, *E. histolytica*.

Statistical analysis. Statistical analyses were performed using a two-tailed Student's *t* test. *P* values of <0.05 were deemed significant.

RESULTS

***The E. histolytica* DNA microarray accurately detected differences in message abundance.** In order to find genes whose expression correlated with virulence, expression profiling of 2,110 amebic genes was performed for the virulent strain *E. histolytica* HM-1:IMSS, the nonvirulent strain *E. histolytica* Rahman, and the nonvirulent species *E. dispar* SAW760. We have previously shown that the microarray we utilized accurately detects message abundance and transcriptionally regulated genes (37). In order to ascertain whether we could accurately detect differences in message abundance from different species/strains, we examined genes previously published as being either absent or having decreased expression in *E. dispar* (Fig. 1). The ratio of hybridization intensities of *E. dispar* SAW760 and *E. histolytica* Rahman to *E. histolytica* HM-1:IMSS were calculated. As expected, genes previously shown to be missing or divergent (*CP1*, *Ariel1*, *KERP1*, and *EhSINE1*) or to have reduced expression (*CP8*) in *E. dispar* all had significantly less signal on the arrays in *E. dispar* SAW760 than in *E. histolytica* HM-1:IMSS (*P* value of <0.05) (12, 13, 49, 57). However, no such differences were observed for *E. histolytica* Rahman. Other genes with known differential expression between the species/strains (the amebapore and Lgl genes and

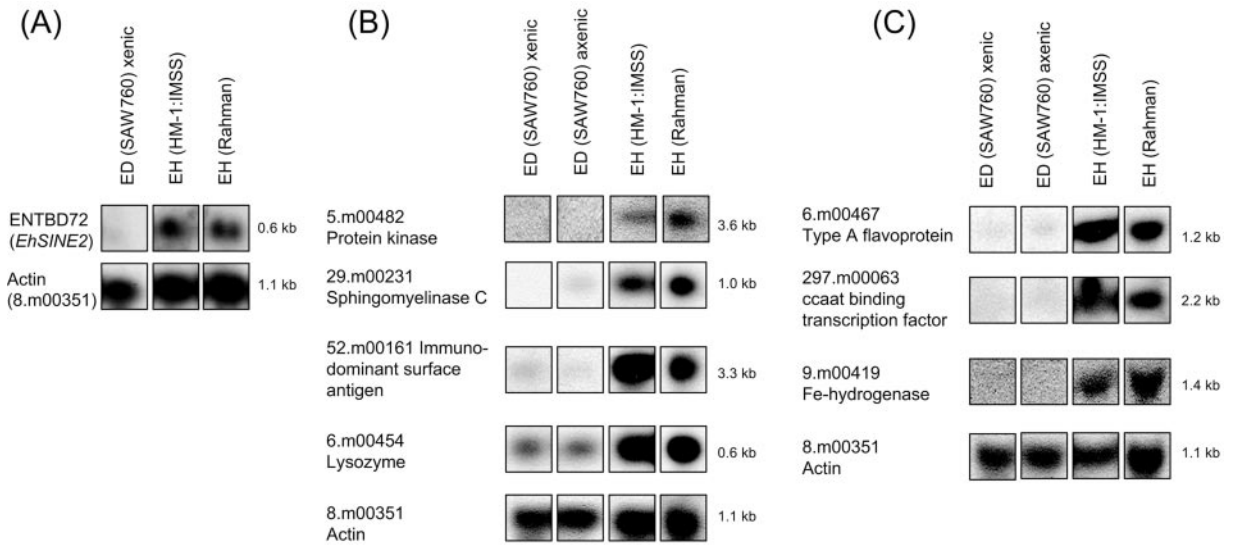


FIG. 2. Northern blot analysis for genes identified as differentially expressed between *Entamoeba* species. (A) Clone ENTBD72 was used to represent *EhSINE2*. (B) The genes 5.m00482, 29.m00231, 52.m00161, and 6.m00454 are shown. (C) The genes 6.m00467, 297.m00063, and 9.m00419 are shown. All of the genes in this figure exhibited significantly less expression in *E. dispar* (ED) SAW760 than in *E. histolytica* (EH) HM-1:IMSS by microarray analysis. Panels A, B, and C represent different blots. *EhActin*, which is equally expressed in all species/strains, is shown for each blot as a loading control.

CP5) were not analyzed, since the clones containing those also contained another potential ORF.

Our arrays were able to detect differential expression levels between *E. histolytica* and *E. dispar* both for genes that are conserved at the genomic level and for those that are genomically divergent. For genes with sequence conservation between *E. dispar* and *E. histolytica*, the lack of array hybridization indicates that the gene is not expressed. In contrast, for genomic loci that are significantly divergent in *E. dispar* compared to *E. histolytica*, the lack of array hybridization is due to sequence differences, and expression data cannot be ascertained. Thus, even if that gene was functionally expressed in *E. dispar*, the significantly divergent sequence would mean that the signal would not cross-hybridize on the array. We and others have previously analyzed a number of genes that are divergent in *E. dispar* at the nucleotide level to assess whether they would make functionally similar proteins (50, 58). For the majority of the genes analyzed, a significantly divergent nucleotide sequence resulted in premature stop codons and/or a highly divergent protein. Overall, this indicates that genes with significant sequence divergence in *E. dispar* are likely to be nonfunctional in comparison to their orthologs in *E. histolytica*.

We used *E. histolytica* HM-1:IMSS parasites from two different laboratories grown in two different medium preparations. We did find some differences in gene expression between the two samples, which were confirmed by Northern blot analyses (data not shown). These differences are most likely due to differences between the media and/or growth conditions; however, the functional relevance of these differences has yet to be determined. Since we were less interested in genes that are specific to growth conditions or media, for our purposes the two *E. histolytica* HM-1:IMSS samples were analyzed together as a set.

