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ORIGINAL ARTICLE

Salmonella transcriptional signature in *Tetrahymena* phagosomes and role of acid tolerance in passage through the protist

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Salmonella enterica **Typhimurium remains undigested in the food vacuoles of the common protist, *Tetrahymena*. Contrary to its interaction with *Acanthamoeba* spp., *S.* Typhimurium is not cytotoxic to *Tetrahymena* and is egested as viable cells in its fecal pellets. Through microarray gene expression profiling we investigated the factors in *S.* Typhimurium that are involved in its resistance to digestion by *Tetrahymena*. The transcriptome of *S.* Typhimurium in *Tetrahymena* phagosomes showed that 989 and 1282 genes were altered in expression compared with that in water and in LB culture medium, respectively. A great proportion of the upregulated genes have a role in anaerobic metabolism and the use of alternate electron acceptors. Many genes required for survival and replication within macrophages and human epithelial cells also had increased expression in *Tetrahymena*, including *mgtC*, one of the most highly induced genes in all three cells types. A Δ*mgtC* mutant of *S.* Typhimurium did not show decreased viability in *Tetrahymena*, but paradoxically, was egested at a higher cell density than the wild type. The expression of *adiA* and *adiY*, which are involved in arginine-dependent acid resistance, also was increased in the protozoan phagosome. A Δ*adiAY* mutant had lower viability after passage through *Tetrahymena*, and a higher proportion of *S.* Typhimurium wild-type cells within pellets remained viable after exposure to pH 3.4 as compared with uningested cells. Our results provide evidence that acid resistance has a role in the resistance of *Salmonella* to digestion by *Tetrahymena* and that passage through the protist confers physiological advantages relevant to its contamination cycle.**
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Introduction

Grazing by phagocytic protozoa is a major factor in shaping bacterial populations in aquatic, soil, and anthropogenic ecosystems (Pace, 1988; Barker and Brown, 1994). Bacteria that resist grazing by protozoa may show increased environmental fitness (Hahn and Hofle, 2001). Grazing resistance may occur through pre-ingestional adaptations involving development of oversized cells, surface masking or microcolony formation, and through post-ingestional adaptations that include development of toxin release, digestional resistance and/or intracellular growth (Matz and Kjelleberg, 2005). Indeed, the intracellular pathogens *Mycobacterium avium*, *Chlamydia pneumoniae*, *Listeria monocytogenes* and *Legionella pneumophila* replicate in the digestive vacuoles (phagosomes) of *Acanthamoeba castellanii* (Ly and Muller, 1990; Cirillo *et al.*, 1997; Essig *et al.*, 1997; Abu Kwaik *et al.*, 1998) whereas *Salmonella enterica* serovars Dublin and Typhimurium multiply in *Acanthamoeba rhysodes* and *Acanthamoeba polyphaga* (Gaze *et al.*, 2003; Tezcan-Merdol *et al.*, 2004).

The resistance of certain intracellular pathogens to digestion by protozoa may coincidentally facilitate their ability to cause disease in their eukaryotic hosts. Phagocytosis in free-living protozoa shares basic mechanisms with that in human phagocytic cells, and conditions within the protozoan food vacuoles overlap with those in the macrophage phagosome (Lock *et al.*, 1987; Jacobs *et al.*, 2006; Cosson and Soldati, 2008). The interaction between pathogens and predatory amoebae has been implicated in the maintenance of bacterial virulence in various pathogens (Molmeret *et al.*, 2005). For example, *L. pneumophila* and *M. avium* both show increased infectivity of human macrophages after passage through *A. castellanii* (Cirillo *et al.*, 1994, 1997). On the basis of similarities in *L. pneumophila* mechanisms of infection of amoebae and mammalian

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cells, Molmeret *et al*. (2005) suggested that the intracellular lifestyle of human pathogens may have resulted from their adaptation to replication within free-living amoebae.

We have reported previously that *S.* Typhimurium can survive digestion by *Tetrahymena* at high rates and is released as viable cells in its fecal pellets in which it has enhanced survival compared with cells remaining undigested and free in suspension (Brandl *et al*., 2005). This is in contrast with *L. monocytogenes*, which is digested by the protist and detected infrequently in its fecal pellets (Brandl *et al*., 2005; Gourabathini *et al*., 2008). In addition, *S.* Typhimurium does not decrease the viability of *Tetrahymena* during its intravacuolar passage (Gourabathini *et al*., 2008). Therefore, its interaction appears to be different than with *Acanthamoeba* spp., which causes death of the protist (Gaze *et al*., 2003; Tezcan-Merdol *et al*., 2004; Feng *et al*., 2009). *S.* Typhimurium is an intracellular pathogen that has evolved specific mechanisms for its persistence and replication in eukaryotic cells. It is currently unclear whether the ability of the pathogen to resist digestion by *Tetrahymena* involves the same adaptations used to survive the phagocytic process of other eukaryotic cells.

In this study, we investigated the interaction of *S.* Typhimurium with *Tetrahymena* by microarray analysis of gene expression in *S.* Typhimurium cells residing in the *Tetrahymena* digestive vacuole. The global transcriptional response of this human pathogen to the *Tetrahymena* vacuolar environment indicates that it experiences conditions in the protozoan phagosome that overlap with those in macrophages and epithelial cells. In addition, cell viability assays showed that *S.* Typhimurium requires acid stress tolerance for survival to phagocytosis by *Tetrahymena*.

Materials and methods

Strains, plasmids and culture conditions

All strains and plasmids used in this study are listed in Table 1. *Tetrahymena* sp. MB125 was isolated from soil in California, belongs to an unknown species of this genus and has been described previously (Brandl *et al*., 2005). It was used in this study because of its release of numerous large fecal pellets containing viable *S. enterica* cells upon grazing on this pathogen (Figure 1).

