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The metabolic enzyme AdhE controls the virulence of Escherichia coli O157:H7

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Introduction

Enterohaemorrhagic Escherichia coli (EHEC) strains cause diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome (HUS), with the young and the elderly being most at risk (Nataro and Kaper, 1998). Cattle can be asymptomatically colonized by EHEC and thus can act as the major reservoir for human infections. Production of Shiga toxin (Stx) is responsible for HUS, the leading cause of acute paediatric renal failure in both the UK and USA (Noris and Remuzzi, 2005). No vaccines are currently available against EHEC infections and antibiotic treatment is associated with increased clinical severity, which can be attributed to increased production and/or release of the Stx toxin.

EHEC strains colonize the intestinal mucosa via a carefully regulated process that involves first expression of flagella and then expression of a Type Three Secretion System (T3SS) (Mahajan et al., 2009). The flagellum is a key motility organelle, while the T3SS is an organelle used widely by Gram-negative bacteria to facilitate interactions with host cells, including invasion, host cell killing and, in the case of EHEC, close attachment (Coburn et al., 2007). T3SS-mediated attachment of the bacterium to host epithelium is characterized by formation of distinctive attaching and effacing (A/E) lesions (Frankel et al., 1998). In EHEC strain O157:H7, the T3SS is encoded by the locus of enterocyte effacement (LEE) (Elliott et al., 2000). The first
three operons (LEE1–3) encode a multi-protein apparatus that spans the bacterial inner and outer membranes (Hueck, 1998). The fourth operon (LEE4) encodes proteins for a needle complex (EscF), a translocation filament (EspA) and pore forming proteins (EspB/D) (Hartland et al., 2000). LEE5 contains the genes for the adhesins Tir and intimin (Sánchez-Samartí et al., 2001).

In addition to motility, flagella mediate initial adhesion to epithelial cells in vitro and in vivo during colonization of the bovine intestine (Mahajan et al., 2009) and, therefore, play a role in the initial stages of the infection process. However, the expression of this virulence factor is tightly regulated. Since flagellin is the main agonist of Toll-like receptor 5 (TLR-5) and thus induces the production of pro-inflammatory cytokines (Hayashi et al., 2001), the ability to switch off expression of this virulence factor is important to evade detection by the immune system. Production of flagella requires expression of three classes of promoters (Chevance and Hughes, 2008). The class I operon includes genes for the master regulator FlhD,FlhC, which binds to and activates class II promoters. One class II operon includes flIA, which encodes the flagella-specific sigma factor (σ43), which is required for the expression of class III gene expression. The class III operon encodes structural proteins (e.g. flagellin) and the chemotactic proteins. Regulation of the flagellar regulon is complex and sophisticated, controlled by many stimuli and regulators (Wolfe and Visick, 2008). Importantly, flagella and the T3SS are cross-regulated to prevent coexpression (Iyoda et al., 2006), an arrangement that fits with their different functions – motility and initial attachment for the flagella (Mahajan et al., 2009), followed by close attachment and cell subversion for the T3SS.

Our previous work identified a set of proteins that are implicated in the regulation of virulence in EHEC; one was AdhE (D. Wang et al., 2011), a bi-functional acetaldehyde-CoA dehydrogenase and alcohol dehydrogenase. Most studies of AdhE have focused mainly on its role in ethanol production and its importance during anaerobic respiration (Clark and Cronan, 1980). In contrast, the role of AdhE under aerobic growth conditions has been poorly studied. However, two studies link AdhE to virulence in Salmonella typhimurium; a transposon insertion into adhE resulted in diminished capacity of S. typhimurium to survive murine macrophages (Baumler et al., 1994), while deletion of adhE affected SPI-1-mediated gene expression and infectivity (Abernathy et al., 2013). In this study, we found that deletion of the adhE from EHEC resulted in a stark pleiotropic phenotype: excretion of acetate into the surrounding environment, strong expression of non-functional flagella, suppression of the T3SS, and reduced binding to host cells. Further analysis of the ΔadhE mutant revealed insights into the molecular mechanism by which deletion of adhE affects gene expression. The identification of AdhE as a protein critical for proper regulation of virulence gene expression paves the way for further studies to specifically target this protein.

