

Escherichia coli O157:H7 Survives within Human Macrophages: Global Gene Expression Profile and Involvement of the Shiga Toxins[∇]

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***Escherichia coli* O157:H7 is an important food-borne pathogen that specifically binds to the follicle-associated epithelium in the intestine, which rapidly brings this bacterial pathogen in contact with underlying human macrophages. Very little information is available about the interaction between *E. coli* O157:H7 and human macrophages. We evaluated the uptake and survival of strain EDL933 during infection of human macrophages. Surprisingly, EDL933 survived and multiplied in human macrophages at 24 h postinfection. The global gene expression profile of this pathogen during macrophage infection was determined. Inside human macrophages, upregulation of *E. coli* O157:H7 genes carried on O islands (such as *pagC*, the genes for both of the Shiga toxins, and the two iron transport system operons *fit* and *chu*) was observed. Genes involved in acid resistance and in the SOS response were upregulated. However, genes of the locus of enterocyte effacement or genes involved in peroxide resistance were not differentially expressed. Many genes with putative or unknown functions were upregulated inside human macrophages and may be newly discovered virulence factors. As the Shiga toxin genes were upregulated in macrophages, survival and cytotoxicity assays were performed with isogenic Shiga toxin mutants. The initial uptake of Shiga toxin mutants was higher than that of the wild type; however, the survival rates were significantly lower at 24 h postinfection. Thus, Shiga toxins are implicated in the interaction between *E. coli* O157:H7 and human macrophages. Understanding the molecular mechanisms used by *E. coli* to survive within macrophages may help in the identification of targets for new therapeutic agents.**

Enterohemorrhagic *Escherichia coli* (EHEC) causes an acute gastroenteritis, bloody diarrhea, and hemorrhagic colitis. Severe complications develop in up to 10% of cases, including the hemolytic-uremic syndrome, which can be fatal (45, 56). EHEC strains share their genetic backbone with the laboratory strain K-12 and also contain 1.34 Mb of extra genetic material called O islands (OI) (46). In the EHEC strain EDL933 (O157:H7), 177 OI were identified; some of them harbor genes for important known virulence factors of EHEC, such as the locus of enterocyte effacement (LEE) and two different Shiga toxins, Stx1 and Stx2. Following the ingestion of contaminated food or water, EHEC enters the gastrointestinal tract, survives the acidic conditions of the stomach, and is released in the intestine. Adhesion of the bacteria to the intestinal wall is the first step in establishing infection. The LEE encodes a type III secretion system (T3SS) that injects effectors in host epithelial cells, creating an intimate binding, involving actin rearrangement and microvillus effacement, which results in the pathological lesions called attaching and effacing lesions (40, 52). In

the large intestine, EHEC specifically adheres to the follicle-associated epithelium of Peyer's patches (47). Binding at the follicle-associated epithelium results in the rapid contact of *E. coli* O157:H7 with underlying human macrophages; however, there is very little information available about the interaction between EHEC and these cells. The interaction between EHEC and murine macrophages was investigated, and it was shown that phagocytosis of EHEC by murine macrophages caused actin rearrangements surrounding the phagosome; EHEC was rapidly killed, and high levels of Shiga toxins were produced (53). However, to our knowledge, the interaction between EHEC and human macrophages has not been investigated. This interaction could be important in the global pathogenesis of human EHEC infections.

One way to understand host-pathogen interactions is to dissect the molecular mechanisms by studying gene expression. There are very few studies on the transcriptional response of bacteria during infection, as there are many factors that make difficult bacterial RNA isolation in infected host cells or tissues, such as the small amount of bacterial RNA in vivo, the short half-life of bacterial mRNA, and the contamination of bacterial mRNA with rRNA and host RNA. Moreover, bacterial mRNA purification is complicated by the fact that there are very few bacterial transcripts that are polyadenylated. To overcome these problems, the selective capture of transcribed

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sequences (SCOTS) was developed to identify bacterial genes expressed during infection (20). This technique has been successfully used with many pathogens, such as *Mycobacterium*, *Salmonella*, *Actinobacillus*, *Helicobacter*, and *Streptococcus* (4, 8, 12, 15, 21, 27). SCOTS was recently used in combination with a DNA microarray in order to provide an effective way to elucidate the global bacterial expression profile, or transcriptome, from infected host cells (13). Until now, no study has investigated the transcriptome of *E. coli* O157:H7 in contact with human cells. In this study, we characterized the interactions between *E. coli* O157:H7 (EDL933) and human macrophages, the uptake and survival rates of the bacteria, and the cytotoxicity to macrophages. We have obtained the global bacterial gene expression profile during infection of human macrophages and evaluated the role of Shiga toxins in phagocytosis.

MATERIALS AND METHODS

Bacterial strains. *E. coli* O157:H7 strain EDL933 (ATCC 700927) and its derivatives were grown routinely in Luria-Bertani (LB) medium at 37°C. Isogenic Stx mutants were described previously (19). The EDL933 green fluorescent protein (GFP)-positive strain was obtained by electroporation of a high-copy plasmid constitutively expressing GFP (pKen2) (7). When necessary, kanamycin or ampicillin was added to the medium at 50 µg/ml.