S. enterica serovar Typhimurium SL1344 strains MB676 and MB681 are derivatives of SL1344 with a deletion in *mgtC* and *adiAY*, respectively. Mutants MB676 and MB681 were created using the lambda Red recombinase system (Datsenko and Wanner, 2000) to replace *mgtC* and *adiAY*, respectively, with the chloramphenicol acetyltransferase (CAT) cassette. All primers used for this procedure and others in this study are listed in Supplementary Table S1. The *mgtC* and *adiAY* deletions were confirmed by PCR. Complemented mutant strains MB692 and MB694 were constructed by transforming MB676

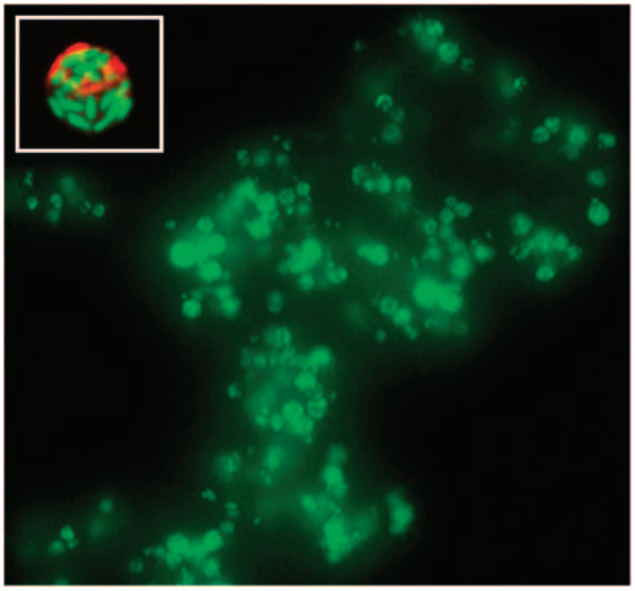

Figure 1 Epifluorescence micrograph of a large aggregate of fecal pellets released by *Tetrahymena* sp. upon feeding on *S.* Typhimurium and stained with SYTO 9 (Invitrogen). The inset shows a single optical scan through a Live/Dead BacLight-stained fecal pellet containing live (green) and dead (red) *S.* Typhimurium cells. The micrograph was captured with a Leica SP5 AOTF confocal microscope (Leica Microsystems, Wetzlar, Germany).

Table 1 Strains and plasmids used in this study

Strain name	*Description*	*Reference*
MB125	*Tetrahymena* spp. isolated from wet soil in California	Brandl *et al*., 2005
MB282	*S. enterica* serovar Typhimurium SL1344	Hoiseth and Stocker, 1981
MB676	SL1344-derived; *mgtC* ORF replaced with CAT cassette	This study
MB681	SL1344-derived; *adiA* and *adiY* ORFs replaced with CAT cassette	This study
MB692	MB676-derived; complemented with pMTB687	This study
MB694	MB681-derived; complemented with pMTB688	This study
Plasmid		
pKD3	Used in lambda Red-mediated recombination; contains CAT cassette	Datsenko and Wanner, 2000
pKD46	Lambda-Red recombinase expression plasmid	Datsenko and Wanner, 2000
pBBR1MCS-5	Broad-range cloning vector; $gent^R$	Kovach *et al*., 1995
pMTB687	pBBR1MCS-5 ligated with *mgtC* ORF	This study
pMTB688	pBBR1MCS-5 ligated with *adiA–adiY* ORFs	This study

and MB681 with the stably maintained plasmid pBBR1MCS-5 (Kovach *et al*., 1995) containing *mgtC* or *adiAY*, respectively. Briefly, *mgtC* or *adiAY* were PCR-cloned from strain SL1344 into pBBR1MCS-5 using primers *Kpn*I + *mgtC*_F/*Pst*I + *mgtC*_R or *Kpn*I + *adiA*_F/*Pst*I + *adiY*_R. Plasmid pBBR1MCS-5 containing either *mgtC* (pMTB687) or *adiAY* (pMTB688) was electroporated into mutant MB676 or MB681, respectively. Transformants were isolated on LB agar containing gentamycin (15 μg ml^{-1}) and X-gal. *S. enterica* SL1344 and derivatives were cultured in LB broth containing streptomycin (30 μg ml^{-1}), and chloramphenicol (20 μg ml^{-1}) and gentamycin (15 μg ml^{-1}), as appropriate.

Co-culture conditions

Tetrahymena MB125 was grown in 2/3 strength Plate Count Broth (2/3 PCB) (Becton Dickinson, Franklin Lakes, NJ, USA) for 2 days at 28 °C, with agitation at 50 r.p.m. It was then centrifuged at 200 × *g* for 2 min and repeatedly washed with half volumes of sterile deionized H_2O (dH_2O), incubating the cells for 15 min during each wash to minimize lysis by osmotic shock followed by centrifugation. The cell concentration was measured with a hemacytometer and was adjusted to 1×10^5 cells ml^{-1}. SL1344 cells were cultured to the mid-log phase of growth in LB broth at 28 °C and washed twice in sterile dH_2O. The OD_{600} of the suspension was adjusted to 0.2 and the suspension was combined with that of washed *Tetrahymena* cells at a bacteria:ciliate ratio of 1000:1 in sterile dH_2O. The mixed suspensions were incubated at 28 °C and 50 r.p.m. for 3 h until most bacteria were ingested by *Tetrahymena*. This was determined by staining the suspension with SYTO 9 (Invitrogen, Carlsbad, CA, USA) (10 μM final) for 30 min at 23 °C in the dark and visualized by epifluorescence microscopy with a Leica DMR microscope.

RNA extraction

Eight co-cultures of 50 ml were centrifuged at 800 × *g* for 2 min. The resulting pellet was enriched for *Tetrahymena* cells containing ingested SL1344 cells as most uningested free bacteria were not pelleted and remained in suspension. The supernatant was quickly removed and the pellet was resuspended in ice-cold lysis buffer (0.5% SDS, 19% ethanol and 1% phenol in H_2O), on the basis of the protocol by Eriksson *et al*. (2003). The suspension containing lysed *Tetrahymena* cells was centrifuged at 3200 × *g* for 5 min at 4 °C. The bacterial pellet was stored at −80 °C. RNA extraction was performed with the Promega SV Total RNA Isolation kit per the manufacturer's specifications, except that bacterial pellets were first treated with 50 μg ml^{-1} of lysozyme (Fisher Scientific, Pittsburgh, PA, USA). RNA from two different sources was used as control. The first control RNA was isolated from 50 ml of SL1344 cells incubated in sterile dH_2O ($OD_{600} = 0.2$) for 3 h, similar to the SL1344 cells mixed with *Tetrahymena*. The second control RNA was isolated from 20 ml of SL1344 cells grown to the mid-log phase of growth in LB broth. Both suspensions were centrifuged at 3200 × *g* for 5 min at 4 °C, followed by addition of lysis buffer, centrifugation and RNA extraction, as described above. Three biological replicates were used for each type of suspension. RNA integrity was assessed with the Agilent 2100 Bioanalyzer and only RNA of high quality was used for microarray hybridizations.

