Differences in adherence and virulence gene expression between two outbreak strains of enterohaemorrhagic *Escherichia coli* O157:H7

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The *Escherichia coli* O157:H7 TW14359 strain was implicated in a multi-state outbreak in North America in 2006, which resulted in high rates of severe disease. Similarly, the O157:H7 RIMD0509952 (Sakai) strain caused the largest O157:H7 outbreak to date. Both strains were shown to represent divergent phylogenetic lineages. Here we compared global gene expression patterns before and after epithelial cell exposure, as well as the ability to adhere to and invade epithelial cells, between the two outbreak strains. Epithelial cell assays demonstrated a 2.5-fold greater adherence of the TW14359 strain relative to Sakai, while whole-genome microarrays detected significant differential expression of 914 genes, 206 of which had a fold change ≥ 1.5 . Interestingly, most locus of enterocyte effacement (LEE) genes were upregulated in TW14359, whereas flagellar and chemotaxis genes were primarily upregulated in Sakai, suggesting discordant expression of these genes between the two strains. The Shiga toxin 2 genes were also upregulated in the TW14359 strain, as were several pO157-encoded genes that promote adherence, including type II secretion genes and their effectors *stcE* and *adfO*. Quantitative RT-PCR confirmed the expression differences detected in the microarray analysis, and expression levels were lower for a subset of LEE genes before versus after exposure to epithelial cells. In all, this study demonstrated the upregulation of major and ancillary virulence genes in TW14359 and of flagellar and chemotaxis genes in Sakai, under conditions that precede intimate bacterial attachment to epithelial cells. Differences in the level of adherence to epithelial cells were also observed, implying that these two phylogenetically divergent O157:H7 outbreak strains vary in their ability to colonize, or initiate the disease process.

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INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is a human pathogen that contributes to an average of 17 outbreaks and 75 000 cases of illness each year in the USA alone (Mead *et al.*, 1999). EHEC O157:H7 strains cause a range of clinical complications including diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome (HUS), which is characterized by thrombotic microangiopathy, haemolytic anaemia and acute renal failure (Tarr *et al.*, 2005).

Abbreviations: A/E, attaching/effacing; EHEC, enterohaemorrhagic *Escherichia coli*; FAS, fluorescent-actin staining; HUS, haemolytic uraemic syndrome; LEE, locus of enterocyte effacement; qRT-PCR, quantitative RT-PCR; TTSS, type three secretion system.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Barrett *et al.*, 2009) and are accessible through GEO Series accession number GSE17389 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17389>).

Two supplementary figures and two supplementary tables are available with the online version of this paper.

In 2006, O157:H7 was implicated in a multi-state North American outbreak, which resulted in high rates of hospitalization (51%), HUS (15%) and death (1.5%) (CDPH, 2007). Another notable outbreak, which occurred in 1996 in Sakai, Japan, was associated with contaminated radish sprouts, and over 5000 individuals (mostly children) were reportedly affected (Fukushima *et al.*, 1999; Higami *et al.*, 1998; Ikeda *et al.*, 1999; Michino *et al.*, 1999). While both outbreaks were severe, recent genomic sequencing and genotyping data have demonstrated that the two O157:H7 strains [TW14359, Spinach; and RIMD0509952, Sakai (Hayashi *et al.*, 2001)] implicated in the outbreaks represent divergent phylogenetic lineages (Laing *et al.*, 2009; Leopold *et al.*, 2009; Manning *et al.*, 2008). Indeed, the Sakai and TW14359 genomes differ by 10% in nucleotide sequence among 2741 conserved genes, while the difference between Sakai and EDL-933 (Perna *et al.*, 2001), another genome strain associated with the first O157:H7 outbreak in 1982 (Riley *et al.*, 1983), was only 3% (Manning *et al.*, 2008). TW14359 also contains a

variant of the Shiga toxin 2 gene (*stx2c*) and the lysogenic bacteriophage 2851 that carries it (Strauch *et al.*, 2008), as well as several putative virulence factor genes absent in the other two genomes (Kulasekara *et al.*, 2009). In addition, TW14359 is a member of clade 8, an O157:H7 subpopulation that was more commonly identified from patients with HUS in one study (Manning *et al.*, 2008), while another study demonstrated that TW14359 caused more severe disease in germ-free mice than Sakai (Eaton *et al.*, 2008).

While variation in genetic content is likely important for some aspects of disease pathogenesis, differences in the expression of key virulence determinants among O157:H7 strains may also be important. Previous studies have observed differential expression of Shiga toxins (Stx1, 2 and variants), which are critical for HUS pathogenesis (Kaper *et al.*, 2004), among strains from distinct O157:H7 subpopulations (de Sablet *et al.*, 2008; Dowd & Williams, 2008). In addition, expression of the locus of enterocyte effacement (LEE) genes, which encode a type III secretion system (TTSS) that mediates intimate attachment and yields attaching/effacing (A/E) lesions (McDaniel *et al.*, 1995), is influenced by distal regulators, chromosomal and plasmid borne (Kaper *et al.*, 2004; Spears *et al.*, 2006). Distinct differences in LEE regulation have been observed between closely related intestinal *E. coli* pathogens (Spears *et al.*, 2006). Genome-wide expression studies have also been performed in O157:H7 to examine expression changes following exposure to eukaryotic cells. One study demonstrated transcription of ~8% of the genome and downregulation of LEE gene expression following association of O157:H7 with erythrocytes, compared to O157:H7 grown in medium alone (Dahan *et al.*, 2004). Another study compared the transcriptomes of O157:H7 co-incubated with epithelial cells to the O157:H7 transcriptome without epithelial cell exposure; differential expression of 131 genes was observed, one of which has important immunomodulatory roles (Jandu *et al.*, 2009). While these studies have focused on within-strain differences, little is known about the variation in gene expression between phylogenetically distinct O157:H7 strains, upon exposure but preceding adherence to host epithelial cells, and how that relates to cell attachment.