Cell culture and infection. The human monocytic cell line THP-1 (ATCC TIB-202) was maintained and infected as described previously (8). A stock culture of these cells was maintained as monocyte-like, nonadherent cells at 37°C in an atmosphere containing 5% CO₂. For macrophage infection, cells were seeded at 5 × 10⁵ cells per well in 24-well tissue culture dishes and were differentiated by addition of 10⁻⁷ M phorbol 12-myristate 13-acetate for 48 h, and then bacteria were added to the cell monolayer at a multiplicity of infection of 10. The plate was centrifuged briefly to synchronize the infection and incubated for 20 min (0 h), and then the cells were washed and fresh medium containing 100 µg/ml of gentamicin was added to kill extracellular bacteria. After 2 h, the medium was discarded, the cells were washed, and the medium was changed to include 12 µg/ml of gentamicin. The infected monolayers were either lysed from the tissue culture dishes by addition of 0.1% sodium deoxycholate in phosphate-buffered saline or further incubated. The number of surviving bacteria was determined by bacterial plate counts (CFU). The level of phagocytosis, including associated bacteria, was calculated as a percentage of the initial inoculum. The survival level corresponds to the percentage of intracellular bacteria at a given time postinfection compared to the percentage at 0 h. *Salmonella* strain ISP1820 and nonpathogenic *E. coli* strain DH5α were used as positive and negative controls, respectively. For flow cytometry analysis, infections were performed as described above using a GFP-positive strain, except for the lysis step. The infected samples were gently scraped and fixed with 4% formaldehyde. The data from 20,000 acquired events on a BD FACSort were analyzed with the CellQuest program.

Cytotoxicity assay. Human macrophages were seeded in 24-well plates and infected as described above. After 24 h of infection, the supernatant was collected and the amount of release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was evaluated with the Cytotox96 kit (Promega) according to the manufacturer's instructions. The percentage of cytotoxicity was expressed as (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100, in which the spontaneous release was the amount of LDH activity in the supernatant of uninfected cells and the maximum release was that when cells were lysed with the provided lysis buffer.

RNA extraction, SCOTS, and quantitative PCR (qPCR). At each time point (2, 8, and 24 h postinfection), macrophage monolayers were washed, and infected macrophages from three different wells were combined and lysed in TRIzol (Invitrogen) for total RNA isolation according to the manufacturer's instructions. Similarly, total RNA was obtained from bacteria grown in the cell culture medium. Five micrograms of each RNA sample were treated with DNase and converted to cDNA using primer RB1-RNA, containing a defined 5' end and a random nonamer in the 3' end (9, 20) (Table 1). The second-strand cDNA was synthesized using the Klenow fragment of DNA polymerase I (NEB) as described by Froussard (17). As there are numerous limitations and difficulties in isolating bacterial RNA during infection to monitor gene expression (26), we

TABLE 1. Primers

Technique and primer	Sequence
SCOTS	
RBI-RNA	CGGGATCCAGCTTCTCACGCANNNN NNNNN
RBI-PCR	CGGGATCCAGCTTCTCACGCA
qPCR	
gyrA-F	TAATGCTGTCTCCGCCGAAGGTA
gyrA-R	TCGCCTAAACGAATACCGCGAACA
ler-F	TAATGTGCCTGATGATGGACTCGC
ler-R	ACAGTGTCTCTTTAAGCCAGCGTG
lexA-F	ATGACGAAGTTACCGTTAAGCGCC
lexA-R	TCAATGGTGAAGCTCTGCTGACGA
modE-F	AAACGCTGACAACCAATTACCGGG
modE-R	AAGTCGCTTCATTTACCGGCATCT
recA-F	GAATGCAACTGCCTGGCTGAAAGA
recA-R	ACAGAGAAATCCGGCGTTGAGTTC
stx1A-F	TAATGCAATTCTGGGAAGCGTGCC
stx1A-R	ATTGTGCGTAATCCCACGGACTCT
stx1B-F	ATCTTCAGTCTCTTCTTCTCAGTGGC
stx1B-R	CCCTCCATTATGACAGGCATTAGT
pagC-F	AGGCCAGCTTATCGTCTGAATGA
pagC-R	ATCAGCACCCACACTGTTTCTCCA
soxR-F	AGAGTGGAAACAGCTTTCGTCCA
soxR-R	AGCCACAACCAATACATCCGTCCA

used SCOTS. Each SCOTS sample represents the cDNAs captured from a mixture of three independent infected macrophage samples. Enrichment of EHEC cDNA from the mixture of host cDNA and bacterial rRNA genes by SCOTS was obtained as described previously (9, 13, 20).

For qPCR analysis, RNA samples from three series of different infections (biological replicates) were extracted as described above, precipitated with 2.5 M LiCl (Ambion), washed with ice-cold 75% ethanol, and resuspended in diethyl pyrocarbonate-treated water. Rigorous DNase treatment was then performed to remove any trace of DNA (DNA-free kit; Ambion). cDNA was synthesized in triplicate using SuperScript II (Invitrogen) with random hexamers (Sigma), according to the manufacturer's instructions. For each sample, a reaction done without reverse transcriptase served as a no-template control. For each qPCR run, the calculated threshold cycle was normalized to the threshold cycle of an internal control gene amplified from the corresponding sample, and the fold change was calculated as previously described (13). The gene coding for the DNA gyrase subunit A (*gyrA*) was used as the reference gene for normalization. Primers are described in Table 1.