Microarray analysis

Custom, whole-genome *S. enterica* LT2 arrays were prepared at the Western Regional Research Center. Each array contained 4360 PCR-generated open-reading frames (including 104 from virulence plasmid pSLT) from *S. enterica* serovar Typhimurium strain LT2 printed onto Ultra-GAPS glass slides (Corning, Corning, NY, USA) with a Gene Machine Omnigrid Accent Arrayer (Genomic Solutions, Ann Arbor, MI, USA). Labeled nucleotides were prepared and hybridized to arrays as described previously (Kyle *et al*., 2010) on the basis of the method developed by Eriksson *et al*. (2003), with a few modifications. Briefly, 20 μg of RNA from co-cultures and controls was reverse-transcribed and Cy3-dCTP (GE Healthcare, Waukesha, WI, USA) incorporated into cDNA using the Fairplay III Microarray Labeling Kit (Stratagene, La Jolla, CA, USA). A 2-μg volume of SL1344 genomic DNA was labeled with Cy5-dCTP (GE Healthcare) using Klenow (New England Biolabs, Ipswich, MA, USA) and served as the reference signal on the arrays. Labeling efficiency was determined with a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). All cDNA solutions were adjusted to the same label concentration in the Pronto! cDNA hybridization solution (Corning) and were hybridized to the arrays overnight at 42 °C according to the manufacturer's protocol. For each experimental condition, cDNA was prepared from three replicate suspensions (biological replicates) and each cDNA was hybridized to three replicate arrays (technical replicates) at random among several slides. The slides were washed and then scanned with an Axon Genepix 4000b scanner and the Axon Genepix Pro 4.1 software (Molecular Devices, Sunnyvale, CA, USA).

To address spatial dye effects or any disparity in the amounts of spotted cDNA, data were normalized by setting the median log (Cy3/Cy5 signals) of all of the spots on the same array to zero. Biological replicates were then analyzed by analysis of variance ($P < 0.05$) with the Genespring version 7 software (Agilent Technologies, Santa Clara, CA, USA). From these data sets, only genes that were differentially regulated at least two-fold and for which the mean change in expression passed the Benjamini–Hochberg False Discovery Rate test ($P < 0.05$) were included in gene lists. Gene lists were generated for comparison of expression in SL1344 cells in *Tetrahymena* phagosomes with that in (1) H_2O and (2) LB broth, and for comparison of expression in H_2O versus that

in LB broth. All final gene lists are presented as Supplementary Table S2. Genes of particular interest were those that were differentially regulated in *S.* Typhimurium in *Tetrahymena* vacuoles versus both in H_2O and in LB broth.

Determination of cell viability and population density in fecal pellets

After co-incubation of SL1344 and *Tetrahymena* MB125 as described above, the suspension was filtered through Millipore black Isopore membranes (0.2 μm pore size, 25 mm diameter) and cell viability assessed as described previously (Brandl *et al.*, 2005). Briefly, filters were gently submerged in H_2O to allow *Tetrahymena* cells to swim away from the filter leaving behind free bacteria and pellets. The filters were removed from the water and drained. For assessment of bacterial acid resistance, the filters were submerged again in H_2O at pH 3.4 for 1 h. The filters were rinsed by submersion in H_2O five times and drained.

Viability staining of cells on the filters was performed with Live/Dead *Bac*Light (Invitrogen) by placing the filter into the stain solution (1.5 μl of each dye per ml) for 25 min at 24 °C in the dark. The filters were rinsed by submersion in H_2O and cells were viewed under an epifluorescence microscope with a fluorescein filter. The ratio of dead cells (red fluorescence) to viable cells (green fluorescence) was estimated in at least 50 fecal pellets in each of three replicate co-cultures. This ratio was estimated also for 10–30 cells external to each fecal pellet to assess the proportion of viable cells among cells remaining uningested and free in the mixed suspension. Figure 1 shows pellets produced by *Tetrahymena* and the presence of live and dead *S.* Typhimurium cells in the pellets, as shown by Live/Dead *Bac*Light stain.

The viability of the Δ*adiAY* and Δ*mgtC* mutants, of the complemented mutants and of the wild-type SL1344 in fecal pellets was assessed as described above. Cell density in pellets was estimated for the Δ*mgtC* mutant, the complemented mutant and the wild-type strain by counting the total cell number per pellet for at least 50 pellets. For strain comparisons, an aliquot of the bacterial inoculum was stained with Live/Dead *Bac*Light to ensure that viability did not differ among strains before preparation of the co-cultures with *Tetrahymena.* Cell viability and density data were analyzed statistically with a two-tailed *t*-test or a one-way analysis of variance followed by Tukey's multiple comparison test, with $P<0.05$. All tests were performed with the Prism software version 5.02 (GraphPad Software, La Jolla, CA, USA). Experiments were performed at least twice.

Results

Transcriptional profile of S. *Typhimurium in* Tetrahymena *phagosomes*

The gene expression profile of *S.* Typhimurium SL1344 residing in *Tetrahymena* food vacuoles was compared with that of cells incubated in H_2O or in LB broth. Because *S.* Typhimurium cells were harvested from the food vacuoles 3 h after the start of co-incubation of the two microorganisms in water, it is likely that a considerable percentage of pathogen cells spent a significant amount of time in water before falling prey to the protist. Therefore, we considered incubation of *S.* Typhimurium in water without the protist as the most appropriate control to identify genes differentially regulated in *Tetrahymena* phagosomes. However, because microarray studies of the *S.* Typhimurium transcriptome in other eukaryotic cells used LB broth as a control (Eriksson *et al.*, 2003; Hautefort *et al*, 2008), we included LB broth as an additional control to compare our results with previously published data. Our microarray analysis showed that expression of 989 and 1282 genes changed at least two-fold in the *Tetrahymena* phagosome compared with in water and in LB medium, respectively (Supplementary Table S2). Overall, more genes were downregulated (520 and 811) than upregulated (469 and 471) in the *Tetrahymena* phagosome versus in H_2O and in LB broth, respectively (Figure 2 and Supplementary Table S2). Notable exceptions, as illustrated by Categories of Orthologous Genes (COG) categories with H_2O as the control environment, include upregulated genes involved in energy production and conversion, nucleotide transport and metabolism, translation, cell motility and intracellular trafficking and secretion (Figure 2). Also of interest is the higher number of upregulated than downregulated genes that have a role in replication, recombination and repair, and in cell wall/membrane biogenesis. A close examination of the detailed gene lists showed that phagosome conditions induced numerous genes involved in anerobiosis. These included the hydrogenase operons *hyc*, *hyd* and *hyp*, and the reductase genes *dmsAB* and *frdBA* (Table 2).

We also observed increased expression of various virulence genes, several of which are associated with the *Salmonella*-containing vacuole of the macrophage (Eriksson *et al.*, 2003; Faucher *et al.*, 2006). These included the PhoP/PhoQ-regulated genes *pagC*, *pagK*, *envE*, *virK*, *mgtB* and *mgtC* (Table 2). Additional *Salmonella*-containing vacuole-associated virulence genes upregulated in the *Tetrahymena* phagosome were those encoding the *Salmonella* Pathogenicity Island-2 (SPI-2) type-III secretion system apparatus protein SsaV and the secreted effector proteins SifB and SopB (Faucher *et al.*, 2006) (Table 2).

