Tree, J.J. and Wang, D. and McInally, C. and Mahajan, A. and Layton, A. and Houghton, I. and Elofsson, M. and Stevens, M.P. and Gally, D.L. and Roe, A.J. (2009) *Characterisation of the effects of salicylidene acylhydrazide compounds on type three secretion in Escherichia coli O157:H7*. Infection and Immunity, 77 (10). pp. 4209-4220. ISSN 0019-9567

http://eprints.gla.ac.uk/7526/

Deposited on: 23 September 2009

Enlighten – Research publications by members of the University of Glasgow
http://eprints.gla.ac.uk

Characterisation of the effects of salicylidene acylhydrazide compounds on type three secretion in *Escherichia coli* O157:H7

Jai J. Tree[1], Dai Wang[2], Carol McInally[2], Arvind Mahajan[3], Abigail Layton[4], Irene Houghton[2], Mikael Elofsson[5], Mark P. Stevens[4], David L. Gally[1] and Andrew J. Roe[2]*

[1]Zoonotic and Animal Pathogens Research Laboratory, Immunity and Infection Division, The Roslin Institute and R(D)SVS, Chancellor's Building, University of Edinburgh, 49 Little France Crescent, Edinburgh, EH16 4SB

[2]Microbiology Research Group, Infection and Immunity, Glasgow Biomedical Research Centre, Faculty of Biomedical & Life Sciences, Glasgow, G12 8QQ.

[3]Cellular Microbiology Group, Division of Infection and Immunity, The Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Penicuik, EH25 9RG, UK.

[4]Division of Microbiology, Institute for Animal Health, Compton, Newbury, Berkshire, RG20 7NN, UK.

[5]Department of Chemistry, Umea University, SE-90187, Umea, Sweden.

*Communicating author: Andrew J Roe, e-mail: andrew.roe@bio.gla.ac.uk

Keywords: T3SS, inhibitor, EHEC, O157.

Running title: Effect of T3SS inhibitors on O157

Abstract

Recent work has highlighted a number of compounds that target bacterial virulence by affecting gene regulation. In this work, we show that small-molecule inhibitors affect the expression of the type III secretion system (T3SS) of *Escherichia coli* O157:H7 in liquid culture and when the bacteria are attached to bovine epithelial cells. The inhibition of T3SS expression resulted in a reduction in the capacity of the bacteria to form attaching and effacing lesions. Our results show a marked variation in the ability of four structurally-related compounds to inhibit the T3SS of a panel of isolates. Using transcriptomics, we provide a comprehensive analysis of the conserved- and inhibitor-specific transcriptional responses to the four compounds. These analyses of gene expression show that numerous virulence genes, located on horizontally-acquired DNA elements, are affected by the compounds but the number of genes significantly affected varied markedly between the compounds. Overall, we highlight the importance of assessing the effect of such "anti-virulence" agents on a range of isolates and discuss the possible mechanisms which may lead to the co-ordinate down-regulation of horizontally acquired virulence genes.

Introduction

Outbreaks of Enterohaemorrhagic *E. coli* (EHEC) infection occur sporadically in both the developed and developing world causing diarrhoeal disease that can progress to haemorrhagic colitis and haemolytic uremic syndrome (HUS) (22). *E. coli* O157:H7 is the dominant serotype in North America, parts of Europe and Japan, and its pathogenicity has been studied extensively at the molecular level. A number of key virulence factors are known to be required for disease progression including the Shiga toxins and the type III secretion system (T3SS) required for characteristic attaching and effacing (A/E) lesion formation (13). The T3SS is encoded on the locus of enterocyte effacement (LEE) pathogenicity island containing 41 genes on 5 main operons (18). Deletion of the LEE4 operon prevents A/E lesion formation and prevents colonisation of the terminal rectum, the principle colonisation site in cattle (24). To this extent, vaccines that target EHEC T3SS are being developed for application in cattle (28). Furthermore, given the central role of the T3SS for bovine colonization, it is highly likely that inhibition of a functional T3SS could prevent colonisation of humans, thereby limiting disease.

As the prevalence of antibiotic resistant strains increases, targeting virulence determinants of pathogenic bacteria has become an increasingly attractive alternative to bactericidal antibiotics. The major advantage of these compounds is significantly less positive selection on resistant mutants as the non-pathogenic normal flora are unaffected by treatment (14). Such a class of 'virulence blockers' has been described that inhibit T3SS in *Yersinia* spp. and have been shown to be broadly effective in a number of pathogenic bacteria that utilise T3SS including *Chlamydia* spp., *Salmonella enterica* serovar Typhimurium, and Enteropathogenic *E. coli* (EPEC). The mechanism of inhibition in each is incompletely understood. However, it is clear that transcription of T3S genes in these pathogens is reduced. Whether this is due to feedback from the T3S machinery or by direct effects on transcription factors remains to be determined. Treatment of bacteria with μM concentrations of inhibitors is sufficient to reduce *S.* Typhimurium invasion of cultured epithelial cells and induction of enteritis *in vivo* (11), arrest maturation of *Chlamydia* sp. inclusions (37), and reduce proliferation of *Yersinia pseudotuberculosis* (25). Further experiments have shown that the antichlamydial

activity of this family can be reversed by the addition of iron suggesting that the activity of these compounds may be linked to iron availability in the cell, although the mechanisms behind this reversal are unknown. In *Vibrio cholera* a 'virulence blocker' has been described that reduces expression of cholera toxin (CT) and the toxin co-regulated pilus (TCP). This inhibitor has been shown to act by post-translationally inactivating the AraC family regulator, ToxT, suggesting regulators of virulence genes may be an important target for this class of inhibitors (36). A recent study has also shown that a structurally distinct compound, LED209, can prevent the phosphorylation of the *E. coli* O157 QseC membrane protein, a sensor of host adrenergic molecules (30). This inhibition of QseC function leads to impaired expression of virulence factors and affects colonization of tissue culture cells by downregulation of Ler, the master regulator of the T3SS of *E. coli* O157 (30). Of particular importance to this study is a previous report that described the screening of virulence blockers in EPEC (9). This previous study showed that the addition of the compound, with structural similarity to the ones described in this work, transcriptionally down-regulated the major operons of the EPEC LEE. However, the extent to which other genes were affected was not examined. Here we characterize the effects of four structurally-related salicylidene acylhydrazide virulence blockers on global gene expression in *E. coli* O157:H7. These analyses show that transcriptional repression of the T3SS is also accompanied by repression of virulence genes encoded on mobile genetic elements demonstrating global effects on virulence gene regulation. We also show that these inhibitors effectively reduce formation of actin pedestals *in vitro*.

MATERIALS AND METHODS

Bacterial strains and plasmids

Unless otherwise stated, all experiments were performed using *E. coli* O157:H7 strain TUV-930, a Shiga-toxin negative derivative of strain EDL933 (3). To examine the effect of the inhibitors across a range of strains and phage-types (PTs) six additional *E. coli* O157 strains were used: ZAP 3 (PT 2), ZAP 11 (PT 21/28), ZAP 12 (PT 21/28), ZAP

229, (PT 2), ZAP 231 (PT 4) and ZAP 243 (PT 8). The green fluorescent protein (GFP) reporter plasmids pAJR71-75 used in this study have been described previously (32, 34).

Inhibitors and media

The compounds (ME0052-0055; Figure 1), selected on the basis of their ability to inhibit the T3SS of related pathogens (25) (2) (37), were prepared and supplied by Mikael Elofsson. The compounds have been previously reported using a number of different nomenclatures: ME0052 (INP0010 & compound 8), ME0053 (INP0403 & compound 11), ME0054 (INP0401 & compound 10) and ME0055 (INP0031 & compound 17) (25). Stock solutions were prepared in DMSO, aliquotted and stored at -20°C in the dark. The final DMSO concentration added to the bacteria was kept below 2% in all experiments. Minimal essential media with HEPES modification (MEM-HEPES, Sigma M7278) was used in this study to promote expression of the LEE pathogenicity island. This was supplemented with 250 nM $Fe(NO_3)_2$ and glucose to give a final concentration of 0.2 %. Antibiotics were included when required at the following concentrations: 12.5 $\mu g.ml^{-1}$ chloramphenicol and 25 $\mu g.ml^{-1}$ kanamycin. To examine the effects of higher concentrations of iron supplementation on the effects of the inhibitors, 200 µM $FeSO_4$ was added to the growth medium when stated.

Protein secretion and Western Blot analyses

Bacteria were cultured overnight in LB and diluted to an $OD_{600} \leq 0.05$ in prewarmed MEM-HEPES medium, supplemented with 250 nM $Fe(NO_3)_2$ and glucose to give a final concentration of 0.2 %. When required, the specific growth rate was reduced by addition of 0.1M or 0.2M NaCl to this media. Cultures were grown at 37 °C to an OD600 of 0.8 and then secreted proteins were extracted by TCA precipitation as described previously (35). For protein-localization experiments, whole-cell fractions were prepared by centrifugation (20 min, 4000 x ***g***), washed twice in 20 ml PBS, sonicated and resuspended in 400 µl protein A buffer (35). Proteins were analyzed by SDS-PAGE, and Western blotting for Tir, EspD and GroEL was carried out as described previously (35). The

Western blots were quantified using Scion image software allowing comparisons between different samples.

Transcriptomics

Transcriptional profiling of cultures treated with the inhibitors was carried out essentially as described previously (33). In brief, overnight cultures of *E. coli* TUV-930 grown in MEM-HEPES (Sigma) supplemented with 250 nM $Fe(NO_3)_2$ and glucose to a final concentration of 0.2% and then diluted to an OD600 of 0.1 in the same media. Inhibitor was added to test cultures at a final concentration of 20 μM and an equal volume of DMSO added to reference cultures. At an OD600 of 0.8, 15 ml cultures were stabilized in an equal volume of RNAprotect (Qiagen) and RNA extracted using a Qiagen RNeasy Mini kit. DNA contamination was removed by DNaseI treatment (Ambion). Total RNA was assessed for quality and quantified using an Aglient 2100 Bioanalyser. Synthesis of cDNA and labeling of total RNA was performed using an Amersham CyScribe Post-Labelling Kit as per manufactures instructions. The cDNA was hybridized to 70mer spotted oligonucleotide arrays containing ORFs from *E. coli* K-12, *E. coli* Sakai VT2, and *E. coli* EDL933 (University of Birmingham, UK) using a Genomic Solutions GeneTac hybridization machine. Hybridized slides were scanned using a Genepix 4000A scanner and GenePix 7.0 software (Axon Instruments, Union City, Calif.). Data were analyzed using Genespring GX 7.3.1 (Agilent). The MIAME compliant data are deposited on the Gene Expression Omnibus (GEO) at NBCI under the GEO accession number (GSE10319).

Regulon analysis for global regulators

In order to determine if post-translational modification of regulators was contributing to regulation by ME0052-55, the regulons for H-NS, LexA, FUR and PchA were defined from the literature and analysed in Genespring 7.3.1. The H-NS, LexA and FUR regulons have not been elucidated for *E. coli* O157 but have been described for K-12 and provides an indicator of activity within the *E. coli* common backbone. The H-NS regulon has previously been described by Oshima *et al* (26) and the LexA regulon has been described

for K-12 using transcriptomics and chromatin-immunoprecipitation (ChIP) studies (40) . For both of these regulons, K-12 gene designations were converted to O157 “z” numbers using Genespring 7.3.1 and by BLAST analysis. The FUR regulon has been described by Gamma-Castro *et al* (8) and was acquired from RegulonDB (http://regulondb.ccg.unam.mx/) and similarly converted to “z” numbers using Genespring 7.3.1 and BLAST. The PchA and Ler regulons were recently defined by transcriptomics and ChIP studies for *E. coli* O157 Sakai, a sequenced O157 outbreak strain from Japan. The Sakai PchA and Ler regulons were converted to EDL933 gene designations using the nucmer genomic alignment tool at coliBASE (http://xbase.bham.ac.uk/colibase/) and by BLAST analysis. Analysis of overlap between these regulons and genes affected by inhibitor treatment were performed in Genespring 7.3.1.

Determination of LEE expression using GFP reporter fusions

Expression of the LEE was determined by using a series of plasmid-based promoter::GFP reporter fusions, described previously (pAJR71-75) (35). To measure population fluorescence, triplicate 100 µl aliquots of transformed bacteria were dispensed into 96-well black microtitre plates and analysed using a BMG Fluostar plate reader at 37 °C. Cultures were also monitored at OD_{600} to monitor growth. Fluorescence was plotted against optical density using Microsoft Excel software and the line of best fit obtained. The promoter-less plasmid, pAJR70 (35) was employed to correct for background fluorescence of the strain and media. Comparisons between treated and culture treated with DMSO alone were determined at an OD_{600} of 0.8 to provide the best comparison with the data obtained by transcriptomics.

Fluorescence microscopy analysis of LEE1 and flagella expression

Embryonic bovine cells (German Collection of Microorganisms and Cell Cultures, no. ACC192) were prepared and cultured as described previously (32). TUV-930 pAJR71 (LEE1::GFP) was cultured in MEM-HEPES to OD_{600} 0.6 at 37 °C with 20 µM ME0055 or the same volume of DMSO. Bacteria were added to the multi-chamber slide and

incubated at 37°C. The samples were fixed at intervals by removal of the culture and addition of 4 % paraformaldehyde (PFA). Detection of flagella expression was carried out as described previously (10). The adherent bacteria were stained with rabbit anti-O157 and anti-H7 polyclonal antiserum (Mast Diagnostics; diluted 1:500 in PBS) at RT for 30 min, washed then incubated with TRITC-labeled secondary antibody (Sigma anti-rabbit; diluted 1:1000). Time points analyzed were 60, 120, 180 and 300 minutes after bacterial addition. To measure single-cell expression by fluorescence microscopy, a Z-stack of 20 images was captured at a spacing of 0.1 μm on a Zeiss Axioimager M1 fluorescence microscope equipped with a Hamamatsu ORCA AG camera using Volocity software (Improvision). These images were used to create a composite image that reduced the spatial effects of bacteria in different focal planes. GFP intensity was determined using Volocity software with a threshold of 2000 relative fluorescence units as the cut-off point to separate GFP "positive" and GFP "negative" bacteria.

Detection and quantification of A/E lesions

To detect host cell actin, cells were permeabilized using 0.2% Triton X100 then treated for 20 min at RT with TRITC-Phalloidin (5μg.ml^{-1}; Sigma) washed twice with PBS and mounted in fluorescent mounting medium (DAKO). Improvision Volocity software (Quantification) was used to examine 3 independent replicates of the experiment and sampling 15 separate fields per replicate. Bacteria and areas of concentrated actin were identified using size and fluorophore as selection parameters. The percentage of total bacteria in each field associated with condensed actin was then determined.