Microarray, labeling, hybridization, and data acquisition and analysis. Corning Ultra-Gap II slides (Corning, Acton, MA) were spotted with the MWG *E. coli* O157:H7 array set (Ocimum) at the National Microbiology Laboratory (Winnipeg, Canada). The MWG array consists of 6,167 50-mer oligonucleotides covering the genomes of *E. coli* K-12 (MG1655) and *E. coli* O157:H7 strains Sakai (RIMD 0509952) and EDL933 (ATCC 700927), and 5,419 oligonucleotides correspond to the EDL933 genome, including 83 genes from the plasmid pO157. Four hybridization replicates were used for each time point (2 h, 8 h, and 24 h postinfection) in the microarray experiments, and the coefficient of correlation was calculated between each replicate. As the interaction between EHEC and macrophages was never previously reported, the experimental design was to sample a variety of conditions rather than to make repeat observations, an approach previously shown to be more valuable to get an overall picture (11). SCOTS cDNA or genomic DNA (400 ng) was labeled with the Bioprime DNA labeling system kit (Invitrogen) according to the manufacturer's instructions. SCOTS cDNA was labeled with Cy5, and genomic DNA was labeled with Cy3 (Amersham). The templates were purified, and 3 µg of each labeled SCOTS cDNA and genomic DNA sample were mixed in warmed digoxigenin hybridization buffer (Roche). The templates were denatured and added to the microarray, which had been prehybridized with a digoxigenin-5% bovine serum albumin buffer. The microarray was incubated at 42°C for 16 h and was then washed three times in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate for 5 min each at 37°C and one last time in 0.1× SSC. Data acquisition and normalization were performed as described elsewhere (13, 49). Bacterial genomic DNA was used as the reference channel on each slide to

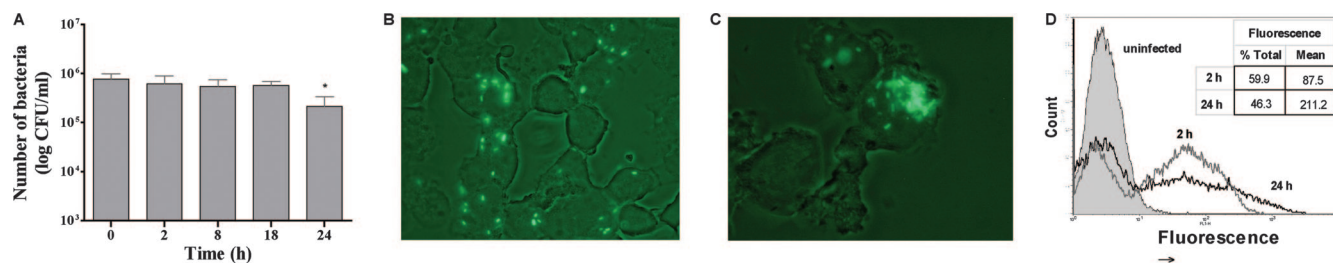


FIG. 1. Infection of THP-1 macrophages with EDL933. (A) Quantification of intracellular bacteria at different times postinfection, where 0 h corresponds to 20 min of phagocytosis. (B and C) Fluorescence microscopy of infected macrophages, with EDL933-GFP and human macrophages at 2 h (B) or 24 h (C) postinfection (magnification, $\times 1,000$). (D) Cytometry analysis of EDL933-GFP in contact with human macrophages at 2 h (gray) and 24 h (black) postinfection. All assays were conducted in duplicate and repeated independently at least three times. Results are expressed as means \pm standard deviations for the replicate experiments, and the Student *t* test was used for statistical analysis of the data.

allow comparison of each time point and different samples (54). Statistical analysis was performed with TIGR Multiexperiment Viewer software by using the analysis of variance (ANOVA) function with default parameter (<http://www.tmt4.org>). A *P* value of less than 0.0001 was considered statistically significant. Only the significant genes were considered for further studies, unless otherwise indicated. Moreover, for a gene to be considered upregulated or downregulated, the expression level of the gene needed to be two times superior or inferior, respectively, to the expression level in the control condition (bacteria grown in cell culture medium).

Microarray data accession number. The microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO accession number GSE9671) (<http://www.ncbi.nlm.nih.gov/projects/geo/>).

RESULTS

Uptake, survival, and multiplication of *E. coli* O157:H7 within human macrophages. The level of phagocytosis and survival of *E. coli* O157:H7 strain EDL933 in contact with human macrophages was determined (Fig. 1A). Twelve percent of the initial bacterial inoculum was associated with the macrophages after 20 min of interaction (0 h) (data not shown). The number of internalized bacteria remained relatively constant during the infection, at approximately 5.5×10^5 CFU/ml, and the level of survival decreased at 24 h postinfection but still remained high, at about 1.9×10^5 CFU/ml (Fig. 1A). EDL933 constitutively expressing GFP was used in order to visualize bacteria within macrophages by fluorescence microscopy. Initially, many macrophages were infected with a single bacterium or a few bacteria (Fig. 1B). However, at 24 h postinfection, only a few macrophages were infected, suggesting that many of the macrophages managed to clear the bacteria, but the infected macrophages contained many bacterial clusters, suggesting that the bacteria had replicated (Fig. 1C). This observation was quantified by cytometry in order to estimate the percentage of macrophages infected with GFP-expressing bacteria, as well as the mean level of fluorescence. A higher number of fluorescent macrophages (60%) was observed at the beginning of the infection than at 24 h postinfection (46%) (Fig. 1D). However, the mean fluorescence was twice as high at 24 h postinfection (Fig. 1D). This corroborates the fluorescence microscopy data and indicates that later in the infection, fewer macrophages were infected but infected macrophages contained more bacteria.