Here we investigated whether the TW14359 and Sakai outbreak strains, which represent the two most divergent O157:H7 lineages in a prior study (Manning *et al.*, 2008), vary in the ability to colonize epithelial cells and express shared virulence genes preceding adherence to epithelial cells. Resolving phenotypic and transcriptional differences among O157:H7 outbreak strains is important for advancing our understanding of the molecular mechanisms that underlie the variation in clinical illness.

METHODS

Bacterial strains and growth conditions. *E. coli* O157:H7 strains RIMD0509952 (Sakai) (Hayashi *et al.*, 2001) and TW14359 (Manning

et al., 2008) were used. *E. coli* K-12 MG1655 (Blattner *et al.*, 1997) was used as a control. Strains were grown in MOPS-buffered minimal medium as described previously (Bergholz *et al.*, 2007). Prior to infection of MAC-T epithelial cells, stationary-phase MOPS cultures were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) (pH 7.4, without phenol red, 0.45% glucose, 0.37% NaHCO₃) (transfer ratio 1:40) for 3 h at 37 °C with shaking (~200 r.p.m.) for medium adaptation. Cell densities of O157:H7 cultures were $(5 \pm 0.5) \times 10^8$ c.f.u. ml⁻¹ based on standard plate counts of DMEM culture samples. Reproducible growth of O157:H7 cultures in MOPS and DMEM was confirmed by optical density (OD₆₀₀) readings (see Supplementary Fig. S1, available with the online version of this paper).

MAC-T cells. Bovine mammary epithelial (MAC-T) cells (Huynh *et al.*, 1991) are commonly used in studies of adherent and invasive *E. coli* (Dogan *et al.*, 2006; Dopfer *et al.*, 2000), and *E. coli* O157:H7 was previously shown to induce A/E lesions on MAC-T cells (Matthews *et al.*, 1997). MAC-T cells were grown in 75 cm² tissue flasks with DMEM [10% fetal bovine serum (FBS) and 2% Antibiotic-Antimycotic solution (Invitrogen)], at 37 °C with 5% CO₂. For microarray experiments, MAC-T cells were grown to confluence in 75 cm² flasks. Approximately 12 h before experiments, MAC-T cells were washed with DMEM and the culture medium was replaced by DMEM (2% FBS) without antibiotics. DMEM was replaced again prior to experiments, with DMEM without FBS.

Fluorescence microscopy. The ability of Sakai and TW14359 strains to form A/E lesions on MAC-T cells was examined using the previously described fluorescent-actin staining (FAS) test (Knutton *et al.*, 1989; Shariff *et al.*, 1993), with modification. Briefly, MAC-T monolayers growing on 12 mm glass coverslips (Fisher Scientific), in a 24-well plate (Corning), were infected with 100 µl Sakai, TW14359 or K-12 MG1655 culture in DMEM. After 3 h incubation (37 °C, 5% CO₂) and three washes with PBS, samples were fixed and permeabilized with chilled acetone for 2 min, on ice. After washing in PBS, a 30 min blocking step with 1% BSA/PBS was applied to inhibit non-specific staining. After washing in PBS, samples were stained with phalloidin-conjugated Alexa Fluor 488 (Molecular Probes), according to the manufacturer's instructions, to detect filamentous actin. Samples were counterstained with propidium iodide (Molecular Probes), which stains nucleic acids. Samples were analysed using a Zeiss 510 Meta ConfoCor3 LSM confocal laser scanning microscope with a 63× plan apochromat numerical aperture 1.4 objective, with oil immersion (Carl Zeiss MicroImaging). Images were imported and handled with Zeiss LSM Image Browser Version 3.2.0.115; confocal slices were merged in Adobe Photoshop 6. The experiment was performed in triplicate for two independent cultures of each strain.

Association and invasion assays. Association assays, a measurement of adherence and invasion, were performed in 24-well plates by a previously described method (Dibb-Fuller *et al.*, 2001), with modification. Monolayers of MAC-T cells (~1 × 10⁶ cells per well) were infected with 1 ml of Sakai or TW14359 culture in DMEM at an m.o.i. (the ratio of bacteria to host cells), of 500:1; concentrations of O157:H7 starting inocula were $(5 \pm 0.4) \times 10^8$ c.f.u. ml⁻¹. After 30 min or 1 h of incubation (37 °C, 5% CO₂), wells were washed three times with PBS, and MAC-T cells were disrupted with 0.1% Triton X-100 (Sigma-Aldrich) for 30 min at 37 °C. Lysate dilutions were plated on LB agar with the Autoplate 4000 Spiral Plater (Spiral Biotech) and enumerated following overnight incubation (37 °C), using the Q-Count instrument (Spiral Biotech). O157:H7 strains were assayed in quadruplicate and the experiment was repeated with three independent cultures of each strain. The c.f.u. ml⁻¹ of Sakai and TW14359 recovered from all wells of a biological replicate were normalized against the c.f.u. ml⁻¹ recovered from the first well of the corresponding biological replicate infected with Sakai. Normalized data from three biological replications of the experiment were

analysed with a PROC MIXED ANOVA model (SAS v9.1), relative association = strain + replicate (strain) + error, where biological replicate was nested within the strain effect. The difference in association was expressed as the mean $\pm$ standard error of TW14359 relative to Sakai. For invasion assays, 1 h after incubation wells were washed with PBS and the medium were replaced by DMEM containing 200 μ g gentamicin ml⁻¹ to kill extracellular bacteria (Dibb-Fuller *et al.*, 2001). After 2 h additional incubation, bacteria were recovered and enumerated as described above. For invasion, each O157:H7 strain was assayed in triplicate and the experiment was repeated twice.