## Results

**AdhE affects virulence factor expression**

To evaluate whether AdhE regulates virulence factor expression, an adhE deletion mutant in the EHEC strain O157:H7 was generated by allelic exchange. No difference in the growth rate was observed between the wild-type (WT) parent and its isogenic ΔadhE mutant. To assess any changes in the expression of secreted virulence proteins, bacteria were cultured in MEM-HEPES media (Roe et al., 2003), and the secreted protein fraction was isolated. Secreted proteins were analysed by SDS-PAGE, tandem mass spectrometry (MS) and immunoblotting. The WT and ΔadhE mutant showed marked differences in their secreted protein profiles (Fig. 1A). Most starkly, the ΔadhE mutant increased expression of a ~60kDa protein. Tandem mass-spectrometry identified this protein as FlIC, the major structural subunit of the flagellar filament (Fig. 1A), a result confirmed by immunoblot analysis (Fig. 1B). Correspondingly, the ΔadhE mutant exhibited markedly elevated expression of σ28 (Fig. 1C), the flagellar-specific sigma factor required for fliC transcription. In contrast, the chaperone GroEL was expressed at similar levels in the WT and ΔadhE mutant (Fig. 1C). That these defects were the consequence of the adhE deletion was verified by allelic exchange with the WT allele; the resultant strain suppressed FlIC secretion (Fig. S1) and fully restored secretion of effector proteins.

Given that flagella and T3SS expression are cross regulated, we sought changes in secreted effector proteins and found that T3SS-associated proteins, including EspA and Tir, were markedly reduced in the ΔadhE mutant (Fig. 1A and B). To determine if this phenotype was due to a defect in assembly of the T3SS, whole cell lysates from WT and ΔadhE were analysed for the presence of EspJ, one of the basal apparatus proteins of the T3SS. EspJ (Fig. 1C) was detected only in WT bacteria, suggesting expression of the entire T3SS was reduced. Since the effector and the T3SS apparatus proteins were not detected in the ΔadhE mutant (Fig. 1C), we conclude that the entire T3SS regulon is downregulated in the ΔadhE mutant.

To visualize the pleiotropic phenotype, indirect immunofluorescence microscopy of EspA and FlIC was performed. The ΔadhE mutant produced diminished levels of EspA protein, but increased levels of FlIC (Fig. 1D). Indeed, the mutant had assembled multiple intact flagellar filaments on its surface. In summary, these data showed that the deletion of adhE results in a stark switch in phenotype: suppression of expression of the T3SS and strong upregu-
The ΔadhE mutant displays a 'paralysed' phenotype

Given that the ΔadhE mutant exhibited multiple apparently intact flagella, we wondered whether the mutant also displayed enhanced motility. Instead, the ΔadhE mutant was unable to migrate through semi-solid agar (Wolfe and Berg, 1989) (Fig. 2A). Changing the composition of the agar and incubation at different temperatures had no effect; the mutant did not migrate (data not shown). To determine whether this lack of migration resulted from a lack of motility, we visualized bacterial behaviour following culture in tryptone broth (TB) at 30°C, conditions that normally promote motility. Previous work has extensively characterized *E. coli* motility, which consists of directional 'swimming' that is interspersed by brief periods of 'tumbling' to facilitate chemotaxis (Porter et al., 2011). We used AlexaFluor 488 dye esters to assess the arrangement of the flagellar filaments on the bacterial cell surface (Turner et al., 2000). This enabled us to assess both swimming behaviour and flagellar motion. A polarized flagellar bundle, required for normal swimming activity, was visualized for WT EHEC (Fig. 2B and Movie S1). Transforming the WT with a plasmid encoding red fluorescent protein (RFP) enabled imaging for extended periods and showed...
both swimming and tumbling (Movie S2). In contrast, the ΔadhE mutant displayed a ‘paralysed’ phenotype (Fig. 2C and Movie S3). The flagella were dispersed over the surface of the bacterium, as expected of a peritrichous organism. Unlike WT flagella (Fig. 2B), the adhE flagella did not form a bundle (Fig. 2C). Collectively, these data show that the ΔadhE mutant can express and assemble flagella, but imply that the assembled flagella do not rotate.