Transcriptome of EHEC within macrophages. To determine the global bacterial gene expression profile of *E. coli* O157:H7 in human macrophages, strain EDL933 was used to infect macrophages. Three intracellular postinfection time points

were chosen, i.e., 2 h, 8 h, and 24 h. Bacteria grown in RPMI, the culture medium for human macrophages, were used as an *in vitro* control. In order to obtain a complex mixture of bacterial transcripts, three rounds of SCOTS were performed on each of these samples, as described previously (9, 20). The bacterial cDNAs were hybridized to the *E. coli* microarray, which includes the entire genome of EDL933. The percentage of detected genes was approximately 78% for each condition tested, confirming that SCOTS allowed the recovery of a great variety of transcripts. The intracellular expression profiles were analyzed by comparison with the expression profile of bacteria grown in RPMI. A total of 1,221 genes of EDL933 were significantly differentially expressed within macrophages (ANOVA, $P < 0.0001$). For each intracellular time point, approximately 600 genes were upregulated (11%) and 400 genes downregulated (7%) (Fig. 2A). Interestingly, most of the differentially expressed genes showed a similar expression profile at all intracellular time points, as more than 60% (405) of upregulated genes and more than 50% (222) of downregulated were conserved at each of the intracellular time points (Fig. 2B). Similar detection profiles for many of the transcripts at three different time points validated the use of SCOTS cDNA to reveal the EHEC transcriptome from infected host cells. Because the transcription profile observed was comparable for numerous genes throughout the infection course and the largest number of genes were expressed at 24 h postinfection, our analysis focused mainly on this time point. *E. coli* O157:H7 differentially expressed genes were classified according to their orthologous groups (COG) in the TIGR annotation (www.cmr.tigr.org) (Fig. 2C). The profile of highly upregulated or downregulated genes was not found to be specific to an orthologous group. The microarray data were confirmed by qPCR, and a correlation of 0.9 was obtained between the expression ratios determined by microarrays versus qPCR (Fig. 3). Two different technologies applied to different biological samples were consistent, supporting the validity of SCOTS-microarray analysis as an effective way to study host-pathogen interaction.

Differential expression of OI. OI represent about 20% of the EDL933 genome, and 13% of the genes within OI were upregulated, whereas 6% were downregulated, during infection. The OI that harbor at least six genes and contain at least 15% of differentially expressed genes were selected (Fig. 4 and 5). OI-148, carrying the LEE, did not meet these criteria, as only

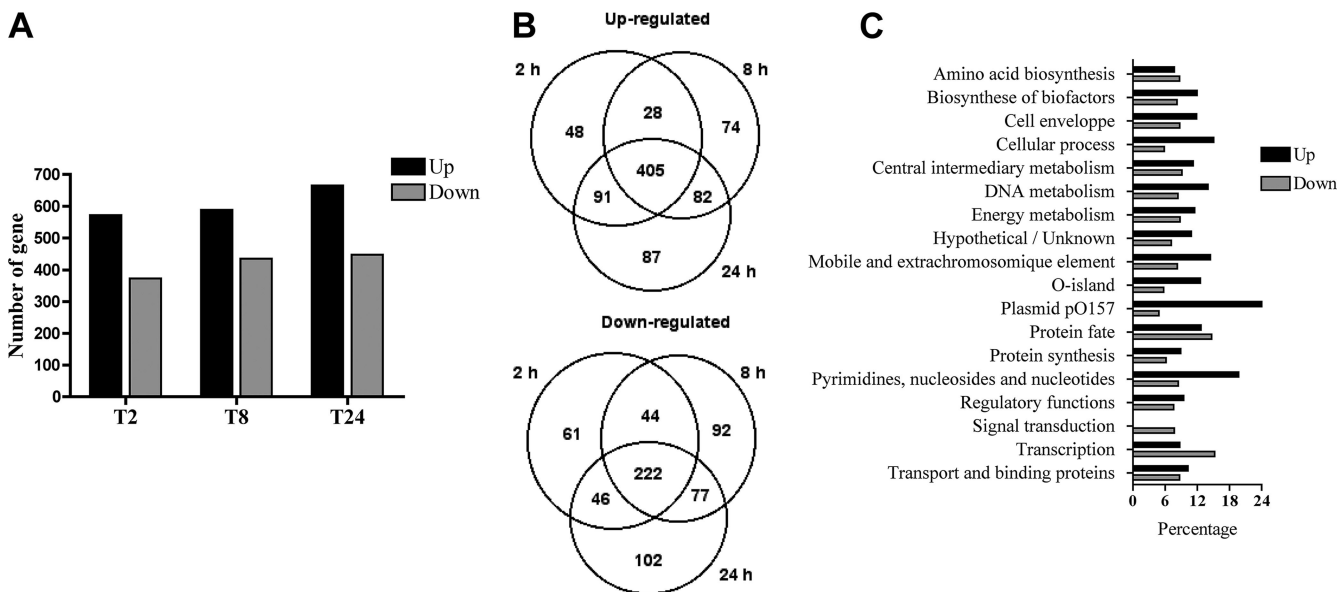


FIG. 2. Global analysis of differentially expressed genes. (A) Number of upregulated or downregulated genes at each intracellular time point of macrophage infection. (B) Venn diagram of the number of up- or downregulated genes at each intracellular time point. (C) Distribution of *E. coli* genes in functional categories (COG). Percentages of upregulated and downregulated genes for each functional class at 24 h postinfection are shown. Data are the means from four replicates.

