Minireview

Virulence regulation in *Citrobacter rodentium*: the art of timing

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Summary

The mouse enteric pathogen *Citrobacter rodentium*, like its human counterpart, enteropathogenic *Escherichia coli*, causes attaching and effacing lesions in the intestinal epithelium of its host. This phenotype requires virulence factors encoded by the locus for enterocyte effacement (LEE) pathogenicity island. For timely expression of these virulence determinants at the site of infection and for efficient delivery of some virulence factors into epithelial cells, *C. rodentium* utilizes a positive regulatory loop involving the LEE-encoded regulatory proteins Ler, GrlA and GrlR to control LEE expression. Several transcription factors not encoded by LEE, some of which respond to specific environmental signals, also participate in this regulatory loop. Recently, we identified a non-LEE encoded, AraC-like regulatory protein, RegA, which plays a key role in the ability of *C. rodentium* to colonize the intestine. RegA functions by activating the transcription of a number of horizontally acquired operons encoding virulence-associated factors, such as autotransporters, fimbriae, a dispersin-like protein and its transporter. In addition, RegA represses transcription of a number of housekeeping genes. Importantly, RegA requires a gut-specific environmental signal, bicarbonate, to exert its effects on gene expression. In our proposed model, when *C. rodentium* senses bicarbonate ions in the gastrointestinal tract, RegA directs the bacterium to reduce the production of proteins involved in normal cellular functions, while enhancing the production of factors required for colonization and virulence.

Introduction

*Citrobacter rodentium* is a Gram-negative enteric bacterium that is a natural pathogen of mice. In its natural host, *C. rodentium* causes colonic epithelial hyperplasia accompanied by mild diarrhoea (for reviews, see Luperchio and Schauer, 2001; Mundy et al., 2005). Its principal importance, however, is that infection of mice with *C. rodentium* provides a convenient small animal model to investigate the molecular and cellular pathogenesis of infections with the human pathogens, enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC). This is because all three pathogens produce virtually indistinguishable attaching and effacing lesions in the intestinal epithelium (Frankel et al., 1998) (Fig. 1), due to the fact that they carry the locus for enterocyte effacement (LEE), a highly conserved pathogenicity island (PAI), which is required for the development of these lesions (McDaniel et al., 1995; Elliott et al., 1998; Deng et al., 2001). Thus, studies of the regulation of virulence genes in *C. rodentium* are likely to shed new light on the molecular pathogenesis of EPEC and EHEC, and permit the development of testable hypotheses regarding virulence gene regulation in these important pathogens.

The association of *C. rodentium* with its natural host has led to the evolution of complex regulatory networks, which are the result of recruiting and adapting pre-existing regulatory mechanisms to act in concert with newly acquired regulatory genes carried on mobile elements. These sophisticated regulatory networks bring about precise control of virulence gene expression in response to different environments within and outside the host, and ensure the survival of *C. rodentium* in the environment and the extreme conditions encountered in the stomach and small intestine.

Colonization of the intestinal epithelium by *C. rodentium* is thought to occur in two stages: initial loose attachment...
expression of LEE by upregulating transcription of regulatory proteins, Ler and GrlR/A, which play an important role in the transcriptional control of LEE and several virulence determinants not encoded by LEE (Elliott et al., 2000; Deng et al., 2004; Torres et al., 2007; Abe et al., 2008). In addition, RegA appears to modulate expression of LEE by upregulating transcription of grlR/A.

The regA gene was initially identified as a virulence determinant of C. rodentium by using signature-tagged transposons to create a library of C. rodentium mutants that were screened for their ability to colonize mouse intestine (Kelly et al., 2006; Hart et al., 2008). In a mixed-infection experiment with wild-type C. rodentium, a C. rodentium mutant, which carried a disrupted regA gene, was significantly out-competed by the wild type, indicating an impaired ability to colonize mouse intestine. Additional infection experiments using the regA mutant showed that the mean maximum number of the regA mutant in faeces was only 0.02% that of the wild type. Fulfilment of molecular Koch’s postulates was achieved by trans-complementing the mutant with a plasmid carrying a wild-type regA gene that restored its colonizing ability, thus confirming that C. rodentium requires regA to colonize mouse intestine.

