RcsB determines the locus of enterocyte effacement (LEE) expression and adherence phenotype of *Escherichia coli* O157:H7 spinach outbreak strain TW14359 and coordinates bicarbonate-dependent LEE activation with repression of motility

Jason K. Morgan, Khoury W. Vendura, Stanley M. Stevens Jr and James T. Riordan

The 2006 US spinach outbreak of *Escherichia coli* O157:H7, characterized by unusually severe disease, has been attributed to a strain (TW14359) with enhanced pathogenic potential, including elevated virulence gene expression, robust adherence and the presence of novel virulence factors. This study proposes a mechanism for the unique virulence expression and adherence phenotype of this strain, and further expands the role for regulator RcsB in control of the *E. coli* locus of enterocyte effacement (LEE) pathogenicity island. Proteomic analysis of TW14359 revealed a virulence proteome consistent with previous transcriptome studies that included elevated levels of the LEE regulatory protein Ler and type III secretion system (T3SS) proteins, secreted T3SS effectors and Shiga toxin 2. Basal levels of the LEE activator and Rcs phosphorelay response regulator, RcsB, were increased in strain TW14359 relative to O157:H7 strain Sakai. Deletion of *rcsB* eliminated inherent differences between these strains in *ler* expression, and in T3SS-dependent adherence. A reciprocating regulatory pathway involving RcsB and LEE-encoded activator GrlA was identified and predicted to co-ordinate LEE activation with repression of the *flhDC* flagellar regulator and motility. Overexpression of *grlA* was shown to increase RcsB levels, but did not alter expression from promoters driving *rcsB* transcription. Expression of *rcsDB* and RcsB was determined to increase in response to physiological levels of bicarbonate, and bicarbonate-dependent stimulation of the LEE was shown to be dependent on an intact Rcs system and *ler* activator *grvA*. The results of this study significantly broaden the role for RcsB in enterohaemorrhagic *E. coli* virulence regulation.

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is a virulent human pathogen attributed to sporadic cases and large outbreaks of bloody diarrhoea (haemorrhagic colitis) (Rangel *et al.*, 2005). In 2006, an outbreak in the United States of O157:H7 due to the consumption of tainted spinach was associated with unusually high rates of hospitalization and life-threatening sequelae (i.e. haemolytic uraemic syndrome) (Manning *et al.*, 2008). Phylogenetic analysis of over 500 clinical O157:H7 isolates suggested that the strain which caused this outbreak, TW14359, belongs to a discrete genetic group referred to as clade 8, members of which are highly virulent (Manning *et al.*, 2008). Consistent with this hypothesis, DNA sequencing of this strain has revealed the presence of virulence factors that are absent in the reference genomes of sequenced O157:H7 strains Sakai (1996 Japan) and EDL933 (1982 USA) (Kulasekara *et al.*, 2009; Manning *et al.*, 2008). In addition, a virulence expression phenotype has been described for strain TW14359 characterized by increased basal transcription of locus of enterocyte effacement (LEE) genes, as well as Shiga toxin 2 genes (*stx2AB*) and Stx2 protein relative to other O157:H7 strains (Abu-Ali *et al.*, 2010a, b; Neupane *et al.*, 2011). Elevated basal LEE expression in TW14359 has
further been correlated with increased adherence to bovine epithelial cells (Abu-Ali et al., 2010a).

The LEE is a 35.6 kb pathogenicity island that encodes a type III secretion apparatus required for competitive colonization of the intestine and attactting/effacing lesion formation in EHEC and enteropathogenic E. coli (EPEC) (Elliott et al., 1998; McDaniel & Kaper, 1997; Pernia et al., 1998), and in the mouse pathogen Citrobacter rodentium (Schauer & Falkow, 1993). Regulation of LEE expression has been extensively studied (Mellies et al., 2007; Tree et al., 2009), but is still not fully understood. Four LEE-encoded proteins are currently known to control its expression: Ler (LEE-encoded regulator), Mcp (multiple point controller), GrIR (global regulator of LEE repressor) and GrIA (global regulator of LEE activator). ler is the first gene of the LEE1 operon, and is a master regulator of the LEE, activating transcription from all five LEE operons (Elliott et al., 1998; Sperandio et al., 1999). Mcp, when overexpressed, downregulates LEE expression through interaction with Ler, and GrIA directly activates ler transcription (and thus the LEE), whereas GrIR represses ler transcription through interactions with GrIA (Creasey et al., 2003; Deng et al., 2004; Huang & Syu, 2008; Lio & Syu, 2004). In addition, a myriad of non-LEE-encoded regulators modulate LEE expression through ler in response to environmental cues such as growth phase, bicarbonate and stress (Abe et al., 1997, 2002; Bergholz et al., 2007b; Friedberg et al., 1999; Grant et al., 2003; Kenny et al., 1997; Shin et al., 2001; Sperandio et al., 1999; Tobe et al., 2005; Umanski et al., 2002).