MAC-T challenge experiments and RNA extraction. Confluent monolayers of MAC-T cells in 75 cm² tissue flasks with 15 ml DMEM were infected with 3 ml O157:H7 Sakai or TW14359 culture in DMEM, at a concentration of $(5 \pm 0.5) \times 10^8$ c.f.u. ml⁻¹. Following incubation for 30 min (37 °C, 5% CO₂), with rocking at 1.5 r.p.m. on a rocking platform (VWR International) to mimic gut peristalsis, 8 ml aliquots of non-adherent, suspended bacteria were mixed with 1/10 volume of 10% phenol/ethanol buffer to stabilize the RNA (Bhagwat *et al.*, 2003), and centrifuged at 4500 g, 4 °C, for 30 min to pellet cells. The supernatant was decanted and pellets were suspended in 5 ml buffer (2 mM EDTA, 20 mM NaOAc, pH 5.2). RNA purification, RNA integrity assessment, cDNA conversion and Cy dye labelling were performed as previously described (Bergholz *et al.*, 2007).

Microarray hybridization conditions, data collection and analysis. cDNAs were hybridized onto microarray slides that target 5978 ORFs from O157:H7 Sakai (Hayashi *et al.*, 2001), O157:H7 EDL-933 (Perna *et al.*, 2001) and K-12 MG1655 (Blattner *et al.*, 1997), and 110 ORFs from the O157:H7 plasmid (pO157) (Burland *et al.*, 1998), under previously described conditions (Bergholz *et al.*, 2007; Kailasan Vanaja *et al.*, 2009). Each microarray slide was loaded with a pair of alternately Cy-dye-coupled cDNA samples from Sakai and TW14359 strains. In total, 10 dye-swap hybridizations, corresponding to five independent RNA extractions each from Sakai and TW14359 strains, were carried out. Microarrays were scanned with an Axon 4000B scanner (Molecular Devices), probe intensities were retrieved with Genepix 6.0 software (Molecular Devices), and data were analysed as described by Bergholz *et al.* (2007). Briefly, LOWESS normalization (Quackenbush, 2002) was applied to raw intensity values for all probes on each array using the R/MAANOVA package (version 0.98-8) in R (version 2.2.1) (R Development Core Team, 2005). Because all probes were printed in duplicate, signal intensities from probes were averaged before they were log₂ transformed. Transformed data were fitted to a mixed model ANOVA in R/MAANOVA [$y_{\text{intensity}} = \text{Array} + \text{Dye} + \text{Strain} + \text{Sample}$ (Sample = biological replicate)], which identifies fixed (Dye, Strain) and random (Array, Sample) sources of variation (Cui & Churchill, 2003; Kerr, 2003). Relative gene expression differences between Sakai and TW14359 strains were determined with the F_3 test (Cui *et al.*, 2005), using 1000 random permutations to generate P -values. P -values were adjusted for multiple comparisons using the Benjamini-Hochberg correction included in R/MAANOVA. Differences in gene expression with an adjusted P -value (adj. P -value) < 0.05 were considered significant.

Quantitative RT-PCR (qRT-PCR). For qRT-PCR, three independent replications of the MAC-T challenge experiment were performed with both Sakai and TW14359, and O157:H7 RNA was isolated as described above. O157:H7 RNA was also isolated from strains prior to infection of MAC-T cells, to compare gene expression before and after exposure to MAC-T cells. Gene-specific oligonucleotide primers and annealing temperatures are given in Supplementary Table S1. The primers for the *stx2B* gene were specifically designed to discriminate between Stx2 and Stx2c variants of the B subunit gene. The forward primer is not specific whereas the reverse primer anneals to 103–120 bp downstream of the first nucleotide of the *stx2B* gene sequence; this is the variable region of *stx2B* between the Stx2 and Stx2c

variants. Reverse transcription of RNA, qRT-PCR volumes and cDNA amplification conditions were performed as previously described (Bergholz *et al.*, 2007). qRT-PCRs were performed with cDNA populations from three independent RNA extractions, from each O157:H7 strain, and each cDNA sample was tested in quadruplicate, across fivefold serial dilutions ($1 \times$ to $0.008 \times$). The 16S rRNA (*rrsH*) gene was used as an internal control for within-sample normalization of mRNA abundance (Tasara & Stephan, 2007), and transcription differences between Sakai and TW14359 strains were determined using a previously described method (Pfaffl, 2001).

ELISA for Stx quantification. The Premier EHEC ELISA kit (Meridian Biosciences) was used for relative quantification (OD₄₅₀) of Stx production by TW14359 and Sakai cultures grown in DMEM, according to the manufacturer's instructions. The assay was performed three times with three independent cultures of each strain.

RESULTS

Interaction of O157:H7 with MAC-T bovine epithelial cells

The FAS test showed that infection of MAC-T cells with TW14359 and Sakai O157:H7 strains induced marked cytoskeletal rearrangement characterized by filamentous actin emanating from the cells, which is typical of A/E lesions (Fig. 1). Propidium iodide counter-staining of specimens confirmed co-localization of actin pedestals and bacteria. Both strains demonstrated the ability to form densely packed microcolonies, or 'localized adherence' (Dibb-Fuller *et al.*, 2001; Matthews *et al.*, 1997; Naylor *et al.*, 2005; Nougayrede *et al.*, 2003), on the surface of MAC-T cells. A/E lesions were not observed following infection with *E. coli* K-12 (Fig. 1).