RESULTS

Effects of salicylidene acylhydrazide inhibitors on growth rate.

Our initial work aimed to determine the effects of the compounds on bacterial growth rate. We were aware that marked changes in growth rate result in changes of global gene expression making any specific effects of the compounds hard to determine. Bacteria were cultured in MEM-HEPES media containing a range (10 – 50 μM) of the four compounds. At both 10 and 20 μM we observed only minor changes in growth rate

(Table 1) for all 4 compounds. Addition of higher concentrations (50 μM) of the compounds had more marked effects on specific growth rate (Table 1). We therefore focused our studies the 20 μM concentration to minimize any effects of altered growth rate on gene expression.

Effect of salicylidene acylhydrazide inhibitors on secretion of T3SS effector proteins.

In order to characterize the effects of the four inhibitors on the secretion of effector proteins by the T3SS, total secreted proteins were prepared from strain TUV-930 cultured in MEM-HEPES medium containing 20 μM of the inhibitor or the equivalent volume of DMSO. Secreted protein profiles were analysed by Colloidal blue staining of the total secreted proteins produced by the bacteria as well as Western blotting to quantify both the whole cell expression and secretion of the translocated intimin receptor (Tir) secretion. Addition of 20 μM of the compounds resulted in inhibition of Tir expression for all four compounds with ME0053 and ME0055 being the most effective, reducing Tir expression in the whole cell fraction by >90% (Figure 2). Analysis of the secreted fractions resulted in no detectable Tir when the bacteria were treated with ME0055, which correlates with the major reduction in whole cell expression by this compound. Treatment with ME0053 gave a reduced but detectable signal implying that, while whole cell expression of Tir was reduced, its secretion was not strongly inhibited. ME0052 and ME0054 were less effective, reducing Tir expression by 39% and 54% respectively. ME0054 resulted in strong inhibition of Tir secretion whereas ME0052 did not appear to reduce secretion of Tir and the secreted fraction correlated closely to that of the whole cell fraction.

The same samples were also tested for the translocator protein, EspD that is transcribed from a different operon within the LEE. Addition of ME0054 or ME0055 gave strong inhibition of whole cell EspD expression (>95% reduction) and correspondingly low (ME0054) or undetectable (ME0055) levels of EspD in the supernatant fraction (Figure 2). ME0053 was less effective at blocking whole cell expression although the level of secreted EspD was markedly reduced. ME0052 was the least effective inhibitor and both

whole cell expression and secreted levels showed similar reductions to those observed for Tir.

These data were confirmed by Colloidal blue visualization of total secreted proteins which showed a concentration-dependent inhibitory effect on the overall secretion profile that varied between compounds with ME0055 being most effective, followed by ME0054, ME0053 and ME0052 (data not shown). As a control, intracellular GroEL levels were also assessed by Western analysis (Figure 2). These results showed that the compounds do not affect general protein expression or stability and less than 15% variation between the samples was observed.

To examine if the observed inhibition of translocator and effector protein expression and secretion was due to the slight reduction of bacterial growth rate caused by addition of the compounds, we tested the relationship between growth rate and secretion phenotype. To reduce bacterial growth rate independently of the salicylidene acylhydrazide inhibitors, NaCl was added to increase the osmolarity of the growth medium. When compared to the bacteria cultured in MEM-HEPES alone, the addition of NaCl caused an increase in Tir protein secretion (Figure 2). Additional of 0.1M NaCl reduced bacterial growth by 18% (data not shown) and increased the level of secreted Tir by 34% (Figure 2). Addition of 0.2M NaCl reduced the growth rate by 44% leading to an increased level of Tir secretion by 61%. These data suggest that the decrease in growth rate seen by addition of the salicylidene acylhydrazide inhibitors is unlikely to account for the inhibition of T3S.

To examine if the compounds would act across a range of strains, six further *E. coli* O157 isolates were tested against all four compounds (Table 2). These consisted of three isolates from human outbreaks (ZAP 229, ZAP 231 and ZAP 243) and three isolates sourced from bovine samples (ZAP3, ZAP 11 and ZAP 12). These strains cover 4 different phage types (2, 4, 8, and 21/28), all commonly associated with disease outbreaks in the UK. For these assays, any variation in the extent of the secretion inhibition was determined by blotting for secreted Tir (Table 2). Addition of ME0052, whilst largely ineffective in TUV-930, resulted in inhibition of Tir secretion in the additional 6 strains

(Table 2). ME0053 showed inhibition against TUV-930 and the addtional 6 strains, but the extent of inhibition varied considerably. ME0054 was the most effective inhibitor across the entire strain collection. In comparison, ME0055 which was highly effective against 4 strains was far less inhibitory when tested against ZAP229, ZAP231 and ZAP243, reducing Tir secretion by only 43-60% (Table 2).

Analysis of transcriptional changes resulting from inhibitor addition.

In an effort to understand the molecular mechanism by which the inhibitory compounds function we carried out transcriptional profiling experiments. Strain TUV-930 was grown to an OD_{600} of 0.8 in MEM-HEPES media in the presence or absence of 20 μM of inhibitor and RNA harvested and labeled for microarray analysis. Table 3 shows cumulative results for genes with >2 fold change in all 4 inhibitor samples. The data show that all 4 inhibitors reduce the expression of all LEE-encoded genes (Figure 3). Furthermore, in addition to repressing the LEE, all tested inhibitors also repress transcription of the non-LEE encoded effectors *nleA*, *espJ*, and *espN*, and pO157 ORFs *L7027* and *L7037*. The compounds predominantly repressed gene expression and they varied in the total number of genes affected. For example, using a cut-off of a >3 fold change in transcription, ME0052 significantly ($p \leq 0.05$) affected the transcription of 181 genes whereas this was reduced to 24 genes for ME0055 (Tables 3, 4 & 1S).

Repression of cryptic prophage encoded genes was observed across all inhibitors with 52% (ME0052), 57% (ME0053), 78% (ME0054) and 19% (ME0055) of affected genes encoded on cryptic prophage (Table 4). Genes with >2 fold or >3 fold change for each inhibitor were found to be significantly biased towards laterally acquired O-island (OI) encoded DNA (27) for compounds ME0052, ME0053 and ME0054 using a Chi squared test ($P<0.0001$, Table 4). ME0055 fell short of this level of significance, probably due to the small subset of genes affected by this compound.

The finding that the transcriptional changes induced by salicylidene acylhydrazide compounds are biased towards a subset of OI-encoded genes suggested that a global regulator of horizontally-acquired DNA was being affected by the inhibitory compounds.

We therefore examined the array data to determine if any of the established regulators of the LEE and other horizontally-acquired genes was affected by the compounds. 17 previously-characterised regulators (see (19) for an excellent review) were analysed to determine if the compounds resulted in transcriptional changes to their expression. For several of these, the analysis was expanded to examine the genes known to be controlled by these regulators. Figure 4 shows the effect of all four compounds on the regulators. It is clear that the majority (*hha*, *sdiA*, *recA*, *rpoS*, *qseA*, *fis*, *yhiF*, *yhiE*, *lexA* and *hns*) show no marked changes in their transcription. To examine if compounds could be affecting the regulators post-transcriptionally, the regulons of H-NS, LexA and RecA were examined.

The histone-like nucleoid structuring protein (H-NS) is a pleiotropic regulator of gene expression that plays an important role in transcriptional inactivation of horizontally acquired DNA (7, 23) . We used the *E. coli* K-12 H-NS regulon described by Oshima *et al* (26) to look for transcriptional changes within the *E. coli* K-12 "backbone" (genes that are common to both K-12 and O157) that would be indicative of changes in H-NS regulation. Of the 63 genes affected by deletion of H-NS we found that 10 genes were significantly ($p \leq 0.05$) up or down regulated at least two fold, however the majority of the regulon (53 genes) were unaffected by addition of inhibitor (Suppl. Figure 1SA & B). These data suggest that the compounds do not function by affecting global H-NS regulation.

LexA and RecA mediate the SOS response to DNA damage in *E. coli* K-12. The SOS response is known to be a key regulator of lambdoid bacteriophage transcription as RecA stimulates auto-cleavage of the phage repressor, cI in response to DNA damage, triggering a cascade that leads to transcription of the Q antiterminator transcript and the lytic cycle. LexA has also been shown to regulate the LEE in Enteropathogenic *E. coli* placing both the LEE and potentially phage regulated transcripts under the control of the SOS response (21). However, transcripts within the LexA regulon were not significantly repressed in inhibitor treated cultures. It therefore seems unlikely that inhibition of LexA de-repression is the mechanism of transcriptional silencing of T3SS (Suppl. Figure 1SC).

Whereas the majority of the regulators showed no changes in expression by addition of the compounds, the LEE-located regulators *ler* (encoding Ler), *grlA* and *grlR* all show consistent down-regulation upon treatment with all four inhibitors. The finding that *ler* and *grlA/R* show co-ordinate down-regulation is consistent with previous reports (6) (12). The genes encoding *pchA-C* also showed some changes, particularly after addition of ME0055, the most potent inhibitor of Tir secretion. The T3S regulators, Ler and PchA, have been shown to bind and positively activate transcription of a number of genes encoded on O-islands (1). Comparison of genes affected by inhibitor treatment and the Ler/PchA regulon defined by Abe and co-workers (2008, L1 and L2) indicates that significant overlap exists between these regulons (Supp. Table 1S). Fifty two of 100 genes repressed by ME0052, 39/60 repressed by ME0053, 11/21 repressed by ME0054, and 6/19 repressed by ME0055 are bound or regulated by Ler or PchA. PchA binds and positively regulates a number of secreted effectors encoded on horizontally acquired elements such as cryptic prophage. Supplementary Table 1S shows that a number of the non-LEE encoded effectors are repressed by inhibitor treatment indicating that in addition to repressing transcription of the T3SS machinery, translocated effectors encoded outside of the LEE are also down regulated.

Iron starvation is proposed to play a key role in the virulence attenuating properties of these T3S inhibitors in *Chlamydia pneumoniae* (37). Related compounds have been shown to sequester iron and addition of high concentrations (200 μM) of iron sulfate or iron chloride to inhibitor treated cultures is able to reverse the inhibitory effects of these compounds. Our own studies support this finding in that addition of 200 μM $FeSO_4$ reverses the effect of ME0053 in TUV-930 (data not shown). In order to assess the effects of the inhibitors on iron availability within the cell, we used the RegulonDB database definition of the Fur regulon in *E. coli* K-12 (http://regulondb.ccg.unam.mx/) (8). Of 16 genes known to be Fur regulated none were significantly affected by addition of inhibitor (Suppl. Figure 1SD). Similarly, using the GO ontology “iron transport” sub-set of 29 genes (GO6826), no genes involved in iron transport were affected by all four inhibitors indicating intracellular iron supplies are not affected at the concentration of inhibitor used in this study (Suppl. Fig. 1S panel E). It should be noted that the growth

media contained 250 nM $Fe(NO_3)_2$ which might mask any effects on iron availability. Finally, to confirm that the 20 μM concentration of inhibitor was not modulating gene expression by perturbing bacterial growth rate, we analysed both the *rpoS* (z4049) and *groEL* genes (z5748) and found them to be not significantly affected by addition of the four compounds (data not shown).

Whilst the majority transcriptional change conserved across all four inhibitors were down regulated, *pspDC* and *yjiY* were significantly upregulated by inhibitor treatment ($p \leq 0.05$). *pspBC* is part of the phage shock protein response and analysis of the transcriptional data indicates that the rest of the operon encoding *pspAB* and *pspE* is induced albeit to a lesser degree. The phage shock protein response has previously been reported to be induced by loss of proton motive force, notably through the insertion of filamentous phage pIV secretin. It is also essential for membrane insertion of the YscC secretin of the Yersinia T3SS (5). *yjiY* was also consistently induced in inhibitor treated cultures however there has been little characterisation of this protein apart from its induction under conditions of stress such as growth in urine and pH stress (17, 38)

Reporter gene analysis

As an independent verification of the array data we used a series of GFP reporter fusions to analyze the effects of the compounds on the promoters of the LEE and the *rpsM* gene as a control. Previous work has shown that the majority of LEE genes are transcribed by promoters that encompass 5 polycistronic operons named LEE1-5 (20). We therefore used LEE1-5 plasmid-based GFP reporter fusions (35) to verify the transcriptomic data and show inhibition of LEE transcription. The same 20 μM concentration of each compound was used as in the transcriptional profiling to provide a direct comparison of these data. Expression of LEE1-5 was repressed in the presence of the inhibitors, consistent with microarray results (Table 5). The control fusion, consisting of the *rpsM* promoter which drives expression of the small ribosomal RNA protein, was unaffected by addition of the compounds consistent with the microarray findings (Table 4).

Effect on A/E lesion formation

The Western analyses demonstrated that compound ME0055 was the most effective of the four compounds at reducing expression and, therefore, secretion of the key virulence-associated effector protein, Tir. However, all the data presented above consisted of *in vitro* assays, the majority of which used tissue culture media to stimulate expression of the LEE. This raised the question of whether the compound would affect the ability of *E. coli* O157 to bind host cells and form A/E lesions. Using a bovine epithelial cell line (EBL) we analysed the ability of TUV-930 to form A/E lesions. Following a 6 hour infection period, we observed that the bacteria cultured in the presence of 20 μM ME0055 were still capable of condensing host cell actin and forming distinctive pedestals (Figure 5b). However, it was evident that the proportion of bacteria forming A/E lesions was reduced compared to the culture treated with DMSO alone (Figure 5a). In order to quantify any differences, we used Volocity Quantification (Improvision) to identify bacteria associated with areas of dense actin within the 3-D images. This was achieved using size and fluorescence intensity thresholds to identify bacteria, condensed actin and then areas of clear co-localisation. This automated approach allowed us to collate the data from some 15 individual fields collected across triplicate repeats of the experiment. These analyses indicated that after 6 hours, 28% (± 3%) of bacteria treated with ME0055 were associated with A/E lesions (Figure 5b). This was significantly less ($P<0.001$) than the culture treated with DMSO alone for which some 69% (± 16%) of bacteria formed A/E lesions (Figure 5a).