In this study, a role for RcsB, a response regulator of the Rcs phosphorelay system, in the virulence expression phenotype of strain TW14359 was examined. In particular, the importance of RcsB to enhanced LEE expression and adherence characteristic of this strain was investigated. In addition, the study sought to define the regulatory contribution of RcsB to bicarbonate-dependent activation of the LEE and LEE-dependent repression of motility.

METHODS

**Bacterial strains and culture conditions.** The strains and plasmids used in this study are listed in Table 1. Strains were stocked at −80 °C in glycerol diluted (15%, v/v, final concentration) in Luria broth (LB), and were maintained in LB or on LB with 1.5% agar (LBA). Unless otherwise noted, overnight (18–20 h) cultures grown in LB were used to inoculate fresh LB or LB buffered with sodium bicarbonate (44 mM NaHCO3) or fresh Dulbecco’s modified Eagle’s medium (DMEM) (4 g glucose l−1, 4 mM glutamine, 44 mM NaHCO3, pH 7) to a final OD600 of 0.05. Cultures were grown at 37 °C in a rotary shaker (200 r.p.m.) using a 1:10 media-to-flask volume. Growth of strains was monitored by taking OD600 readings at 1 h intervals for 11 h (Fig. S1, available in Online). The strains and plasmids used in this study are listed in Table 1. Strains were stocked at −80 °C in glycerol diluted (15%, v/v, final concentration) in Luria broth (LB), and were maintained in LB or on LB with 1.5% agar (LBA).

**RNA purification and qRT-PCR.** Primers used for qRT-PCR are provided in Table S1. RNA purification, cDNA synthesis, qRT-PCR cycling

**Protein extraction, SDS-PAGE and Western blots.** Protein extraction, purification and Western blots were performed as described previously (Mitra et al., 2012). To extract and purify secreted proteins, mid-exponential phase (OD600 of 0.5) cultures were centrifuged at 5000 g for 5 min and supernatants were passed through sterile 0.22 μm Millex-GV syringe filters (Millipore). Filtrates were precipitated overnight (18–20 h) in 15% (v/v) trichloroacetic acid at 4 °C and then centrifuged at 15,000 g for 30 min at 4 °C. Protein pellets were washed twice with 100% ice-cold acetone before resuspension in 1 M triethyl ammonium bicarbonate. The amount of protein loaded on SDS-PAGE gels for Western blots was measured using a Bradford protein assay standard curve, and equal loading was validated by Western blots for GroEL using anti-GroEL mAbs (Bio-Rad). mAbs against FLAG (Sigma-Aldrich) were also used. Each experiment was repeated a minimum of three times in independent trials. Densitometry was used to estimate differences in protein levels for select experiments using a ChemiDoc XRS+ Imaging System and Image Lab 3.0 (Bio-Rad).

**Proteomic analysis.** Isobaric tag for relative and absolute quantification (iTRAQ)-based MS was carried out on strains TW14359 and Sakai following a previously described protocol (Rivera et al., 2012). DMEM cultures (n=4 for cytosolic; n=3 for secreted) of strains TW14359 and Sakai were grown to an OD600 of 0.5 (2.5–3 h) before sampling for protein extraction (see above Methods). Peptide labelling with isobaric tags (FLAG) that can be detected by Western blots using anti-FLAG mAbs (Uzzau et al., 2001). All genetic constructs were validated using a combination of PCR and restriction mapping, quantitative real-time PCR (qRT-PCR) and DNA sequencing (Eurofins MWG Operon). DNA was purified using the QIAquick PCR Purification kit (Qiagen).
conditions and data analysis followed previously described protocols (Riordan et al., 2010). For ler mRNA stability, cultures were grown to mid-exponential phase (OD600 of 0.4) before addition of a subinhibitory concentration of the transcription inhibitor rifampicin (Rif; 300 μg ml−1 final concentration). Sampling for RNA extraction was performed immediately before addition of Rif, and at 4 min intervals thereafter for 12 min. qRT-PCR was performed using a Realplex2 Mastercycler (Eppendorf). Cycle threshold (Ct) data were normalized to 16S rRNA gene and normalized cycle threshold values (ΔΔCt) were transformed to arbitrary transcript expression levels using 2−ΔΔCt/10−6 as described (Livak & Schmittgen, 2001; Riordan et al., 2010). Expression levels were compared using the appropriate t-test or by Tukey’s honestly significant difference (HSD) test following a significant F-test (n≥3, x = 0.05) (R. ver. 2.13.0).