Cell-association assays demonstrated that there was no significant difference in association levels with MAC-T cells between TW14359 and Sakai ($P = 0.28$) at 30 min post-infection. After 1 h, however, infection with TW14359 resulted in significantly higher levels of association compared to Sakai ($P = 0.02$): the number of adherent bacteria recovered after 1 h was 2.5 ± 0.3 -fold higher for strain TW14359 (Fig. 2). In contrast, there was no significant difference in the ability of the two strains to invade MAC-T cells ($P = 0.33$), and the number of recovered bacteria was considerably lower than in the association assays (Supplementary Fig. S2). Because the growth rates of each bacterial culture were similar (Supplementary Fig. S1) and the c.f.u. ml⁻¹ of the starting inocula varied by less than 10%, differences in association levels were not attributable to variation in growth phase or cell densities among strains. Consequently, these results indicate that TW14359 has a greater ability to adhere to, but not invade, epithelial cells relative to Sakai.

Microarray expression profiling

The TTSS is the main O157:H7 colonization factor; therefore increased adherence of TW14359 to MAC-T epithelial cells, 1 h post-infection, implies that there may be differences in LEE

Fig. 1. Fluorescence micrographs of MAC-T epithelial cells infected with *E. coli* K-12, O157:H7 TW14359 and O157:H7 Sakai. The FAS test was performed to detect pedestal formation on MAC-T cells, a characteristic of A/E lesions, following 3 h infection with O157:H7 strains and K-12 MG1655. Filamentous actin was stained green (phalloidin-conjugated Alexa Fluor 488); nucleic acid was stained red (propidium iodide). Merging the green and red fluorescence demonstrated co-localization of actin pedestals and bacteria. Scale bar represents 10 μ m. Magnification, 63 $\times$ with 2.7 $\times$ scan zoom for K-12 and TW14359, and 1.8 $\times$ for Sakai.

transcription between the two strains. Gene expression was measured using whole-genome microarrays among TW14359 and Sakai cells exposed to MAC-T monolayers for 30 min. Since prior studies have demonstrated that LEE transcription is downregulated after bacterial adherence to the eukaryotic cell membrane (Beltrametti *et al.*, 1999; Dahan *et al.*, 2004), we examined differential gene expression following exposure to epithelial cells, but prior to attachment, to determine whether these outbreak strains respond differently to the vicinity of the host cell. The F_5 test identified 914 genes with significant differential expression between TW14359 and Sakai (adj. P -value <0.05) (Fig. 3, Supplementary Table S2), 41 of which are encoded on the pO157 plasmid. In TW14359, 440 genes were downregulated, and 475 were upregulated, relative to Sakai. Relative differences in gene expression greater than 1.5-fold were detected for 206 of the 914 genes, 98 of which encode hypothetical proteins. Of the 206 genes, 105 were upregulated in TW14359.

Differential expression of adherence and motility genes

Of major interest were the expression ratios from 36 of 41 LEE genes that were upregulated in TW14359 (Table 1),

including the positive LEE regulators *ler* and *grlA*. LEE genes were almost uniformly upregulated from the LEE1 to LEE4 operon (Fig. 4), which encodes the EspADB needle complex that inserts into the host cell and allows translocation of effector proteins (Garmendia *et al.*, 2005). The five LEE genes without significantly elevated transcript levels in TW14359 were *escT* and *escU*, which encode inner-membrane-bound constituents of the TTSS export structure, and two genes of unknown function, *orf1* and *rorf3*. Although *grlA* and *grlR* constitute a single operon in the LEE island, no difference in the expression of *grlR* was detected between strains. Significant differences in gene expression ratios were validated by qRT-PCR for five LEE genes (Table 4). Together with the seven LEE-encoded effector proteins (Tir, Map, EspF, EspG, EspH, EspB and SepZ), the TTSS serves to export another 39 non-LEE-encoded effectors (Tobe *et al.*, 2006). Genes coding for 24 of these effectors were differentially expressed between TW14359 and Sakai, 16 of which were upregulated in TW14359 and 8 were upregulated in Sakai (Table 2).

The expression of several genes relevant to adherence, but not associated with the TTSS, was also significantly different between the two strains (Table 1). The pO157-

Fig. 2. Association of O157:H7 Sakai and TW14359 with MAC-T epithelial cells. Association of O157:H7 strains with MAC-T cells was quantified by standard plate counts of bacteria that were recovered 30 min (left panel) and 1 h (right panel) post-infection of MAC-T epithelial cells. Error bars indicate the standard deviation of the mean number of c.f.u. ml⁻¹ recovered from four wells. Each plot represents an independent experiment.

borne type II secretion system, encoded by the *etpC-O* polycistron, is represented by 12 probes on the microarray, nine of which indicated upregulation of target genes (*etpEFGHIJKMN*) in TW14359. The type II apparatus secretes several factors that influence colonization (Sandkvist, 2001). The transcription of one such gene, *stcE* (*tagA*), which encodes a metalloprotease that contributes to intimate adherence (Grys *et al.*, 2005), was elevated in TW14359. Expression of *adfO* (ECs1772), which encodes another type II secretion effector that promotes adherence of O157:H7 to epithelial cells (Ho *et al.*, 2008), was also elevated in TW14359. Overall, these results indicate that the transcription of major and accessory colonization factors, preceding intimate adherence of O157:H7 to MAC-T epithelial cells, is higher in TW14359 than Sakai. This result is consistent with the finding that TW14359 adhered to MAC-T cells at significantly higher levels than Sakai.

To investigate the effect of epithelial cell exposure on O157:H7 LEE expression, transcript levels of *eae*, *tir*, *espA* and *espB* were compared between and within strains before

and after exposure to MAC-T monolayers. The qRT-PCR results, summarized in Table 4, reveal that LEE expression differences between TW14359 and Sakai are smaller before versus after MAC-T exposure for the genes assayed. In contrast, within-strain comparisons indicate that LEE gene expression was considerably higher following exposure to MAC-T cells (Table 4) in both TW14359 and Sakai.