Effect on LEE1 gene expression and flagellae production during contact with bovine epithelial cells

The reduction in the ability of TUV-930 to form A/E lesions implied that addition of compound ME0055 resulted in suppression of LEE expression even during contact with host epithelial cells. To examine this result further, we performed cell binding assays using TUV-930 transformed with a LEE1::GFP reporter plasmid (pAJR71) to allow measurement of the *ler* expression during contact with epithelial cells. Additionally, our observation that the compounds affected *grlA* and *grlR* expression implied that flagella production may be increased as these regulators have been shown to cross-regulate the LEE and flagellae (12). Furthermore, recent work has shown that during the cell infection

process, *E. coli* O157 undergo a rapid temporal shift from the expression of H7 flagella to production of the T3SS (15). We therefore questioned if the inhibitory compounds would affect this transition. To test this, immuno-fluorescence antibody straining was used to examine any effects on the expression of H7 flagellae. After addition of the bacteria to the EBL cell line, the expression of LEE1::GFP was determined at fixed time points by addition of 4% PFA. Figure 6 shows that 60 minutes after addition of TUV-930 to the EBL cell line there was a significant difference in LEE1::GFP expression when ME0055 treated cells are compared with the culture treated with DMSO alone. Both the proportion of bacteria expressing GFP and the GFP intensity of bacteria which were GFP "positive" were measured to be significantly different. Over the time course, GFP levels are seen to reduce in the bacteria treated with DMSO alone. The ME0055 treated cells follow a similar pattern in that GFP levels fall throughout the time course but both the proportion of GFP positive bacteria and the GFP intensity of these bacteria were observed to be consistently reduced compared to the culture treated with DMSO alone. Staining for H7 revealed that the addition of ME0055 led to an increase in flagellal expression compared to the bacteria treated with DMSO alone. Over the time course, H7 expression increased from 20% of the population at 60 minutes to 40% at 120 minutes and was still high (30%) some 5 hours after addition of the bacteria to the eukaryotic cell line. These data indicate that addition of ME0055 suppresses LEE1 expression, even during host cell contact and leads to an increase in H7 expression. Clearly, both these observations correlate well with the reduction in A/E lesion formation.

DISCUSSION

The central role of T3SSs in the pathogenesis of many bacterial species provides the impetus to develop strategies to interfere with their function. To this effect, 'virulence blockers' have been described that selectively target expression or function of T3SSs in a number of bacterial species including *Y. pseudotuberculosis*, *S.* Typhimurium, *C. pneumoniae*, and EPEC. There exists marked conservation of elements of the T3SS between EPEC and EHEC, yet the transcriptional control of these systems is varied with many regulators of T3S being encoded on horizontally acquired DNA in this polylysogenic pathogen (31, 41, 42). The variation in T3S regulation observed between

isolates of EHEC O157 has also been suggested to play an important role in the epidemiology of this pathogen (16). As salicylidene acylhydrazide inhibitors of T3S repress transcription of the T3SS, detailed investigation of the effects of these compounds on EHEC O157 is warranted. In this study we attempted to address two questions: firstly, is the T3S inhibition demonstrated in other pathogens observed in EHEC O157 and is there serotype- and strain- specific variation in these responses. Secondly, are there conserved- and/or inhibitor-specific transcriptional responses that may help us to understand their mode of action?

In agreement with these previous studies examining repression of bacterial T3SS, we observed that all four compounds were capable of inhibiting secretion of virulence proteins from *E. coli* O157:H7. Addition of higher concentrations of the compounds had marked effects on growth rate and were not used in subsequent experiments. At 20 μM inhibition of Tir and EspD was observed without major effects to GroEL levels or growth rate. Further, relative transcription levels of *rpoS* (z4049) or *groEL* genes (z5748) were not found to be significantly ($p \leq 0.05$) affected by addition of any of the 4 compounds. This indicated that the transcriptional changes observed could not be simply attributed to changes in bacterial growth rate.

The repression of T3S in a diverse range of pathogens suggests that there exists a common, conserved target. The most conserved region of the T3SS lies in the basal apparatus proteins and has recently been proposed to be the target of related inhibitors in *Shigella flexneri* (39). We note that the results presented in this study are not contradictory to our findings as these authors show that treatment with inhibitor decreases the amount of basal apparatus found in the membrane, suggesting that inhibition occurs at a very early stage of assembly and insertion, or at the level of transcription. We would anticipate that if these inhibitors bind proteins of the T3S complex, related strains with conserved basal apparatus proteins would likely all be affected by the compounds to a similar, if not the same extent. In the present study we found significant variation in the level of inhibition of T3S between strains. We propose that this reflects inhibition through a more variable route such as the repertoire of LEE-regulators in any one strain. Indeed we previously reported that the EHEC O157:H7 Sakai strain differed from EHEC

O26:H- strain 193 in the presence of the ETT2-encoded negative regulators of LEE z3720 and z3734 (42), and variability in expression of the *pchABCX* genes has recently been described (41). We therefore favor a model of inhibition involving transcriptional repression of T3S that would be consistent with the strain variation and the global transcriptional changes we observe.

To examine the effect of the compounds on gene regulation we examined the transcriptional profile of cells treated with all four inhibitors. The results presented indicate that ME0052-55 represses transcription of the LEE and non-LEE encoded virulence factors. Surprisingly, ME0052 which was the least effective compound at limiting Tir secretion, produced the greatest effect on global gene transcription. Conversely, ME0055 showed much better repression of protein secretion but more limited global repression of transcription. Perhaps critically, *espA*, encoding the T3 translocation filament, was strongly repressed by ME0055 and may indicate that repression of the translocon can have a more significant overall effect on protein secretion.

Related salicylidene acylhydrazide compounds have been shown to repress expression of T3SS in *C. pneumoniae*, a phenotype that can be reversed by addition of high concentrations of iron. These findings are supported by our own results which show that addition of 200 μM $FeSO_4$ is able to reverse the effect of compound ME0053 in TUV-930. In order to assess the effects of these compounds on iron availability within the cell, we analyzed the transcriptome for changes that would be indicative of iron starvation. The ferric uptake regulator (Fur) is a key regulator of iron uptake under conditions of iron starvation, repressing expression of iron uptake pathways and virulence determinants such as Shiga toxin under iron replete conditions. We did not observe marked changes in the Fur regulon or in genes associated with iron transport in inhibitor treated cells (Supp. Figure 2D & 2E) indicating that they are not iron starved.

Repressed transcripts were biased towards O-island encoded genes, particularly those encoded on cryptic prophage (Table 4), many of these encode secreted effectors of the T3SS. One explanation for the bias towards repression of the OI genes would be that one

or more key regulators of horizontally-acquired genes are affected by the addition of the compounds. On this basis we examined the expression of regulators known to play a role in co-ordinate expression of virulence genes including H-NS, LexA, Fur, PchA and Ler. There was little indication within the transcriptional data that H-NS or LexA regulons were significantly affected by inhibitor treatment (Figure 1SC). In contrast, PchA and Ler were significantly repressed by inhibitor treatment.

Recent transcriptional profiling and chromatin immunoprecipitation experiments by Abe and co-workers (1) have shown that both Ler and PchA can globally regulate transcription of the LEE and a significant number of non-LEE encoded effectors. For many of these recently identified effectors this is the first indication of a regulatory mechanism that co-ordinates transcription with the LEE. Transcriptional profiling of inhibitor treated cells indicates that repression of transcription is not confined to the LEE but is extended to pathogenicity island encoded virulence determinants such as the non-LEE encoded effectors, elements of the Type II secretion pathway, and haemolysins encoded on pO157. Comparison of the Ler/PchA regulon with genes conserved across all four inhibitor treatments shows that 6 of the 12 non-LEE encoded genes repressed by all inhibitor treatments are bound or regulated by PchA and/or Ler, and 30/166 are significantly repressed by at least one inhibitor treatment. PchA homologues exist in many pathogenic and non-pathogenic bacteria including *Yersinia* (YpsIP31758_0776), *Salmonella* (NP_700416.1), *Shigella* (NP_599072.1), EPEC (PerC), UPEC (NP_754054.1 and NP_755073.1) and non-pathogenic *E. coli* (*yfdN*) (29) and may contribute to expression of horizontally acquired elements in these bacteria. Our results suggest that modulating the activity of the PchA regulon may contribute to the broad anti-virulence effects of these inhibitors in EHEC and, potentially, in other pathogenic bacteria. Clearly, inhibition of Pch function, through changes in its expression or activity would be one possible mechanism for how numerous OI-encoded genes are co-ordinately down-regulated by the addition of the compounds. Simultaneous repression of a number of virulence determinants would contribute to the effectiveness of these inhibitors to limit colonization of cattle and disease in humans.

In conclusion we have shown that salicylidene acylhydrazide inhibitors selectively inhibit the transcription of the T3SS and other OI-encoded genes of *E. coli* O157. Our data suggest that, in *E. coli* O157 these compounds act through regulators rather than directly at the level of the type three secretion system apparatus. We have also shown inhibitor-, and strain-specific effects that should be taken into consideration when determining the mode of action and therapeutic potential of such molecules. Our ongoing studies are using a combination of transposon mutagenesis screening and affinity purification to identify possible protein targets.

ACKNOWLEDGEMENTS

This research was supported by funding from Tenovus Scotland to AJR and a Medical Research Scotland grant (ref. 223 ORG G 0709) to AJR and DW. DLG and JJT are supported by grants VF0304 and VT0102 from the Department for Environment and Rural Affairs (DEFRA). MPS and AL gratefully acknowledge the support of the Biotechnology & Biological Sciences Research Council (BB/D010632/1). Infection assays were performed in conjunction with AM at the Easter Bush. We are grateful for the input of Professor Tim Mitchell and his pathogenesis group at University of Glasgow for helpful ideas and support.

FIGURE LEGENDS

FIG. 1: Structures of ME0052, ME0053, ME0054 and ME0055. The compounds have been previously reported using a number of different nomenclatures: ME0052 (INP0010 & compound 8), ME0053 (INP0403 & compound 11), ME0054 (INP0401 & compound 10) and ME0055 (INP0031 & compound 17).

FIG. 2: ME0052, ME0053, ME0054 and ME0055 inhibit expression and secretion of the effector proteins Tir and EspD, from *E. coli* O157:H7. Secreted (S/N) and whole cell (W/C) fractions were prepared from bacteria cultured in MEM-HEPES. Samples were separated by SDS-PAGE and analysed by Western blotting. The antibodies used for the blots are indicated on the left. To examine the effect of decreased growth rate on Tir secretion, *E. coli* O157:H7 was cultured in the absence (No addition: N/A) or presence of NaCl and secreted Tir detected by Western blotting. The concentration of NaCl added is indicated.

FIG. 3: A: Effect of all ME0052, ME0053, ME0054 and ME0055 on the transcription of the LEE. The fold change ($\log_2$) in transcription of treated versus culture treated with DMSO alone has been plotted for all the genes encoded on the LEE (OI-148A). The strong repressive effect of the compounds on LEE operons 1-5 can be contrasted to the Prophage 933L genes on the left hand side of the figure. B: As a reference, a diagram of the LEE from strain EDL933 is presented showing the 5 polycistronic operons: LEE1-5. The figure was produced from using Colibase (http://xbase.bham.ac.uk/colibase/).

FIG. 4: Effect of ME0052, ME0053, ME0054 and ME0055 on the transcription of known regulators of the LEE. The fold change (log_2) in transcription of treated versus cultures treated with DMSO alone has been plotted for 17 characterised regulators.

FIG. 5: Addition of ME0055 affects formation of attaching and effacing lesions on bovine epithelial cells (EBLs). Bacteria were cultured in the presence of DMSO (Fig. 5a) or 20 μM ME0055 (Fig. 5b) and added to the EBL cell line. After 6 hours, the cells fixed using 4% paraformaldehyde and stained to show host cell actin (red, TRITC-phalloidin) and bacteria (green, α-O157 and Alexifluor 488 secondary conjugate). Composite, overlay images from 15 Z stack sections are presented.

FIG. 6: Effect of *ler* gene expression during contact with bovine epithelial cells (EBLs) in the DMSO (panels A1-A4) or ME0055 (panels B1-B4) treated bacteria. Bacteria transformed with the lee1::GFP reporter plasmid (pAJR71) were added to EBL cells, the samples fixed using 4% PFA, and visualised using α-O157/H7 antibodies and Alexifluor 555 secondary conjugates. Time points analyzed were 60 (A1/B1), 120 (A2/B2), 180 (A3/B3) and 300 (A4/B4) minutes after bacterial addition. Composite, overlay images from 20, 0.1 μM Z stack sections are presented. Panel C: quantification of lee1::GFP expression during bacterial contact with host cells. Samples marked "-" and "+" contained DMSO or ME0055 respectively. Panel C1-C4 shows the quantified level of GFP per bacterial cell (relative fluorescence units) for the same time points as described in FIG. 6A and B. The proportion of bacteria that showed GFP expression above a threshold level and the proportion of bacteria that were found to express flagellae are also shown (red bars).

FIG. 1S: Analysis of H-NS, LexA and iron utilization/transport regulons of inhibitor treated cultures. The fold change (log_2) in transcription of treated versus cultures culture treated with DMSO alone has been plotted for the genes within each regulon: A&B, H-NS regulon presented on RegulonDB v6.2 (regulondb.ccg.unam.mx/). C, LexA regulon. D, FUR regulon. E, GO "iron uptake" associated genes.