Construction of lacZ transcriptional promoter fusions and β-galactosidase assays. Construction of lacZ reporter transcriptional fusions to the promoters of ler, rcsB, rcsD and flhD followed a previously described protocol using vector pRS551 (Simons et al., 1987). For lerPCR951-lacZ, a 904 bp BamHI/EcoRI-digested PCR fragment generated using primers ler-905/EcoRI and ler-1/BamHI was cloned into similarly digested pRS551 using T4 DNA ligase (Fisher Scientific) to produce pRM-2. Similarly, rcsB, rcsD and flhD promoter fragments were cloned into pRS551 following BamHI/EcoRI digestion using primers rcsB-1/BamHI and rcsD-1000/EcoRI (rcsB1000−lacZ), rcsD-501/EcoRI and rcsD-1/BamHI (rcsD951−lacZ), and flhD-1/BamHI and flhD-1000/EcoRI (flhD1000−lacZ). ler-, rcsB-, rcsD- and flhD−lacZ transcriptional fusion plasmids were transformed into wild-type (WT) and derivative backgrounds of strains TW14359 and Sakai. All lacZ fusion constructs were confirmed by PCR and sequencing (Eurofins MWG Operon). β-Galactosidase activity (Miller units) was measured as previously described and compared between strains using a Student’s t-test or by Tukey’s HSD test following a significant F-test (n≥3, x = 0.05) (R) (Miller, 1972; Mitra et al., 2012).

Adherence assays. Adherence to epithelial cells was determined following the method of Abe et al. (2002). Briefly, human HT-29 (ATCC HTB-38) colonic epithelial cells were grown to confluence on polylysine-treated glass coverslips placed within the wells of 24-well culture plates at 37 °C with 5 % CO2. Overnight DMEM cultures were diluted 1:40 (v/v) in fresh DMEM and 0.05 ml of this dilution was used to inoculate each well, which already contained 0.45 ml sterile DMEM was then added before incubating for an additional 3 h. Plate wells were subsequently washed three times in PBS, and then fixed with ice-cold (−20 °C) 100 % methanol for 10 min before staining with Giemsa diluted in PBS (1:20, v/v) for 20 min. Giemsa stain was

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>Vector propagation, recA1 endA1</td>
<td></td>
</tr>
<tr>
<td>Sakai</td>
<td>WT 1996 outbreak, Osaka, Japan</td>
<td>Michino et al. (1999)</td>
</tr>
<tr>
<td>TW14359</td>
<td>WT 2006 outbreak, western USA</td>
<td>Manning et al. (2008)</td>
</tr>
<tr>
<td>EDL933</td>
<td>WT 1982 outbreak, MI and OR, USA</td>
<td>Riley et al. (1983)</td>
</tr>
<tr>
<td>EcRJM-1</td>
<td>pGW14359ArecN</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-2</td>
<td>Sakai ΔrscN</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-3</td>
<td>TW08264ArscB</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-4</td>
<td>TW14359ArscB</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-5</td>
<td>TW14359ArscB</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-6</td>
<td>TW14359ArscC</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-7</td>
<td>TW14359ArscC</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-8</td>
<td>TW08264ArscB-FLAG</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-9</td>
<td>TW14359rasB-FLAG</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-10</td>
<td>TW14359rasB-FLAG</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-11</td>
<td>TW14359AgrrA</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-12</td>
<td>TW14359ArscB ΔagrA</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-13</td>
<td>TW14359tir-FLAG</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-14</td>
<td>TW08264tir-FLAG</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-15</td>
<td>TW14359ArscB tir-FLAG</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-16</td>
<td>TW08264ArscB tir-FLAG</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-35</td>
<td>TW14359grrR::kan</td>
<td>This study</td>
</tr>
<tr>
<td>EcRJM-59</td>
<td>TW14359ArscB grrR::kan</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD22</td>
<td>Ara inducible expression vector, AmpR, M13</td>
<td>Guzman et al. (1995)</td>
</tr>
<tr>
<td>pBAD-TOPO(R)</td>
<td>Ara inducible expression vector, lacZ, V5-His, pBR322</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pSU312</td>
<td>FLAG epitope template, AmpR, KanR, R6K</td>
<td>Uzzau et al. (2001)</td>
</tr>
<tr>
<td>pRM-1</td>
<td>pACYC177 containing rcsB</td>
<td>This study</td>
</tr>
<tr>
<td>pRM-2</td>
<td>prRS551 containing lerP951-lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pRM-7</td>
<td>prRS551 containing rcsBp1000-lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pRM-8</td>
<td>prRS551 containing rcsDp1001-lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pRM-15</td>
<td>pBAD22 containing grlA</td>
<td>This study</td>
</tr>
<tr>
<td>pRM-17</td>
<td>prRS551 containing flhDp1000−lacZ</td>
<td>This study</td>
</tr>
</tbody>
</table>
aspirated from the wells, and each stained coverslip was then examined at 1000× magnification by oil immersion bright-field microscopy using a binocular microscope (Fisher Scientific). Microcolonies, defined as a pattern of localized adherence (McKee & O’Brien, 1995; Nataro & Kaper, 1998), were scored as discrete clusters of five or more bacterial cells as previously defined (Abe et al., 2002; Iyoda & Watanabe, 2004). For each sample, a minimum of ten viewing frames were observed and the mean number of microcolonies was reported per 80 HT-29 cells. Microcolony counts were compared between strains by Tukey’s HSD test following a significant F-test was reported per 80 HT-29 cells. Microcolony counts were compared viewing frames were observed and the mean number of microcolonies Iyoda & Watanabe, 2004). For each sample, a minimum of ten biological replicates per trial.