Finally, another notable result was that several motility genes showed significantly higher expression in Sakai. Fourteen genes that mediate flagellar biosynthesis (*flgCDEFGK*, *fliCDHL* and *fliA*) and chemotaxis (*cheB*, *tsr* and *tar*) were upregulated in the Sakai strain (Table 3). Relative expression differences were confirmed by qRT-PCR for two representatives, *flgE* and *cheB* (Table 4).

Upregulation of Shiga toxin 2 in the O157:H7 Spinach strain

The Sakai strain is lysogenized with Stx1- and Stx2-converting phages (Michino *et al.*, 1999), while strain TW14359 harbours Stx2 and Stx2c variants (Manning *et al.*,

Fig. 3. Significant differential expression of 914 genes between TW14359 and Sakai, detected with microarrays. Genes were sorted according to chromosomal position in Sakai (y-axis), and gene expression ratios between TW14359 and Sakai were plotted as fold change differences (x-axis). On the left side of the figure are genes that were found to be upregulated in strain TW14359, and on the right side are genes that were found to be upregulated in Sakai. The dotted line indicates a fold change of 1.5. Genes for which the fold change difference in expression was >8 were excluded from the figure; expression ratios for all 914 genes are provided in Supplementary Table S2. Genes located on the same element (prophage, plasmid or backbone chromosome) are depicted by the same colour and symbol. ECs, O157:H7 Sakai gene number; Sp, Sakai prophage; SpLE, Sakai prophage-like element; pO157, plasmid genes.

Table 1. Significant differences in expression of LEE and other adherence-associated genes between TW14359 (Spinach) and Sakai, as detected by microarrays

ECs*	Gene	Function†	Fold change‡
ECs4550	<i>espF</i>	Effector	1.60
ECs4551	<i>orf29</i>	Unknown	1.23
ECs4552	<i>escF</i>	Needle protein	1.70
ECs4553	<i>cesD2</i>	Chaperone for EspD	1.73
ECs4554	<i>espB</i>	Effector/translocator	1.83
ECs4555	<i>espD</i>	Translocator	1.70
ECs4556	<i>espA</i>	Translocator	1.70
ECs4557	<i>sepL</i>	TTSS	1.44
ECs4558	<i>escD</i>	TTSS	1.65
ECs4559	<i>eae</i>	Intimin adherence protein	1.72
ECs4560	<i>cesT</i>	Chaperone for Tir	1.64
ECs4561	<i>Tir</i>	Translocated intimin receptor	1.76
ECs4562	<i>map</i>	Effector	1.54
ECs4563	<i>cesF</i>	Chaperone for EspF	1.61
ECs4564	<i>espH</i>	Effector	1.59
ECs4565	<i>sepQ</i>	TTSS	1.65
ECs4566	<i>orf16</i>	Unknown	1.32
ECs4567	<i>orf15</i>	Unknown	1.75
ECs4568	<i>escN</i>	TTSS	1.47
ECs4569	<i>escV</i>	TTSS	1.58
ECs4570	<i>orf12</i>	Unknown	1.55
ECs4571	<i>sepZ</i>	Effector	1.77
ECs4572	<i>rorf8</i>	Unknown	1.72
ECs4573	<i>escJ</i>	TTSS	1.42
ECs4574	<i>sepD</i>	TTSS	1.31
ECs4575	<i>escC</i>	TTSS	1.41
ECs4576	<i>cesD</i>	Chaperone for EspD	1.36
ECs4577	<i>grlA</i>	Regulator, positive	1.42
ECs4582	<i>escS</i>	TTSS	1.28
ECs4583	<i>escR</i>	TTSS	1.35
ECs4584	<i>orf5(escL)</i>	Binds to escN	1.50
ECs4585	<i>orf4</i>	Unknown	1.51
ECs4586	<i>orf3</i>	Unknown	1.69
ECs4587	<i>cesAB</i>	Chaperone for EspA and EspB	1.68
ECs4588	<i>ler</i>	Regulator, positive	1.48
ECs4590	<i>espG</i>	Destruction of microtubules	1.38
pO157	<i>stcE/tagA</i>	StcE metalloprotease	1.83
ECs1772	<i>adfO</i>	Putative colonization factor	1.22
pO157	<i>etpE</i>	Type 2 secretion	1.20
pO157	<i>etpF</i>	Type 2 secretion	1.20
pO157	<i>etpG</i>	Type 2 secretion	1.19
pO157	<i>etpH</i>	Type 2 secretion	1.20
pO157	<i>etpI</i>	Type 2 secretion	1.21
pO157	<i>etpJ</i>	Type 2 secretion	1.14
pO157	<i>etpK</i>	Type 2 secretion	1.15
pO157	<i>etpM</i>	Type 2 secretion	1.14
pO157	<i>etpN</i>	Type 2 secretion	1.17

*ECs, O157:H7 Sakai chromosomal gene numbers; numbers ECs4550–ECs4590 denote LEE genes; pO157, plasmid-encoded genes.

†LEE protein functions, summarized from Dahan *et al.* (2004); Garmendia *et al.* (2005) and Pallen *et al.* (2005).

‡Positive values were found to be upregulated in the TW14359 strain; adj. *P*-value <0.05 .

Fig. 4. Heatmap of expression ratios of LEE genes between TW14359 and Sakai. The heatmap was generated based on $\log_2$ expression ratios of Spinach:Sakai, and imposed on a graphic representation of the genetic organization of the LEE island, adapted from Stevens *et al.* (2005). Red colours indicate upregulation in TW14359. Black arrows indicate LEE operons and the direction of transcription.