REFERENCES

1. **Abe, H., A. Miyahara, T. Oshima, K. Tashiro, Y. Ogura, S. Kuhara, N. Ogasawara, T. Hayashi, and T. Tobe.** 2008. Global regulation by horizontally transferred regulators establishes the pathogenicity of Escherichia coli. DNA Research **15:**25-38.
2. **Bailey, L., A. Gylfe, C. Sundin, S. Muschiol, M. Elofsson, P. Nordstrom, B. Henriques-Normark, R. Lugert, A. Waldenstrom, H. Wolf-Watz, and S. Bergstrom.** 2007. Small molecule inhibitors of type III secretion in Yersinia block the Chlamydia pneumoniae infection cycle. Febs Letters **581:**587-595.
3. **Campellone, K. G., and J. M. Leong.** 2003. Tails of two Tirs: actin pedestal formation by enteropathogenic *E. coli* and enterohemorrhagic *E. coli* O157:H7. Current Opinion in Microbiology **6:**82-90.
4. **Chase-Topping, M., D. Gally, C. Low, L. Matthews, and M. Woolhouse.** 2008. Super-shedding and the link between human infection and livestock carriage of Escherichia coli O157. Nat Rev Microbiol **6:**904-12.
5. **Darwin, A. J.** 2005. The phage-shock-protein response. Mol Microbiol **57:**621-8.
6. **Deng, W. Y., J. L. Puente, S. Gruenheid, Y. Li, B. A. Vallance, A. Vazquez, J. Barba, J. A. Ibarra, P. O'Donnell, P. Metalnikov, K. Ashman, S. Lee, D. Goode, T. Pawson, and B. B. Finlay.** 2004. Dissecting virulence: Systematic and functional analyses of a pathogenicity island. Proceedings of the National Academy of Sciences of the United States of America **101:**3597-3602.
7. **Dorman, C. J.** 2004. H-NS: A universal regulator for a dynamic genome. Nature Reviews Microbiology **2:**391-400.
8. **Gama-Castro, S., V. Jimenez-Jacinto, M. Peralta-Gil, A. Santos-Zavaleta, M. I. Penaloza-Spinola, B. Contreras-Moreira, J. Segura-Salazar, L. Muniz-Rascado, I. Martinez-Flores, H. Salgado, C. Bonavides-Martinez, C. Abreu-Goodger, C. Rodriguez-Penagos, J. Miranda-Rios, E. Morett, E. Merino, A. M. Huerta, L. Trevino-Quintanilla, and J. Collado-Vides.** 2008. RegulonDB (version 6.0): gene regulation model of Escherichia coli K-12 beyond transcription, active (experimental) annotated promoters and Textpresso navigation. Nucleic Acids Research **36:**D120-D124.
9. **Gauthier, A., M. L. Robertson, M. Lowden, J. A. Ibarra, J. L. Puente, and B. B. Finlay.** 2005. Transcriptional inhibitor of virulence factors in enteropathogenic Escherichia coli. Antimicrobial Agents and Chemotherapy **49:**4101-4109.
10. **Giron, J. A., A. G. Torres, E. Freer, and J. B. Kaper.** 2002. The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. Molecular Microbiology **44:**361-379.
11. **Hudson, D. L., A. N. Layton, T. R. Field, A. J. Bowen, H. Wolf-Watz, M. Elofsson, M. P. Stevens, and E. E. Galyov.** 2007. Inhibition of type III secretion in Salmonella enterica serovar typhimurium by small-molecule inhibitors. Antimicrobial Agents and Chemotherapy **51:**2631-2635.
12. **Iyoda, S., N. Koizumi, H. Satou, Y. Lu, T. Saitoh, M. Ohnishi, and H. Watanabe.** 2006. The GrlR-GrlA regulatory system coordinately controls the expression of flagellar and LEE-encoded type III protein secretion systems in enterohemorrhagic Escherichia coli. Journal of Bacteriology **188:**5682-5692.
13. **Jerse, A. E., J. Yu, B. D. Tall, and K. J.B.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87:**7839-7843.
14. **Keyser, P., M. Elofsson, S. Rosell, and H. Wolf-Watz.** 2008. Virulence blockers as alternatives to antibiotics: type III secretion inhibitors against Gram-negative bacteria. Journal of Internal Medicine **264:**17-29.
15. **Mahajan, A., C. G. Currie, S. Mackie, J. Tree, S. McAteer, I. McKendrick, T. N. McNeilly, A. Roe, R. M. La Ragione, M. J. Woodward, D. L. Gally, and D. G. Smith.** 2009. An

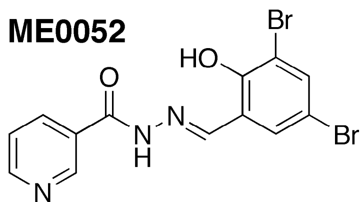
investigation of the expression and adhesin function of H7 flagella in the interaction of Escherichia coli O157 : H7 with bovine intestinal epithelium. Cell Microbiol **11:**121-37.

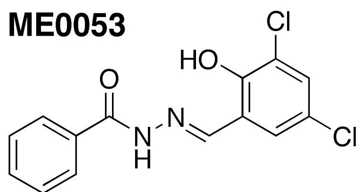
16. **Matthews, L., J. C. Low, D. L. Gally, M. C. Pearce, D. J. Mellor, J. A. Heesterbeek, M. Chase-Topping, S. W. Naylor, D. J. Shaw, S. W. Reid, G. J. Gunn, and M. E. Woolhouse.** 2006. Heterogeneous shedding of Escherichia coli O157 in cattle and its implications for control. Proc Natl Acad Sci U S A **103:**547-52.
17. **Maurer, L. M., E. Yohannes, S. S. Bondurant, M. Radmacher, and J. L. Slonczewski.** 2005. pH regulates genes for flagellar motility, catabolism, and oxidative stress in Escherichia coli K-12. J Bacteriol **187:**304-19.
18. **McDaniel, T. K., K. G. Jarvis, M. S. Donnenberg, and J. B. Kaper.** 1995. A genetic-locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proceedings of the National Academy of Sciences of the United States of America **92:**1664-1668.
19. **Mellies, J. L., A. M. S. Barron, and A. M. Carmona.** 2007. Enteropathogenic and enterohemorrhagic Escherichia coli virulence gene regulation. Infection and Immunity **75:**4199-4210.
20. **Mellies, J. L., S. J. Elliott, V. Sperandio, M. S. Donnenberg, and J. B. Kaper.** 1999. The Per regulon of enteropathogenic *Escherichia coli*: identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). Molecular Microbiology **33:**296-306.
21. **Mellies, J. L., K. R. Haack, and D. C. Galligan.** 2007. SOS regulation of the type III secretion system of enteropathogenic Escherichia coli. Journal of Bacteriology **189:**2863-2872.
22. **Nataro, J. P., and J. B. Kaper.** 1998. Diarrheagenic *Escherichia coli*. Clinical Microbiology Reviews **11:**403.
23. **Navarre, W. W., M. McClelland, S. J. Libby, and F. C. Fang.** 2007. Silencing of xenogeneic DNA by H-NS - facilitation of lateral gene transfer in bacteria by a defense system that recognizes foreign DNA. Genes & Development **21:**1456-1471.
24. **Naylor, S. W., A. J. Roe, P. Nart, K. Spears, D. G. E. Smith, J. C. Low, and D. L. Gally.** 2005. *Escherichia coli* O157:H7 forms attaching and effacing lesions at the terminal rectum of cattle and colonization requires the *LEE4* operon. Microbiology **151:**2773-2781.
25. **Nordfelth, R., A. M. Kauppi, H. A. Norberg, H. Wolf-Watz, and M. Elofsson.** 2005. Small-molecule inhibitors specifically targeting type III secretion. Infection and Immunity **73:**3104-3114.
26. **Oshima, T., S. Ishikawa, K. Kurokawa, H. Aiba, and N. Ogasawara.** 2006. Escherichia coli histone-like protein H-NS preferentially binds to horizontally acquired DNA in association with RNA polymerase. DNA Research **13:**141-153.
27. **Perna, N. T., G. Plunkett, 3rd, V. Burland, B. Mau, J. D. Glasner, D. J. Rose, G. F. Mayhew, P. S. Evans, J. Gregor, H. A. Kirkpatrick, G. Posfai, J. Hackett, S. Klink, A. Boutin, Y. Shao, L. Miller, E. J. Grotbeck, N. W. Davis, A. Lim, E. T. Dimalanta, K. D. Potamousis, J. Apodaca, T. S. Anantharaman, J. Lin, G. Yen, D. C. Schwartz, R. A. Welch, and F. R. Blattner.** 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. Nature **409:**529-33.
28. **Peterson, R. E., T. J. Klopfenstein, R. A. Moxley, G. E. Erickson, S. Hinkley, D. Rogan, and D. R. Smith.** 2007. Efficacy of dose regimen and observation of herd immunity from a vaccine against Escherichia coli O157 : H7 for feedlot cattlet. Journal of Food Protection **70:**2561-2567.
29. **Porter, M. E., P. Mitchell, A. Free, D. G. E. Smith, and D. L. Gally.** 2005. The LEE1 promoters from both enteropathogenic and enterohemorrhagic Escherichia coli can be activated by PerC-like proteins from either organism. Journal of Bacteriology **187:**458-472.
30. **Rasko, D. A., C. G. Moreira, R. Li de, N. C. Reading, J. M. Ritchie, M. K. Waldor, N. Williams, R. Taussig, S. Wei, M. Roth, D. T. Hughes, J. F. Huntley, M. W. Fina, J. R. Falck, and V. Sperandio.** 2008. Targeting QseC signaling and virulence for antibiotic development. Science **321:**1078-80.
31. **Roe, A. J., and D. L. Gally.** 2000. Enteropathogenic and enterohaemorrhagic Escherichia coli and diarrhoea. Current Opinion in Infectious Diseases **13:**511-517.
32. **Roe, A. J., S. W. Naylor, K. J. Spears, H. M. Yull, T. A. Dransfield, M. Oxford, I. J. McKendrick, M. Porter, M. J. Woodward, D. G. Smith, and D. L. Gally.** 2004. Co-ordinate

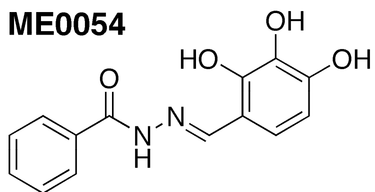
single-cell expression of LEE4- and LEE5-encoded proteins of Escherichia coli O157:H7. Mol Microbiol **54:**337-52.

33. **Roe, A. J., L. Tysall, T. Dransfield, D. Wang, D. Fraser-Pitt, A. Mahajan, C. Constandinou, N. Inglis, A. Downing, R. Talbot, D. G. E. Smith, and D. L. Gally.** 2007. Analysis of the expression, regulation and export of NleA-E in Escherichia coli O157 : H7. Microbiology-Sgm **153:**1350-1360.
34. **Roe, A. J., Tysall, L., Dransfield, T., Wang, D., Fraser-Pitt, D., Mahajan, A., Constandinou, C., Inglis, N., Downing, A., Talbot, R., Smith D.G.E., & Gally D. L.** 2007. Analysis of the expression, regulation and export of NleA-E in Escherichia coli O157:H7. Microbiology **153:**1350-1360.
35. **Roe, A. J., H. Yull, S. W. Naylor, M. J. Woodward, D. G. E. Smith, and D. L. Gally.** 2003. Heterogeneous surface expression of EspA translocon filaments by *Escherichia coli* O157:H7 is controlled at the posttranscriptional level. Infection and Immunity **71:**5900-5909.
36. **Shakhnovich, E. A., D. T. Hung, E. Pierson, K. Lee, and J. J. Mekalanos.** 2007. Virstatin inhibits dimerization of the transcriptional activator ToxT. Proceedings of the National Academy of Sciences of the United States of America **104:**2372-2377.
37. **Slepenkin, A., P. A. Enquist, U. Hagglund, L. M. de la Maza, M. Elofsson, and E. M. Peterson.** 2007. Reversal of the antichlaraydial activity of putative type III secretion inhibitors by iron. Infection and Immunity **75:**3478-3489.
38. **Snyder, J. A., B. J. Haugen, E. L. Buckles, C. V. Lockatell, D. E. Johnson, M. S. Donnenberg, R. A. Welch, and H. L. Mobley.** 2004. Transcriptome of uropathogenic *Escherichia coli* during urinary tract infection. Infect Immun **72:**6373-81.
39. **Veenendaal, A. K., C. Sundin, and A. J. Blocker.** 2009. Small-molecule type III secretion system inhibitors block assembly of the Shigella type III secreton. J Bacteriol **191:**563-70.
40. **Wade, J. T., N. B. Reppas, G. M. Church, and K. Struhl.** 2005. Genomic analysis of LexA binding reveals the permissive nature of the Escherichia coli genome and identifies unconventional target sites. Genes & Development **19:**2619-2630.
41. **Yang, Z., J. Kim, C. Zhang, M. Zhang, J. Nietfeldt, C. M. Southward, M. G. Surette, S. D. Kachman, and A. K. Benson.** 2009. Genomic instability in regions adjacent to a highly conserved pch prophage in Escherichia coli O157:H7 generates diversity in expression patterns of the LEE pathogenicity island. J Bacteriol **191:**3553-68.
42. **Zhang, L. H., R. R. Chaudhuri, C. Constantinidou, J. L. Hobman, M. D. Patel, A. C. Jones, D. Sarti, A. J. Roe, I. Vlisidou, R. K. Shaw, F. Falciani, M. P. Stevens, D. L. Gally, S. Knutton, G. Frankel, C. W. Penn, and M. J. Pallen.** 2004. Regulators encoded in the *Escherichia coli* type III secretion system 2 gene cluster influence expression of genes within the locus for enterocyte effacement in enterohemorrhagic *E. coli* 0157:H7. Infection and Immunity **72:**7282-7293.