**RESULTS**

**LEE expression phenotype of strain TW14359**

qRT-PCR of LEE transcript levels revealed the upregulation of LEE regulatory (ler, 3.8-fold), structural (espA, 2.3-fold), effector (tir, 2.7-fold) and effector chaperone (cesT, 2.3-fold) genes in TW14359 when compared with strains Sakai and EDL933 during exponential growth (OD_{600} of 0.5) (P<0.05) (Fig. 1a), consistent with studies of LEE expression in TW14359 grown in DMEM when co-cultured with bovine mammary epithelial (MAC-T) cells (Abu-Ali et al., 2010a). Although expression of the LEE activator gene grlA was elevated 1.6-fold in TW14359, levels did not differ significantly when compared with Sakai and EDL933. Transcript levels for all LEE genes did not differ between Sakai and EDL933. The expression of LEE genes decreased markedly in stationary phase (OD_{600} of 3.0) for all strains and did not differ between them (Fig. 1a). This pattern of expression in DMEM is consistent with previous observations of LEE gene expression during exponential and stationary growth phases in MOPS minimal media (Bergholz et al., 2007a).

Expression from the ler promoter, as measured by β-galactosidase activity from ler_{9063}–lacZ, increased in both the TW14359 and the Sakai backgrounds during exponential growth, and decreased as cultures transitioned into stationary phase (OD_{600} of 3.0) for all strains and did not differ between them (Fig. 1b). This pattern of expression in DMEM is consistent with previous observations of LEE gene expression during exponential and stationary growth phases in MOPS minimal media (Bergholz et al., 2007a).

The expression of Ler protein was measured by Western blotting. In TW14359, the Ler protein was detected at a lower level than in Sakai. The Ler protein was not detected in the Western blot of WT strain Sakai (Fig. 2a). This suggests that basal levels of ler are intrinsically upregulated in TW14359 in a manner that is dependent on a promoter(s) that is intracstronic to rcsD. Consistent with a role for RcsB in the LEE expression phenotype of TW14359, ler transcript levels were higher in TW14359 than in Sakai (P<0.001) (Fig. 2b); however, rcsD, which is transcribed as a dicistron with rcsB (i.e. rcsDB) from an upstream rcsDp promoter (Krin et al., 2010; Pescaretti et al., 2009), was not altered in expression between strains (Fig. 1b). This suggests that basal levels of rcsB are intrinsically upregulated in TW14359 in a manner that is dependent on a promoter(s) that is intracstronic to rcsD. Consistent with a role for RcsB in the LEE expression phenotype of TW14359, ler transcript levels were higher in TW14359 than in Sakai (P<0.001), but did not differ between TW14359ΔrcsB and SakaiΔrcsB (Fig. 3a). Complementation of TW14359ΔrcsB and SakaiΔrcsB with rcsB did not restore differential ler expression as observed for WT strains (Fig. 3a), supporting the hypothesis that the elevated basal expression of rcsB in TW14359 relative to Sakai is responsible for the LEE expression phenotype. The fact that ler transcript levels were higher in rcsB complementation strains relative to WT (Fig. 3a) probably reflects expression...
from pACYC177. Although this plasmid is low-copy, rcsB transcript levels were still 20-fold higher in complement strains compared with WT (data not shown). In strain Sakai, the activation of ler by rcsB overexpression in trans requires an intact global regulator of virulence (grvA) gene, the product of which activates transcription from the LEE1 promoter (Tobe et al., 2005). As such, this study sought to determine if in TW14359, which intrinsically overexpresses rcsB, ler activation also required grvA, or if activation was through a different pathway. Deletion of grvA reduced ler

---

**Fig. 1.** The LEE expression phenotype of strain TW14359. (a) Transcript levels for LEE genes are plotted for exponential (OD<sub>600</sub> of 0.5) and stationary (OD<sub>600</sub> of 3.0) phase DMEM cultures of strains TW14359 (filled), Sakai (empty) and EDL933 (grey). (b) β-Galactosidase activity in Miller units for TW14359 (squares) and Sakai (triangles) containing a ler<sub>p903</sub>−lacZ fusion plotted against time during growth in DMEM. TW14359 containing an empty vector (pRS551) control is denoted by the hatched line. Asterisks denote significant differences in transcript levels of TW14359 compared with other strains by Tukey’s HSD test following a significant F-test (a) or Student’s t-test (b) (*P<0.05, **P<0.01, n≥3). Error bars denote SD.
expression to the same extent as observed for TW14359ΔrcsB when compared with TW14359 (Fig. 3b) (P<0.05). Moreover, deletion of grvA in TW14359ΔrcsB did not further significantly alter ler expression when compared with TW14359ΔrcsB or TW14359ΔgrvA. The expression of ler was, however, slightly but significantly higher in the TW14359ΔrcsBΔgrvA backgrounds when compared with Sakai (P<0.05). Therefore, increased basal expression of rcsB in TW14359 and interaction with grvA is required for the LEE expression phenotype of this strain.