2008); the microarray used in this study contains probes for the *stxA* and *stxB* subunits of *stx1* and *stx2*, but not *stx2c*. As expected, the expression of genes encoding Stx1, *stx1a* and *stx1b* was elevated in Sakai, by 36.4-fold and

29.7-fold, respectively. The transcription of *Stx2*, which is shared by both strains, was increased in TW14359 relative to Sakai by 1.70-fold and 2.4-fold for *stx2a* and *stx2b*, respectively, which was confirmed by qRT-PCR (Table 4). Upregulation of *stx2a* and *stx2b* expression in TW14359, however, was not dependent on exposure to MAC-T cells since there was no difference in expression levels, relative to Sakai, before and after MAC-T cell exposure, as determined by qRT-PCR (Table 4). Also, the quantification of Stx in TW14359 and Sakai prior to MAC-T exposure by ELISA demonstrated that TW14359 produced ~30 % more Stx than Sakai. In addition, expression of the *q* antiterminator (ECs1203) of the *Stx2*-converting phage was also significantly higher in TW14359 (1.27-fold). The phage-borne *q* antiterminator, located upstream of *stx2*, is critical for Stx expression and initiates transcription at the *pR'* late promoter site (Wagner *et al.*, 2001).

DISCUSSION

An increasing body of phylogenetic evidence has shown that the extant *E. coli* O157:H7 population is characterized by considerable genetic variation (Laing *et al.*, 2009; Leopold *et al.*, 2009; Manning *et al.*, 2008). Strain TW14359 belongs to an ancestral lineage compared to that of Sakai (Leopold *et al.*, 2009). TW14539 also differs by 10 % of nucleotide sequence in 2741 conserved genes (Manning *et al.*, 2008) and contains more unique sequence (70 kb) than both Sakai and EDL-933 outbreak strains (Kulasekara *et al.*, 2009). Given that EDL-933 is so closely related to Sakai (Laing *et al.*, 2009; Leopold *et al.*, 2009; Manning *et al.*, 2008), we chose to make comparisons between the two strains representing the most divergent phylogenetic lineages. Here we have demonstrated that following infection of epithelial cells, TW14359 adhered better and expressed higher levels of shared virulence determinants relative to Sakai.

Table 2. Significant differential expression of non-LEE encoded effector genes, as detected by microarrays

ECs	Effector*	Fold change†
ECs0472	EspY3	−1.35
ECs1127	EspV'	1.45
ECs1567	EspO1-1	1.18
ECs1810/1	NleG2-1'	−1.26
ECs1812	NleA	−1.30
ECs1814	NleH1-2	1.30
ECs1824	NleG	1.23
ECs1994	NleG2-2	1.59
ECs1996	NleG5-1	1.46
ECs2154	NleG5-2	1.33
ECs2155	NleG6-2	1.36
ECs2156	NleG2-3	1.43
ECs2226	NleG7'	1.26
ECs2228	NleG3'	3.13
ECs2229	NleG2-4'	1.51
ECs2714	EspJ	−1.34
ECs2715	TccP	−1.40
ECs3485	EspM2	−1.48
ECs3487	EspW	1.20
ECs4642/3	EspL3'	1.17
ECs4653	EspY4	1.20
ECs4657	EspY5'	−1.25
ECs5048	EspX5	−1.20
ECs5295	EspX6	1.46

*Nomenclature adopted from Tobe *et al.* (2006).
†Positive values were found to be upregulated in the TW14359 (Spinach) strain; negative values indicate upregulation in the Sakai strain; adj. *P*-value <0.05.

Table 3. Upregulation of flagellar genes in Sakai relative to TW14359 (Spinach), as detected by microarrays

ECs	Gene	Function	Fold change*
ECs0256	<i>fliA</i>	Flagellar, putative motility protein	1.13
ECs1452	<i>flgC</i>	Flagellar, cell-proximal portion of basal-body rod	1.51
ECs1453	<i>flgD</i>	Flagellar, initiation of hook assembly	1.16
ECs1454	<i>flgE</i>	Flagellar, hook protein	2.27
ECs1455	<i>flgF</i>	Flagellar, cell-proximal portion of basal-body rod	1.31
ECs1456	<i>flgG</i>	Flagellar, cell-distal portion of basal-body rod	1.60
ECs1460	<i>flgK</i>	Flagellar, hook-filament junction protein 1	1.15
ECs2662	<i>fliC</i>	Flagellar; flagellin, filament structural protein	1.23
ECs2663	<i>fliD</i>	Flagellar, capping protein for filament assembly	1.15
ECs2683	<i>fliL</i>	Flagellar biosynthesis	1.32
ECs2679	<i>fliH</i>	Flagellar, export of flagellar proteins	1.19
ECs2593	<i>cheB</i>	Response regulator for chemotaxis (<i>cheA</i> sensor)	1.58
ECs2596	<i>tar</i>	Methyl-accepting chemotaxis protein II	1.18
ECs5315	<i>tsr</i>	Methyl-accepting chemotaxis protein I	1.74

*Positive values indicate upregulation in Sakai, adj. *P*-value <0.05.