Other genes with increased expression in *Tetrahymena* have a role in acid stress-response (*adiAY*) and in antibiotic or antimicrobial resistance (*marRAB*, *emrA* and *yddG*) (Table 2). *yddG* codes for a porin involved in the efflux of methyl viologen, which generates oxygen radicals (Santiviago *et al.*, 2002). In addition, a small number of upregulated genes are involved in osmotic stress, namely *kdpA*

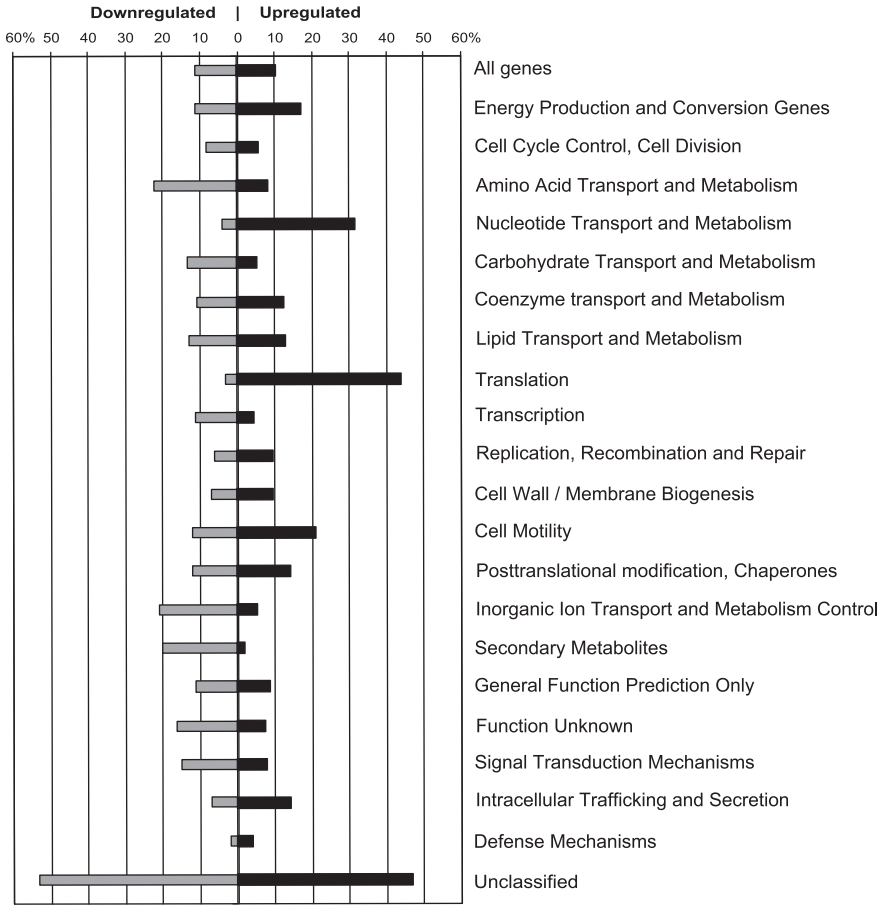

Figure 2 Differential expression of genes within Categories of Orthologous Genes (COG) in *S.* Typhimurium cells residing in *Tetrahymena* vacuoles compared with that in H_2O, as shown by microarray analysis. The bars represent the percentage of genes with a change in transcription of at least two-fold within a given category.

and *kpdB*, and *ompW*. The *kdp* operon is induced upon osmotic upshift through loss of turgor (Balaji *et al.*, 2005), whereas *ompW* encodes a porin involved in osmoregulation. However, there is also evidence that similar to YddG, OmpW exports methyl viologen and may work in conjunction with YddG (Gil *et al.*, 2007).

When examining expression changes in *S.* Typhimurium in *Tetrahymena* phagosomes with LB broth as a control environment, we identified additional genes that are upregulated also in macrophages and epithelial cells. Supplementary Table S3 lists a subset of 146 and 92 genes that are upregulated in *Tetrahymena* versus in LB and which were reported previously to also increase in expression after 8 h in J774-A.1 macrophages and after 2 h in HeLa cells, respectively (Supplementary Table S1 in reference Hautefort *et al.*, 2008). Commonalities in differentially regulated genes in the latter cells and in *Tetrahymena* were observed at other incubation times as well, but the number of overlapping upregulated genes was smaller. Of the genes that showed increased expression in both *Tetrahymena* and macrophages, 53% were upregulated also in HeLa cells (Supplementary Table S3). Common *Tetrahymena*- and macrophage-upregulated genes in comparison to LB included, but were not limited to, the following categories: SPI-2 (*ssrA*, *ssaB/G/H/I/L/V/R* and *sscA*); oxidative stress (*ycfR*, *trxC* and *ibpB*—in addition to *dps*, *yfiA* and *katG*, which are involved in oxidative stress response also but were not upregulated in macrophages); osmotic stress (*osmB*); SOS response (*uvrB* and *umuC*); multidrug resistance (*emrD*); phosphate starvation (*psiF*); Mg^{2+} transport (*mgtB*); anerobic metabolism (*hydN*, *hycA* and *fhlA*) and 49 hypothetical proteins (Supplementary Table S3). It is noteworthy that as observed in macrophages, the iron acquisition genes *entABCE* were highly downregulated in *Tetrahymena*. Along with downregulation of *sitABCD*, this suggests the presence of iron and manganese in the protozoan phagosome.

Passage through Tetrahymena *induces an acid stress response*

To determine whether the acidic conditions in *Tetrahymena* digestive vacuoles induce an adaptive tolerance to low pH in *S.* Typhimurium, we measured the viability of strain SL1344 cells in

Table 2 Select categories of *S.* Typhimurium genes upregulated at least two-fold in the *Tetrahymena* phagosome compared with that in water and in LB broth, as determined by microarray analysis