Deletion of adhE reduces binding to host cells and increases activation of TLR-5

When added to epithelial cells, WT EHEC bacteria use their T3SS to condense host cell actin, facilitating close attachment – a process known as attaching and effacing (A/E) lesion formation. This process was visualized using immunofluorescence microscopy (Fig. 3A). After 4 h incubation of bacteria with host cells, WT bacteria clearly produced A/E lesions, a phenotype that was absent in the ΔadhE mutant. Indeed, both the total number of attached bacteria and the condensation of host cell actin were significantly reduced (P < 0.005) in the ΔadhE mutant relative to its WT parent (Fig. 3B and C). These results confirm that the mutant does not express the T3SS. In contrast to the WT, the ΔadhE mutant retained its flagella. Thus, it did not perform the normal switch from flagellar to T3SS expression, even in the presence of host cell signals or following direct contact with host cells.

Flagellin plays an important role in the activation of innate immune responses raising the possibility that the ΔadhE mutant might increase activation of TLR-5 signalling. This signalling was assessed using a HEK-293 TLR-5 cell line that produces secreted alkaline phosphatase in response to appropriate stimuli. Supernatants from WT EHEC, the ΔadhE mutant, and a ΔfliC mutant were added to the reporter cells across a range of dilutions and the extent of TLR-5 stimulation determined. The ΔadhE mutant exhibited a greater than 100-fold increase in TLR-5 stimulation compared with WT EHEC (Fig. 3D). The specificity of the reporter was confirmed by addition of supernatants prepared from a ΔfliC mutant; they produced no detectable TLR-5 activity (Fig. 3D).

Deletion of adhE affects colonization and clinical disease in vivo

Given that the ΔadhE mutant dysregulated expression of both the T3SS and flagella, we tested its ability to colonize the mammalian intestine, using the infant rabbit model of *E. coli* O157:H7 infection (Ritchie et al., 2003). In this model, *E. coli* O157:H7 colonization is dependent on a functional T3SS system (Ritchie et al., 2003; Ritchie and Waldor, 2005). Furthermore, rabbits develop diarrhoea and intestinal inflammation when infected by WT EHEC, but not by mutants lacking intimin or Tir. Thus, EHEC pathogenesis also depends on the T3SS (Ritchie et al., 2003). Litters of 3-day-old New Zealand White infant rabbits were oro-gastrically inoculated with either WT EHEC or the adhE mutant. Rabbits were scored for diarrhoea and the number of cells present in the intestine enumerated at 5 days post infection. As expected, all rabbits infected with WT EHEC developed severe diarrhoea, as evident by the presence of extensive areas of faecal staining on their hind legs and tails. In contrast, approximately 70% of the rabbits infected with the adhE mutant did not develop any visible signs of diarrhoea and the remaining animals exhibited only mild diarrhoea (P < 0.05) (Table 1). Furthermore, significantly fewer bacteria were recovered in intestinal homogenates
obtained from rabbits infected with the adhE mutant compared with rabbits given WT EHEC. The number of organisms recovered from adhE-infected rabbits was >10-fold less in the mid-colon (P < 0.05) and distal colon (P < 0.01) at 5 days post infection (Fig. 4A and B). Thus, the adhE mutant is less able to colonize the mammalian intestine and cause disease than WT EHEC. We conclude that AdhE plays a key role in virulence.

Deletion of adhE increases transcription of the flagellar regulon

To investigate the molecular basis for this phenotype, we used RNA-sequence analysis (RNA-seq), which provided us with a global view into the differences in gene expression between the ΔadhE mutant and its WT. The results were extremely specific. Members of the flagellar regulon were the most markedly affected genes (Fig. 5A). Transcripts from all three flagellar gene classes were significantly upregulated in the mutant (P < 0.005). This was true of the regulatory genes flhC (14-fold), flhD (16-fold) and fliA (5134-fold), of structural components such as fliF (138-fold), flgE (503-fold) and fliC (2143-fold), of the chemotaxis machinery, such as cheA (502-fold) and cheY (207-fold), and of the energy transduction system motA (384-fold) and motB (319-fold) (Table S1). In contrast, expression of several ‘housekeeping’ controls was similar in the ΔadhE mutant and its WT parent (Table S1). These results support the conclusion that the ΔadhE mutant synthesizes and assembles flagella.