The tight attachment of O157:H7 to epithelial cells occurs through binding of intimin with its receptor, Tir (Garmendia *et al.*, 2005). This binding triggers actin rearrangements and the formation of pedestal structures, representative of A/E lesions. In this study, both O157:H7 strains were positive by the FAS reaction, confirming that colonization of MAC-T bovine epithelial cells is also mediated through the intimin–Tir interaction, consistent with prior findings (Matthews *et al.*, 1997). However, TW14359 adhered to epithelial cells in significantly greater numbers than Sakai, within 1 h post-infection. The lack of a difference in invasion levels between TW14359 and Sakai is also in agreement with previous studies (Dibb-Fuller

et al., 2001; Matthews *et al.*, 1997). Despite the magnitude of the difference between strains, it is important to note that these data were generated using a monoculture of bovine epithelial cells, which do not accurately represent the complexity of the intestinal environment and the multicellular composition of the mucosal lining in both humans and bovines. However, Dibb-Fuller *et al.* (2001) demonstrated no difference in the ability of O157:H7 to adhere to epithelial cells of human origin (HEp-2) when compared to several distinct cell lines of bovine origin. Consequently, it is likely that the differences in adherence levels between the two strains would be similar if alternative human-derived cell lines were used. Because

Table 4. Differences in gene expression between TW14359 and Sakai as determined by qRT-PCR

Gene	Fold change* TW14359/Sakai	<i>E</i> †	Fold change‡ TW14359/Sakai	<i>E</i> †	Fold change§ TW14359/ TW14359	<i>E</i> †	Fold change Sakai/Sakai	<i>E</i> †
<i>eae</i>	1.94 ± 0.2	1.93	1.53 ± 0.2	1.99	2.75 ± 0.2	2.00	2.65 ± 0.2	1.91
<i>tir</i>	2.08 ± 0.6	2.21	1.65 ± 0.3	1.91	5.11 ± 1.3	2.01	6.95 ± 1.6	2.06
<i>espA</i>	2.13 ± 0.3	2.10	1.57 ± 0.3	1.93	2.70 ± 0.3	2.02	2.80 ± 0.6	1.92
<i>espB</i>	2.04 ± 0.6	2.01	1.29 ± 0.5	1.98	6.20 ± 1.2	2.02	5.14 ± 1.1	1.97
<i>sepZ</i>	1.80 ± 0.3	2.02	ND	ND	ND	ND	ND	ND
<i>stx2A</i>	4.86 ± 1.4	2.06	5.17 ± 1.5	1.96	1.61 ± 1.0	2.01	1.41 ± 0.6	2.00
<i>stx2B</i>	4.30 ± 0.2	2.08	5.24 ± 0.5	1.95	1.06 ± 0.2	2.03	1.49 ± 0.1	2.02
<i>flgE</i>	−8.33 ± 1.8	2.00	ND	ND	ND	ND	ND	ND
<i>cheB</i>	−3.41 ± 0.5	2.02	ND	ND	ND	ND	ND	ND

ND, Not determined.

*Mean ± standard deviation of relative fold change difference in gene expression between TW14359 (*n*=3) and Sakai (*n*=3) strains 30 min following exposure to MAC-T cells. Negative sign indicates increased expression in the denominator strain.

†Mean qRT-PCR efficiency (*n*=6).

‡Prior to MAC-T cell exposure.

§After and before TW14359 exposure to MAC-T cells.

||After and before Sakai exposure to MAC-T cells.

intimate attachment to host epithelia is a key step in the infection process, increased aptitude for adherence possibly gives O157:H7 TW14359 a competitive edge to avoid clearance by host defences and competitive exclusion by indigenous microbiota.

To compare transcription of colonization genes between TW14359 and Sakai following epithelial cell exposure, gene expression was examined in bacteria that had not yet intimately adhered to epithelial cells. The *in vitro* conditions that most clearly mimic the intestinal environment, including contact with epithelial cells (Beltrametti *et al.*, 1999) and physiological bicarbonate ion availability (Abe *et al.*, 2002), were used since these conditions are considered optimal for LEE expression. Although the number of genes shown to be up- or downregulated varies in previous transcriptome profiling studies, these results are typically dependent on the experimental design, conditions and cutoff values used. Dahan *et al.* (2004), for example, reported 404 genes with >2-fold increased expression following O157:H7 Sakai association with erythrocytes, compared to Sakai grown in DMEM. One study, which examined the transcriptome of O157:H7 after 3 h of attachment to HT-29 epithelial cells, describes 611 upregulated and 384 downregulated O157:H7 genes relative to the same strain grown in media alone (Kim *et al.*, 2009). Conversely, another study identified only 131 differentially expressed genes following 6 h of O157:H7 co-incubation with HEP-2 cells compared to unexposed O157:H7 (Jandu *et al.*, 2009). These data, however, were generated from pooled RNA that included both adherent and non-adherent O157:H7 (Jandu *et al.*, 2009). Here, after a 1.5-fold cutoff was applied, above which the change in expression is considered meaningful (Hughes *et al.*, 2000), we found 206 genes to be differentially expressed between two phylogenetically distinct O157:H7 strains exposed to epithelial cells, but prior to attachment.

Following infection of MAC-T epithelial cells, LEE genes were upregulated in the TW14359 strain relative to Sakai, thereby providing transcriptional support for the increased level of TW14359 adherence to MAC-T cells. Only the magnitude of the change varied when strains were examined without epithelial cell exposure. Regulation of the LEE is complex and is influenced by multiple environmental factors that act on several chromosomal genes, which in turn fine-tune LEE expression through LEE-encoded regulators, Ler, GrlA and GrlR (Deng *et al.*, 2004; Spears *et al.*, 2006). Once activated, Ler leads to induction of operons LEE2, LEE3, LEE5 and LEE4 (Barba *et al.*, 2005; Haack *et al.*, 2003). More so, GrlA can stimulate expression of Ler (Deng *et al.*, 2004) as well as of LEE2 and LEE4 independently of Ler (Russell *et al.*, 2007). Also, the promoter for LEE4, which encodes the EspADB translocon, was previously shown to be activated upon bacterial contact with epithelial cells (Beltrametti *et al.*, 1999). The upregulation of *espADB* in strain TW14359 may therefore result from a more efficient response to the host cell, compared to Sakai. This could be due in part to an

unrecognized gene or set of genes unique to TW14359. Indeed, the recent comparison of TW14359 and Sakai genomes identified several genes exclusive to TW14359, including putative TTSS effectors and an intact *norV* gene, the deletion of which is hypothesized to decrease colonization of the large intestine (Kulasekara *et al.*, 2009). Future work should focus on comparing TTSS and effector protein levels between these strains.