Figure 1







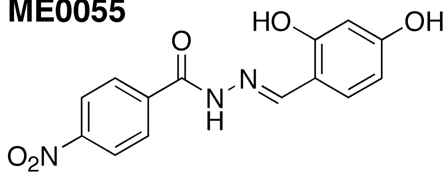
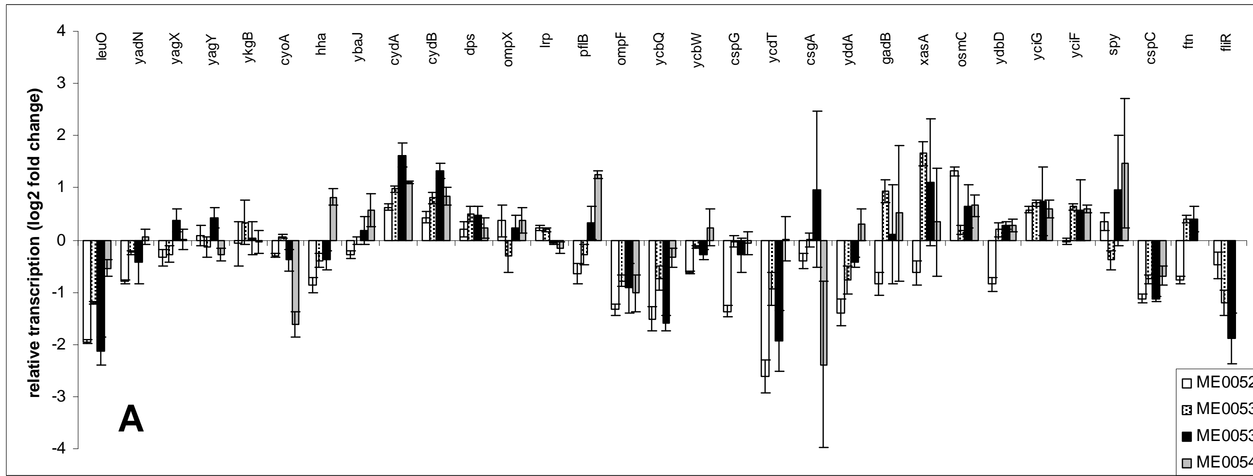
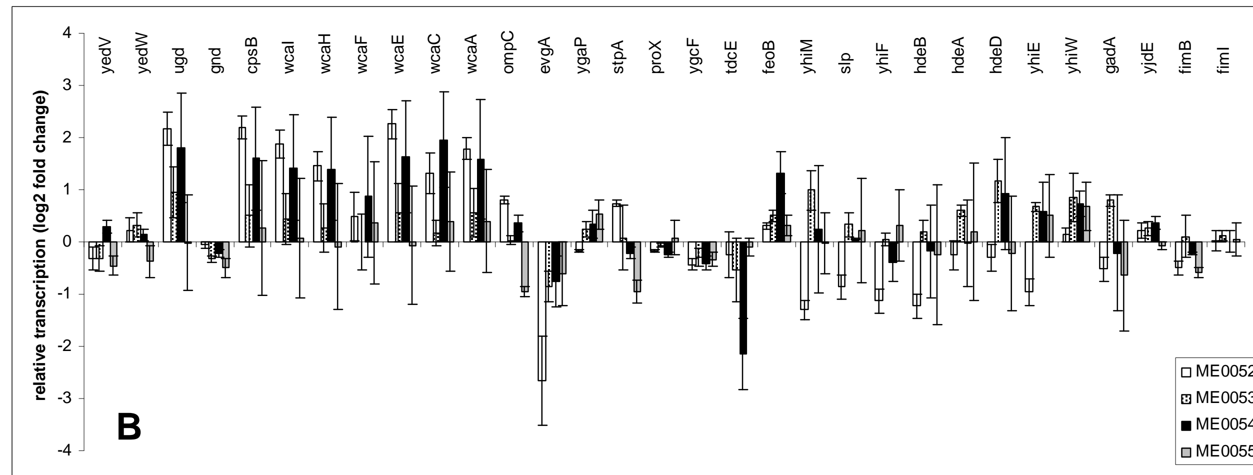
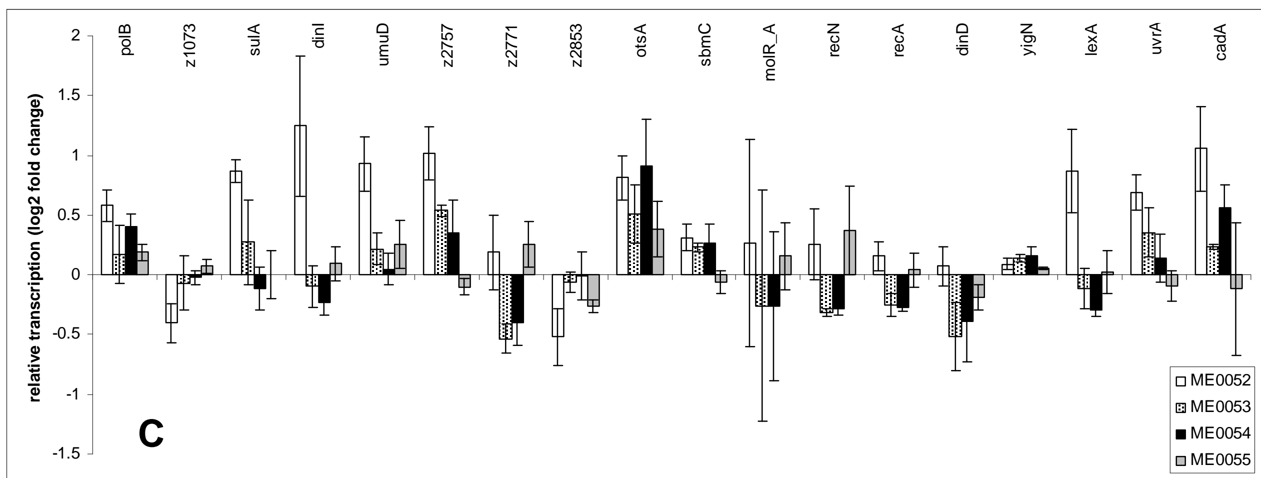


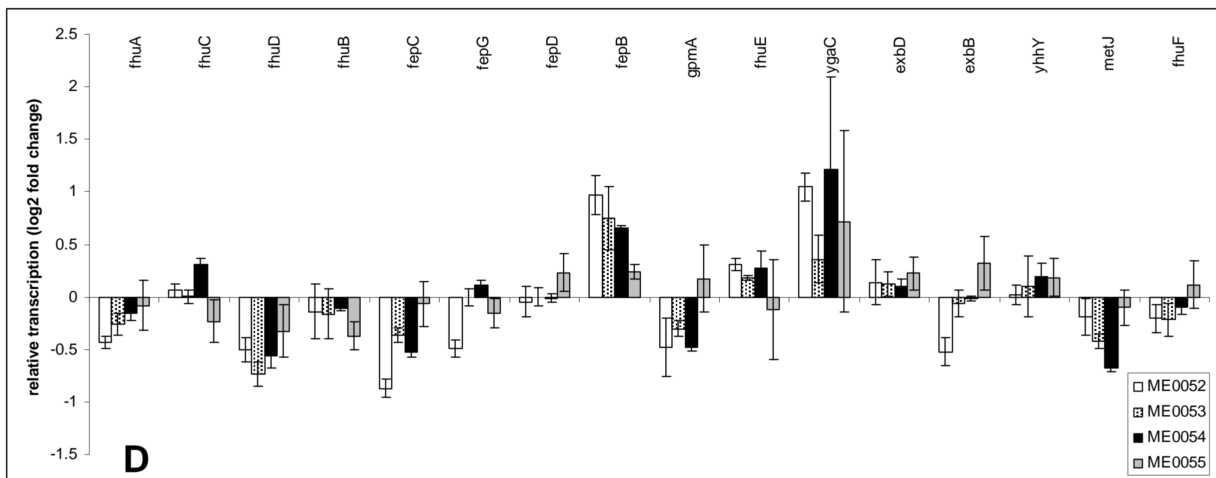
Figure 1S



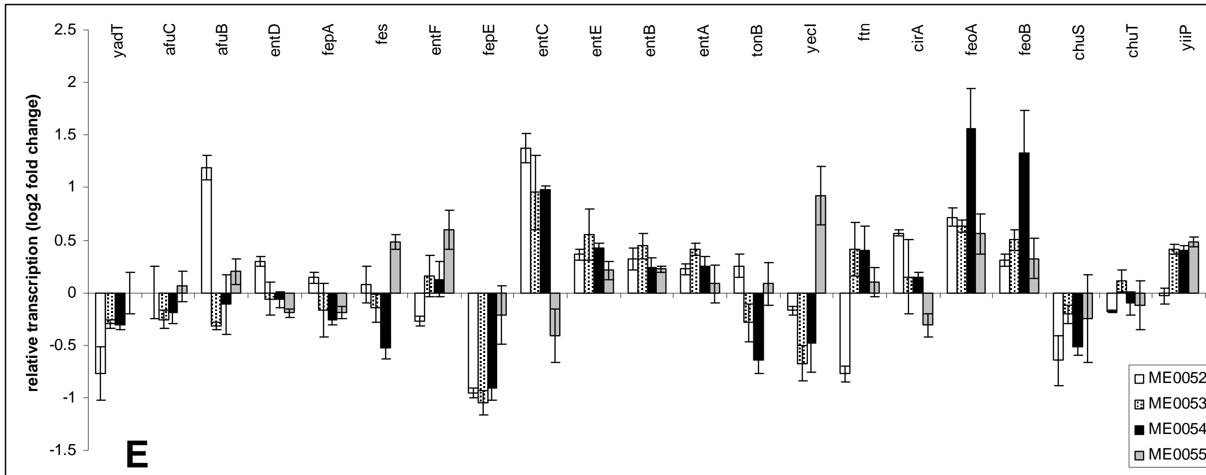




relative transcription (log2 fold change)
2
1.5
1
0.5
0
-0.5
-1
-1.5
polB
z1073
sulA
dinI
umuD
z2757
z2771
z2853
otsA
sbmC
molR_A
recN
recA
dinD
yigN
lexA
uvrA
cadA
ME0052
ME0053
ME0054
ME0055
C



relative transcription (log2 fold change)
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
fhuA
fhuC
fhuD
fhuB
fepC
fepG
fepD
fepB
gpmA
fhuE
ygaC
exbD
exbB
yhhY
metJ
fhuF
ME0052
ME0053
ME0054
ME0055
D



relative transcription (log2 fold change)
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
yadT
afuC
afuB
entD
fepA
fes
entF
fepE
entC
entE
entB
entA
tonB
yecI
ftn
cirA
feoA
feoB
chuS
chuT
yiiP
ME0052
ME0053
ME0054
ME0055
E

Figure 2

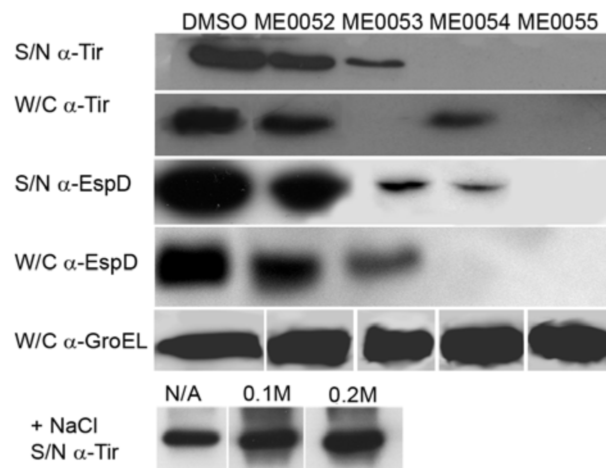
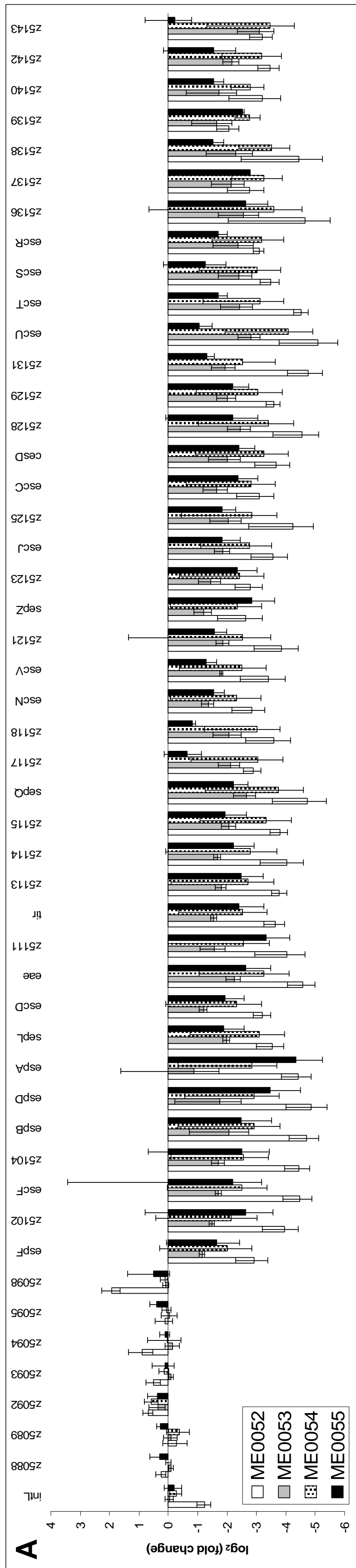


Figure 3



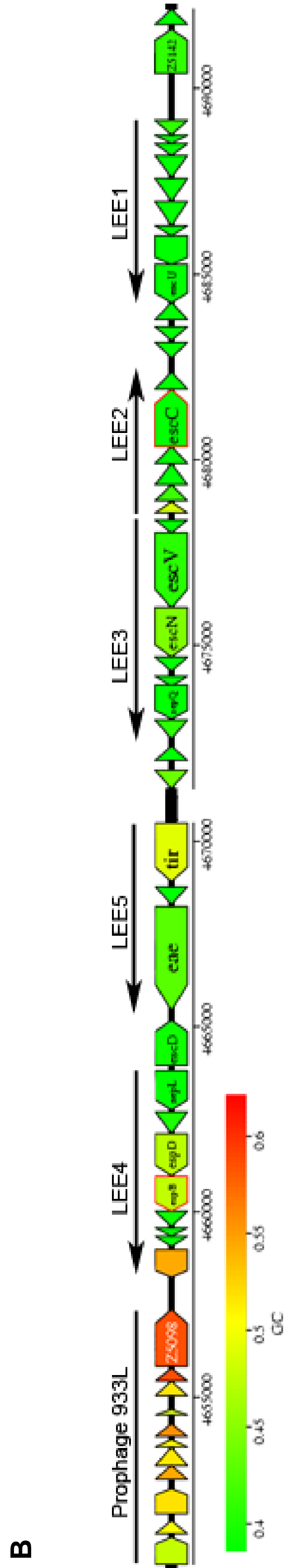


Figure 4

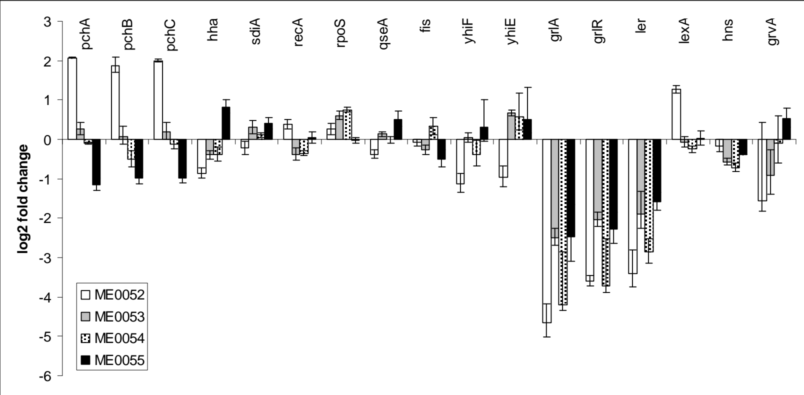
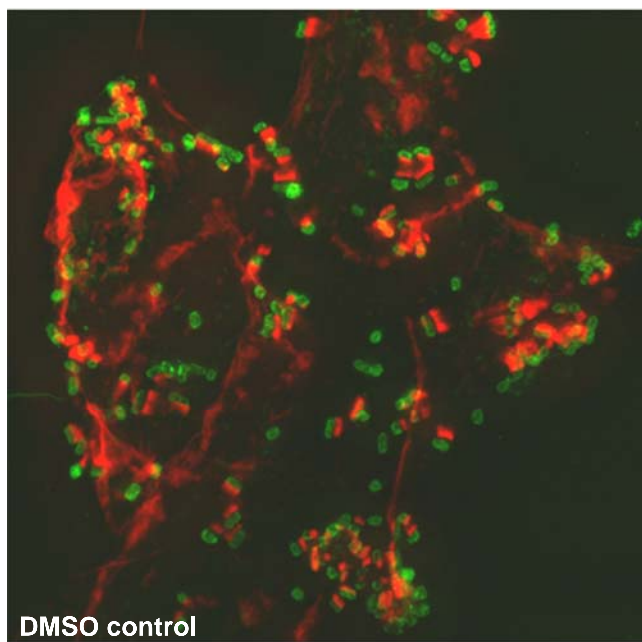