4 genes were downregulated (*sepZ*, *espD*, *tir*, and Z5094) and 2 genes were upregulated (Z5095 and Z5088), of a total of 48 genes. In most of the selected OI, genes were upregulated (Fig. 4). OI-122, OI-123, and OI-140 contain many genes that were upregulated (Fig. 4 and 5). Among upregulated genes in OI-122, the virulence gene *pagC* was upregulated at all intracellular time points. The upregulated genes of OI-123 include the putative iron transport operon *fit* (44), and the upregulated genes of OI-140 include the iron uptake operon *chu* (39). In OI-93, harboring genes of phage CP-933V as well as Stx1, many genes were downregulated, with the exception of *stx1B*, which was upregulated. In OI-45, encoding genes of phage BP-933W and Stx2, genes were mostly upregulated, including *stx2B*. In OI-43, encoding the tellurite resistance- and adherence-conferring island (55), genes for tellurite resistance (*terA* and *terC*) were upregulated. Most of the other islands with differentially expressed genes harbor prophage genes or genes encoding hypothetical proteins with unknown functions.

Genes involved in stress responses and metal ion availability. As the macrophage environment is hostile, expression of

genes involved in the bacterial response to DNA damage (SOS response), to superoxide, and to peroxide and in acid resistance were analyzed. Genes related to DNA damage, such as *recA*, *recB*, *recC*, *recN*, *ruvC*, *dinF*, *dinD*, and *sulA* (14), as well as genes involved in superoxide stress, such as *soxRS*, *hmpA*, and *zwf* (48), were upregulated (Fig. 6). The peroxide resistance genes *oxyR*, *katP*, *katE*, and *katG* were not differentially expressed. The acid regulator genes *yhiF* and *gadE* were upregulated, as were many acid resistance genes such as *hdeA*, *hdeD*, *gadA*, *adiY*, *asr*, and *adiC*. Metal ion-related genes for transport and utilization are important, as metal ions are scarce in the host. The transcription of ion uptake systems *mntH*, *chu* (OI-140), *fit* (OI-123), *bfrA*, and *fieF* was upregulated in macrophages, whereas transcription of *feo*, *zupT*, *ent*, or *flu*, involved in metal ion transport, was not upregulated (Fig. 6).

Role of Shiga toxins in *E. coli* O157:H7 survival in human macrophages. As the Shiga toxins are very important virulence

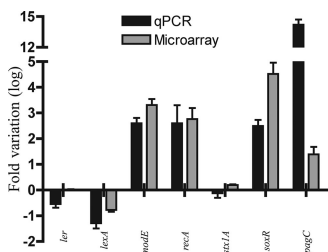


FIG. 3. Real-time qPCR and microarray results for a set of seven representative genes at 24 h postinfection compared to RPMI. Data are the means and standard deviations for RNA extracted from three different infections. See text for details.

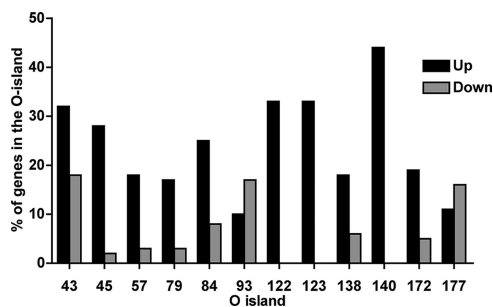


FIG. 4. Percentage of upregulated and downregulated genes for selected OI (OI containing more than six genes and showing a minimum of 15% of differentially expressed genes) at 24 h postinfection. Data are the means from four technical replicates.