Locus	*Name*	*Fold change*		*Function*
		vs H_2O	*vs LB*	
Anaerobic energy generation/alternate electron acceptors				
STM0964	*dmsA*	17.87	14.40	Anaerobic DMSO reductase subunit-A
STM0965	*dmsB*	11.79	18.94	Anaerobic DMSO reductase subunit-B
STM1538	*hydA*	2.36	4.31	Putative Ni-Fe hydrogenase-1 large subunit
STM1539	*hydB*	3.16	6.22	Putative Ni-Fe hydrogenase-1 small subunit
STM2063	*phsC*	2.33	3.50	H_2S production from thiosulfate
STM2065	*phsA*	4.78	5.46	H_2S production from thiosulfate
STM2529		7.27	7.14	Putative anaerobic DMSO (dimethylsulfoxide) reductase
STM2530		8.69	8.51	Putative anaerobic DMSO reductase
STM2843	*hydN*	5.30	6.85	Formate dehydrogenase-H, [4Fe-4S] ferredoxin subunit
STM2845	*hycI*	5.07	5.80	Protease involved in processing the C-terminal end of HycE
STM2846	*hycH*	6.62	5.59	Processing of HycE (part of the formate–hydrogen–lyase (FHL) complex)
STM2848	*hycF*	5.66	7.87	Hydrogenase-3, putative quinone oxidoreductase
STM2850	*hycD*	3.42	5.64	Hydrogenase-3, membrane subunit (part of FHL complex)
STM2852	*hycB*	4.86	7.62	Hydrogenase-3, Fe-S subunit (part of FHL complex)
STM2853	*hycA*	3.78	6.13	Transcriptional repressor of *hyc* and *hyp* operons
STM2854	*hypA*	5.57		Functions as nickel donor for HycE of hydrogenlyase-3 in FHL complex
STM2855	*hypB*	8.84	2.79	Hydrogenase-3 accessory protein, assembly of metallocenter
STM2856	*hypC*	3.93	2.80	Putative hydrogenase expression/formation protein
STM2857	*hypD*	8.62	3.09	Putative hydrogenase expression/formation protein
STM2858	*hypE*	3.77	2.98	Putative hydrogenase expression/formation protein
STM2859	*fhlA*	2.69	2.55	Formate hydrogenlyase transcriptional activator for *fdhF*, *hyc* and *hyp* operons
STM3143	*hybG*	2.20	2.25	Hydrogenase-2 operon protein
STM3144	*hybF*	3.81	2.84	Putative hydrogenase-2 expression/formation protein
STM3145	*hybE*	3.28	2.64	Hydrogenase-2 operon protein
STM3146	*hybD*	4.70	3.52	Putative processing element for hydrogenase-2
STM3147	*hybC*	4.64	2.95	Hydrogenase-2, large subunit
STM3148	*hybB*	8.96	5.20	Putative cytochrome Ni/Fe component of hydrogenase-2
STM3149	*hybA*	9.29	5.92	Putative hydrogenase operon protein
STM3150	*hypO*	11.29	4.61	Hydrogenase-2, small subunit
STM4285	*fdhF*	17.52	14.14	Formate dehydrogenase
STM4342	*frdB*	2.54	3.18	Fumarate reductase, Fe-S subunit
STM4343	*frdA*	3.20		Fumarate reductase, flavoprotein subunit
Virulence and antimicrobial resistance genes				
Virulence plasmid				
pSLT012	*orf7*	4.07	3.43	Putative bacterial regulatory protein, *luxR* family
pSLT013	*pefI*	5.25	6.94	Transcriptional regulator of *pef* operon
pSTL096	*trbE*	2.29	2.16	Conjugative transfer
PhoP–PhoQ-activated genes				
STM0628	*pagP*	3.56		Lipid-A palmitoyl transferase required for resistance to antimicrobial peptides
STM1242	*envE*	2.41		Putative envelope protein
STM1246	*pagC*	2.73	2.16	Putative envelope protein required for survival in macrophages
STM1254		4.37	5.51	Putative outer membrane lipoprotein
STM1867	*pagK*	2.23	2.47	Putative virulence protein
STM2781	*virK*	6.67		Putative virulence protein
STM2782	*mig-14*	6.94		Required for virulence and resistance to antimicrobial peptides
STM3763	*mgtB*	3.32	4.46	Mg^{2+}-transporting ATPase
STM3764	*mgtC*	81.98	73.67	Putative ion homeostasis protein required for persistence in macrophages
Other virulence genes				
STM1091	*sopB*	2.02		Inositol polyphosphatase required for entry into intestinal epithelial cells
STM1414	*ssaV*	3.87	5.66	Type-III secretion system apparatus protein
STM1602	*sifB*	2.84	3.08	Secreted effector
Antimicrobial resistance				
STM1518	*marB*	2.33		Multiple antibiotic resistance protein
STM1519	*marA*	5.76		Transcriptional activator of defense systems, multiple antibiotic resistance protein
STM1520	*marR*	8.58	3.89	Transcriptional repressor of *mar* operon, multiple antibiotic resistance protein
STM1571	*yddG*	4.17	3.73	Required for efflux of methyl viologen, a quaternary ammonium compound
STM2814	*emrA*	5.06		Multidrug resistance protein

Table 2 (Continued)

Locus	*Name*	*Fold change*		*Function*
		vs H_2O	*vs LB*	
Osmotic stress				
STM0705	*kdpB*	5.15	9.31	P-type ATPase, high-affinity potassium transport system, B-chain
STM0706	*kdpA*	9.01	11.67	P-type ATPase, high-affinity potassium transport system, A-chain
STM1732	*ompW*	9.99		Porin involved in osmoregulation
Acid stress				
STM4295	*adiY*	4.12	6.81	Transcriptional activator of *adiA* (AraC/XylS family)
STM4296	*adiA*	6.20	5.71	Arginine decarboxylase, catabolic; inducible by acid

fecal pellets released by the protist and subsequently exposed to acidic conditions. The mean proportion of viable cells in pellets after acid treatment at pH 3.4 was 67.7% with a standard deviation (s.d.) of 1.47, and was significantly greater than that among cells remaining uningested and free in suspension (7.7%; s.d., 0.95) (*t*-test, $P<0.0001$). The frequency distribution of percentage viability among the two cell types clearly illustrates a shift toward greater viability of SL1344 in the pellets as compared with that of free cells upon exposure of both to acid stress (Figure 3). Although the percentage of viable cells in individual pellets was variable after exposure to pH 3.4, with the exception of one outlier pellet, it did not reduce below 31% (Figure 3a). By contrast, the percentage viability among free cells after acid treatment was lower than 31% in 90% of the areas sampled on the filter (Figure 3b).