Deletion of adhE does not affect transcription of the LEE

Surprisingly, transcription of the LEE was not significantly (P < 0.05) affected in the ΔadhE background, with levels equivalent to those of the WT parent (Fig. 5B). Of the 42 LEE-encoded genes, 36 showed less than a twofold change in expression when the WT and adhE mutant were compared (Table S1) and none of these differences were statistically significant. These results were verified using reporter plasmids that carried four key LEE promoters (LEE1, LEE2, LEE3 and LEE5) fused to gfp. As a control, we monitored expression of rpsM, which encodes a small ribosomal protein. When cultured under T3SS-inducing conditions, all four of the LEE-encoded promoters transcribed at similar levels in both the WT parent and its ΔadhE mutant, as did the ribosomal control (Fig. 5C–G). In contrast, transcription from the fliC::gfp reporter was strongly elevated in the ΔadhE mutant, consistent with the RNA-seq and immunoblotting data (Fig. 5H). Thus, the adhE mutant exhibits a fascinating phenotype: strong transcriptional upregulation that produces assembled but non-functional flagella coupled with post-transcriptional regulation of the LEE.

Suppression of the T3SS is controlled post-transcriptionally in the adhE mutant

In the ΔadhE mutant, there was a stark contrast between expression of the LEE, which was at WT levels, and T3SS

Table 1. Diarrheal status of rabbits infected with WT EHEC or the adhE mutant.

<table>
<thead>
<tr>
<th>Diarrhoea</th>
<th>WT</th>
<th>ΔadhE</th>
</tr>
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<tbody>
<tr>
<td>Severe</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

Fishers exact test P < 0.05

a. Diarrhoea is scored as follows: severe – extensive contamination of ventral surfaces with liquid faeces; mild – localized areas of faecal contamination and observation of soft, formed faecal material; none – no faecal staining on animals’ ventral surfaces with hard, formed stool occasionally present.

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protein secretion, which was strongly inhibited. Several studies have shown that the expression of the LEE can be controlled in a post-transcriptional manner. One key regulator that has been shown to affect LEE expression is Hfq, a chaperone that binds small regulatory RNA (sRNAs) and mRNAs to facilitate translational regulation in response to a variety of stresses. We therefore investigated Hfq levels by immunoblotting and found that Hfq expression was markedly increased in the \( \Delta \text{adhE} \) mutant (Fig. 6A), eightfold as determined by scanning densitometry of the blots. This result suggested that elevated levels of Hfq might be central to the regulation of the T3SS. To test this hypothesis, we raised Hfq levels in the WT parent by transformation with a plasmid that expresses Hfq, and found that secretion of T3SS effector proteins decreased (Fig. 6B). These included intimin (encoded by \( \text{LEE5} \)) and EspP (encoded by pO157) (Fig. 6B). However, overexpression did not result in a complete loss of T3SS secretion and did not cause a switch to flagella expression, as seen in the \( \Delta \text{adhE} \) mutant. These data raised the possibility that Hfq inhibits production of the T3SS in the \( \Delta \text{adhE} \) mutant in response to strongly elevated flagellar expression. To test this hypothesis, we transformed the WT strain with a plasmid carrying both \( \text{flhD} \) and \( \text{flhC} \), thereby creating a scenario similar to the \( \Delta \text{adhE} \) mutant: concurrent transcription of both the flagella regulon and the LEE operons. Expression of \( \text{flhDC} \) in a WT background raised Hfq levels (Fig. 6A) and reduced production of the effector proteins from the T3SS compared with the WT, especially intimin and Tir (Fig. 6B). However, \( \text{flhDC} \) overexpression did not result in a complete switch to flagella expression observed in the \( \Delta \text{adhE} \) mutant.