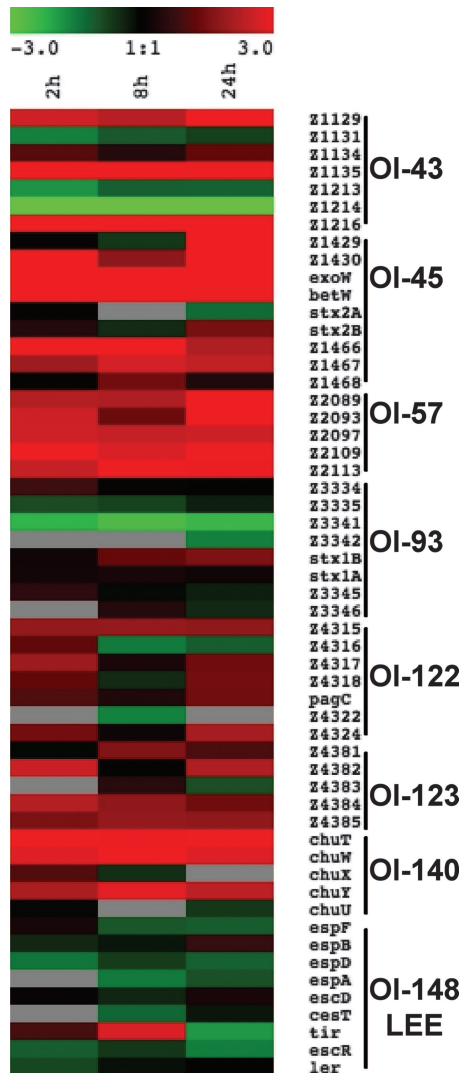


FIG. 5. Heat map of expression levels of predicted open reading frame selected from specific OI over time during macrophage infection. Expression values were determined from the ANOVA and are represented colorimetrically, with red representing upregulation (ratio of 3) and green representing downregulation (ratio of -3) on a log₂ scale. Data are the means from four technical replicates.

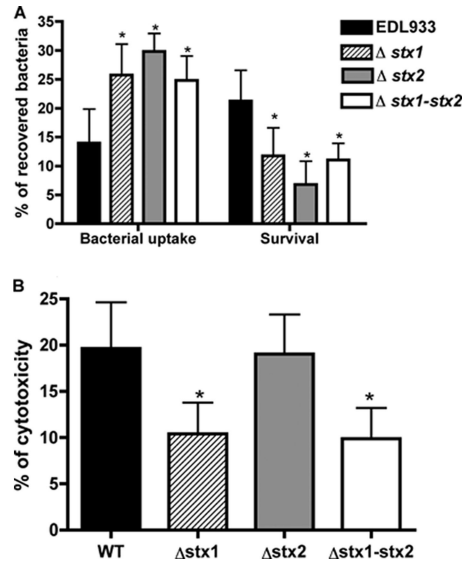


FIG. 7. Role of Stx during macrophages infection. (A) Bacterial uptake (percent compared to inoculum) and survival (percent compared to 0 h) of the *stx* mutants. (B) Cytotoxicity of strain EDL933 O157:H7 and isogenic mutants measured by LDH release after 24 h of infection. The figures show the means \pm standard deviations from at least three independent experiments. An asterisk indicates a significant difference compared to EDL933 ($P < 0.05$).

factors in EHEC and as its gene transcription was modulated during infection of macrophages, we investigated the impact of Shiga toxins on the phagocytosis, survival, and cytotoxicity level of *E. coli* O157:H7 in human macrophages. The absence of either or both of the Shiga toxins significantly increased the level of phagocytosis compared to the wild-type level (Fig. 7A). The survival rate of each *stx* mutant significantly decreased at 24 h postinfection compared to that of the wild type and was significantly more affected by the absence of Stx2 than by that of Stx1 (Fig. 7A). As the macrophages' viability could be affected by toxins, the cytotoxicity level of infected macrophages was evaluated. A twofold decrease of cytotoxicity with the *stx1* mutant or the *stx1 stx2* mutant was observed, whereas the cytotoxicity of the *stx2* mutant was similar to that of the wild-

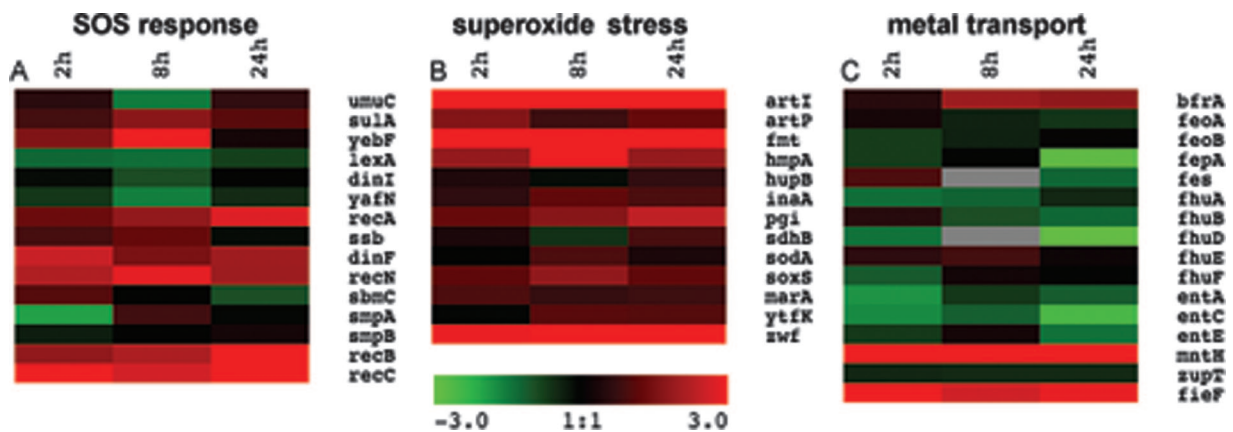


FIG. 6. Heat map of gene expression from the SOS response, oxidative stress, and metal transport gene classes. Data are the means from four technical replicates.

type strain (Fig. 7B). Shiga toxins appear to play an important role during interaction with macrophages: they reduced bacterial uptake by macrophages and promoted bacterial survival.