The regA gene codes for a protein of 276 amino acids (Hart et al., 2008) which exhibits sequence similarity to AraC-like virulence transcriptional regulators from other intestinal bacterial pathogens. Homologues of RegA include PerA from EPEC (Gomez-Duarte and Kaper, 1995), Rns from enterotoxigenic E. coli (Caron et al., 1989), AggR from enteraggregative E. coli (Natario et al., 1994) and ToxT from Vibrio cholerae (Higgins et al., 1992). Adjacent to the regA gene on the C. rodentium chromosome are two divergently transcribed loci, adcA and kfc, which encode a putative autotransporter involved in diffuse adherence, and a K99-like fimbrial operon respectively (Hart et al., 2008). Real-time PCR analysis has shown that transcription of the adcA gene and the kfc cluster is strongly activated by RegA (Yang et al., 2008). Further studies showed that expression of the adcA gene in E. coli K12 caused the recipient bacteria to self-aggregate and enabled them to adhere to mammalian tissue culture cells in vitro (Hart et al., 2008). Although inactivation of kfc resulted in a slight, but significant, reduction in the duration of intestinal colonization by C. rodentium, deletion of both adcA and kfc from C. rodentium did not lead to the same reduction in colonizing ability as that exhibited by the regA mutant alone (Hart et al., 2008). This suggested that there are other RegA-regulated genes, which also contribute to the ability of C. rodentium to colonize mice.

Bicarbonate-mediated activation of the adcA and kfc promoters by RegA

Interestingly, RegA is the only known AraC-like transcriptional regulator from enteric bacteria that responds
directly to a gut-specific environmental signal, namely bicarbonate ions (Yang et al., 2008). In mammals, bicarbonate is responsible for whole-body homeostasis (Guyton and Hall, 2006). In the gastrointestinal tract, bicarbonate ions are secreted by the pancreas into the duodenum to neutralize stomach acid (Kauñitz and Akiba, 2006). In the duodenum, the concentration of bicarbonate can exceed 50 mM (Meyer et al., 1970). We have shown that C. rodentium can sense such bicarbonate-rich environments and respond by activating genes required for intestinal colonization.

In the presence of 40 mM bicarbonate ions, RegA enhances expression of the divergent adcA and kfcC promoters in an E. coli K12 background approximately 100-fold compared with 10-fold when bicarbonate is absent (Yang et al., 2008). This is because in the absence of bicarbonate, RegA binds only weakly to a DNA fragment carrying the regulatory region of adcA and kfcC, but binding is greatly enhanced when bicarbonate is present. In addition, DNA footprinting analysis has shown that RegA protects an approximately 80 bp portion of the region between the promoters for adcA and kfcC from digestion by DNase I. The interaction of RegA with this sequence causes a conformational change in DNA, which resembles that observed in eukaryotic nucleosomes (Yang et al., 2008).

The intergenic region between the divergent adcA and kfcC promoters is extremely AT-rich (~70%) and is predicted to be highly curved. Like many virulence genes of enteric bacteria that were acquired horizontally, transcription of adcA and kfcC is subject to repression control by the global, histone-like regulatory proteins, H-NS and StpA (Yang et al., 2008). Under inducible conditions, RegA stimulates transcription by counteracting the repressive effect of these proteins.

The RegA protein

By analogy with the AraC protein of E. coli, RegA is predicted to constitute two structural domains: an N-terminal domain [amino acid residues (aa) 1–180] and a C-terminal domain (aa 190–276) (Fig. 2). Using secondary structure prediction programmes, we showed that the N-terminal domain includes an α-helical arm (aa 1–32) followed by a region consisting of six β-sheets (aa 34–90). The downstream portion of the N-terminal domain (aa 92–180) is predicted to comprise five α helices. The C-terminal domain contains predominantly α helices, which, like other members of the AraC family, includes two helix–turn–helix, DNA-binding motifs.