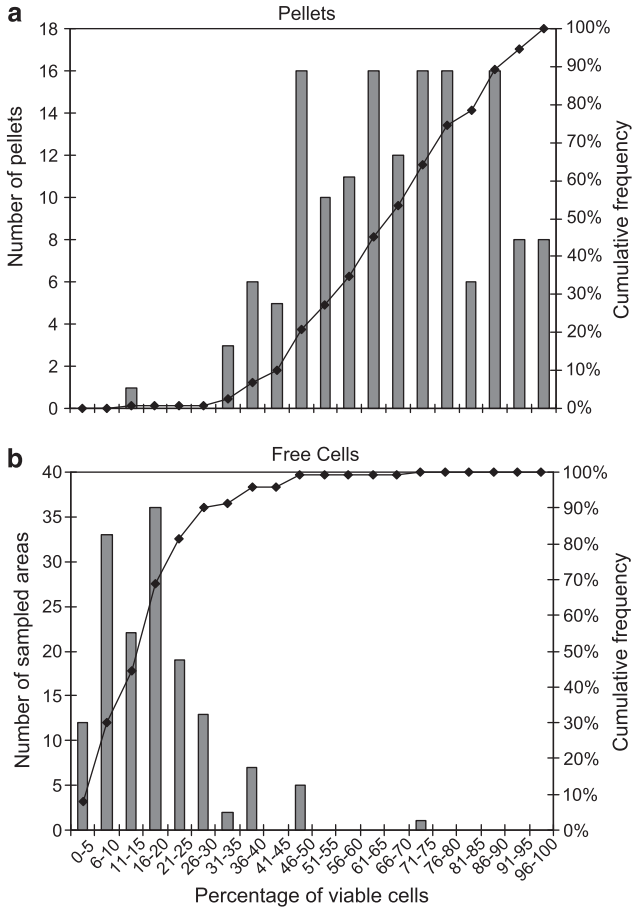

Figure 3 Frequency distribution of percent viable *S.* Typhimurium cells in individual *Tetrahymena* fecal pellets and in sampled areas of free uningested cells after exposure to pH 3.4. Cell viability was assessed with the Live/Dead BacLight stain. The bars represent the number of pellets (**a**) and the number of sampled areas (for free cells) (**b**), with a proportion of viable *S.* Typhimurium cells in a given range. The data on the solid line, plotted against the right *y*-axis, represent the cumulative frequency of observations in each distribution.

Arginine-dependent acid resistance in S. *Typhimurium within* Tetrahymena *phagosomes*

The viability of the SL1344 Δ*adiAY* mutant (MB681) and of its complemented strain (MB694) was compared to that of the wild-type strain in *Tetrahymena* fecal pellets. After a 3-h incubation of these strains with *Tetrahymena*, MB681 showed a mean percent viability in pellets of 66.03% (s.d., 3.53), versus 84.82% (s.d., 2.49) and 95.50% (s.d., 1.49) for MB694 and the wild-type strain, respectively. Although complete mortality of MB681 was not observed, MB681 had lower survival in the *Tetrahymena* phagosome than MB694 and the wild-type strain (Tukey's multiple comparison test, $P<0.05$). The percentage viable cells among free cells uningested by *Tetrahymena* did not differ significantly between the three strains (analysis of variance, $P=0.59$). The distribution of percentage viable MB681 cells in pellets was broad, ranging from 100% mortality in one pellet to over 96% viable cells in others (Figure 4a). By contrast, the proportion of viable cells of the complemented mutant (MB694) in the pellets did not reduce below 46% (with the exception of one outlier), and in half of the pellets the percentage of viable cells was greater than 85% (Figure 4b). The wild-type strain had the highest viability of all three strains, with 76% of the pellets containing at least 96% of viable cells (Figure 4c).

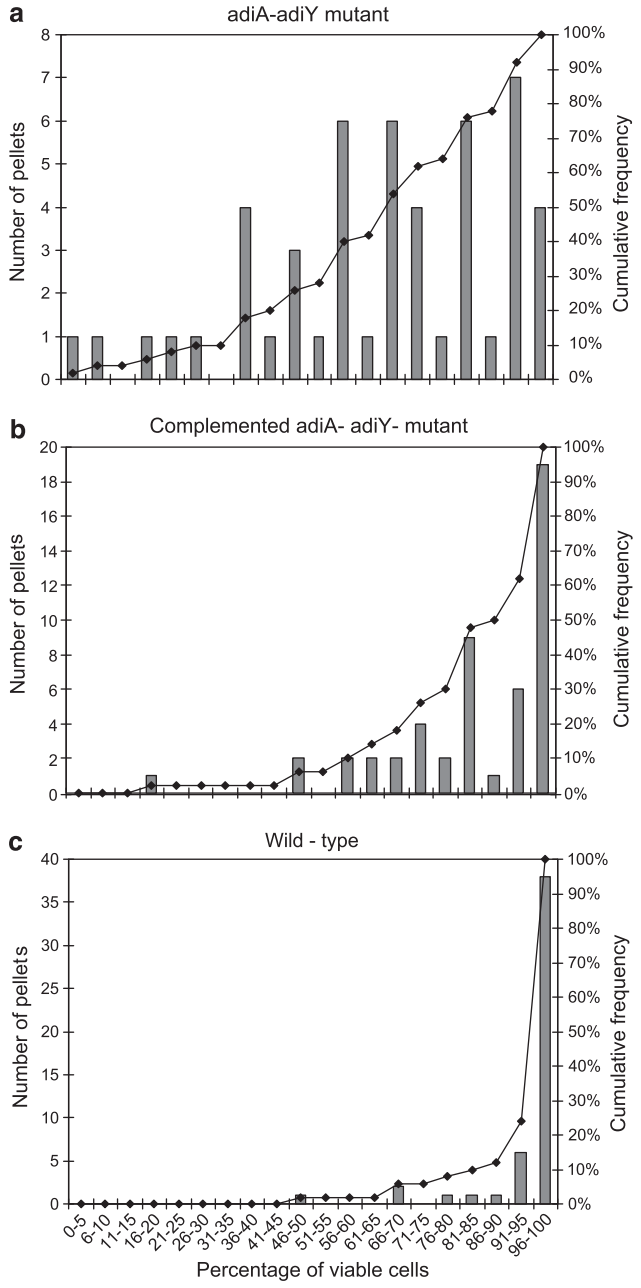

Figure 4 Frequency distribution of percent viable *S.* Typhimurium cells in individual fecal pellets released by *Tetrahymena* during co-culture of the two microorganisms, as assessed with the Live/Dead BacLight stain. The number of individual pellets with a proportion of viable *S.* Typhimurium cells in a given range (bars) and the cumulative frequency of pellets across the range of percent viable cells (solid line, right *y*-axis) are illustrated for the *S.* Typhimurium Δ*adiA* Δ*adiY* mutant (MB681) (**a**), the complemented mutant (MB694) (**b**) and the wild-type strain (**c**).