DISCUSSION

EHEC of serotype O157:H7 interacts with intestinal cells and causes microvillus effacement, which results in mild to bloody diarrhea. A strong inflammatory response is observed during EHEC infection, and cytokines produced by infected macrophages were previously shown to contribute to the severe inflammation associated with hemolytic-uremic syndrome (58). Thus, the interaction between EHEC and human macrophages could be important in the global pathogenesis of EHEC during human infections, but this interaction is very poorly characterized. In this study, we have examined the phagocytosis and survival rate of *E. coli* O157:H7 and of its isogenic Shiga toxins mutants during infection of human macrophages, as well as the global bacterial gene expression profile. We observed that *E. coli* O157:H7 can survive in human macrophages and was even able to replicate in this milieu. Effectively, even if many macrophages manage to clear the bacteria, some infected macrophages contain higher numbers of bacteria after 24 h of infection than at earlier time points, suggesting replication of the bacteria (Fig. 1). There was a 20% mortality of macrophage cells following 24 h of infection (Fig. 7), which may be due to lysis of cells that were killed by replicating bacteria or may be due to toxin release. Although not reported for *E. coli* O157:H7, it was previously shown that some extracellular bacterial pathogens are able to survive within professional phagocytes, such as uropathogenic *E. coli* (42), group A *Streptococcus* (37, 59, 60), *Staphylococcus aureus* (22), and *Enterococcus faecalis* (3, 18). However, only *E. faecalis* has been shown to multiply in macrophages at 24 h postinfection (18). We have shown that EHEC O157:H7 can resist macrophage killing and can also multiply, although the total number of viable bacteria decreased throughout the course of infection (Fig. 1). There may be two distinct populations of bacteria inside the macrophages, one that is able to survive and multiply and another that is killed. This hypothesis of a “double population” has been proposed for other pathogens infecting macrophages, such as *E. faecalis*, *Salmonella*, and *Listeria monocytogenes* (1, 10, 18). Our microarray analysis cannot distinguish between these populations. Yet, EHEC has not been detected in blood or organs, and intestinal infection does not appear to lead to a systemic infection; however, prolonged survival in macrophages may be important in the global course of infection and may significantly influence the intestinal inflammatory response.

During infection of human macrophages, *E. coli* O157:H7 faces a hostile environment and adapts by modifying its global transcriptome in order to survive and multiply. There was 22% of the genome of EDL933 that was differentially regulated intracellularly. Interestingly, up to one-third of the upregulated genes (210) encode proteins with unknown functions, and these could potentially be new virulence genes. OI are regions of the EDL933 genome that are present in *E. coli* O157 but not in *E. coli* K-12, and some OI are known to encode virulence determinants. Genes in some OI were upregulated in macrophages. OI-122 has been shown to be present in Stx-producing

E. coli strains associated with epidemic and/or serious disease, which suggests that genes of this region could be important for virulence (28). One of the OI-122 genes, *pagC*, was constitutively upregulated. A *pagC* mutant of *Citrobacter rodentium*, which causes attaching and effacing lesions in mice, had a significantly lower competitive index, indicating that this gene is important for the establishment of in vivo infection (61). In *Salmonella*, PagC is important for bacterial survival in macrophages (38) and was upregulated in macrophages (13), and PagC antibodies were detected in human blood (24). Thus, *pagC* might also be involved in survival of *E. coli* O157:H7 in human macrophages.

The iron uptake system operons *fit* and *chu*, harbored by OI-123 and OI-140, respectively, were upregulated. The *fit* (“ferrous or ferric iron transport”) system was previously found to be expressed during mouse infection with a human strain of *E. coli* O25:H causing septicemia (30). *Chu* is an iron uptake system based on heme and is sufficient for bacterial survival when the heme is the only available iron source (39, 62). In uropathogenic *E. coli*, the transcription of *chuW*, a member of the *Chu* system, was found to be upregulated in the urine of mice and, moreover, antibodies against the hemin receptor, *ChuA*, were detected in infected mice (23). In avian-pathogenic *E. coli*, a *chuA* mutant did not survive in a chicken infection model (32). These iron uptake genes are therefore important during in vivo infections and may also confer a survival advantage to *E. coli* O157:H7 during macrophage infection. Other metal ion uptake systems, such as *mntH*, *bfrA*, *fieF*, *feo*, *zupT*, *ent*, and *fhu*, are located outside OI. *MntH* is a metal ion transporter involved in the uptake of Mn^{2+} , specifically in acidic conditions, and to a lesser extent of Fe^{2+} and was upregulated, as were *bfrA* and *fieF*. *BfrA* is transcribed under conditions of iron abundance, and *FieF* is believed to pump iron out from the cytoplasm in the extracellular environment. In human THP-1 macrophages, *E. coli* O157:H7 does not seem to face iron-limiting conditions but may use heme or other metal ion sources. Similarly, transcriptional profiles of intracellular pathogens such as *Salmonella* and *Mycobacterium* in THP-1 macrophages did not correspond with a predicted low-iron environment either (13, 16).