Northern blot analysis confirmed the array data of differentially expressed genes. A subset of genes identified as differentially expressed between *Entamoeba* strains and species was confirmed by Northern blot analysis (Fig. 2 and 3). Eight genetic loci (ENTBD72 or *EhSINE2*, 5.m00482, 29.m00231, 52.m00161, 6.m00454, 6.m00467, 297.m00063, and 9.m00419) which exhibited significantly decreased hybridization in only the *E. dispar* SAW760 versus *E. histolytica* HM-1:IMSS comparison were confirmed by Northern blot analysis (Fig. 2). Five genetic loci (194.m00115, 238.m00054, 29.m00210, 2.m00567, and ENTOB31 or *EhLINE1*) which exhibited significantly decreased hybridization in both nonvirulent species/strains were confirmed by Northern blot analysis (Fig. 3). All of the probes gave results that matched the array data (absent or markedly reduced signal in the appropriate species/strains) and the expected transcript sizes based on the TIGR gene annotation.

Genes with lower expression in *E. dispar* SAW760 than in *E. histolytica* HM-1:IMSS. In order to identify genes with lower expression in the nonvirulent *E. dispar* than in the virulent *E. histolytica* HM-1:IMSS, we used the Significance Analysis of Microarrays program (53). We found 415 unique genes expressed at significantly lower levels in *E. dispar* SAW760 than in *E. histolytica* HM-1:IMSS (Tables 2 and 3; see Table S1 in the supplemental material). Approximately two-thirds of the genes identified as having lower expression in *E. dispar* SAW760 did not have a highly conserved ortholog in the *E. dispar* database (Table 2; see Table S1 in the supplemental material). This subset contains genes already described as being divergent in *E. dispar* SAW760, including *CP1* (242.m00078 and 79.m00156), *Ariel1* (160.m00087), *AIG1* (565.m00023) (50), and *KERP1* (77.m00174). Other genes in this category included stress response genes such as those encoding a multidrug resistance protein (151.m00094), a DNA repair protein (151.m00093), and heat shock proteins (53.m00209, 92.m00150, and 136.m00105). Several members of the endoplasmic reticulum (ER)-associated translocon that were first identified in yeast secretory mutants (19), including *Sec24* (1.m00597

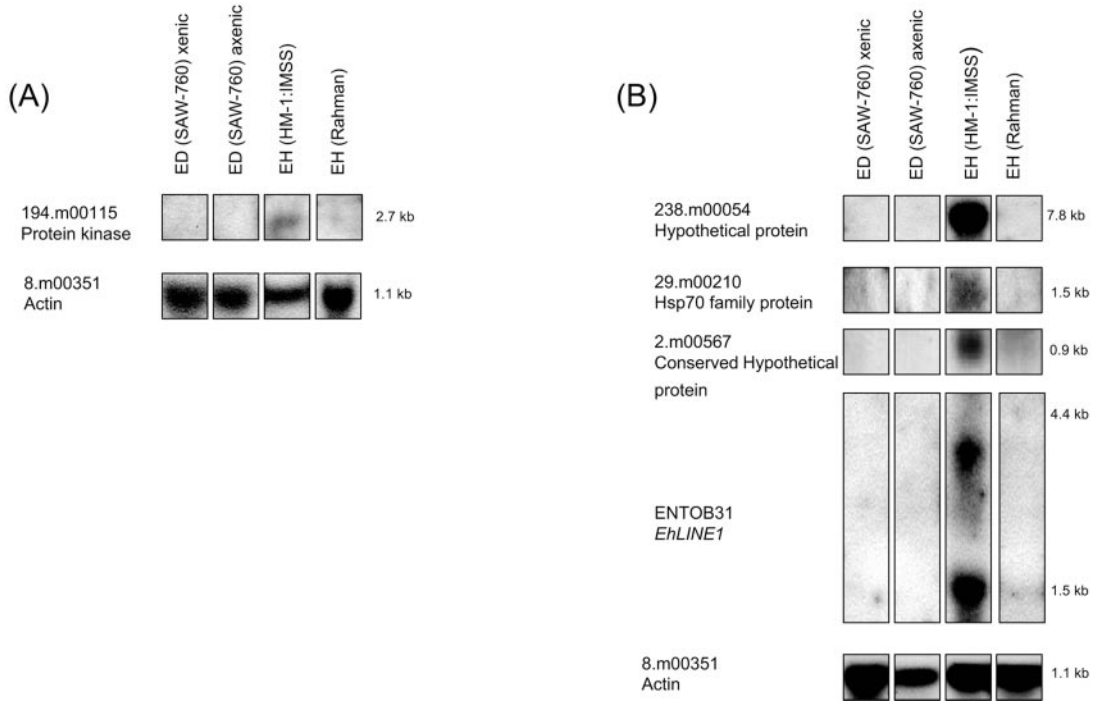


FIG. 3. Northern blot analysis for genes identified as differentially expressed in both nonvirulent species/strains. (A) The gene 194.m00115 is shown. (B) The genes 238.m00054, 29.m00210, 2.m00567, and *EhLINE1*, represented by the clone ENTOB31, are shown. All of the genes in this figure exhibited significantly less expression in both *E. dispar* (ED) SAW760 and *E. histolytica* (EH) Rahman than in *E. histolytica* HM-1:IMSS by microarray analysis. Panels A and B represent different blots. *EhActin*, which is equally expressed in all species/strains, is shown for each blot as a loading control.

and 178.m00100) and *Sec6* (7.m00480), had decreased expression in *E. dispar*.