This study further ascertained the contribution of intrinsic rcsB overexpression to the TW14359 adherence phenotype using the human colonic cell line HT-29. As observed for bovine MAC-T cells (Abu-Ali et al., 2010b), adherence to HT-29 cells, as measured by microcolony formation, was significantly higher for TW14359 than for Sakai (P=0.006) (Fig. 3c). Consistent with ler expression data, adherence did not differ between Sakai and SakaiΔrcsB, but was reduced in TW1359ΔrcsB to a level comparable to Sakai and

---

**Fig. 2.** Expression of rcsB in Sakai and TW14359. (a) Representative Western blot for FLAG-tagged RcsB in strains Sakai and TW14359 grown in DMEM (OD600 of 0.5). Equal loading was controlled for by Western blots for GroEL. (b) (Top) Transcript levels of rcsB and rcsD plotted for Sakai (filled) and TW14359 (empty) grown in DMEM. The asterisk denotes significant differences between strains by Student's t-test (P<0.001, n≥3). Error bars denote SD. (Bottom) Graphic depicting the rcsDB and rcsC ORFs with their respective promoters, the location of qRT-PCR priming sites, and the location of amplified promoter lacZ fusion fragments for rcsD and rcsB in pRS551.

**Fig. 3.** Expression of rcsB, grvA and the LEE and adherence phenotype of TW14359. (a) Transcript levels of ler plotted for WT, ΔrcsB and complement ΔrcsB þrcsB versions of Sakai (solid) and TW14359 (empty) grown in DMEM (OD600 of 0.5). (b) Transcript levels of ler plotted for WT strains and mutant derivative strains of TW14359 during growth in DMEM. (c) Adherence to HT-29 cells as measured by microcolony formation plotted for WT, ΔrcsB and ΔescN versions of Sakai (solid) and TW14359 (empty). For (a), the asterisk denotes a significant difference between Sakai and TW14359 by Student's t-test (P<0.017, n≥3). For (b) and (c), plots with different lower-case letters differ significantly by Tukey's HSD test following a significant F-test (P<0.05, n≥3). Error bars denote SD.
SakaiΔrcsB. As anticipated, the adherence of T3SS-deficient TW14359ΔescN and SakaiΔescN strains to HT-29 cells was significantly reduced when compared with WT and rcsB isogenic backgrounds (P<0.05), but not between escN isogenic strains (Fig. 3c), indicating that the adherence phenotype of TW14359 is dependent on intrinsic rcsB upregulation, as well as a functional T3SS.

**Control of rcsB by the LEE-encoded regulator GrlA, and GrlA-RcsB-dependent repression of motility**

In TW14359, genes associated with motility, including structural and regulatory genes for flagellar biosynthesis (flg and fli genes), and chemotaxis (cheB, tsr and tar) are reduced in expression compared with Sakai (Abe et al., 2002; Abu-Ali et al., 2010b). In the present study, this has been observed to correspond with a 30–40% reduction in the lateral growth of TW14359 on motility plates relative to Sakai (Fig. 4a). Importantly, both RcsB and LEE-encoded activator GrlA are known to negatively regulate motility (Francez-Charlot et al., 2003; Iyoda et al., 2006), and both are increased in expression in TW14359. It was therefore of interest to investigate the potential genetic interaction of rcsB and grlA, and their contribution to the regulation of motility in TW14359.

As expected, deletion of rcsB in TW14359 enhanced the lateral growth of TW14359 on motility plates by 30–40%.