Figure 5

A



B

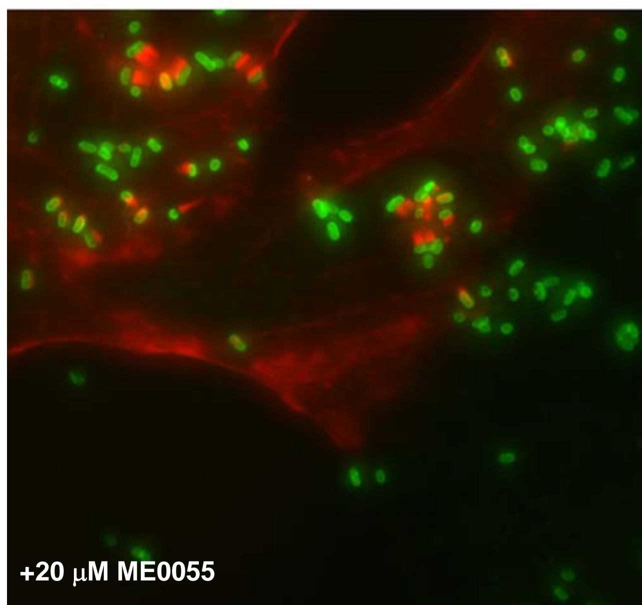


Figure 6

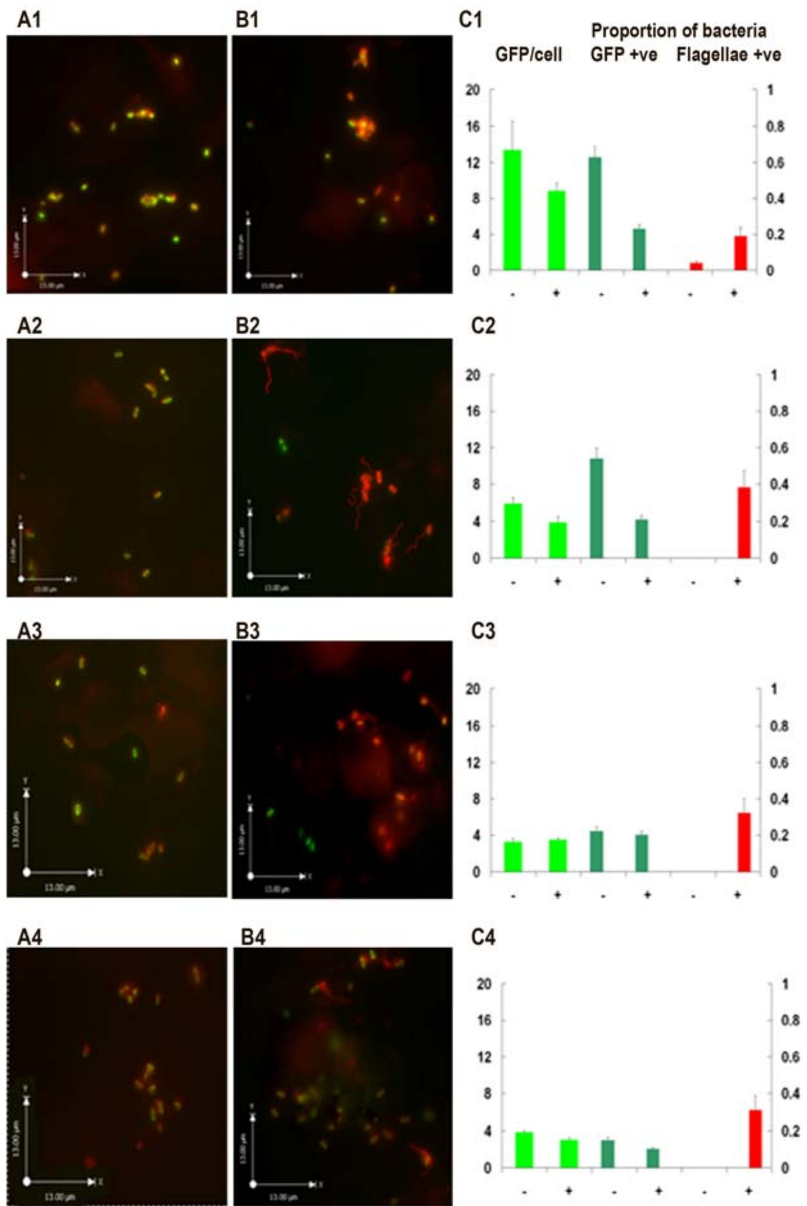


TABLE 1: Growth rates of strain TUV-930 ± inhibitors.

Compound (concentration)	Specific growth rate h^{-1} (mean ± SD)	% growth inhibition
None	0.54 ± 0.01	-
ME0052 (10 μM)	0.49 ± 0.01	10
ME0052 (20μM)	0.44 ± 0.01	12
ME0052 (50μM)	0.37 ± 0.02	46
ME0053 (10 μM)	0.49 ± 0.02	10
ME0053 (20 μM)	0.44 ± 0.01	12
ME0053 (50 μM)	0.06 ± 0.01	900
ME0054 (10 μM)	0.54 ± 0.03	0
ME0054 (20 μM)	0.50 ± 0.02	8
ME0054 (50 μM)	0.49 ± 0.01	10
ME0055 (10 μM)	0.54 ± 0.02	0
ME0055 (20 μM)	0.50 ± 0.02	8
ME0055 (50 μM)	0.44 ± 0.03	12

TABLE 2: Relative inhibition of T3S by salicylidene acylhydrazide compounds in different *E. coli* O157 strains. The amount of secreted Tir effector protein was determined by Western analysis and compared to the strain when cultured in DMSO alone to determine the percentage inhibition. "100" is therefore complete inhibition of Tir secretion.

			Inhibitor			
Strain	Source	Phage type	ME0052	ME0053	ME0054	ME0055
TUV-930	Human	Not typed	23	68	98	99
ZAP3	Bovine	2	100	100	100	100
ZAP11	Bovine	21/28	100	100	100	100
ZAP12	Bovine	21/28	100	100	100	100
ZAP229	Human	2	80	59	97	60
ZAP231	Human	4	79	75	95	43
ZAP243	Human	8	86	94	91	44

.

TABLE 3. Genes with >2 fold change in all inhibitor treatments and $p<0.05$ (Benjamini & Hochberg FDR) in 3 of 4 treatments. Transcription of *rpsM* and *fliC* have been included as controls. Genes upregulated by inhibitor treatment are shaded grey.

Systematic name	Common name	OI#*	Effector**	mobile element*	Ler and PchA ***	ME0052		ME0053		ME0054		ME0055	
						Ratio	p-value	Ratio	p-value	Ratio	p-value	Ratio	p-value
L7027	L7027			pO157		0.361	3.08E-03	0.483	1.62E-01	0.328	5.89E-02	0.337	8.44E-02
L7031	L7031			pO157	reg	0.141	1.27E-03	0.464	3.68E-02	0.151	1.05E-01	0.32	6.75E-02
Z0955	Z0955	36		CP-933K	P+L	0.083	2.13E-04	0.214	4.11E-02	0.096	9.60E-02	0.155	6.75E-02
Z1823	Z1823	50		CP-933N	P	0.277	3.70E-05	0.349	8.09E-02	0.298	4.37E-02	0.218	2.86E-05
Z1824	Z1824	50	espN	CP-933N	P	0.147	5.33E-04	0.214	1.42E-03	0.17	7.79E-03	0.435	3.50E-04
Z2478	pspD					3.648	6.53E-05	2.338	6.46E-02	2.45	1.03E-01	3.567	5.04E-03
Z2479	pspC					3.9	5.05E-05	2.444	5.19E-02	2.512	9.44E-02	3.97	7.69E-03
Z3071	Z3071	79	espJ	CP-933U	P+L	0.152	3.49E-04	0.236	9.20E-03	0.168	3.01E-03	0.478	2.45E-01
Z3572	argT					0.494	1.35E-03	0.492	1.00E-01	0.387	1.48E-02	0.262	6.02E-03
Z5100	espF	148	espF1	CP-933L	L	0.126	8.48E-03	0.452	4.74E-04	0.145	1.70E-01	0.268	8.42E-02
Z5102	Z5102	148		CP-933L	L	0.062	4.34E-03	0.357	2.87E-05	0.103	1.92E-01	0.125	5.12E-02
Z5103	escF	148		CP-933L	L	0.044	1.85E-03	0.306	3.23E-05	0.087	1.54E-01	0.162	7.38E-02
Z5104	Z5104	148		CP-933L	L	0.045	1.80E-03	0.305	5.35E-04	0.085	1.48E-01	0.138	5.03E-02
Z5105	espB	148	espB	CP-933L	L	0.037	1.01E-03	0.216	3.90E-02	0.071	1.16E-01	0.122	7.56E-02
Z5106	espD	148		CP-933L	L	0.033	3.62E-03	0.261	5.87E-02	0.072	1.17E-01	0.061	4.71E-02
Z5107	espA	148		CP-933L	L	0.045	3.04E-03	0.437	2.11E-01	0.074	1.23E-01	0.037	2.42E-02
Z5108	sepL	148		CP-933L	L	0.083	3.97E-05	0.252	1.21E-03	0.065	1.01E-01	0.238	5.33E-02
Z5109	escD	148		CP-933L	P+L	0.107	5.47E-04	0.435	3.59E-05	0.105	1.59E-01	0.237	3.92E-02
Z5110	eae	148		CP-933L	P+L	0.040	2.24E-03	0.211	4.34E-05	0.061	9.04E-02	0.132	3.86E-02
Z5111	Z5111	148		CP-933L	P+L	0.056	7.15E-03	0.329	1.64E-02	0.084	1.48E-01	0.081	3.12E-02
Z5112	tir	148	tir	CP-933L	L	0.079	2.12E-03	0.344	1.40E-05	0.103	1.24E-01	0.156	5.16E-02
Z5113	Z5113	148		CP-933L	P+L	0.072	3.23E-04	0.287	1.06E-03	0.082	1.26E-01	0.155	3.80E-02
Z5114	Z5114	148		CP-933L	P+L	0.057	5.71E-03	0.312	1.13E-03	0.075	1.24E-01	0.191	3.64E-02
Z5115	Z5115	148	espH	CP-933L	P+L	0.071	2.57E-04	0.235	2.21E-03	0.065	6.92E-02	0.224	6.52E-02
Z5116	sepQ	148		CP-933L	P+L	0.033	9.11E-03	0.156	3.88E-04	0.047	6.05E-02	0.204	1.28E-02
Z5119	escN	148		CP-933L	P+L	0.134	7.62E-03	0.388	1.47E-04	0.123	1.32E-01	0.333	1.32E-02
Z5120	escV	148		CP-933L	P+L	0.088	1.01E-02	0.285	5.95E-04	0.114	1.06E-01	0.398	1.91E-02
Z5121	Z5121	148		CP-933L	P+L	0.064	1.08E-02	0.273	2.96E-03	0.092	1.27E-01	0.328	8.38E-03
Z5123	Z5123	148		CP-933L	P+L	0.139	5.37E-03	0.361	1.69E-02	0.118	1.16E-01	0.176	3.22E-02
Z5124	escJ	148		CP-933L	P+L	0.081	4.69E-03	0.272	5.05E-04	0.111	7.00E-02	0.259	4.04E-02
Z5125	Z5125	148		CP-933L	P+L	0.047	1.05E-02	0.233	1.79E-02	0.085	1.01E-01	0.27	9.86E-03
Z5126	escC	148		CP-933L	P+L	0.112	6.86E-03	0.307	7.42E-03	0.087	1.04E-01	0.173	2.91E-02
Z5127	cesD	148		CP-933L	P+L	0.075	6.20E-03	0.237	2.02E-02	0.062	8.64E-02	0.178	1.73E-02
Z5128	Z5128	148		CP-933L	P+L	0.04	5.09E-03	0.177	5.26E-03	0.056	7.90E-02	0.179	5.51E-02
Z5129	Z5129	148		CP-933L	P+L	0.083	1.06E-03	0.244	8.03E-04	0.076	8.62E-02	0.204	2.14E-02
Z5131	Z5131	148		CP-933L	P+L	0.035	3.05E-03	0.256	1.10E-02	0.076	1.09E-01	0.397	3.56E-03
Z5132	escU	148		CP-933L	P+L	0.025	1.07E-02	0.14	4.94E-03	0.044	2.57E-02	0.463	7.15E-02
Z5133	escT	148		CP-933L	P+L	0.043	8.33E-07	0.178	1.54E-02	0.088	4.77E-02	0.3	1.82E-03
Z5134	escS	148		CP-933L	P+L	0.088	1.57E-03	0.18	1.67E-02	0.085	7.07E-02	0.374	4.24E-02
Z5135	escR	148		CP-933L	P+L	0.117	2.84E-04	0.179	2.74E-02	0.086	4.95E-02	0.304	4.41E-04
Z5137	Z5137	148		CP-933L	P+L	0.14	1.12E-02	0.216	2.29E-02	0.091	2.44E-02	0.148	1.09E-05
Z5139	Z5139	148		CP-933L	P+L	0.233	2.46E-03	0.295	4.66E-02	0.143	4.93E-03	0.173	3.92E-05
Z5142	Z5142	148	espG	CP-933L	P+L	0.089	9.46E-04	0.221	1.72E-03	0.092	3.57E-02	0.298	7.90E-02
Z5953	yjiY					12.27	2.22E-06	4.792	2.84E-03	4.997	2.60E-02	2.653	1.66E-01
Z6024	Z6024	71	nleA	CP-933P	P+L	0.077	1.49E-03	0.196	3.13E-03	0.075	8.08E-02	0.38	6.11E-02
Z4668	rpsM					0.673	0.015	0.926	0.159	1.103	0.219	0.965	0.585

Z3013	fliC			4.305	0.061	33.830	0.020	5.064	0.111	0.311	0.340

TABLE 4. Comparison of total number of genes with >2 fold change* in transcription for each inhibitor and number of genes encoded on O-islands and cryptic prophage (CP-933).

	Total # genes	# OI encoded	*p***	# CP-933 encoded	*p***
ME0052	181	118	$7.2e^{-32}$	94	$1.3e^{-52}$
ME0053	72	46	$8.2e^{-13}$	41	$1.5e^{-25}$
ME0054	23	18	$2.2e^{-08}$	18	$1.2e^{-19}$
ME0055	36	7	0.22	7	0.41
Genome***	5481	1449	0.98	727	0.98

*>3 fold change was used as the threshold for inhibitors ME0052 and ME0054.
** *p*-values were calculated using a chi squared test with Yates correction. Expected values were generated from the total number of OI or CP-933 genes encoded within the genome as described by Perna *et al* (2001) and expressed as a relative proportion of the total # genes with 2 fold change. *p* values represent the significance of the bias toward OI or CP-933 encoded genes.
***Total number of genes encoded by *E. coli* O157 EDL933 in each category. Annotation taken from Perna *et al* (2001).