We identified 146 genes that, although highly conserved in *E. dispar* SAW760, had significantly lower expression in *E. dispar* than in *E. histolytica* HM-1:IMSS (Table 3; see Table S1 in the supplemental material). Since the sequences of these genes in *E. histolytica* and *E. dispar* are highly similar, we can definitively ascribe the differences in the microarray signal to decreased transcript abundance. Importantly, these genes would not have been identified as being different between *E. histolytica* and *E. dispar* on the basis of comparative genomic hybridization or genome sequencing alone. This group includes genes potentially involved in virulence, including those encoding lysozyme (6.m00454), sphingomyelinase C (29.m00231), and several transcription regulators (251.m00088 and 297.m00063). Additionally, in this group were several putative stress response genes, including those encoding Fe hydrogenase (9.m00419), peroxiredoxin (176.m00112), and a type A flavoprotein (6.m00467). In other systems, these genes have been shown to be involved in a response to reactive oxygen species and may play similar roles in *Entamoeba* (9, 17, 24, 31). Additionally, a peptidyl-prolyl *cis-trans* isomerase gene (15.m00331) was identified. This gene (originally identified by accession number NP189160) has previously been shown to be induced in *E. histolytica* by exposure to high oxygen levels by differential-display PCR (1).

Genes with expression restricted to a virulent strain of *E. histolytica*. We found 29 genes (~1% of the total number examined) with significantly decreased expression in both the nonvirulent *E. histolytica* Rahman strain and *E. dispar* SAW760 (Table 4). Most genes in this category were genes for hypothetical proteins; however, genes encoding an endoplasmic reticulum-associated heat shock protein (29.m00210), a cell surface protein (80.m00165), and serine palmitoyltransferase (32.m00218) were also identified. The majority (~80%) of genes in this category did not have highly conserved homologues in the *E. dispar* SAW760 database, and none of the genes had been identified in previous studies relating to amebic virulence. Interestingly, five of the identified genes (147.m00110, 543.m00021, 864.m00008, 296.m00047, and 460.m00024) showed homology to the open reading frame found in the SSE58 repeat region identified as encoding a stress-dependent polymorphic charged antigen (Ehssp1) (48). We confirmed a lack of expression in *E. dispar* SAW760 and *E. histolytica* Rahman for one gene family member (460.m00024) by Northern blot analysis (data not shown; P. Vanchinathan, personal communication). One of the hypothetical proteins identified as having decreased expression in both the avirulent strain and the species has homology to a *Plasmodium* STARP antigen as well as a hemagglutinin from *Staphylococcus epidermidis*. The gene for this protein (238.m00054) belongs to a gene family consisting of three members (238.m00054, 21.m00228, and 312.m00036) which are each ~7.8 kb in size. Two other loci (16.m00292 and 126.m00120), which may represent truncated versions (1.7 kb and 1.2 kb, respectively) of the gene, also exist and share homology with the 3′ region of the gene family.

Genes with lower expression in the nonvirulent strain *E. histolytica* Rahman than in *E. histolytica* HM-1:IMSS. In a comparison of *E. histolytica* Rahman to *E. histolytica* HM-1:IMSS,

TABLE 2. Subset of genes with lower expression in *E. dispar* SAW760 than in *E. histolytica* HM-1:IMSS and with loci not highly conserved in the *E. dispar* SAW760 database[a]

TIGR locus[b]	TIGR gene product	Microarray hybridization ratio		Gene size (nt)	% Nucleotide identity[c]	% of locus found in database[d]
		E. dispar/ *E. histolytica* HM-1:IMSS	*E. histolytica* Rahman/ *E. histolytica* HM-1:IMSS			
320.m00035	Pseudogene, Gal/GalNAc lectin heavy subunit	0.22	1.16	3,870	88	54
151.m00094	Multidrug resistance protein, putative	0.38	1.18	4,398	97	39
242.m00078	Cysteine protease 1	0.31	1.27	948	85	60
79.m00156	Cysteine protease 1	0.46	1.18	1,005	85	57
191.m00117	Cysteine proteinase, putative	0.09	1.07	957	82	65
13.m00317	Sucrose transporter, putative	0.15	1.10	1,386	No hits	No hits
116.m00130	Ras GTPase activating protein, putative	0.35	0.73	3,551	96	12
52.m00161	**Immunodominant variable surface antigen**	**0.22**	**1.01**	**3,342**	**90**	**26**
11.m00326	tRNA intron endonuclease, putative	0.54	0.77	492	86	28
5.m00482	**Protein kinase, putative**	**0.31**	**4.52**	**3,594**	**88**	**34**
114.m00128	Rab family GTPase	0.21	0.93	667	95	6
310.m00070	BspA-like leucine-rich repeat protein, putative	0.23	0.80	1,197	89	67
129.m00157	DNA primase large subunit, putative	0.14	0.73	1,374	90	47
501.m00019	Cysteine protease 8	0.10	1.14	948	82	57
39.m00237	Cortexillin, putative	0.14	0.76	2,376	93	18
442.m00024	Receptor protein kinase, putative	0.36	1.70	6,617	96	28
151.m00093	DNA repair protein, putative	0.41	1.07	1,982	88	19
2.m00588	Protein kinase, putative	0.51	0.94	1,227	92	6
126.m00100	Rho GTPase activating protein, putative	0.10	0.75	1,242	93	46
565.m00023	AIG1 family protein, putative	0.07	0.57	1,095	86	47
7.m00480	Sec6 protein, putative	0.39	0.65	2,301	95	28
53.m00209	Heat shock protein 70, putative	0.44	0.82	1,898	88	62
16.m00343	Ubiquitin carboxyl-terminal hydrolase, putative	0.31	0.87	2,421	89	100
136.m00105	70-kDa heat shock protein, putative	0.37	0.83	1,727	85	26
77.m00174	Hypothetical protein (KERP1)	0.22	0.68	552	No hits	No hits
338.m00048	Leucyl-tRNA synthetase, putative	0.48	0.71	3,219	96	32
130.m00115	Importin beta subunit, putative	0.25	1.27	3,115	90	25
92.m00150	Heat shock protein 90, putative	0.17	0.58	2,157	98	37
77.m00153	Glycogen debranching enzyme, putative	0.32	1.13	4,332	94	19
178.m00100	Sec24 protein, putative	0.35	0.59	2,283	93	47
14.m00281	Pseudogene, beta-adaptin	0.24	0.90	2,592	98	30
57.m00152	CCR4/NOT complex transcription factor subunit 4	0.58	0.67	2,112	96	49
60.m00136	Conserved hypothetical protein	0.55	1.25	2,597	86	21
296.m00051	Rho family GTPase	0.35	0.86	746	97	41
1.m00597	SEC-24 protein, putative	0.51	0.95	1,944	94	19
309.m00046	Phospholipid-transporting P-type ATPase, putative	0.43	1.66	3,297	94	38
160.m00087	Surface antigen ariel1 related	0.28	1.31	648	No hits	No hits