Role of MgtC in S. *Typhimurium cell density in* Tetrahymena *fecal pellets*

The macrophage virulence factor MgtC was investigated for its potential role in the digestion resistance of the pathogen. Whereas *mgtB* encodes an Mg^{2+} transporter, *mgtC* is involved in regulating membrane potential (Gunzel *et al.* 2006). With an increase in expression of 82- and 74-fold compared with that in water and LB broth, respectively, *mgtC* was one of the most highly induced genes in *Tetrahymena* (Table 2). The cell density and viability of the Δ*mgtC* mutant (MB676), its complemented strain (MB692) and the wild-type strain of SL1344 were compared in the fecal pellets produced by co-culture of each strain with *Tetrahymena*. Live/Dead staining of the cells in the fecal pellets showed that their viability did not differ between each strain (data not shown). However, the mean cell density per pellet for the mutant MB676 (24.3; s.d., 1.5) was significantly greater than that of the wild-type (17.8; s.d., 0.7) and the complemented mutant MB 692 (17.3; s.d., 0.7) (Tukey's multiple comparison test, $P<0.05$). Compared with the wild-type and the complemented mutant, the frequency distribution of the number of mutant MB676 cells in the pellets was shifted toward a higher cell density (Figure 5).

Discussion

As intracellular pathogens have evolved to resist or escape phagosomal conditions, grazing protozoa may represent an environmental reservoir for these pathogens. Understanding the interaction between pathogenic bacteria and protozoan grazers may further our understanding of the factors that allow persistence of pathogens in the environment. We have reported previously that the intracellular pathogen, *S. enterica*, can remain undigested in *Tetrahymena* and that most cells remain viable upon their release in its fecal pellets (Brandl *et al.*, 2005). The viability of cells within nascent fecal pellets is thus a direct result of survival within the *Tetrahymena* phagosome.

Bacteria that resist degradation in *Tetrahymena* phagosomes must counter harsh conditions such as acidification from proton-translocating ATPases, oxidative stress caused by reactive oxygen species, the presence of hydrolytic enzymes and reduced oxygen tension (Fok and Allen, 1975; Jacobs *et al.*, 2006). Our global transcriptional analysis of *S.* Typhimurium SL1344 cells in *Tetrahymena* phagosomes showed extensive upregulation of the *hyc*, *hyp*, *hyd* and *hyb* hydrogenase operons, which function in anerobiosis (Vignais and Colbeau, 2004), thus, indicating a metabolic shift to an anaerobic lifestyle. Hyc and Hyp are involved in fermentative H_2 evolution as part of the formate H_2 lyase (FHL) complex, whereas Hyd and Hyb are linked to respiratory fumarate reduction (Richard *et al.*, 1999; Zbell *et al.*, 2007; Zbell and Maier, 2009). Deletion of *hyd* and *hyb* in *S.* Typhimurium results in colonization deficiency in a mouse model (Maier *et al.*, 2004). Upregulation of the terminal reductases for fumurate and dimethylsulfoxide in the phagosome suggests that oxygen is highly limiting and alternate terminal electron acceptors may be preferred by *S.* Typhimurium. Overall, this

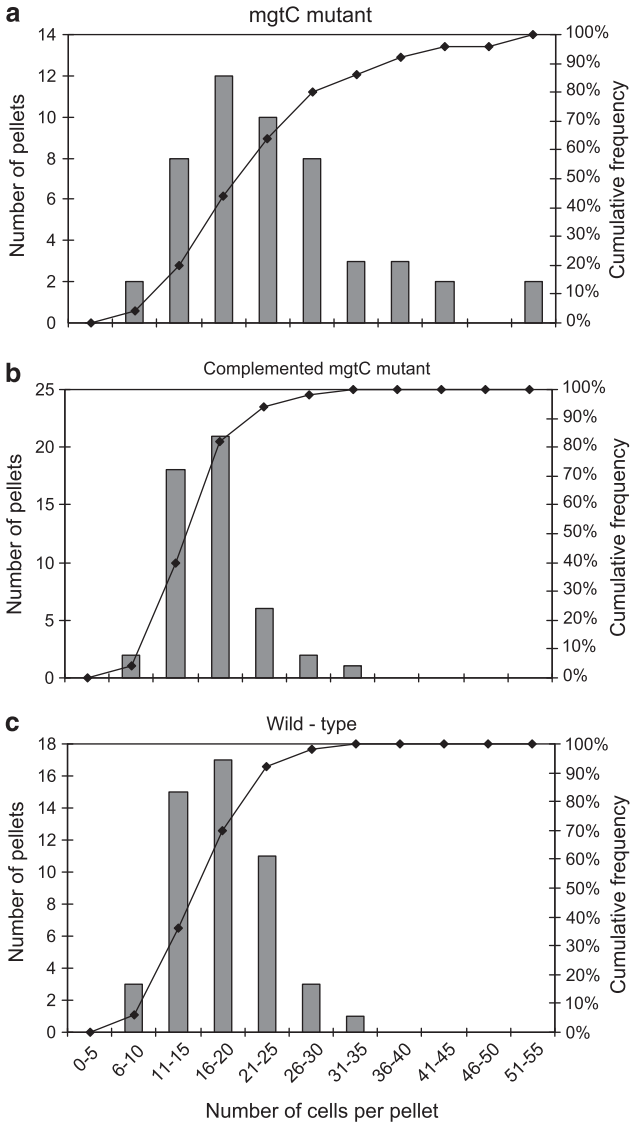

Figure 5 Frequency distribution of the number of *S.* Typhimurium cells in individual fecal pellets released by *Tetrahymena* during co-culture of the two microorganisms, as assessed with the Live/Dead BacLight stain. The number of individual pellets with an *S.* Typhimurium cell density in a given range (bars) and the cumulative frequency of pellets across the range of cell density (solid line, right *y*-axis) are illustrated for the *S.* Typhimurium Δ*mgtC* mutant (MB676) (**a**), the complemented mutant (MB692) (**b**) and the wild-type strain (**c**).

metabolic adaptation, along with increased translation and expression of genes that function in cell replication and wall/membrane biogenesis, may be indicative of the ability of *S.* Typhimurium to grow in the *Tetrahymena* phagosome. Further evidence of such a phenomenon still needs to be obtained.