We have used mutational analysis to show that the N-terminal arm of RegA is required for its dependence on bicarbonate ions to activate transcription. In particular, deletion of the four amino acids between positions 2 and 5, or changing the asparagine residue at position 16 to an alanine significantly enhance the ability of RegA to activate transcription in the absence of bicarbonate (J. Yang and R. Robins-Browne, in preparation). These observations are consistent with other AraC-like proteins, which also use the N-terminal arm to modulate protein function (Saviola et al., 1998; Basturea et al., 2008; Dominguez-Cuevas et al., 2008). For example, in the absence of the effector 3-methylbenzoate, residues in the N-terminal arm of the XylS protein of Pseudomonas putida, interact with its C-terminal domain and inhibit binding of XylS to its DNA targets (Dominguez-Cuevas et al., 2008).

![Fig. 2. Amino acid sequence and predicted secondary structure of the RegA protein.](image-url)

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Interestingly, RegA also appears to use self-contact inhibition to modulate its DNA-binding activity in response to its effector – bicarbonate. Thus, binding of their respective effectors to XylS and RegA or deletion of their N-terminal arm appears to relieve the inhibition of the DNA-binding activity of these proteins.

Analysis of the crystal structure of the N-terminus of AraC indicates that the arabinose-binding pocket is imbedded within an eight-stranded, antiparallel β-barrel. The corresponding region in RegA, which comprises six successive β-sheets (aa 33–90), is also likely to be involved in effector binding, as disrupting this region by inserting an alanine and a serine into each of the β-sheets completely eliminates bicarbonate-dependent activation via RegA (J. Yang and R. Robins-Browne, in preparation).

Two AraC monomers are able to dimerize via anti-parallel coiled-coils. Dimerization requires the packing of three leucine residues at positions 150, 151 and 161 in a knobs-into-holes-like manner (Soisson et al., 1997). As shown in Fig. 2, RegA contains two leucine residues at positions 150 and 151 and a valine at position 161. Replacing leucine-150 with an alanine abolishes the activation function of RegA, indicating the probable involvement of this leucine in dimerization (J. Yang and R. Robins-Browne, in preparation). Thus, although the primary sequence of the N-terminal domain of RegA has diverged considerably during evolution from those of other members of the AraC family, it retains certain structural and functional features, which these proteins employ to control interactions with their target promoters.

Global gene regulation by RegA

Microarray analysis has revealed that RegA is a global transcriptional regulator of C. rodentium. RegA strongly activates transcription (between 4- and 126-fold) of 19 open reading frames organized in 11 separate operons at widely different locations on the bacterial chromosome (Table 1). Interestingly, all of the RegA-upregulated operons are flanked by mobile genetic elements such as transposons, insertion sequences and remnants of bacteriophages, indicating that they were horizontally acquired. Several of the RegA-regulated transcriptional units identified in this way code for homologues of known virulence factors of intestinal pathogens, such as the Aap dispersin and its transporter (Aat) from enterotoaggregative E. coli (Sheikh et al., 2002; Nishi et al., 2003), the CexE virulence factor from enterotoxigenic E. coli (Pilonieta et al., 2007) and the SfpA porin (systemic factor protein A) from Yersinia enterocolitica (Mildiner-Earley and Miller, 2006). As with adcA and kfc, activation of these putative virulence determinants of C. rodentium by RegA is bicarbonate-dependent and therefore is likely to be directly regulated by this protein.

RegA also effects the transcriptional repression of 24 operons. As with upregulation, repression is also bicarbonate-dependent (Table 1). All of the downregulated genes identified by using microarray analysis are housekeeping genes located on the chromosomal backbone of enteric pathogens and non-pathogenic bacteria alike. These genes encode amino acid transporters, regulatory proteins and enzymes involved in various cellular functions. In contrast to its potent stimulatory effect on the transcription of virulence genes, RegA exerts only a moderate degree of repression on housekeeping genes (less than fivefold). Whether the negative control of these genes is directly or indirectly affected by RegA is yet to be determined. Microarray analysis also revealed that the flhC-flhD operon, which codes for a master regulatory complex (FlhC-D), responsible for stimulating the expression of flagella (Liu and Matsumura, 1994), is significantly downregulated by RegA (Table 1). Although C. rodentium is non-motile, it nevertheless carries an intact flhC-flhD operon, which can affect the expression of a number of gene targets apart from those required for flagella (Pruss et al., 2001).