Genes involved in nucleotide synthesis and in DNA synthesis and repair were upregulated, which could correspond to the multiplication of the bacteria or their encounter with different stressors. During macrophage infection, expression of genes of the SOS response, such as *recA*, *recB*, *recC*, *recN*, *sulA*, *ruvC*, *dinD*, and *dinF*, was upregulated, whereas the expression of *lexA*, the repressor of the SOS system, was downregulated. Activation of the SOS response will lead to induction of phage lytic cycles, including that of Stx prophages, which will lead in turn to an increase in production of Stx, corresponding with the phenotypes of *stx* mutants observed during macrophage infection and with *stx* upregulation detected by microarray analysis. We identified many upregulated genes that belong to acid resistance systems. The two extreme acid resistance systems, as well as a group of genes called the “acid fitness island,” were activated during macrophage infection. Furthermore, it was also observed that some of these acid resistance genes were specifically upregulated in human macrophages by *Shigella dysenteriae* (35). *E. coli* O157:H7 may face unsuitable acidic conditions within human macrophages, particularly late

in infection. Interestingly, no peroxide stress response was noted, as the transcription of critical genes such as *oxyR*, *katP*, *katE*, and *katG* was not upregulated.

In other pathogens, T3SSs are implicated in bacterial survival in macrophages, such as the T3SS located on SPI-2 in *Salmonella* (43) or on the plasmid in *Shigella* (2). In enteropathogenic *E. coli*, the T3SS encoded by the LEE was shown to inhibit phagocytosis by murine macrophages and to inhibit uptake by M cells (36), which can be a way of escaping underlying macrophages. Our results show that the majority of LEE genes were not differentially regulated during macrophage infection. Tissue culture media may be an inappropriate control in this situation for gene expression comparison, as LEE genes are expressed in Dulbecco modified Eagle medium (29, 31). LEE genes may be already expressed in RPMI, our control, explaining the absence of differential expression. Moreover, two genes, *yhiF* and *gadE* (*yhiE*), which are known repressors of the LEE (57), were upregulated, suggesting that the LEE may not be involved in EHEC survival in human macrophages, but further studies are needed to confirm this observation.

Shiga toxins are important virulence factors of EHEC that recognize the Gb3 receptor on host cells. It was previously shown that differentiated THP-1 human macrophages express Gb3 (50). At this stage of the study, we cannot say if the Gb3 status of the macrophages is important for the observed effect of Shiga toxin during interaction with macrophages. However, the Shiga toxins are also known to affect epithelial cells that do not express Gb3 (51). In this study, we notice that the transcription of *stx1B* and *stx2B* was induced in human macrophages compared to growth in RPMI. A concomitant upregulation of genes encoding the Stx subunit A, *stx1A* and *stx2A*, was not observed. Differential regulation of Shiga toxin subunit genes was previously observed during growth in vitro. Effectively, in a study that investigated global gene expression during growth transitions of *E. coli* O157:H7, the transcript levels of *stx2A* decreased 1.5-fold from late exponential to stationary phase, while levels of *stx2B* remained higher in stationary phase than in exponential phase (5). In contrast, when global expression of EDL933 in response to norfloxacin was obtained, the transcript levels of *stx2A* were at least twice as high as those of *stx2B* (25).

The involvement of Shiga toxins in phagocytosis and survival level in THP-1 human macrophages was examined. We have shown that there is a correlation between the level of phagocytosis of EDL933 by macrophages and the presence of Shiga toxins. The Stx mutants, lacking one or both toxins, were phagocytosed to a greater extent than the wild-type strain. The Shiga toxins were also involved in bacterial survival in macrophages, as Stx mutants showed a lower survival rate than the wild type. The *stx2* mutant has a significantly lower survival rate than the *stx1* mutant or the double *stx1 stx2* mutant. Interestingly, both the *stx1* and *stx1 stx2* mutants were less cytotoxic to the macrophages than the *stx2* mutant, whose cytotoxicity was similar to that of the wild-type strain. Thus, the presence of both toxins contributed to bacterial survival in macrophages, and Stx1 is more cytotoxic than Stx2 for macrophages. Differential activities of the two Shiga toxins have been previously shown on cultured human endothelial cells (34) and epithelial intestinal cells (41). The mechanism by which these toxins influence the uptake and survival of *E. coli* O157:H7 and its

cytotoxicity on human macrophages is unknown, and more research will be needed to answer this question.

EHEC preferably binds to Peyer's patches (47), where it encounters subadjacent macrophages. EHEC infections are known to induce a strong inflammatory response. The presence of EHEC and Shiga toxins in the gut recruits and activates cells of the immune system. The activated cells produce proinflammatory cytokines that intensify the inflammatory response and lead to the disruption of the intestinal barrier, permitting transport of the Stx toxins into the circulatory system (6). Furthermore, these cytokines have also been shown to increase Gb3 expression on endothelial cells, allowing more binding of Stx and thus further exacerbating the disease process (33, 58). Thus, the extended survival of EHEC in underlying macrophages could have many functions. First, EHEC survival may enhance inflammatory responses responsible for intestinal epithelial barrier disruption, which could lead to an increase of Shiga toxins entering the bloodstream. Second, as Shiga toxins are expressed and participate in bacterial survival in macrophages, the production of Shiga toxins within macrophages may be used to increase the toxin concentration in the bloodstream, as it was shown that bacteria residing in macrophages release these toxins (53). Understanding the interactions between host and pathogen is of fundamental importance in developing vaccines and other new therapeutic agents, particularly in the case of *E. coli* O157:H7, as no effective treatment is presently available for humans infected with this bacterium.

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