[a] Based on <90% nucleotide identity and/or <50% of the locus.
[b] Genes confirmed by Northern blot analysis are shown in bold.
[c] Compared to *E. dispar* SAW760 ortholog.
[d] *E. dispar* SAW760 database.

we found only three genes that had uniquely lower expression levels in *E. histolytica* Rahman (Table 5). These included the genes encoding the oxidoreductase aldo/keto reductase family of proteins (248.m00073), ubiquitin ligase (195.m00092), and a Rap/Ran GTPase-activating protein (putative 20.m00318). In addition, there were a number of clones that had homology to the non-LTR retrotransposable elements *EhLINE*s that also had lower expression in *E. histolytica* Rahman.

A number of retrotransposons have decreased expression in the nonvirulent *Entamoeba* species/strains. The *E. histolytica* genome has a number of repetitive elements (5, 6, 38). These include short interspersed nuclear elements (SINEs) and long interspersed nuclear elements (LINEs) that represent non-LTR retrotransposons. Three SINE genes (*EhSINE1*, *EhSINE2*, and *EhSINE3*) and three LINE genes (*EhLINE1*, *EhLINE2*, and *EhLINE3*) have been identified, and each (with the exception of *EhSINE3*) is present in multiple copies in the genome. The consensus sequence for *EhLINEs* contains one or two ORFs: the protein encoded by ORF1 (1.5 kb) contains a putative coiled-coil domain, and the protein encoded by ORF2 (3 kb) contains endonuclease and reverse transcriptase domains (5, 6, 38). We used the consensus sequence for the conserved regions of each element to identify clones on our array that represent each of these elements (5). The 50 clones on the array with the greatest similarity to the consensus sequence for each LINE or SINE were used to determine the expression levels (Fig. 4). For each of the SINEs and LINEs, we had clones on the array with a high similarity to the consensus sequence (median E values, 9E-83 to E = 0), with the exception of *EhLINE2*, for which we could not find clones highly similar to the currently defined consensus sequences for ORF1 and ORF2 (median E values, E-18 and 3E-12). This is apparently due to the fact that the consensus sequence, as identified by Bakre, is significantly different from genomic copies, as we

TABLE 3. Subset of genes with lower expression in *E. dispar* SAW760 than in *E. histolytica* HM-1:IMSS and with loci with significant orthologs in the *E. dispar* SAW760 database[a]

TIGR locus[b]	TIGR gene product	Microarray hybridization ratio		Gene size (nt)	% Nucleotide identity[c]	% of locus found in database[d]
		E. dispar/ *E. histolytica* HM-1:IMSS	*E. histolytica* Rahman/ *E. histolytica* HM-1:IMSS			
20.m00330	CXXC-rich protein	0.25	0.82	3,379	92	76
6.m00454	**Lysozyme, putative**	**0.47**	**0.92**	**639**	**96**	**99**
9.m00419	**Fe hydrogenase, putative**	**0.39**	**1.42**	**1,407**	**91**	**65**
18.m00300	Protein kinase, putative	0.45	2.42	1,188	94	99
171.m00098	Ankyrin repeat protein, putative	0.58	1.34	828	93	100
836.m00014	ARP2/3 complex 21 kDa subunit, putative	0.46	0.79	586	93	100
110.m00129	Rho family GTPase	0.56	0.67	639	96	96
32.m00230	BspA-like leucine rich repeat protein, putative	0.54	1.16	1,278	93	99
344.m00046	Ser/Thr protein phosphatase, putative	0.25	0.63	1,551	93	100
283.m00063	DEAD/DEAH box helicase, putative	0.37	1.20	1,260	94	100
94.m00134	Glycogen synthase, putative	0.34	0.96	4,128	96	76
50.m00199	Sec61 protein, putative	0.43	0.81	246	96	86
30.m00257	Rab family GTPase	0.31	0.57	648	92	101
1.m00628	Protein disulfide isomerase, putative	0.32	1.33	990	92	91
251.m00088	Sir2 family transcriptional regulator, putative	0.48	0.63	1,079	93	68
297.m00063	**CCAAT-box-binding transcription factor, putative**	**0.40**	**1.07**	**2,157**	**94**	**100**
87.m00163	Potassium transporter, putative	0.35	1.17	2,202	94	65
76.m00156	Rab family GTPase	0.50	0.98	760	95	68
176.m00112	**Pseudogene, peroxiredoxin**	**0.42**	**0.68**	**696**	**94**	**91**
8.m00352	Phospholipid-transporting P-type ATPase, putative	0.28	0.62	4,124	93	66
95.m00149	Protein phosophatase 2C, putative	0.32	1.35	2,925	91	66
143.m00082	Protein kinase, putative	0.53	0.93	1,686	96	100
16.m00300	Gal/GalNAc lectin heavy subunit	0.24	0.97	3,861	90	54
103.m00174	Conserved hypothetical protein	0.24	1.26	2,586	91	57
67.m00102	Phosphatidylinositol 3-kinase, putative	0.45	0.73	3,330	95	63
95.m00133	Rab GTPase activating protein, putative	0.48	0.92	1,954	94	60
29.m00231	**Sphingomyelinase C, putative**	**0.17**	**0.51**	**975**	**95**	**100**
6.m00467	**Type A flavoprotein**	**0.23**	**0.87**	**1,221**	**92**	**99**
15.m00331	Peptidyl-prolyl *cis-trans* isomerase, putative	0.41	0.78	1,185	93	69

[a] Based on ≥90% nucleotide identity and/or ≥50% of the locus.
[b] Genes confirmed by Northern blot analysis are shown in bold.
[c] Compared to *E. dispar* SAW760 ortholog.
[d] *E. dispar* SAW760 database.

were unable to find many highly similar sequences even in the *E. histolytica* HM-1:IMSS genomic database (data not shown).