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Deletion of adhE affects acetate production

AdhE performs two enzymatic functions: the conversion of acetyl-CoA to acetaldehyde, followed by the conversion of acetaldehyde to ethanol (Fig. 7A). AdhE works in conjunction with the Pta-AckA pathway to recycle NAD+ and CoA with the generation of ATP by substrate phosphorylation (Wolfe, 2005). Deletion of AdhE would presumably affect levels of the metabolites directly associated with these two pathways, such as acetyl-CoA, acetyl phosphate and acetate. Thus, we hypothesized that virulence factor dysregulation might be related to changes in one or more of these metabolites. Using highly quantitative 1H-NMR, extracellular acetate levels were assessed in WT EHEC and its ΔadhE mutant. In the WT, extracellular acetate was measured at 4.41 ± 0.04 mM, a level that was raised by 18% in the mutant to 5.21 ± 0.05 mM. Because acetyl-CoA and acetyl phosphate are high energy central metabolites (Wolfe, 2005) that can function as acetyl donors for Nε-lysine acetylation in E. coli (Hu et al., 2010; Thao and Escalante-Semerena, 2011; Weinert et al., 2013; Kuhn et al., 2014), we also examined the acetylation status of the ΔadhE mutant and its parent. An anti-acetyl lysine immunoblot showed greater cross-reactivity for several different bands in the ΔadhE mutant, suggesting that numerous proteins were acetylated compared with the WT control (Fig. 7B). Furthermore, the ΔadhE mutant expressed 100-fold more cAMP receptor protein (CRP) than its WT parent, as determined by scanning densitometry of triplicate immunoblots (Fig. 7C). This result is intriguing because acetate accumulation induces flhDC and thus flagellar expression via CRP (Soutourina et al., 1999).

Finally, to test whether the metabolic effects of adhE deletion were specific to the AdhE and Pta-AckA pathways, we conducted a global metabolomic analysis using a mass-spectrometry based approach, which enabled the detection and relative quantification of more than 300 metabolites that originated from diverse cellular pathways, including amino acid synthesis, lipid and carbohydrate metabolism, and nucleic acid synthesis. No significant differences were detected in the ΔadhE mutant relative to its WT parent (data not shown), suggesting that deletion of AdhE specifically affects metabolites immediately associated with these two pathways.

Discussion

That central metabolism plays a role in regulating virulence factors is becoming increasingly evident. From a biological viewpoint this makes sense, as the metabolic status of a cell should determine its ability to effectively adapt to environmental changes encountered during the course of an infection. An early report suggested that acetyl-CoA or a derivative was responsible for the control the T3SS of Pseudomonas aeruginosa (Rietsch et al., 2005; Rietsch and Mekalanos, 2006), while a more recent report linked Vibrio cholerae virulence to acetyl-CoA (Minato et al., 2013). Several other reports provided evidence that other metabolic products induce expression of virulence factors, including the Shiga toxin and flagella (Fukuda et al., 2011;
Deletion of AdhE, a metabolic enzyme linked to acetyl-CoA, offered an opportunity to further these findings. The anaerobic role of AdhE is well-characterized; it is essential for ethanol fermentation (Clark and Cronan, 1980). Since AdhE also is expressed under aerobic conditions (Echave et al., 2003), its role is likely not limited to anaerobic conditions, but the nature of that aerobic role has remained unknown. Here, we deleted adhE and observed a novel pleiotropic phenotype: (i) overexpression of the flagellar regulon that results in assembled but non-functional flagella coupled to (2) a complete lack of the T3SS, including the structural proteins that comprise the secretory apparatus and its effector proteins. Thus, the mutant was immotile. It also stimulated activation of innate immune responses through TLR-5, presumably because of the flagella that it assembles on its surface. Finally, it bound to host cells in fewer numbers, likely because it lacks the T3SS. Collectively, these phenotypes rendered the ΔadhE mutant a less effective pathogen. In fact, when tested in vivo using the rabbit model of EHEC infection, the ΔadhE mutant colonized poorly and caused less diarrhoea than the WT, traits that are dependent on a functional T3SS (Ritchie et al., 2003).