Autogenous regulation of regA

The regA gene is expressed from multiple promoters (Fig. 3). Promoter P1 is located immediately upstream of the regA structural gene and is constitutively expressed (A. Tan and R. Robins-Browne, in preparation). Promoters P2 and P3 are located in the regulatory region of an open reading frame of unknown function (orfU), situated immediately upstream of regA (Fig. 3). Microarray and transcriptional analysis using promoter–lacZ fusions have shown that RegA activates transcription of P2 and P3 in the absence of bicarbonate (A. Tan and R. Robins-Browne, in preparation). In addition, bicarbonate enhances RegA-mediated activation of orfU from 15- to 50-fold (Table 1). A fourth promoter, P4, which also contributes to regA expression, is the promoter of the adcA gene.
gene (Fig. 3), which is strongly upregulated by RegA in the presence of bicarbonate.

Constitutive expression from the P1 promoter ensures that trace amounts of RegA are produced when bicarbonate is absent. Under these conditions, RegA would exert little effect on the expression of genes of the RegA regulon. Once C. rodentium reaches the proximal small intestine of its host, however, the presence of bicarbonate leads to induction of RegA synthesis through stimulation of promoter P4. The resultant elevated levels of RegA lead to further RegA synthesis from promoters P2 and P3, which results in the synchronous activation of multiple virulence genes and the downregulation of some housekeeping genes.

Transcriptional regulation of the LEE PAI

The Ler protein

The Ler regulatory protein plays a central role in controlling transcription of the entire LEE PAI (Mellies et al., 1998; 2007). Ler proteins from EPEC, EHEC and C. rodentium are highly homologous in terms of their structure, function and mechanism of action. Although there was some uncertainty about which of the three methionine codons at the N-terminus is the start site of the ler structural gene, this is now known to be the methionine at position 7 of the previously predicted Ler open reading frame, reducing the predicted length of Ler from 127 to 121 amino acids (Yerushalmi et al., 2008).

Table 1. Genes activated/repressed by RegA identified by microarray analysis.