Non-LEE effectors were previously suggested to antagonize host immune defences, including inhibition of host phagocytosis and suppression of proinflammatory cytokine production (Celli *et al.*, 2001; Deng *et al.*, 2004; Hauf & Chakraborty, 2003). In addition, the expression of several of these factors was shown to be under the influence of the GrlA regulator (Deng *et al.*, 2004; Spears *et al.*, 2006). The contribution of non-LEE effectors, which were differentially expressed in this study, to the pathogenesis of O157:H7 is not clear. The *espJ-tccP* operon (Garmendia & Frankel, 2005), for instance, was downregulated in TW14359 (<1.5-fold), an unexpected result given that TccP plays a critical role in EHEC pedestal formation (Garmendia *et al.*, 2005). However, *espJ* is not required for pedestal formation, and the deletion of *espJ* in *Citrobacter rodentium* and O157:H7 resulted in more persistent colonization of mice and lamb, respectively (Dahan *et al.*, 2005). Indeed, the expression of the *espJ-tccP* operon is influenced by multiple environmental signals, and its regulation is unknown (Garmendia & Frankel, 2005). Given the substantial difference in genomic content between TW14359 and Sakai, it is possible that unknown regulatory cues are differentially affecting transcription in the two strains under the experimental conditions used; this may have resulted in the weak discrepancy in expression of the *espJ-tccP* operon between the two strains.

Increased adherence of TW14359 may also be facilitated by upregulation of the type II secretion machinery. Mutation of *etpC*, the first gene in the *etp* operon, has been shown to result in a significant reduction of O157:H7 colonization in an infant rabbit model of disease (Ho *et al.*, 2008). In this study, two effectors of type II secretion previously suggested to contribute to adherence, StcE (Grys *et al.*, 2005) and AdfO (Ho *et al.*, 2008), were upregulated in TW14359. The StcE metalloprotease, the expression of which is positively influenced by Ler (Lathem *et al.*, 2002), is hypothesized to promote adherence by cleaving proteins in the glycocalyx and mucin layers atop the intestinal epithelium, facilitating close contact with the intestinal mucosa (Grys *et al.*, 2005). The contribution of AdfO to adherence is not clear, as an *adfO* mutation has been shown to markedly decrease epithelial cell adherence *in vitro*, but does not attenuate colonization following oral challenge of infant rabbits (Ho *et al.*, 2008).

Shiga toxin is a key virulence factor of EHEC and can solely trigger HUS, which is evident from the observation that LEE-negative Shiga toxin-producing *E. coli* also cause HUS (Gyles, 2007; Paton & Paton, 1998). Stx2 has been

associated with severe disease more frequently than Stx1 (Boerlin *et al.*, 1999; Ethelberg *et al.*, 2004), and was shown to be 400 times more potent than Stx1, in mice (Tesh *et al.*, 1993). Mitomycin C treatment also led to increased induction of *stx2* expression in TW14359 compared to Sakai (Kulasekara *et al.*, 2009). Here we show that in the absence of DNA-damaging agents, baseline expression of *stx2* is markedly higher in TW14359 relative to Sakai before and after epithelial cell exposure, suggesting that increased Stx2 production in this strain may play a role in HUS development. These transcriptional differences are in agreement with ELISA-based quantification in this study and a prior one in germ-free mice that were orally challenged with both TW14359 and Sakai (Eaton *et al.*, 2008).

Conversely, microarray measurements revealed the upregulation of motility genes involved in flagellar biosynthesis (*flg* and *fli* operons) in Sakai, and of genes that encode chemotaxis molecules. In both enteropathogenic *E. coli* (EPEC) O127:H6 (Giron *et al.*, 2002) and *Salmonella enteritidis* (Allen-Vercoe & Woodward, 1999; Dibb-Fuller *et al.*, 1999), flagella were demonstrated to be important for initial contact with host cells. EPEC O127:H6 bacteria are flagellated in the early stages of adherence to epithelial cells, but flagella are retracted after pedestal formation and intimate attachment of bacteria (Yona-Nadler *et al.*, 2003). GrlA was shown to inhibit cell motility by repressing flagellar gene expression in O157:H7 (Iyoda *et al.*, 2006). The upregulation of *grlA* and LEE genes in TW14359 with concurrent downregulation of flagellar genes is consistent with the study by Iyoda *et al.* (2006). Strict regulation of flagella and the TTSS is necessary for synchronized expression of motility and attachment, as simultaneous expression of both flagella and the TTSS might hinder efficient adhesion, and is energetically expensive. However, the reasons for discrepant expression of LEE and flagella between TW14359 and Sakai cannot be explained by the transcriptional snapshot of O157:H7 described in this study. A time-course investigation would reveal if there is a temporal lag characterized by an unsynchronized switch from flagellar to LEE expression between the two strains; mere intrinsic upregulation of flagella in Sakai upon exposure to epithelial cells is a less likely explanation of these results.

Conclusions

The present study demonstrates that in addition to gene content differences between the O157:H7 Spinach (TW14359) and Sakai outbreak strains, the two strains vary in their ability to colonize the epithelium and express certain genes. Specifically, TW14359 adhered to epithelial cells in greater numbers and expressed higher levels of shared virulence genes when compared to Sakai. Future large-scale functional genomics studies that examine a larger strain set representing more O157:H7 lineages and include additional time points are required to fully understand the extent of variation within the O157 pathogen population.

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