Deletion of adhE should perturb the pathways associated with acetyl-CoA. Indeed, 1H-NMR analysis showed that the ΔadhE mutant excretes about 20% more acetate than its WT parent. Since the undissociated form of acetate easily permeates the membrane and distributes according to the ΔpH across the membrane (Booth, 1985), this 18% difference corresponds to a 9 mM increase in the intracellular pools of both acetyl-CoA and acetyl phosphate, high-energy molecules that can function as acetyl donors for protein acetylation (Hu et al., 2010; Weinert et al., 2013; Kuhn et al., 2014). Indeed, the increased signal detected using the anti-acetyl lysine antibody suggested that numerous proteins were strongly acetylated in the ΔadhE mutant compared with the WT control. While we have not yet determined the identity of the acetylated proteins, it should be noted that recent work has shown that acetylation is quite extensive in both E. coli (Zhang et al., 2009; Zhao et al., 2010; Wang, 2013; Weinert et al., 2013; Kuhn et al., 2014) and in Salmonella enterica (Q. Wang, 2013). The lack of motility by the adhE mutant might result from acetylation of one or more flagellar proteins. Alternatively, the flagellum might be assembled incorrectly, thereby blocking its function. A candidate for this role could be YcgR. This flagellar regulon member acts as a brake when bound by the second messenger cyclic-di-GMP (Boehm et al., 2010; Paul et al., 2010).
Our preferred model is that deletion of the AdhE pathway leads to increased flux through the Pta-AckA pathway, which leads to increased intracellular acetate, acetyl phosphate, and acetyl-CoA. The elevated intracellular acetate increases expression of CRP and σ28. CRP activates flhDC (which encodes the master regulator of the flagellar regulon), while σ28 boosts expression of Class III proteins, such as flagellin. The assembled flagella do not rotate either because a key component is misassembled. YcgR is overexpressed, and/or some flagellar component is inappropriately acetylated.

The other aspect of the phenotype was a lack of production of the T3SS, including the structural proteins required for secretion and the effector proteins themselves. Whereas flagella expression was activated at the level of transcription, it was clear that a different mechanism was inhibiting production of the T3SS. One important caveat of our study was that it was largely performed in media that induces expression of the T3SS. Therefore, the ΔadhE mutant, despite activating flagella expression, was still subject to the environmental signals that stimulated LEE transcription. This was shown very clearly by both RNA-seq and reporter fusion analyses. However, this led to a paradox: LEE transcription in the mutant was equivalent to that in the WT parent, yet expression or secretion of T3SS effector proteins was virtually undetectable.

One strong candidate protein with the capacity to sequester and turn over RNA species is Hfq. Binding of Hfq to an mRNA transcript can either promote its stabilization or degradation, depending on the specific interaction (Massé and Gottesman, 2002; Vytvytska et al., 1998; Q. Wang, 2013). Deletion of hfq in E. coli O157 strain EDL933 leads to increased levels of LEE-encoded proteins by negatively controlling levels of the regulators GrlA and GrlR post-transcriptionally (Hansen and Kaper, 2009). It should be noted that Hfq-mediated effects can be quite strain specific and can be pleiotropic: deletion of hfq in E. coli O157 strain 86-24 led to quite different phenotypes, including a transcriptional downregulation of the LEE (Kendall et al., 2011). The strains in this work were derivatives of EDL933 leading to the hypothesis that, in the ΔadhE mutant, an increase in Hfq expression could suppress LEE expression. While previous studies have focused on the role of Hfq on the master regulator Ler and GrlA (Hansen and Kaper, 2009), ongoing studies have revealed a much wider set of LEE encoded transcripts that can be affected by Hfq, including LEE4 and LEE5 (J. Tree and D. Gally, pers. comm.). This is consistent with our previous research showing that these particular operons are controlled post-transcriptionally (Roe et al., 2003; 2004) and with data in the current work showing that Hfq regulation of T3SS in the ΔadhE mutant is a critical factor after Ler induction of the system. This is apparent from the marked phenotypic impact of the ΔadhE mutant on the T3SS despite very little effect on transcript levels, including those induced by Ler (LEE2/3 and LEE5, Fig. 5). Immunoblotting confirmed that Hfq was elevated in the ΔadhE mutant. Mimicking the concurrent transcription of flagella and LEE operons by overexpression of flhDC in a WT background also raised Hfq levels. These data suggest that Hfq acts as a final ‘safety net’ to mop up transcripts that are expressed, but selectively not translated into proteins.