---

**Fig. 4. GrlA–RcsB-dependent repression of motility.** (a) Motility as measured by lateral growth on representative motility plates for Sakai and TW14359 (top), and mutant derivatives of TW14359 (bottom); EHEC O157:H– strain 493/89 is included as a non-motile control. (b) β-Galactosidase activity in Miller units for TW14359 and mutant derivatives containing an fhlD(1000–lacZ) fusion during growth in DMEM. (c) Representative Western blot for FLAG-tagged RcsB in LB plotted for TW14359 (WT) with and without 44 mM HCO₃⁻, and for grlA overexpression strains of TW14359 (grlA+++, and grlR::kan). Equal loading was controlled for by Western blots for GroEL. (d) β-Galactosidase activity in Miller units plotted for TW14359 (filled) and TW14359grlR::kan (empty) grown in LB and containing rcsD(501–lacZ), rcsB(1000–lacZ) or ler(903–lacZ) fusions. For (b), plots with different lower-case letters differ significantly by Tukey’s HSD test following a significant F-test (P<0.05, n>3). For (d), the asterisk denotes significance by Student’s t-test (P=0.010, n>3). Error bars indicate SD.
(Fig. 4a, Table S3), suggesting that the motility deficiency of TW14359 when compared with Sakai is connected to intrinsic $rcsB$ overexpression. Also, the inactivation of grlR (strain TW14359grlR::kan) leading to grlA overexpression (Fig. S4) (Iyoda et al., 2006) substantially impaired lateral growth on motility plates (Fig. 4a). Most importantly, deletion of $rcsB$ in the TW14359grlR::kan background restored lateral growth on motility plates to the level observed for TW14359acrB, revealing that the control of motility by grlA is epistatic to $rcsB$. Negative regulation of motility by RcsB has been shown to result from direct transcriptional repression of the global regulator of motility genes $flhDC$ (Franchez-Charlot et al., 2003). Consistent with this, expression from the $flhD_P$ promoter, as measured by $\beta$-galactosidase activity from $flhD_{P1000-lacZ}$, was significantly increased in TW14359$Delta rcsB$, but decreased in TW14359grlR::kan when expression did not differ, however, between TW14359grlR::kan and TW14359acsB and TW14359acrB, indicating that RcsB-dependent repression of $flhDC$ and motility is positively regulated by GrlA. In further support of this, levels of RcsB were increased (~2.4-fold) in TW14359grlR::kan and in a grlA overexpression strain compared with WT (Fig. 4c). Interestingly, however, expression from $rcsD_P$ or $rcsB_P$ promoters was not altered in TW14359grlR::kan (Fig. 4d), indicating that control of RcsB by GrlA is at the post-transcriptional level; increased expression from the $ler_P$ promoter was included as a positive control for grlA overexpression. Collectively, these experiments reveal a reciprocating regulatory mechanism in which RcsB and LEE-encoded GrlA co-ordinate LEE activation with repression of $flhDC$ and motility.

**Role for Rcs phosphorelay and grvA in bicarbonate-dependent activation of the LEE**

In the preceding experiments the addition of bicarbonate to TW14359 cultures growing in LB, and added as a positive control for LEE stimulation (Abe et al., 2002), was also observed to increase RcsB levels ~5.4-fold (Fig. 4c). The bicarbonate ion ($\text{HCO}_3^-$) has been shown to activate LEE expression and adherence in a dose-dependent manner in EHEC (Abe et al., 2002). This has been reported to be through increased transcription from the grlRA promoter in C. rodentium (Tauschek et al., 2010), although the mechanism by which bicarbonate stimulates LEE expression in EHEC is unknown. In this study, the increase in RcsB levels with bicarbonate was higher than that observed for grlA overexpression strains (Fig. 4c), suggesting that this added bicarbonate stimulation of RcsB was, at least in part, independent of grlA. It was therefore predicted that the Rcs phosphorelay system was in some way involved in bicarbonate-directed activation of LEE expression. In agreement with the dose dependence of bicarbonate for LEE stimulation, the addition of bicarbonate up to 44 mM (a physiologically relevant molarity) (Feldman, 1983) to LB was observed to increase RcsB levels incrementally (Fig. 5a). Also, $rcsB$ was shown to be required for full activation of LEE expression in response to bicarbonate, as the levels of Tir protein were increased substantially in TW14359 grown with bicarbonate, but only marginally increased in the TW14359$Delta rcsB$ background (Fig. 5a). In addition, growth with bicarbonate increased $ler$ transcript levels 5-fold in the WT ($P = 0.001$) compared with only 1.24-fold in TW14359$Delta rcsB$ (Fig. 5b). Bicarbonate stimulation of $ler$ was restored in complement strain TW14359$acrB$ $pcsB$.

Expression from both $rcsD_P$ and $rcsB_P$ promoters was significantly increased in the presence of bicarbonate ($P < 0.05$) (Fig. 5c), revealing that bicarbonate is stimulating transcription of $rcsDB$ from the $rcsD_P$ promoter, as well as $rcsB$ alone from at least one of two mapped promoters intracistronic to $rcsD$. Expression from the $ler_P$ promoter was, as expected, increased with bicarbonate addition for TW14359 ($P < 0.05$), but not for TW14359$Delta rcsB$ (Fig. 5d). In addition, deletion of the Rcs phosphorelay sensor kinase $rscC$ significantly reduced bicarbonate stimulation of $ler_P$ promoter activity compared with WT ($P < 0.05$). And consistent with an RcsB-GrvA-dependent pathway of LEE activation, $ler_P$ promoter expression with bicarbonate addition was reduced in TW14359$agrA$ and TW14359$acsBagrA$ when compared with WT ($P < 0.05$), but not compared with TW14359$Delta rcsB$ or TW14359$acsC$ (Fig. 5d). Collectively, this reveals that bicarbonate-dependent stimulation of LEE expression in EHEC is at least partly dependent on components of the Rcs phosphorelay system, and on the RcsB-GrvA pathway of LEE activation.