TABLE 5. Analysis of promoter-GFP constructs cultured in MEM-HEPES in presence and absence of the small molecule inhibitors. Ratio values are calculated from the fluorescence value of the strain cultured with the inhibitor divided by the strain cultured in the absence of the compound. All values were corrected for background by deducting the fluorescence value for the strain transformed with pAJR70 (promoterless *gfp*) measured at the same optical density.

Plasmid	Promoter	ME0052	ME0053	ME0054	ME0055
		Ratio	Ratio	Ratio	Ratio
pAJR71	LEE1	0.23	0.25	0.25	0.38
pAJR72	LEE2	0.38	0.55	0.21	0.120
pAJR73	LEE3	0.21	0.41	0.16	0.328
pAJR74	LEE4	0.21	0.34	0.17	0.17
pAJR75	LEE5	0.19	0.45	0.19	0.25
pAJR145	rpsM	0.98	0.99	1.1	0.98

TABLE 1S: Genes with altered expression in inhibitor treated *E. coli* TUV93.0

ME0052 >3 fold change p<0.05 B&H

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
L7004	L7004			pO157		putative hemolysin expression modulating protein	0.183	0.00598
L7024	L7024			pO157		regulatory protein	0.317	0.0074
L7029	L7029			pO157		putative acyltransferase	0.31	0.00105
L7031	L7031			pO157	reg	hypothetical protein	0.141	0.00127
L7032	etpC			pO157	reg	type II secretion protein	0.173	0.000124
L7033	etpD			pO157	reg	type II secretion protein	0.247	7.02E-06
L7036	etpG			pO157		type II secretion protein	0.25	5.50E-07
L7038	etpI			pO157		type II secretion protein	0.323	0.000189
L7047	hlyC			pO157		hemolysin transport protein	0.326	0.00128
L7048	hlyA			pO157		hemolysin toxin protein	0.287	0.000169
L7050	hlyD			pO157	reg	hemolysin transport protein	0.104	0.000116
L7094	L7094			pO157		transposase	0.228	0.00438
Z0012	htgA					positive regulator for sigma 32 heat shock promoters	3.456	0.00356
Z0016	gef					Gef protein interferes with membrane function when in excess	0.222	0.000294
Z0086	leuO					probable transcriptional activator for leuABCD operon	0.26	7.54E-05
Z0157	sfsA					probable regulator for maltose metabolism	0.303	0.000306
Z0367	Z0367	10		IS		unknown protein encoded in ISEc8	3.712	0.00393
Z0371	Z0371	11				putative LysR-like transcriptional regulator	0.316	0.00986
Z0395	Z0395	14			P+L		0.237	0.00623
Z0415	Z0415	17				putative periplasmic binding protein, probable substrate ribose	4.337	0.00954
Z0461	Z0461	20				putative permease; hexosephosphate transport	3.332	0.000333
Z0757	citG					orf, hypothetical protein	3.127	0.00307
Z0878	sdhB					succinate dehydrogenase, iron sulfur protein	0.273	0.00187
Z0893	Z0893	35				putative glutamate mutase subumit E	3.036	0.00211
Z0919	nadA					quinolinate synthetase, A protein	5.096	3.48E-06
Z0920	pnuC					required for NMN transport	4.4	0.00105
Z0950	Z0950	36		CP-933K		unknown protein encoded by prophage CP-933K	3.239	0.00529
Z0955	Z0955	36		CP-933K	P+L	unknown protein encoded by prophage CP-933K	0.083	0.000213
Z0964	Z0964	36		CP-933K		putative DNA packaging protein of prophage CP-933K	3.032	0.00273
Z0978	Z0978	36		CP-933K		putative tail component of prophage CP-933K	3.411	0.00535
Z0985	Z0985	36	nleB-2	CP-933K	P+L	unknown protein encoded by prophage CP-933K	0.31	0.00635

Z1047	ybiY					putative pyruvate formate-lyase 2 activating enzyme	3.112	0.000369
Z1122	Z1122	43					3.011	0.00206
Z1131	Z1131	43				unknown protein encoded in ISEc8	4.417	0.00268

ME0052 cont.

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z1223	Z1223	43					3.242	0.00104
Z1246	pflA					pyruvate formate lyase activating enzyme 1	0.329	0.00051
Z1350	Z1350	44		CP-933M		putative holin protein of cryptic prophage CP-933M	3.128	0.0109
Z1362	Z1362	44		CP-933M		unknown protein encoded by cryptic prophage CP-933M	4.422	0.00329
Z1366	Z1366	44		CP-933M		unknown protein encoded by cryptic prophage CP-933M	4.082	0.00903
Z1372	Z1372	44		CP-933M		unknown protein encoded by cryptic prophage CP-933M	3.821	1.00E-05
Z1375	Z1375	44		CP-933M		tail component encoded by cryptic prophage CP-933M; partial	3.559	0.00931
Z1377	Z1377	44		CP-933M		putative tail component encoded by cryptic prophage CP-933M	5.397	0.00208
Z1378	Z1378	44		CP-933M		tail component encoded by cryptic prophage CP-933M; partial	3.134	0.000326
Z1382	Z1382	44		CP-933M		tail component encoded by cryptic prophage CP-933M; partial	3.083	0.00145
Z1468	Z1468	45		BP-933W		putative lysis protein S of bacteriophage BP-933W	5.177	3.42E-06
Z1471	Z1471	45		BP-933W		putative antirepressor protein Ant of bacteriophage BP-933W	4.361	0.00341
Z1526	ycdS					putative outer membrane protein	0.297	0.00689
Z1527	ycdT					orf, hypothetical protein	0.163	0.0107
Z1538	Z1538	47				putative pilin subunit	0.287	0.009
Z1576	Z1576	48					4.271	0.000537
Z1765	xisN	50		CP-933N		putative excisionase for prophage CP-933N	0.131	0.000278
Z1771	ydaS	50		CP-933N		unknown protein encoded by prophage CP-933N	0.16	0.00027
Z1774	Z1774	50		CP-933N		unknown protein encoded by prophage CP-933N	0.33	0.00367
Z1778	Z1778	50		CP-933N		unknown protein encoded by prophage CP-933N	0.3	8.15E-05
Z1779	Z1779	50		CP-933N		unknown protein encoded by prophage CP-933N	0.279	3.68E-05
Z1798	Z1798	50		CP-933N		putative endopeptidase of prophage CP-933N	3.301	0.0086
Z1807	Z1807	50		CP-933N		unknown protein encoded by prophage CP-933N	5.282	0.000793
Z1814	Z1814	50		CP-933N		unknown protein encoded by prophage CP-933N	3.429	0.00275
Z1822	Z1822	50	espX7	CP-933N	P	unknown protein encoded by prophage CP-933N	0.095	2.80E-05
Z1823	Z1823	50		CP-933N	P	unknown protein encoded by prophage CP-933N	0.277	3.70E-05
Z1824	Z1824	50	espN	CP-933N	P	unknown protein encoded by prophage CP-933N	0.147	0.000533
Z1901	Z1901	52		CP-933X		unknown protein encoded by prophage CP-933X	4.966	0.00767
Z1912	Z1912	52		CP-933X		unknown protein encoded by prophage CP-933X	3.184	0.00116

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z1924	Z1924	52		CP-933X		unknown protein encoded by prophage CP-933X	8.707	0.000286
Z1928	Z1928	52		CP-933X		unknown protein encoded by ISEc8 in prophage CP-933X	5.1	0.00758
Z1985	ychA					orf, hypothetical protein	0.253	0.00262
Z2008	purU					formyltetrahydrofolate deformylase	0.263	0.0071

ME0052 cont.

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z2130	Z2130	57		CP-933O		putative IS encoded protein encoded within prophage CP-933O	3.371	1.84E-05
Z2170	marA					multiple antibiotic resistance; transcriptional activator	4.2	0.000298
Z2339	Z2339	hypervariable	nleG2-2	CP-933R	P	unknown protein encoded by prophage CP-933R	0.211	0.00375
Z2361	Z2361	hypervariable		CP-933R		putative capsid assembly protein of prophage CP-933R	5.304	1.49E-05
Z2372	Z2372	hypervariable		CP-933R		unknown protein encoded within prophage CP-933R	5.653	0.000141
Z2389	Z2389	hypervariable		CP-933R		DNA modification methyltransferase within prophage CP-933R	0.292	0.000419
Z2414	ydaQ	hypervariable		CP-933R		unknown protein encoded within prophage CP-933R	4.269	9.95E-05
Z2478	pspD					phage shock protein	3.648	6.53E-05
Z2479	pspC					phage shock protein: activates phage shock-protein expression	3.9	5.05E-05
Z2480	pspB					phage shock protein, putative inner membrane protein	4.701	0.00518
Z2482	pspA					phage shock protein, putative inner membrane protein	3.197	0.000673
Z2744	pheM					phenylalanyl-tRNA synthetase (pheST) operon leader peptide	0.26	0.00468
Z2841	yeaS					orf, hypothetical protein	0.213	0.00272
Z2866	yebH					putative enzyme	0.302	0.00766
Z2879	Z2879					orf, hypothetical protein	0.27	0.00135
Z2943	motB					enables flagellar motor rotation	3.186	0.000705
Z2967	Z2967	76		CP-933T		unknown protein encoded by prophage CP-933T	0.278	0.000205
Z2970	coxT	76		CP-933T		putative regulator for prophage CP-933T	0.167	4.17E-05
Z2971	Z2971	76		CP-933T		unknown protein encoded by prophage CP-933T	0.156	1.17E-06
Z2972	Z2972	76		CP-933T		unknown protein encoded by prophage CP-933T	0.137	0.00928
Z2973	Z2973	76		CP-933T		unknown protein encoded by prophage CP-933T	0.204	8.34E-05
Z3071	Z3071	79	espJ	CP-933U	P+L	unknown protein encoded within prophage CP-933U	0.152	0.000349
Z3082	Z3082	79		CP-933U		putative tail fiber component L of prophage CP-933U	3.604	0.00286
Z3106	Z3106	79		CP-933U		putative holin protein of prophage CP-933U	3.885	0.000573

Z3154	Z3154	81		IS		unknown protein encoded by ISEc8	4.937	0.00205
Z3190	ugd					UDP-glucose 6-dehydrogenase	4.535	0.0105
Z3202	wbdO	84				glycosyl transferase	0.293	0.00315
Z3208	wcaL					putative colanic acid biosynthesis glycosyl transferase	4.373	0.00146
Z3213	cpsB					mannose-1-phosphate guanyltransferase	4.578	0.00741
Z3216	wcaG					putative nucleotide di-P-sugar epimerase or dehydratase	3.924	0.000397
Z3217	gmd					GDP-D-mannose dehydratase	6.292	0.000967
Z3219	wcaE					putative colanic acid biosynthesis glycosyl transferase	4.791	0.006
Z3223	wcaA					putative regulator	3.457	0.00771

ME0052 cont.

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z3251	Z3251					orf, hypothetical protein	0.28	0.0108
Z3314	Z3314	93		CP-933V		putative tail component of prophage CP-933V	4.181	0.000641
Z3319	Z3319	93		CP-933V		unknown protein encoded within prophage CP-933V	4.695	0.00033
Z3320	Z3320	93		CP-933V		unknown protein encoded within prophage CP-933V	3.644	0.00313
Z3326	Z3326	93		CP-933V		unknown protein encoded within prophage CP-933V	4.217	0.00837
Z3331	Z3331	93		CP-933V		unknown protein encoded within prophage CP-933V	3.683	0.000556
Z3332	Z3332	93		CP-933V		unknown protein encoded within prophage CP-933V	3.574	0.000295
Z3333	Z3333	93		CP-933V		unknown protein encoded within prophage CP-933V	3.691	0.0107
Z3770	Z3770					putative membrane protein	0.306	0.00527
Z3856	nadB					quinolinate synthetase, B protein	4.356	2.09E-05
Z3921	Z3921	108	nleG6-3'	CP-933Y	reg		0.305	0.00104
Z3931	Z3931	108		CP-933Y	P	unknown protein encoded by prophage CP-933Y	0.299	0.000618
Z4085	ygcW					putative oxidoreductase	4.326	0.00906
Z4118	fucP					fucose permease	3.36	0.00898
Z4119	fucI					L-fucose isomerase	3.078	0.000533
Z4326	Z4326	122	espL2	IS	P	putative enterotoxin	0.276	0.000501
Z4328	Z4328	122	nleB1	IS	P		0.171	0.000348
Z4329	Z4329	122	nleE	IS	P		0.157	0.00385
Z4330	Z4330	122		IS		putative transposase	0.216	0.0035
Z4331	Z4331	122		IS			0.321	0.000919
Z4332	Z4332	122		IS		putative cytotoxin	0.101	0.000891
Z4493	agaC					PTS system N-acetylgalactosamine-specific IIC component 1	3.793	0.00935
Z4937	yhjG					orf, hypothetical protein	3.136	0.00108

Z4939	yhjH					orf, hypothetical protein	3.348	0.00132
Z4988	yiaB					orf, hypothetical protein	3.34	0.0106
Z5051	waaJ	145				putative LPS biosynthesis enzyme	0.269	0.000337
Z5052	waaY	145				putative LPS biosynthesis protein	0.319	0.000978
Z5053	waaI	145				putative LPS biosynthesis enzyme	0.319	4.82E-05
Z5098	Z5098	148		CP-933L		unknown protein encoded by ISEc8 within prophage CP-933L	3.736	0.003
Z5100	espF	148	espF1	CP-933L		espF	0.126	0.00848
Z5102	Z5102	148		CP-933L	L		0.062	0.00434
Z5103	escF	148		CP-933L	L	escF	0.044	0.00185
Z5104	Z5104	148		CP-933L	L		0.045	0.0018
Z5105	espB	148	espB	CP-933L	L	secreted protein EspB	0.037	0.00101
Z5106	espD	148		CP-933L	L	secreted protein EspD	0.033	0.00362

ME0052 cont.