EhSINE1 has previously been shown to be divergent in *E. dispar*, and the array data confirmed those results (Fig. 1 and 4). Using a probe for *E. histolytica EhSINE2*, we determined that it had significantly decreased expression in *E. dispar* (Fig. 2A and 4). Sequence analysis against the *E. dispar* databases revealed that *EhSINE2* is missing or highly degenerate (75% identity over 54% of the locus) in *E. dispar*. Thus, the lower array hybridization and the absent signal on the Northern blot may be due to the inability of the *E. histolytica*-specific probe to cross-hybridize with the *E. dispar* gene. *EhSINE3*, originally identified as UEE1 in *E. dispar*, is present once in the *E. histolytica* genome (5, 51). Our arrays contained only one clone with similarity to *EhSINE3*, and it did not show significant hybridization for any of the species/strains tested.

Clones containing *EhLINE1* consistently gave lower signals on the microarrays in both nonvirulent species/strains than in *E. histolytica* HM-1:IMSS (Fig. 3B and 4). Using clone ENTOB31 (with homology to *EhLINE1*) in a Northern blot analysis, we identified the expected 1.5-kb and 3-kb bands, and confirmatory to the array data, both bands showed dramatically less, if any, hybridization in both *E. dispar* SAW760 and *E. histolytica* Rahman (Fig. 3B). Additionally, *EhLINE3* ORF2 (there is no ORF1 in *EhLINE3*) also displayed significantly lower expression in both *E. histolytica* Rahman and *E. dispar* SAW760 than in *E. histolytica* HM-1:IMSS. The expression levels of both ORFs of *EhLINE2* were lower in *E. dispar* SAW760 (although not statistically significant for ORF1) than in *E. histolytica* HM-1:IMSS. *EhLINE2* ORF1 and ORF2 were equally expressed in *E. histolytica* Rahman. Whether *EhLINE2* is unique among the LINE genes and is expressed in *E. histolytica* Rahman or whether these data are misleading due to the lack of representative consensus sequences for *EhLINE2* is not clear at present. Interestingly, both Northern blot and array data revealed that strains of *E. histolytica* HM-1:IMSS from different laboratories have various levels of expression of *EhLINE*s and *EhSINE*s (data not shown). Despite the variability, however, the expression levels for *EhLINE1* and *EhLINE3* for all *E. histolytica* HM-1:IMSS isolates were always higher than those of *E. histolytica* Rahman and *E. dispar* SAW760 (data not shown). Additionally, it has been postulated that some members of *EhLINE1* contain a single ORF instead of two ORFs, and our Northern blots confirmed this hypothesis (data not shown) (5, 54). The roles of these LINEs and SINEs in *E. histolytica* are not clear at present, although diverse roles,

TABLE 4. Genes identified as having lower expression in *E. dispar* SAW760 and *E. histolytica* Rahman than in *E. histolytica* HM-1:IMSS[a]

TIGR locus[b]	TIGR gene product	Microarray hybridization ratio		Gene size (nt)	% Nucleotide identity[c]	% of locus found in database[d]
		E. dispar/ *E. histolytica* HM-1:IMSS	*E. histolytica* Rahman/ *E. histolytica* HM-1:IMSS			
32.m00218	Serine palmitoyltransferase	0.37	0.39	2,649	94	62
2.m00567	**Conserved hypothetical protein**	**0.62**	**0.40**	**912**	**91**	**85**
343.m00074	26S proteasome regulatory subunit	0.52	0.49	1,071	94	68
269.m00084	Mitochondrial carrier protein	0.25	0.44	831	95	77
143.m00082	Protein kinase, putative	0.23	0.35	1,686	96	100
312.m00036	**Hypothetical protein**	**0.07**	**0.08**	**7,866**	**89**	**35**
238.m00054	**Hypothetical protein**	**0.10**	**0.09**	**7,815**	**89**	**35**
196.m00089	Conserved hypothetical protein	0.31	0.47	863	89	56
296.m00047	Hypothetical protein	0.17	0.31	1,092	87	99
7.m00396	Hypothetical protein	0.04	0.13	621	No hits	No hits
209.m00109	Hypothetical protein	0.30	0.31	288	81	56
225.m00057	Hypothetical protein	0.18	0.16	828	No hits	No hits
394.m00058	Hypothetical protein	0.08	0.15	843	82	19
11.m00354	Hypothetical protein	0.24	0.28	1,242	91	7
120.m00092	Hypothetical protein	0.36	0.29	630	88	25
112.m00118	Acyl-coenzyme A synthetase, putative	0.27	0.44	1947	95	48
612.m00020	Hypothetical protein	0.10	0.23	678	89	26
29.m00210	**hsp70 family protein**	**0.55**	**0.45**	**1,570**	**88**	**61**
209.m00108	Hypothetical protein	0.50	0.32	243	No hits	No hits
864.m00008	Conserved hypothetical protein	0.55	0.30	1,172	87	39
80.m00165	Hypothetical protein	0.10	0.30	2,988	89	16
194.m00115	**Protein kinase, putative**	**0.41**	**0.34**	**2,727**	**95**	**23**
27.m00257	Reverse transcriptase, putative	0.21	0.16	1,011	89	85
393.m00037	Hypothetical protein	0.30	0.36	786	No hits	No hits
147.m00110	Conserved hypothetical protein	0.37	0.27	1,152	89	42
543.m00021	Hypothetical protein	0.44	0.44	321	90	85
244.m00078	Hypothetical protein	0.06	0.12	612	No hits	No hits
711.m00021	Hypothetical protein	0.24	0.33	342	84	56
460.m00024	Conserved hypothetical protein	0.10	0.32	612	85	78

[a] The first five loci listed are highly conserved in *E. dispar* SAW760 (≥90% nucleotide identity over ≥50% of the locus).
[b] Genes confirmed by Northern blot analysis are shown in bold.
[c] Compared to *E. dispar* SAW760 ortholog.
[d] *E. dispar* SAW760 database.