Our finding that dysregulation of flux from the acetyl-CoA pool regulates the switch from the motile flagellated state to the attached T3SS-expressing state may be help explain why EHEC display a tropism to one distinct site in the bovine host. Previous work has shown that EHEC use their T3SS to colonize the recto-anoal junction of cattle (Naylor et al., 2003). Cattle contain very high concentrations of short chain fatty acids (SCFAs, e.g. acetate) that vary along the gastrointestinal tract. In the bovine rumen, it is common for acetate concentrations to vary from 60 to 150 mM, with levels at their lowest in the stomach and at the rectum (Bergman, 1990). It is attractive to propose that the low levels of acetate present at the rectum correspond with expression of the T3SS and colonization of the recto-anal tissue. Overall, it seems clear that the relevance of both host and bacterial-derived metabolites can play a major role in influencing virulence gene expression and that this markedly affects the interaction of the pathogen with its host. Recent work has also shown that the effects of Shiga toxin can be modulated in vivo by manipulation of acetate and butyrate levels (Fukuda et al., 2012; Zumbrun et al., 2013). It is worth noting that the strains used in this study were Shiga toxin negative; therefore, toxin activity would not have influenced the clinical symptoms observed in our experiments.

In summary, we have shown that expression of AdhE, a bi-functional acetaldehyde-CoA dehydrogenase and alcohol dehydrogenase, is central for appropriate expression of the genes for motility and attachment in E. coli O157:H7 both in vitro and in vivo. The molecular basis to the phenotype was found to be multifactorial involving perturbations to acetate levels that directly influenced flagella expression, post-transcriptional regulation of the T3SS through Hfq, and effects on protein acetylation. The importance of AdhE for appropriate expression of virulence genes paves the way for further studies to specifically target this protein.

**Experimental procedures**

**Strains and media**

The E. coli O157:H7 wild-type strain used in this study was TUV93-0 (Campellone et al., 2004). The ΔadhE mutant was generated using allelic exchange (Emmerson et al., 2006). To verify that all phenotypes were due to the mutation, it was subsequently repaired by allelic exchange of the WT adhE
allele back into the chromosome at the native locus (ΔadhE + adhE). Bacteria were cultured overnight in LB media and the T3SS induced by culturing in MEM-HEPES (Sigma). Motility assays were carried out using tryptone broth (1% tryptone and 0.5% sodium chloride) with 0.25% agar. Bacteria were inoculated onto the centre of the plate and incubated at 34°C overnight (Wolfe and Berg, 1989).

Animal protocols

Experiments were performed as described previously (Ritchie and Waldor, 2005) using the TU93-0 strain and the ΔadhE mutant. Bacterial doses of 5 × 10^8 colony-forming units (cfu) per 90 g of rabbit body weight were used in the infections. Post inoculation, the infant rabbits were weighed daily and observed twice daily for clinical signs of illness. Diarrhoea was scored as follows: ‘none’, no fecal staining on animals’ ventral surfaces with hard, formed stool occasionally present; ‘mild’, localized areas of faecal contamination and observation of soft, formed faecal material; ‘severe’ extensive contamination of ventral surfaces with liquid faeces. To limit any litter-specific effects, two different litters were used to test each type of inoculum studied.

Ethics statement

This study was carried out in strict accordance with the recommendations in the United Kingdoms Home Office Animals (Scientific Procedures) Act of 1986, which outlines the regulation of the use of laboratory animals for the use of animals in scientific procedures. The experiments described were subject to approval by the University of Surrey Ethics Committee and by a designated Home Office Inspector. All experiments were subject to the 3 R consideration (refine, reduce and replace) and all efforts were made to minimize suffering.

RNA sequencing

Total RNA was prepared as described previously (Tree et al., 2009) and depleted for ribosomal sequences using a Microexpress kit (Ambion). Sequencing of cDNA was carried out on an Illumina Genome Analyser Ix using single ended reads and 6 samples per lane. Raw transcript data were analysed using CLC Genomics Workbench 4 using the E. coli EDL933 genome as a reference. The sequence reads reported in this study have been deposited in the European Nucleotide Archive under study PRJEB6365 (ERS462727-ERS462730).