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z5107	espA	148		CP-933L	L	secreted protein EspA	0.045	0.00304
Z5108	sepL	148		CP-933L	L	sepL	0.083	3.97E-05
Z5109	escD	148		CP-933L	P+L	escD	0.107	0.000547
Z5110	eae	148		CP-933L	P+L	intimin adherence protein	0.040	0.00224
Z5111	Z5111	148		CP-933L	P+L		0.056	0.00715
Z5112	tir	148	tir	CP-933L	L	putative translocated intimin receptor protein	0.079	0.00212
Z5113	Z5113	148		CP-933L	P+L		0.072	0.000323
Z5114	Z5114	148		CP-933L	P+L		0.057	0.00571
Z5115	Z5115	148	espH	CP-933L	P+L		0.071	0.000257
Z5116	sepQ	148		CP-933L	P+L	sepQ	0.033	0.00911
Z5117	Z5117	148		CP-933L	P+L		0.133	0.00148
Z5118	Z5118	148		CP-933L	P+L		0.078	0.00856
Z5119	escN	148		CP-933L	P+L	escN	0.134	0.00762
Z5120	escV	148		CP-933L	P+L	escV	0.088	0.0101
Z5121	Z5121	148		CP-933L	P+L		0.064	0.0108
Z5123	Z5123	148		CP-933L	P+L		0.139	0.00537
Z5124	escJ	148		CP-933L	P+L	escJ	0.081	0.00469
Z5125	Z5125	148		CP-933L	P+L		0.047	0.0105
Z5126	escC	148		CP-933L	P+L	escC	0.112	0.00686
Z5127	cesD	148		CP-933L	P+L	cesD	0.075	0.0062
Z5128	Z5128	148		CP-933L	P+L		0.04	0.00509
Z5129	Z5129	148		CP-933L	P+L		0.083	0.00106

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z5131	Z5131	148		CP-933L	P+L		0.035	0.00305
Z5132	escU	148		CP-933L	P+L	escU	0.025	0.0107
Z5133	escT	148		CP-933L	P+L	escT	0.043	8.33E-07
Z5134	escS	148		CP-933L	P+L	escS	0.088	0.00157
Z5135	escR	148		CP-933L	P+L	escR	0.117	0.000284
Z5139	Z5139	148		CP-933L	P+L		0.233	0.00246
Z5142	Z5142	148	espG	CP-933L	P+L		0.089	0.000946
Z5143	Z5143	148		CP-933L			0.106	6.79E-05
Z5245	asnA					asparagine synthetase A	4.712	5.53E-07
Z5367	fadB					4-enzyme protein: 3-hydroxyacyl-CoA dehydrogenase	0.234	0.00123
Z5401	Z5401						4.126	0.00109
Z5449	rhaS					positive regulator for rhaBAD operon	3.21	0.000746
Z5503	yijI					orf, hypothetical protein	3.624	0.000757

ME0052 cont.

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z5506	frwB					PTS system fructose-like IIB component 1	3.092	0.00023
Z5615	Z5615	166				putative sorbose PTS component	3.007	0.00138
Z5624	yjbE					orf, hypothetical protein	20.24	0.00277
Z5675	nrfG					part of formate-dependent nitrite reductase complex	3.666	0.000125
Z5690	Z5690	167				putative permease of ribose ABC transport system	3.172	0.00848
Z5694	Z5694						3.209	3.21E-05
Z5730	yjdK					orf, hypothetical protein	0.177	0.00642
Z5953	yjiY					putative carbon starvation protein	12.27	2.22E-06
Z6024	Z6024	71	nleA	CP-933P	P+L	unknown protein encoded by cryptic prophage CP-933P	0.077	0.00149
Z6033	Z6033	71		CP-933P		putative tail component of cryptic prophage CP-933P	3.941	0.0107
Z6035	Z6035	71		CP-933P		putative tail assembly protein of cryptic prophage CP-933P	3.355	5.27E-06
Z6046	Z6046	71		CP-933P		putative terminase encoded by cryptic prophage CP-933P	3.003	0.01

ME0053 >2 fold change p<0.05 B&H

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
L7003	L7003			pO157		hypothetical protein 15.6 kDa protein in finO 3' region precursor	0.363	4.03E-05
L7004	L7004			pO157		putative hemolysin expression modulating protein	0.455	6.22E-05
L7005	L7005			pO157		hypothetical protein	0.472	1.03E-05
L7024	L7024			pO157		regulatory protein	0.371	9.27E-05
L7025	L7025			pO157			0.437	6.07E-05

L7030	L7030			pO157			0.395	0.00071
L7032	etpC			pO157	reg	type II secretion protein	0.425	3.31E-06
L7033	etpD			pO157	reg	type II secretion protein	0.385	0.00162
L7050	hlyD			pO157	reg	hemolysin transport protein	0.414	0.00162
L7051	L7051			pO157		hypothetical protein	0.486	0.000645
Z0394	Z0394	14			P+L		0.47	0.000406
Z0395	Z0395	14			P+L		0.413	3.82E-05
Z0701	Z0701	30					0.484	0.000234
Z0728	fepE					ferric enterobactin (enterochelin) transport	0.486	0.000189
Z0840	Z0840	32					0.427	0.000549
Z0920	pnuC					required for NMN transport	3.555	0.000547
Z0989	Z0989	36	nleH1-1	CP-933K	P+L	unknown protein encoded by prophage CP-933K	0.347	0.000179
Z1012	ybhR					orf, hypothetical protein	2.225	0.00185
Z1386	Z1386	44		CP-933M		unknown protein encoded by cryptic prophage CP-933M	0.461	6.01E-05

ME0053 cont.

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z1678	Z1678					orf, hypothetical protein	2.867	0.000267
Z1824	Z1824	50	espN	CP-933N	P	unknown protein encoded by prophage CP-933N	0.221	2.39E-05
Z1829	Z1829	50		CP-933N	P		0.484	0.000209
Z1925	Z1925	52		CP-933X		unknown protein encoded by prophage CP-933X	0.238	2.98E-06
Z2075	Z2075	57		CP-933O		unknown protein encoded by prophage CP-933O	0.38	8.97E-06
Z2149	Z2149	57	nleG2-3	CP-933O	P		0.366	0.000422
Z2150	Z2150	57	nleG6-2	CP-933O			0.317	2.54E-06
Z2184	uxaB					altronate oxidoreductase	2.26	0.00191
Z2189	Z2189					putative LACI-type transcriptional regulator	2.355	0.000139
Z2338	Z2338	hypervariable	nleG6-1	CP-933R	P	unknown protein encoded by prophage CP-933R	0.359	0.000276
Z2339	Z2339	hypervariable	nleG2-2	CP-933R	P	unknown protein encoded by prophage CP-933R	0.234	0.000261
Z2914	yebB						0.491	0.00136
Z3071	Z3071	79	espJ	CP-933U	P+L	unknown protein encoded within prophage CP-933U	0.24	0.00156
Z3202	wbdO	84				glycosyl transferase	0.443	0.000895
Z3593	yfcB					putative adenine-specific methylase	0.179	0.000907
Z3921	Z3921	108	nleG6-3'	CP-933Y	reg		0.242	0.000232
Z4771	malQ					4-alpha-glucanotransferase (amylomaltase)	2.118	0.000686
Z4996	malS					alpha-amylase	2.252	0.00027

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z5100	espF	148	espF1	CP-933L		espF	0.453	0.000123
Z5102	Z5102	148		CP-933L	L		0.357	4.40E-06
Z5103	escF	148		CP-933L	L	escF	0.307	7.23E-06
Z5104	Z5104	148		CP-933L	L		0.308	9.49E-06
Z5108	sepL	148		CP-933L	L	sepL	0.252	0.000119
Z5109	escD	148		CP-933L	P+L	escD	0.437	4.33E-06
Z5110	eae	148		CP-933L	P+L	intimin adherence protein	0.213	1.01E-06
Z5112	tir	148	tir	CP-933L	L	putative translocated intimin receptor protein	0.345	1.28E-06
Z5113	Z5113	148		CP-933L	P+L		0.288	0.000125
Z5114	Z5114	148		CP-933L	P+L		0.313	0.000148
Z5115	Z5115	148	espH	CP-933L	P+L		0.238	0.000173
Z5116	sepQ	148		CP-933L	P+L	sepQ	0.159	4.09E-07
Z5117	Z5117	148		CP-933L	P+L		0.232	0.000489
Z5118	Z5118	148		CP-933L	P+L		0.238	7.61E-05
Z5119	escN	148		CP-933L	P+L	escN	0.391	4.29E-05
Z5120	escV	148		CP-933L	P+L	escV	0.285	8.34E-05
Z5121	Z5121	148		CP-933L	P+L		0.275	0.00049

ME0053 cont.

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z5124	escJ	148		CP-933L	P+L	escJ	0.275	5.79E-06
Z5126	escC	148		CP-933L	P+L	escC	0.316	0.000229
Z5127	cesD	148		CP-933L	P+L	cesD	0.25	0.00167
Z5128	Z5128	148		CP-933L	P+L		0.182	0.000255
Z5129	Z5129	148		CP-933L	P+L		0.248	6.15E-06
Z5131	Z5131	148		CP-933L	P+L		0.263	0.00178
Z5132	escU	148		CP-933L	P+L	escU	0.143	0.000275
Z5133	escT	148		CP-933L	P+L	escT	0.188	0.0012
Z5134	escS	148		CP-933L	P+L	escS	0.191	0.00105
Z5142	Z5142	148	espG	CP-933L	P+L		0.224	0.000256
Z5143	Z5143	148		CP-933L			0.117	0.000877
Z5631	malF					part of maltose permease, periplasmic	3.805	0.000167
Z5632	malE					periplasmic maltose-binding protein	3.282	0.000148
Z5634	lamB				reg	phage lambda receptor protein; maltose high-affinity receptor	5.17	0.000558
Z5635	malM					periplasmic protein of mal regulon	4.756	0.000564
Z5727	yjdH					putative 2-component sensor protein	0.479	0.000279
Z5957	yjjM						2.174	0.000419

Z6024	Z6024	71	nleA	CP-933P	P+L	unknown protein encoded by cryptic prophage CP-933P	0.199	0.00047

ME0054 >3 fold change p<0.1 B&H

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
L7030	L7030			pO157			0.32	0.00749
Z0086	leuO					probable transcriptional activator for leuABCD operon	0.228	0.00153
Z0394	Z0394	14			P+L		0.238	0.00692
Z0985	Z0985	36	nleB-2	CP-933K	P+L	unknown protein encoded by prophage CP-933K	0.255	0.000895
Z0989	Z0989	36	nleH1-1	CP-933K	P+L	unknown protein encoded by prophage CP-933K	0.317	0.00701
Z1765	xisN	50		CP-933N		putative excisionase for prophage CP-933N	0.148	5.73E-05
Z1766	Z1766	50		CP-933N		unknown protein encoded by prophage CP-933N	0.299	6.69E-05
Z1774	Z1774	50		CP-933N		unknown protein encoded by prophage CP-933N	0.258	6.54E-05
Z1775	Z1775	50		CP-933N		unknown protein encoded by prophage CP-933N	0.279	0.00123
Z1776	Z1776	50		CP-933N		unknown protein encoded by prophage CP-933N	0.26	0.000571
Z1777	Z1777	50		CP-933N		unknown protein encoded by prophage CP-933N	0.263	0.00102
Z1822	Z1822	50	espX7	CP-933N	P	unknown protein encoded by prophage CP-933N	0.267	0.00187
Z1824	Z1824	50	espN	CP-933N	P	unknown protein encoded by prophage CP-933N	0.17	0.00779

ME0054 cont.

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z2149	Z2149	57	nleG2-3	CP-933O	P		0.283	0.00619
Z2150	Z2150	57	nleG6-2	CP-933O			0.292	0.00189
Z2339	Z2339		nleG2-2	CP-933R	P	unknown protein encoded by prophage CP-933R	0.241	0.00224
Z2909	yebL					putative adhesin	15.79	0.00811
Z2910	yebM					putative ATP-binding component of a transport system	9.666	1.37E-05
Z3066	Z3066						62.22	0.00737
Z3071	Z3071	79	espJ	CP-933U	P+L	unknown protein encoded within prophage CP-933U	0.168	0.00301
Z3921	Z3921	108	nleG6-3'	CP-933Y	reg		0.178	0.00978
Z5138	Z5138	148		CP-933L	P+L		0.080	0.00756
Z5139	Z5139	148		CP-933L	P+L		0.143	0.00493
Z5140	ler	148		CP-933L			0.138	0.00493

ME0055 >2 fold change p<0.05 B&H

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z0002	thrA					aspartokinase I, homoserine dehydrogenase I	0.424	5.52E-05
Z0003	thrB					homoserine kinase	0.458	0.000383

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z0004	thrC					threonine synthase	0.463	0.000149
Z0232	yafD					orf, hypothetical protein	2.054	0.0148
Z0900	cydA					cytochrome d terminal oxidase, polypeptide subunit I	2.155	2.42E-06
Z0923	Z0923					putative homeobox protein	3.04	0.0345
Z1248	pflB					formate acetyltransferase 1	2.438	0.0095
Z1748	ndh					respiratory NADH dehydrogenase	3.661	0.0987
Z1823	Z1823	50		CP-933N	P	unknown protein encoded by prophage CP-933N	0.219	2.52E-06
Z1824	Z1824	50	espN	CP-933N	P	unknown protein encoded by prophage CP-933N	0.402	0.00206
Z2224	Z2224					putative transport system permease protein	0.285	0.000125
Z2225	Z2225					putative transport protein	0.361	0.00158
Z2227	Z2227					putative ATP-binding component of a transport system	0.332	2.13E-05
Z2425	ydaJ					putative aminohydrolase (EC 3.5.1.14)	0.357	0.000325
Z2477	pspE					phage shock protein	2.5	0.00463
Z2478	pspD					phage shock protein	3.718	0.0209
Z2479	pspC					phage shock protein: activates phage shock-protein expression	4.096	0.039
Z2480	pspB					phage shock protein, putative inner membrane protein	3.956	0.0479
Z2482	pspA					phage shock protein, putative inner membrane protein	4.751	0.0529
Z2548	trpD					anthranilate synthase component II	0.328	8.04E-05

ME0055 cont.

Systematic name	Common name	OI#	effector	mobile element	Ler/PchA*	Product	normalised	p-value
Z2646	ydgR					putative transport protein	2.472	0.000956
Z2707	ynhA					orf, hypothetical protein	2.103	0.0337
Z2711	ynhE					orf, hypothetical protein	2.562	0.0388
Z2712	ydiC					orf, hypothetical protein	3.152	0.00648
Z3147	nac					nitrogen assimilation control protein	0.481	1.99E-06
Z3549	lrhA					NADH dehydrogenase transcriptional regulator, LysR family	2.134	0.00351
Z3558	ackA					acetate kinase	2.195	0.0318
Z3572	argT					lysine-, arginine-, ornithine-binding periplasmic protein	0.266	0.00094
Z4631	yhdY					putative transport system permease protein	0.357	0.00182
Z5131	Z5131	148		CP-933L	P+L		0.356	4.59E-05
Z5133	escT	148		CP-933L	P+L	escT	0.292	0.000935
Z5137	Z5137	148		CP-933L	P+L		0.151	4.41E-07
Z5138	Z5138	148		CP-933L	P+L		0.302	0.00249
Z5139	Z5139	148		CP-933L			0.175	2.09E-06
Z5678	fdhF					formate dehydrogenase	0.391	0.00145
Z5855	pyrI					aspartate carbamoyltransferase, regulatory subunit	3.161	0.000152

Z5856	pyrB	aspartate carbamoyltransferase, catalytic subunit	3.462	0.0549