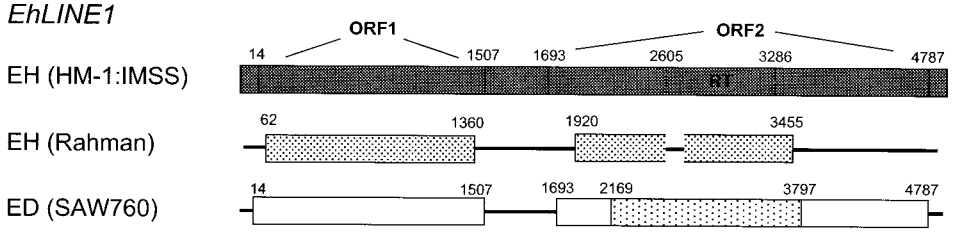
including effects on genome structure and organization, gene expression (30, 43), and response to stress (32, 35), have been described for other systems.

Genomic characterization of genes with decreased expression. Five genetic loci identified as having lower expression in both nonvirulent species/strains were sequenced to determine if the differences in expression levels could be attributed to genetic loss, genetic drift, or other phenomena (Fig. 4 and 5). The hypothetical gene 238.m00054 was sequenced in three segments, covering the majority of the gene. In *E. histolytica* Rahman, the sequences were 95 to 99% identical for the regions sequenced, with a relatively conserved protein structure. For *E. dispar* SAW760, the sequence identity was significantly less (81% over 73% of locus), with a number of predicted stop codons. A gene for an Hsp70 family protein (29.m00210) was also sequenced; again, the sequence was nearly identical (96% identity) in *E. histolytica* Rahman to that in *E. histolytica* HM-1:IMSS. The primers used for this gene incorporated the start and stop codons, so the sequence identity in this region could not be ascertained. In *E. dispar* SAW760, two contigs (98673 and 98651) showing homology to this gene can be found. One contig (98673) showed 85% identity over 100% of the locus; however, the start and stop codons were both mutated. The other contig (98651) showed 82% identity over 100% of the locus and had both start and stop codons conserved; however, it contained many internal stop codons. The gene for the hypothetical protein (2.m00567) was also nearly identical to that in *E. histolytica* Rahman (99% identity); again, the primers used incorporated the stop codon, and thus, conclusive sequence data for the very end of the gene could not be determined. The promoter region of this gene in *E. histolytica* Rahman was sequenced and was identical to the *E. histolytica*

TABLE 5. Genes with significantly lower expression in *E. histolytica* Rahman

TIGR locus	TIGR gene product	Microarray hybridization ratio		Gene size (nt)
		E. dispar/ *E. histolytica* HM-1:IMSS	*E. histolytica* Rahman/ *E. histolytica* HM-1:IMSS	
195.m00092	Ubiquitin-protein ligase e3 component	0.75	0.45	4,158
248.m00073	Oxidoreductase, aldo/keto reductase family	0.72	0.54	918
20.m00318	Rap/Ran GTPase activating protein, putative	1.28	0.40	2,012

(A) *EhLINE1*



(B)

Element	Copy number	Median E-value	EH (Rahman) Relative Expression	EH (Rahman) Sequence similarity	ED (SAW760) Relative Expression	ED (SAW760) Sequence similarity
EhLINE1	409					
ORF1		-102	0.26*	> 90%	0.17*	< 75%
ORF2		0	0.21*	> 90%	0.23*	~85%
EhLINE2	290					
ORF1		-18	0.60	N/A	0.29	< 65%
ORF2		3.00E-12	0.73	N/A	0.18*	< 64%
EhLINE3	52					
ORF2		0	0.26*	N/A	0.22*	< 71%
EhSINE1	272	9.00E-83	0.90	N/A	0.13*	< 63%
EhSINE2	117	-139	0.78	N/A	0.15*	< 75%
EhSINE3	1	0	N/A	N/A	N/A	~ 90%

FIG. 4. *EhLINE*s and *EhSINE*s have altered expression in the nonvirulent *Entamoeba* species/strains. (A) Diagrammatic representation of the sequence data for *EhLINE1*. *E. histolytica* Rahman data obtained by sequence analysis and *E. dispar* SAW760 obtained from BLASTN analysis versus genome sequence data. The numbers above the lines represent nucleotide positions, and the reverse transcriptase domain in ORF2 is noted. The shading is indicative of the nucleotide identity (▩, ≥95% identity; ▨, >90% nucleotide identity; ▤, >85% nucleotide identity; □, <80% nucleotide identity). No high-homology hit was found for ORF1 in *E. dispar* SAW760; however, a 1,600-bp region encompassing the reverse transcriptase domain showed 85% identity in *E. dispar* SAW760. (B) The average expression levels of *EhLINE* and *EhSINE* in *E. histolytica* Rahman and *E. dispar* SAW760 relative to those in *E. histolytica* HM-1:IMSS are shown. For each element, 50 clones with the highest similarity to the consensus sequence for each *EhLINE* or *EhSINE* were used. The copy number (adapted from Bakre et al. [5]), median BLASTN E-value for the 50 clones with highest homology, and genomic sequence similarity are also displayed. Expression levels that are significantly different from that of *E. histolytica* HM-1:IMSS are labeled by an asterisk and denote a P value of <0.05.