**DISCUSSION**

It is predicted that $rcsB$, encoding the response regulator of the Rcs system, is intrinsically upregulated in TW14359, and that this is responsible for the enhanced LEE expression and adherence phenotype of this strain. It is not yet clear why $rcsB$ is upregulated in TW14359. The $rcsB$ gene is co-transcribed as the second gene of a dicistron with $rcsD$ (Krin et al., 2010) and yet $rcsD$ transcript levels by qRT-PCR (Fig. 3) and protein levels by iTRAQ proteomics did not differ in TW14359 when compared with Sakai. This is consistent with the observation that $rcsB$ overexpression can upregulate the LEE independent of $rcsD$ (Tobe et al., 2005). Alternatively, $rcsB$ can be expressed as a monocistron from at least two promoters which are intracistronic to $rcsD$ (i.e. $rcsB_P1$ and $rcsB_P2$) (Krin et al., 2010), but upstream of the priming sites used for qRT-PCR in this study. As there are no differences in the sequence of these promoters between TW14359 and Sakai, it is predicted that intrinsic upregulation of $rcsB$ is therefore dependent on some trans-factor(s) acting on the $rcsB_P1$ and/or $rcsB_P2$ promoters. Unfortunately, how these promoters are expressed and regulated is unknown, and needs to be examined further. A genetic polymorphism unique to strain TW14359 was initially hypothesized in this study to be important for the $rcsB$ expression phenotype of this strain. Kulasekara et al. (2009) had described a 90 bp
Fig. 5. Effect of bicarbonate on RcsB, and rcsB-dependent LEE activation. (a) Representative Western blot for FLAG-tagged RcsB as a function of increasing bicarbonate (HCO₃⁻) molarity (mM) (top), and for FLAG-tagged Tir in TW14359 and TW14359ΔrcsB grown in LB or LB with 44 mM HCO₃⁻ (OD₆₀₀ of 0.5) (bottom). Equal loading was controlled for by Western blots for GroEL. (b) Transcript levels of ler plotted for TW14359, TW14359ΔrcsB and complement TW14359ΔrcsBprcsB strains grown in LB (-) or LB with 44 mM HCO₃⁻ (+) (OD₆₀₀ of 0.5). (c) β-Galactosidase activity in Miller units plotted for TW14359 and TW14359 containing rcsDᵦ₀₁₋lacZ or rcsBᵦ₁₀₀₋lacZ fusions grown in LB (-) or LB with 44 mM HCO₃⁻ (+). (d) β-Galactosidase activity in Miller units plotted for TW14359 (WT) and mutant derivative strains containing lerᵦ₀₁₋lacZ and grown in LB (-) or LB with 44 mM HCO₃⁻ (+). For (b) and (c), the asterisks denote a significant difference between treatments by Student’s t-test (*P<0.05, **P<0.01, n≥3). For (d), plots with different lower-case letters differ significantly by Tukey’s HSD test following a significant F-test (P<0.05, n≥3). Error bars denote SD.

insertion in the ORF of tolA, the product of which is an inner membrane component of the Tol–Pal envelope complex involved in maintaining cell envelope integrity (Bernadac et al., 1998; Cascales et al., 2000). Importantly, mutations in tolA have been reported to substantially upregulate rcsB (Clavel et al., 1996). Indeed, rcsB expression in strains TW14359tolA::kan and SakaitolA::kan was increased compared with their WT counterparts (Fig. S3). However, the level of rcsB transcript was still proportionately increased in TW14359tolA::kan relative to SakaitolA::kan (P=0.016), suggesting that the tolA polymorphism is not responsible for increased basal expression of rcsB in TW14359.

The results also indicated that deletion of rcsB had no effect on ler expression or HT-29 adherence in strain Sakai. This latter finding is inconsistent with a study demonstrating that both the overexpression and deletion of rcsB in Sakai can lead to increased LEE expression and adherence through independent regulatory pathways: RcsB was predicted to upregulate the LEE through activation of grvA, a positive regulator of the LEE (Tobe et al., 2005). The reason for this disparity in results is not yet known. However, the direction and magnitude at which RcsB regulates LEE expression are likely to be sensitive to differences in growth phase, nutrient availability as well as signals which activate Rcs phosphorelay. In this study, the influence of RcsB on ler expression was only examined in mid-exponential cultures (OD₆₀₀ of 0.5) and may differ substantially with even a subtle change in the phase of growth or experimental condition. For example, in Tobe et al. (2005) Caco-2 cells were used for adherence studies, whereas this study utilized HT-29 cells.
The RcsB-dependent LEE expression phenotype of strain TW14359 was further shown to require an intact global regulator of virulence grvA gene. The mechanism by which RcsB controls grvA, and how RcsB–GrvA regulate ler, is unknown. Tobe et al. (2005) observed increased expression of grvA in response to rcsB overexpression and described RcsB boxes in the predicted grvA promoter region with some homoology to the RcsB consensus (Wehland & Bernhard, 2000), suggesting direct regulation of grvA transcription. Alternatively, RcsB may interact with GrvA to directly activate the expression of ler and other genes. There is precedent for this, as RcsB has been shown to regulate transcription as a heterodimer with RcsA (Francez-Charlot et al., 2003; Stout et al., 1991), GadE (Castanié-Cornet et al., 2010) and BglJ (Venkatesh et al., 2010). If RcsB–GrvA activate ler transcription as a heterodimer, they probably bind upstream of the core promoter region (i.e. upstream of −35) as is typical for RcsB promoter activation (Boulanger et al., 2005; Castanié-Cornet et al., 2010; Sturny et al., 2003; Venkatesh et al., 2010). However, there is no RcsB binding site upstream of and including the ler P1 and P2 promoters.