| ORF/gene | Product | Fold increase/decrease$^b$ |
|----------|---------|---------------------------|
| ROD3421  | Homologue of Aap (dispersin) of enterohaemagglutinating E. coli, and virulence factor CexE of enterotoxigenic E. coli | 126.4 |
| ROD3431-81 | Homologue of the Aat transporter of enterohaemagglutinating E. coli | 12.4 |
| ROD15971 | 65% identity : 83% similarity to unknown protein, Z2283, of E. coli O157:H7 | 5.0 |
| ROD16181 | 69% identity : 80% similarity to unknown protein, Z0957, encoded by prophage CP-933K E. coli | 10.7 |
| ROD16201 | Homologue of the porin protein SfpA of Yersinia enterocolitica | 23.4 |
| grlA     | GrlA | 4.6 |
| grlR     | GrlR | 4.6 |
| ROD41031-51 | Gene cluster encoding an unknown protein, and homologues of HlyD and HlyB secretion proteins of Shewanella woodyi | 7.9 |
| klcC-H   | Klc, K99-like fimbriase | 16.4 |
| adcA     | AdcA, autotransporter | 19.6 |
| orfU     | Unknown ORF encoded immediately upstream of regA | 56.0 |
| ROD5001  | Homologue of a putative virulence-related PagC-like membrane protein of E. coli O157:H7 | 4.6 |
| puxA     | gamma-Glu-putrescine oxidase, FAD/NAD(P)-binding | -2.1 |
| ybdR     | Predicted oxidoreductase, Zn-dependent and NAD(P)-binding | -2.3 |
| gltL     | ATP-binding component of an ABC superfamily glutamate/aspartate transporter | -2.6 |
| kC       | Membrane component of an ABC superfamily glutamate/aspartate transporter | -2.4 |
| gti      | Periplasmic-binding component of an ABC superfamily glutamate/aspartate transporter | -2.8 |
| sucA     | 2-oxoglutarate decarboxylase, thiamin-requiring | -2.0 |
| KPN_00795 | Putative urocanase | -2.5 |
| putA     | Multifunctional proline dehydrogenase and DNA-binding transcriptional regulator | -2.1 |
| putP     | Proline : sodium symporter | -2.8 |
| phoH     | Conserved protein with nucleoside triphosphate hydrolyase domain | -3.4 |
| flgB     | Flagellar component of cell-proximal portion of basal-body rod | -4.9 |
| aldA     | Aldehyde dehydrogenase A, NAD-linked | -2.3 |
| treA     | Periplasmic trehalase | -2.3 |
| iblC     | DNA-binding transcriptional dual regulator with FhD | -2.0 |
| fhd      | DNA-binding transcriptional dual regulator with FhC | -2.2 |
| argT     | Periplasmic-binding component of an ABC superfamily lysine/arginine/ornithine transporter | -2.5 |
| fimA     | Major type 1 subunit fimbrin (pilin) | -2.3 |
| yitQ     | Predicted sugar transporter subunit | -2.2 |
| acet     | Acetate permease | -2.5 |
| AcS      | Acetyl-CoA synthetase | -2.3 |
| fadB     | Fatty acid oxidation complex subunit alpha | -2.5 |
| fadA     | 3-ketoyl-CoA thiolase (thiolase I) | -2.6 |
| lidD     | L-lactate dehydrogenase, FMN-linked | -3.1 |
| dppA     | Periplasmic-binding component of an ABC superfamily dipeptide transporter | -2.2 |

a. ORF names are taken from http://www.sanger.ac.uk/Projects/C_rodentium/C_rod_genome_CDS.tab and gene names and product descriptions are taken from EcoCyc (http://www.ecocyc.org). ORF/Gene names are listed in accordance to their predicted positions on the C. rodentium chromosome.

b. Fold increase/decrease derived from the average log2 ratio of transcript levels for a RegA+ strain to those for an isogenic RegA– strain, in the presence of 45 mM bicarbonate (a value of 1 indicates no change). The cut-off value for upregulation is $\geq$ 4-fold and for downregulation is $\geq$ 2-fold. All changes are statistically significant ($P < 0.05$).
In silico analysis and mutational studies of Ler from EPEC have shown that Ler contains three putative functional domains (Sperandio et al., 2000; Yerushalmi et al., 2008). The N-terminal domain includes a coiled-coil region that may be involved in high-order oligomerization, whereas the central and C-terminal domains are responsible for homo-oligomerization and DNA binding respectively.

Ler activates transcription of the LEE2, LEE3 and LEE5 operons, and to a lesser extent the LEE4 operon, by disrupting H-NS-mediated transcriptional silencing (Elliott et al., 2000; Bustamante et al., 2001; Haack et al., 2003). In C. rodentium, Ler also upregulates expression of a small transcriptional unit, located between LEE1 and LEE2, encoding the GrlR and GrlA proteins (Fig. 4) (Deng et al., 2004).

Ler belongs to the H-NS family of DNA-binding proteins. Like H-NS, Ler binds to AT-rich DNA sequences, but so far, no consensus DNA binding sequence has been identified. Because of this, it has been suggested that Ler may recognize a DNA structural motif rather than a distinct sequence (Haack et al., 2003).

Some investigators have reported that transcription of the EHEC LEE1 promoter is autorepressed by Ler when ler expression is induced from a tac promoter by isopropyl-β-D-thiogalactopyranoside (Berdichevsky et al., 2005; Yerushalmi et al., 2008). However, comparison of promoter activity using a LEE1-cat fusion in wild-type C. rodentium has been mapped to a position 174 bp upstream of the start codon of Ler (Deng et al., 2004) that corresponds to the distal LEE1 promoter (P1) of EPEC and EHEC (Porter et al., 2005). In C. rodentium, this is the only LEE1 promoter as nucleotide differences in the region corresponding to the EHEC-specific proximal promoter (P2) abolish both the −35 and extended −10 sequences of this promoter (Deng et al., 2004).