Protein characterization

Secreted proteins were prepared as described in Roe et al. (2003), briefly, bacteria were cultured in MEM-HEPES to OD_600 = 0.8 and cells were pelleted by centrifugation (10 min at 3000 g). The secreted proteins in the supernatant were precipitated by addition of 10% TCA, pelleted by centrifugation (40 min at 10 000 g) and resuspended in 1.5 mM Tris pH 8.8. Bacterial cell pellets were lysed using Bugbuster (Millipore). Whole cell and secreted proteins separated using SDS-PAGE and stained with Coomassie brilliant blue or transferred to nitrocellulose (Amersham) for immunoblotting. Antibodies for the T3SS proteins and Hfq were provided by Prof David Gally (University of Edinburgh) and Profs Susan Gottesman (Centre for Cancer Research, Bethesda) and Elisabeth Sonnleitner (University of Vienna) respectively. The β2M (Mast Assure), GroEL (Enzo), Sigma factors (Neoclon) and anti acetyl lysine antibodies (Abcam) were purchased and used as indicated by the manufacturer. Bands from the gel were excised to permit in-gel trypsin digestion and analysis by liquid chromatography electrospray ionization tandem mass spectrometry (Shevchenko et al., 2006).

Microscopy

Real-time flagellar imaging was performed as described (Turner et al., 2000) on a Zeiss M1 Axioimager microscope using Velocity suite software (Perkin Elmer). T3SS, flagella expression and formation of A/E lesion were analysed as described previously (Tree et al., 2009).

Measurement of acetate concentrations

One-dimensional high-resolution 1H-NMR spectra were acquired on a Brucker AVANCE 600 MHz spectrophotometer at 298 K. The acetate peak at ∼ 1.9 ppm was identified and ∼ 8.8. Bacterial cell pellets were lysed using Bugbuster (Millipore). Whole cell and secreted proteins separated using SDS-PAGE and stained with Coomassie brilliant blue or transferred to nitrocellulose (Amersham) for immunoblotting. Antibodies for the T3SS proteins and Hfq were provided by Prof David Gally (University of Edinburgh) and Profs Susan Gottesman (Centre for Cancer Research, Bethesda) and Elisabeth Sonnleitner (University of Vienna) respectively. The β2M (Mast Assure), GroEL (Enzo), Sigma factors (Neoclon) and anti acetyl lysine antibodies (Abcam) were purchased and used as indicated by the manufacturer. Bands from the gel were excised to permit in-gel trypsin digestion and analysis by liquid chromatography electrospray ionization tandem mass spectrometry (Shevchenko et al., 2006).

Measurement of acetate concentrations

One-dimensional high-resolution 1H-NMR spectra were acquired on a Brucker AVANCE 600 MHz spectrophotometer at 298 K. The acetate peak at ∼ 1.9 ppm was identified and integrated between 1.921 and 1.888 ppm for each sample. This value was compared with a standard curve of known acetate concentrations to quantify the levels in the media. Measurements from triplicate independent experiments were averaged and differences analysed by an unpaired t-test.
form: methanol: water (ratio 1:3:1) followed by vigorous shaking for 60 min at 4°C. Extract mixtures were centrifuged for 2 min at 16 000 RCF, 4°C. The supernatant was collected, frozen and stored at −80°C until further analysis.

Samples were analysed using an UltiMate 3000 RSLC (Thermo Fisher) with a 150 × 4.6 mm ZIC-pHILIC column running at 300 μl min⁻¹ coupled to an Orbitrap Exactive (Thermo Fisher) mass spectrometer. The gradient ran from 20% H₂O 80% acetonitrile to 80% H₂O, 20% acetonitrile in 15 min, followed by a wash at 5% acetonitrile, 95% H₂O for 4 min, and equilibration at 20% H₂O, 80% acetonitrile for 6 min. Raw mass spectrometry data was processed using an in-house pipeline, consisting of XCMS (Smith et al., 2006) (for peak picking), MzMatch (Scheltema et al., 2011) (for filtering and grouping) and IDEOM (for further filtering, post-processing, statistical analysis and identification). Core metabolite identifications were validated against a panel of unambiguous standards by mass and retention time. Additional putative identifications were assigned by mass and predicted retention time (Creek et al., 2011).

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References


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