HM-1:IMSS sequence, with the exception of a single nucleotide polymorphism (A→G change at position −442 relative to the start codon) (7). This single nucleotide polymorphism was also seen in another virulent strain of *E. histolytica* (200:NIH), and so its presence does not correlate with an avirulence phenotype. In *E. dispar* SAW760, the gene 2.m00567 had very high homology over the first 775 bp (contig 98778 with 91% identity); however, the remaining 134 bp had very poor homology (64%) to any *E. dispar* sequences. Sequence data from the two ORFs in *EhLINE1* were also obtained (Fig. 4A). In *E. histolytica* Rahman, the sequence was highly conserved for both ORF1 and the reverse transcriptase domain of ORF2 (>90% identity). In *E. dispar* SAW760, ORF1 is missing or degenerate (74% identity over 24% of locus), whereas ORF2 is somewhat conserved (85% identity over 53% of locus). Our analysis revealed that the genes with decreased expression in *E. histolytica* Rahman were largely conserved at the nucleotide level; in contrast, a majority (~65%) of the genes with decreased expression in *E. dispar* SAW760 had a divergent sequence.

Comparison of genomic and expression differences identified by microarray analysis. We have previously performed comparative genomic hybridizations of *Entamoeba* species and identified a number of loci that are absent, significantly divergent, or significantly decreased in copy number in *E. dispar* SAW760 compared to those in *E. histolytica* HM-1:IMSS. Overall, 22 genes were absent or highly divergent in *E. dispar* SAW760; of these, 8 genes are likely to be expressed in *E. histolytica* HM-1:IMSS under the trophozoite conditions tested. These eight genes had a median expression level in *E. dispar* SAW760 that was nearly 10-fold lower than that in *E. histolytica* HM-1:IMSS. Additionally, 45 genes were significantly divergent in *E. dispar* SAW760; of these, 18 are likely to be expressed in *E. histolytica* HM-1:IMSS. Again, the median expression level for these genes was significantly lower (eightfold) in *E. dispar* SAW760 than in *E. histolytica* HM-1:IMSS. Comparisons between *E. histolytica* Rahman and *E. histolytica* HM-1:IMSS using genomic DNA hybridizations yielded far fewer significantly divergent genes (only five), and none of these genes are likely to be expressed at appreciable levels in *E. histolytica* HM-1:IMSS. Therefore, none of the genes with decreased expression in *E. histolytica* Rahman should be attributed to genomic differences.

DISCUSSION

We have compared the transcriptional profiles of the nonvirulent strain *Entamoeba histolytica* Rahman and the nonvirulent species *E. dispar* SAW760 to that of a virulent *E. histolytica* strain, HM-1:IMSS, using an *Entamoeba histolytica* DNA microarray. We confirmed that the arrays are effective for iden-

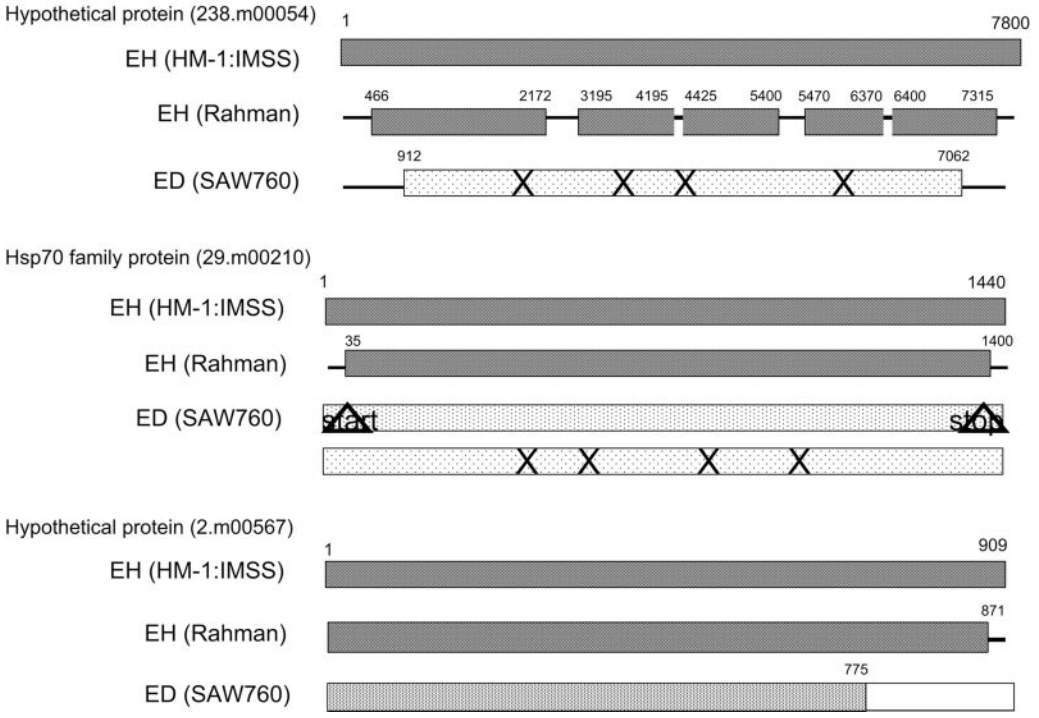


FIG. 5. Diagrammatic representation of the sequence data for the genes with differential expression levels in the nonvirulent species/strains. The shading is indicative of the nucleotide identity (▩, ≥95% identity; ▨, >90% nucleotide identity; ⊡, >85% nucleotide identity; ⊡, >80% nucleotide identity; □, <80% nucleotide identity). ×, stop codons; △, mutated stop or start codons.

tifying differential expression of genes that are missing or degenerate at a genomic level, as well as those genes that are conserved at a genomic level but expressed at differing levels. Additionally, we identified 415 genes with significantly decreased expression levels in *E. dispar* SAW760 and 32 genes with significantly decreased expression levels in *E. histolytica* Rahman compared to those in *E. histolytica* HM-1:IMSS. Importantly, we identified 29 genes with lower expression levels in both the nonvirulent strain *E. histolytica* Rahman and nonvirulent species *E. dispar* SAW760 than in the virulent *E. histolytica* HM-1:IMSS strain.