Transcription from the LEE1 promoters of EHEC, EPEC and C. rodentium is regulated by multiple transcription factors. The nucleotide sequences of the LEE1 promoter region from these strains are highly homologous and the promoters share a number of common regulators. For example, each of the LEE1 promoters is positively regulated by the integration host factor (IHF) and GrlA, and negatively regulated by H-NS. However, unlike the situation with EPEC and EHEC where the LEE1 promoters are upregulated by a plasmid-encoded PerC protein (Gomez-Duarte and Kaper, 1995; Mellies et al., 1999) and chro-

Fig. 4. Transcriptional regulation of the LEE PAI of C. rodentium. The key transcriptional regulators of LEE expression, Ler and GrlA, are shown in red. All of the other regulatory proteins are shown in black. The environmental factors and conditions associated with the functions of these proteins are indicated. Positive regulation is shown by red arrows and ‘+’ signs and downregulation is indicated by dark blue arrows and ‘−’ signs. The thick red arrows show the positive regulatory loop. Whether the effect of RpoS on grlR/A and LEE1 expression (shown in dashed arrows) is direct or indirect is yet to be determined.

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mosomally encoded PerC-like proteins (PchA, B and C) (Iyoda and Watanabe, 2004; Porter et al., 2005) respectively, no homologue of PerC is evident in the C. rodentium genome. Expression of the LEE1 promoter of EHEC is also subject to regulation via quorum sensing (QS) (Russell et al., 2007). This is mediated by the QseA protein, which acts directly on the distal P1 promoter of LEE1. Although there is a QseA homologue in C. rodentium, its involvement in the regulation of the LEE1 promoter remains to be tested.

Coulthurst and colleagues (2007) have reported the presence of an N-aclyhomoserine lactone (AHL) QS system in C. rodentium. This system is directed by CroI, a LuxI homologue. Compared with the wild type, a croI
orf10
that the GrlR and GrlA proteins encoded by rodentium by Deng and colleagues (2004) has revealed grlR/A C. rodentium in vitro mutant strain adhered less efficiently to an abiotic surface in vitro.

The grlR/A operon
Systematic mutational analysis of the LEE PAI of C. rodentium by Deng and colleagues (2004) has revealed that the GrlR and GrlA proteins encoded by orf10 and orf11, respectively, are involved in transcriptional regulation of the LEE PAI. Deletion of grlA (orf11) from C. rodentium results in a major reduction in transcription of LEE1, LEE2 and LEE5, whereas deleting grlR (orf10) leads to a small increase in transcription of LEE1, indicating that GrlA and GrlR are positive and negative regulators of LEE PAI transcription respectively. Barba and colleagues (2005) have identified a cis-acting element upstream of the C. rodentium LEE1 promoter that is responsible for GrlA-mediated activation, indicating that GrlA interacts with the LEE1 promoter directly. In addition, Huang and Syu (2008) have used a GrlA–GST fusion protein in an electrophoretic mobility shift assay to show that GrlA from EHEC forms complexes with DNA fragments carrying the LEE1 promoter region.

Although the GrlR/A proteins from EHEC, EPEC and C. rodentium are highly conserved, they display a different regulatory pattern, in that GrlA of C. rodentium activates LEE1 transcription in both C. rodentium and E. coli K12 (Barba et al., 2005), whereas GrlA from EHEC can only activate LEE1 expression in its native background (Russell et al., 2007). These findings suggest that GrlA-mediated activation of the EHEC LEE1 promoter requires one or more additional factors.