The results of this study suggest that RcsB is involved in the inverse regulation of genes that control motility (i.e. flhDC) and those for intimate colonization (i.e. the LEE) in EHEC. This opposing relationship between colonization and motility may be important for the establishment of E. coli in the gut. For example, constitutive expression of the FlhDC regulon in EHEC markedly reduced adherence to HeLa cells (Iyoda et al., 2006), whereas deletion of flhDC and lack of motility in K12 increased colonization in a murine model (Gauger et al., 2007; Leatham et al., 2005). Note, however, that FlhDC also controls the expression of genes which serve no direct role in motility but which could contribute in some way to colonization. In EHEC, the LEE-encoded activator GrlA indirectly downregulates transcription of flhDC leading to reduced motility (Iyoda et al., 2006). This study has revealed that mutation of rcsB masks the negative regulatory effect of GrlA on motility and flhDC transcription, and that the overexpression of grlA increases RcsB levels. This suggests that RcsB and GrvA are members of a reciprocating regulatory mechanism in EHEC which, at a minimum, co-ordinates the upregulation of LEE genes with a downregulation of the flhDC flagellar regulon. Activation of RcsB by GrlA appears to be at the post-transcriptional level, as increased protein levels did not correspond with increased transcription from promoters driving expression of rcsDB or rcsB.

In EHEC, bicarbonate has been shown to stimulate LEE transcription through ler, and to increase adherence to Caco-2 cells in a pH-independent manner (Abe et al., 2002). It has been hypothesized that bicarbonate produced naturally in the small bowel to neutralize gastric acid may serve as a biological cue for T3SS-dependent colonization (Abe et al., 1997, 2002). In C. rodentium bicarbonate interacts with the AraC-type regulator RegA to directly upregulate grlA expression (Tauschek et al., 2010). In EHEC and EPEC, however, there are no homologues of RegA, and the genetic determinant(s) which regulates bicarbonate-dependent induction of the LEE in these pathogens is unknown. This study has shown that Rcs phosphorelay components RcsB and RcsC and the global regulator of virulence GrvA are required for full stimulation of the LEE in response to bicarbonate in EHEC. Despite the interplay of GrlA and RcsB in the control of flhDC and motility, there is no evidence that grlA is important for bicarbonate-dependent LEE expression through this pathway. For instance, overexpression of grlA did not increase rcsB transcription, whereas bicarbonate addition did enhance rcsB transcription (Figs 4d and 5c). Furthermore, the level of RcsB induction was markedly higher in WT cells grown with bicarbonate than in strains which overexpress grlA alone (Fig. 4c). Therefore, in EHEC it is predicted that bicarbonate stimulates LEE expression through an RcsB–GrvA–Ler activation pathway.

To conclude, the LEE expression phenotype of TW14359 is hypothesized to result from intrinsically increased basal levels of the response regulator RcsB, activating LEE expression through a GrvA–Ler pathway. In addition, increased adherence of strain TW14359 to intestinal cells was shown to be dependent on elevated rcsB expression and a functional T3SS. Whether this dysregulated pathway is responsible for the severe disease attributed to infections with this strain is not yet clear. Furthermore, the LEE-encoded activator GrlA was determined to require rcsB for repression of the flhDC flagellar regulon, suggesting that GrlA and RcsB work together to co-ordinate the activation of genes for colonization with the repression of genes for motility. Finally, bicarbonate was proposed to be a physiological signal for an Rcs phosphorelay- and GrvA-directed pathway activating LEE expression and colonization in EHEC.

ACKNOWLEDGEMENTS

We thank Sergio Uzzau (University of Sassari, Italy) for kindly providing vector pSA312 for FLAG-fusion construction, Edward G. Dudley (Pennsylvania State University) for providing vector pRS551 for lacZ fusions and Robert Buzeo (University of South Florida) for technical assistance with adherence assays. We also thank the University of South Florida Center for Drug Discovery and Innovation (CDDI) for technical assistance with proteomics and MS.

REFERENCES


expression between two outbreak strains of enterohaemorrhagic Escherichia coli O157:H7. Microbiology 156, 408–419.


Edited by: D. Gally