Several *S.* Typhimurium genes that are upregulated in *Tetrahymena* phagosomes as compared with that in H_2O are also induced in macrophages. These included the antimicrobial resistance genes *marRAB* and *emrA*, of which increased expression was observed in the macrophage *Salmonella*-containing vacuole (Eriksson *et al.*, 2003). Also noteworthy is the higher transcription of several virulence genes in the *Tetrahymena* phagosomes, including those of the PhoP–PhoQ regulon (*pagP/C/K*, *envE*, *virK*, *mig-14* and *mgtBC*) and others belonging to SPI-1 (*sopB*) and SPI-2 (*ssaV*, *sifB*). The PhoP–PhoQ system is required for survival of *S.* Typhimurium and expression of SPI-2 genes within macrophages (Miller *et al.*, 1989; Fass and Groisman, 2009). PagP is involved in remodeling of the lipid-A domain of lipopolysaccharide (Bishop, 2005), whereas Mig-14 and VirK promote resistance to antimicrobial peptides produced in macrophages (Brodsky *et al.*, 2005). Low amounts of Ca^{2+} and Mg^{2+} (Groisman, 2001), acidic pH (Prost *et al.*, 2007) and antimicrobial peptides (Bader *et al.*, 2005) are the environmental cues for PhoPQ-mediated regulation. Thus, upregulation of *mgtB*, *adiAY* and a variety of genes involved in antimicrobial resistance correlates well with the activation of the PhoPQ regulon in the *Tetrahymena* phagosome.

Using LB culture medium as a common control environment, we compared the microarray data obtained in this study with that reported by Hautefort *et al.* (2008) regarding *S.* Typhimurium gene expression in macrophages and epithelial cells. This comparative analysis provided evidence that the pathogen experiences physicochemical conditions in the *Tetrahymena* phagosome that overlap with those encountered in macrophages and epithelial cells. Commonalities between the protist vacuoles and vacuoles of at least one of the two other cell types on the basis of transcriptional profiles include conditions of acid, oxidative and osmotic stress, low magnesium and phosphate concentrations, presence of antimicrobials and conditions inducing the SOS response and SPI-2. This overall response of *S.* Typhimurium to a variety of stresses in intravacuolar environments may underlie its ability to resist protozoan digestion. Of particular interest are the 49 hypothetical proteins that are part of the transcriptional signature of the pathogen in both the protist and macrophages, and which may represent proteins with unknown function that are crucial to the survival of *S.* Typhimurium in phagocytic cells.

Despite this overlap in transcriptional profile, the considerably larger sets of *S.* Typhimurium genes upregulated in macrophages and HeLa cells (Hautefort *et al.*, 2008) indicate that significant differences also exist. *S. enterica* does not have any detectable cytotoxic effect in *Tetrahymena* (Brandl *et al.*, 2005; Gourabathini *et al.*, 2008), in contrast to *Acanthamoeba* spp., which are killed by *S. enterica* and other pathogenic species (Abu Kwaik *et al.*, 1998; Gaze *et al.*, 2003; Tezcan-Merdol *et al.*, 2004; Matz *et al.*, 2008; Feng *et al.*, 2009). Hence, the role of SPI-2 and other virulence determinants in its resistance to digestion by *Tetrahymena* is less clear than their potential pathogenic function during interaction with *Acanthamoeba rhysodes*, in which SPI genes are also

induced (Feng *et al.*, 2009). The possibility remains that increased expression of virulence genes in *S.* Typhimurium in *Tetrahymena* is simply a response to environmental signals present also in host phagocytic cells, particularly low pH (Yu *et al.*, 2010). Further investigation of the response of *Tetrahymena* to this enteric pathogen may provide more insight into their interaction.

mgtC is one of the most highly upregulated *S.* Typhimurium genes in macrophages (Eriksson *et al.*, 2003) and in HeLa cells (Hautefort *et al.*, 2008), and is required for its long-term phagosomal survival (Alix and Blanc-Potard, 2007). Similarly, *mgtC* had the highest differential expression in our study, yet the Δ*mgtC* mutant was as viable as the wild-type strain in *Tetrahymena* pellets, suggesting that it was not impaired for survival in its phagosome. Possibly, the passage time of the pathogen before release at the cytoproct, which we estimated to be approximately 1 h (Brandl *et al.*, 2005), is not sufficiently long for this mutation to affect cell survival in *Tetrahymena*. Paradoxically, the Δ*mgtC* mutant had a greater cell density than the wild type in the fecal pellets. Complementation of the mutant caused lower cell density in the pellets, thus supporting a role for MgtC in this phenotype. Because growth in low-Mg^{2+} medium causes cell elongation and aggregation of MgtC-minus mutants (Rang *et al.*, 2007), *Tetrahymena* may have ingested cell aggregates during feeding on this mutant in H_2O, leading to a greater cell density in its food vacuoles. However, it is unclear if *Tetrahymena* would be able to feed on such aggregates because of their size.

In light of the increased expression in *Tetrahymena* phagosomes of two genes belonging to the arginine-dependent acid tolerance pathway, we investigated the effect of *S.* Typhimurium's passage through *Tetrahymena* on its subsequent acid resistance while in fecal pellets. *S. enterica* gains resistance to acid stress at pH 3–4 after adaptation to mild acidic conditions of pH 4.5–5.8 (Foster, 1995; Audia *et al.*, 2001; Audia and Foster, 2003). The digestive process in *Tetrahymena pyriformis* involves a decrease in vacuolar pH to 5.5–6.0 after 5 min, eventually reaching 3.5–4.0 after 1 h (Nilsson, 1977). The viability of *S.* Typhimurium cells exposed to pH 3.4 was enhanced in *Tetrahymena* fecal pellets compared with that of non-ingested cells in the same suspension. This suggested that the pathogen adapted to acidic conditions during its passage through *Tetrahymena* and thereby gained long-term protection from acidic stress in the external environment. The deletion of *adiAY*, which are part of the arginine decarboxylase system for extreme acid resistance in *S. enterica* and function under acidic pH in anaerobic environments (Kieboom and Abee, 2006; Alvarez-Ordonez *et al.*, 2010), significantly decreased the viability of the pathogen in *Tetrahymena*. It is noteworthy that *adiY* is upregulated also under the acidic pH of macrophages (Eriksson *et al.*, 2003). Viability of the Δ*adiAY* mutant in the pellets was partially restored by complementation, supporting a role for this acid stress response in the pathogen's resistance to digestion by *Tetrahymena* and providing further evidence for the presence of anaerobic conditions in *Tetrahymena* food vacuoles.

We have previously reported that ciliated protozoa isolated from bagged leafy vegetables sold at the marketplace can release viable *S. enterica* cells in pellets *in vitro* and that *Tetrahymena* has the ability to produce such pellets while grazing on *S. enterica* inoculated onto plants in the laboratory (Gourabathini *et al.*, 2008). As the postprandial pH in the stomach ranges from 2.5 to 4.9 (Simonian *et al.*, 2005), a gain in acid resistance of *S. enterica* by means of passage through *Tetrahymena* and egestion in its fecal pellets may enhance its survival in the human host. Hence, adaptation to low pH and to a range of other stresses, as shown by transcriptional profiling, may mediate the passage of this food-borne pathogen through *Tetrahymena* and thereby contribute to its contamination cycle.

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