A positive regulatory loop
Transcription of the C. rodentium grlR/A operon is driven by a \( \sigma^70 \) promoter that is repressed by IHF (M. Tauschek and R. Robins-Browne, in preparation) and H-NS but activated by Ler (Barba et al., 2005). Furthermore, Dong and colleagues (2009) have provided evidence that RpoS positively influences expression of the LEE, possibly through activation of the grlR/A and ler genes. The reciprocal activation of the ler (LEE1) promoter by GrlA and the grlR/A promoter by Ler allows a rapid build-up of the two activators that are required to initiate virulence gene expression (Fig. 4). Expression of this positive regulatory loop is further modulated by a number of proteins acting on the LEE1 and grlR/A promoters. Some of these proteins such as H-NS and IHF are able to respond directly to different environmental signals. In C. rodentium, transcription of the grlR/A operon is also upregulated by RegA in the presence of bicarbonate (Table 1). Transcriptional analysis using a grlR/A–lacZ fusion in E. coli K12 and electrophoretic mobility shift assays have confirmed that the RegA-mediated upregulation results from direct binding of the RegA protein to the grlR/A promoter region (M. Tauschek and R. Robins-Browne, in preparation). Thus, through its connection with RegA, the Ler–GrlA positive regulatory loop can be further stimulated by bicarbonate ions, which serve as a gut-specific signal for virulence gene activation.

Although attaching–effacing pathogens must be ready to activate LEE-encoded genes when they reach the site of infection, they also need to maintain appropriate levels of Ler and GrlA within the cell to reduce the metabolic burden that would be imposed by their overexpression. Two mechanisms that may be involved in preventing runaway expression of Ler and GrlA, include (i) concentration-dependent autogenous repression by Ler at the LEE1 promoter (see above) (Berdichevsky et al., 2005), which ensures that transcription of ler is blocked when the Ler concentration reaches a certain threshold, and (ii) inhibition of GrlA via the formation of GrlR–GrlA heterodimers (Jobichen et al., 2007) (Fig. 4).

Conclusion
The mouse model of infection with C. rodentium has proved invaluable in elucidating key features of the pathogenesis of infections with attaching and effacing entero-bacteria in general. In addition, studies of gene regulation in C. rodentium have provided important new insights into how some intestinal pathogens can respond to specific environmental stimuli by producing essential virulence determinants at the appropriate stage of infection. The key role played by bicarbonate sensing in the expression of virulence by C. rodentium has parallels in EPEC and EHEC (Kenny et al., 1997; Yoh et al., 2003), but in E. coli these pathways have not been fully explored.

Although there are some differences in the transcriptional control of the LEE PAIs of EPEC, EHEC and C. rodentium, the principal regulatory mechanisms are the same. Specifically, in all of these bacteria, the evolution of
sophisticated regulatory networks has ensured the rapid production of a key regulatory protein, Ler, which in turn activates other operons within the LEE PAI.

Notwithstanding its essential role in virulence, the LEE is not sufficient on its own for *E. coli* or *C. rodentium* to cause disease. In *C. rodentium*, the RegA regulon, which comprises at least 11 putative virulence operons and 24 genes not directly associated with virulence, also plays a central role in infection. Genes of the RegA regulon appear to be particularly important during the early stages of infection. In addition, through its action on grrA, RegA provides a key link between the sensing of environmental bicarbonate and the expression of virulence determinants encoded by the LEE.

Despite recent advances in our understanding of several important aspects of virulence gene regulation in *C. rodentium*, a number of key questions remain to be resolved, including how bicarbonate ions bind to the RegA protein and what structural changes these ions cause? Is there a consensus nucleotide sequence for RegA binding? What are the gene targets of the AHL QS system in *C. rodentium*? What are the molecular mechanisms of transcriptional and post-transcriptional regulation of the nle genes (non LEE-encoded effectors) (Deng et al., 2004; Kelly et al., 2006; Roe et al., 2007; Wickham et al., 2007; Garcia-Angulo et al., 2008)? What role does each of the newly identified putative virulence genes play in infection? Ongoing investigations into virulence gene regulation in *C. rodentium* will address these specific questions and provide further insights into how pathogenic bacteria are able to persist under widely differing environmental conditions inside and outside their hosts and cause disease.

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