One interesting observation is that a number of genes that had decreased expression in one or both nonvirulent species/strains have roles in pathogenesis or stress response in other systems. Two hypothetical proteins with similarity to both a sporozoite threonine-asparagine-rich protein (STARP) antigen from *Plasmodium* and a hemagglutinin from *Staphylococcus epidermidis* (238.m00054 and 312.m00036) have decreased expression in both *E. dispar* SAW760 and *E. histolytica* Rahman. The STARP antigen from *Plasmodium* is located on the surface of sporozoites and is believed to be involved in pathogenesis, and antibodies against it inhibit invasion of hepatocytes (44). If the gene product functions as a streptococcal hemagglutinin, it may have a role in attachment or erythrophagocytosis. However, functional studies will have to be performed to confirm the roles of these genes in amebic pathogenesis. Serine palmitoyltransferase (locus 32.m00218), also with decreased expression in both nonvirulent *Entamoeba* species/strains, is a membrane-bound endoplasmic-reticulum-associated enzyme, which catalyzes the reaction of L-serine and palmitoyl coenzyme A to 3-ketodihydrosphingosine. This enzyme is essential in the production of ceramides and sphingolipids, which have diverse functions in endocytosis, stress response, adhesion, and trafficking of glycosylphosphatidylinositol-molecules (18, 26, 45). A decrease in the expression of this enzyme also may help explain the previously defined differences in lipophosphoglycans and proteophosphoglycans on the surface of virulent and nonvirulent *Entamoeba* organisms (40).

A number of genes involved in cytolysis were identified as having lower expression levels in *E. dispar* SAW760 than in *E. histolytica* HM-1:IMSS. These include the genes encoding sphingomyelinase C (29.m00231), a cytolytic factor involved in hemolysis in *Listeria* (25), *Staphylococcus* (39), and other pathogenic species, and a putative lysozyme (6.m00454) which has been shown to be involved in cell lysis in many systems, including *Entamoeba* (41).

Many genes classically involved in stress response, particularly the degradation of reactive oxygen species, had decreased expression in the nonvirulent *Entamoeba* species/strains. These included genes encoding a type A flavoprotein (6.m00467), which is potentially important in detoxifying nitric oxide and oxygen, and Fe hydrogenase (9.m00419), which has been shown in certain strains of bacteria to be involved in the response to high oxygen levels (17, 24). Additionally, a gene encoding peroxiredoxin (176.m00112), an important antioxidant involved in detoxifying peroxides, was also identified (9, 31). Choi et al. recently showed by enzyme-linked immunosorbent assay that *E. histolytica* contains as much as 50 times more peroxiredoxin than *E. dispar* (14). Peroxiredoxin is likely to be important in protection from the high oxygen content of the

host after invasion and from reactive oxygen species from host immune cells. During colonization of the colon, the parasites are in an anaerobic environment; however, upon tissue invasion, they are exposed to aerobic conditions. Thus, decreased expression of genes involved in stress response or degradation of reactive oxygen species may potentially provide insights into the ability of certain parasite species/strains to colonize but not cause invasive disease. Five genes with similarity to a gene for stress-induced polymorphic charged antigen (296.m00047, 864.m00008, 147.m00110, 543.m00021, and 460.m00024), as well as a gene previously shown to be induced upon exposure to high oxygen levels (15.m00331), were also identified as having decreased expression levels in nonvirulent *Entamoeba* species/strains.

A number of the LINEs and SINEs, representing non-long-terminal-repeat retrotransposons, had altered expression in the nonvirulent *Entamoeba* species/strains. *EhLINE1* and *EhLINE3* had significantly lower expression in both *E. histolytica* Rahman and *E. dispar* SAW760. Additionally, two SINEs are nonfunctional in *E. dispar* SAW760. Notably, while *E. histolytica* HM-1:IMSS isolates from different labs displayed various levels of *EhLINE* expression, virulent *E. histolytica* species/strains always exhibited higher expression levels of *EhLINE1* and *EhLINE3* than nonvirulent species/strains. Interestingly, in a recent paper, Pritham et al. reported that the two human parasites *Entamoeba histolytica* and *Entamoeba dispar* possessed many copies of retrotransposons but very few DNA transposons, while the opposite was true for the reptilian parasite *Entamoeba invadens* and the free-living *Entamoeba moshkovskii* (46). The authors hypothesized that evolutionary pressures from their human host may be responsible for the phenomenon. A number of diverse roles for similar elements have been described in other systems, including transcriptional regulation, genome organization, and stress response. The roles of these elements in regulating amebic transcription are not well characterized. Interestingly, these LINEs and SINEs are frequently found in close proximity to coding regions in *E. histolytica*, which could allow these elements to influence gene expression (5). A recent observation of fortuitous silencing of the amebapore A gene, by expression driven by an adjacent SINE (10), strongly suggests that in *E. histolytica*, similar to other systems (30, 35), transcriptional regulation is controlled by the LINEs and SINEs. The roles of these elements in affecting transcriptional regulation and genome modulation and their potential roles in the transition of *Entamoeba* from a gut commensal to an invasive pathogen deserve further investigation. Genome-wide comparisons of virulent and nonvirulent species/strains have yielded important results in many pathogen systems (8, 22, 29, 50, 59, 60). In a recent study comparing invasive and noninvasive *Staphylococcus epidermidis* strains, certain genes (including streptococcal hemagglutinin and many transposases) were found to be lacking in noninvasive species/strains (60).

We have performed the first large-scale transcriptional profiling of *E. histolytica* and *E. dispar* and have found differences in the transcriptional profiles of virulent and nonvirulent *Entamoeba* species/strains. A number of genes with roles in pathogenesis and stress response had decreased expression in the nonvirulent *Entamoeba* species/strains. Some of these differentially transcribed genes may represent potential virulence determinants and are important targets for genetic studies.

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