A transcriptome study of the QseEF two-component system and the QseG membrane protein in enterohaemorrhagic Escherichia coli O157: H7

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QseE is a sensor kinase that responds to epinephrine, sulfate and phosphate. QseE constitutes a two-component signalling system together with the QseF σM-dependent response regulator. Encoded within the same operon as qseEF is the qseG gene, which encodes a membrane protein involved in the translocation of a type III secretion effector protein of enterohaemorrhagic Escherichia coli (EHEC) into epithelial cells. The qseEGF genes also form an operon with the glnB gene, which encodes the E. coli nitrogen sensor PII protein. Here we report a transcriptome analysis comparing qseE, qseF and qseG single mutants with the wild-type strain. This study revealed that the proteins encoded by these genes play a modest but significant role in iron uptake. Although QseEFG regulate genes involved in nitrogen utilization, these proteins do not play a notable role in nitrogen metabolism. In addition, QseEFG regulate transcription of the rcsBC and phoPQ two-component systems, linking several signal transduction pathways. The similarity of the microarray profiles of these mutants also indicates that these proteins work together. These data indicate that QseEFG are involved in the regulation of virulence and metabolism in EHEC.

INTRODUCTION

With environmental signalling playing such an important role in the adaptation by bacteria to different niches, the importance of a system to recognize and transduce signals that indicate the external or internal environment is paramount. Histidine sensor kinases are the major environmental sensory proteins in bacteria, and bacterial genomes encode many of these proteins. Histidine sensor kinases usually act in concert with a response regulator protein, constituting a two-component system. The classical two-component systems in Gram-negative bacteria are composed of an integral inner membrane histidine sensor kinase protein and a cytosolic response regulator protein. The periplasmic region of the sensor kinase is able to sense external signals and catalyse an autophosphorylation reaction. Once phosphorylated, the sensor kinase is able to transfer the phosphoryl group to a conserved aspartate residue on the cytosolic response regulator. This enables the regulator to act on downstream genes, most often activating transcription (Hoch, 2000).

As well as adapting to environmental niches, many two-component systems are intimately involved in virulence. In Streptococcus pneumoniae, eight of the 13 known systems are involved in virulence in a mouse model (Throup et al., 2000). Other examples of the roles of two-component systems in virulence are: persistence in infection by Mycobacterium tuberculosis (Zahrt & Deretic, 2001), invasion and antimicrobial resistance in Salmonella typhimurium (Bader et al., 2005; Smith et al., 2008), and adhesion of Escherichia coli (Otto & Silhavy, 2002). In the E. coli K-12 genome there are 29 histidine kinases and 32
response regulators (Mizuno, 1997). The quorum-sensing \textit{E. coli} regulators E, F and G contain the components of a traditional two-component system. These proteins are conserved among numerous enteric organisms, including species of \textit{Shigella}, \textit{Salmonella}, \textit{Yersinia} and \textit{Klebsiella}, and \textit{E. coli}. The genes encoding these proteins are co-transcribed in an operon with \textit{glnB}. QseE (GrlK) is a sensor kinase that responds to the host hormone epinephrine, phosphate and sulfate. It contains two transmembrane domains, a signal histidine kinase domain and an ATPase domain (Reading \textit{et al.}, 2007, 2009; Reichenbach \textit{et al.}, 2009). QseF (GrlR) is a two-component response regulator protein containing a response regulator domain with a key aspartate residue, a \(\sigma^{54}\)-interaction domain including the GAFTGA motif, and a helix–turn–helix DNA-binding domain. QseG is an outer membrane protein and shares homology with many \(\alpha\)-helical proteins (Reading \textit{et al.}, 2007, 2009; Reichenbach \textit{et al.}, 2009). The \textit{glnB} gene encodes the PII protein. PII is a signal transduction protein involved in the modulation of the kinase and phosphatase activities of the nitrogen sensor kinase NtrB in \textit{E. coli}. Upon autophosphorylation, NtrB phosphorylates the NtrC \(\sigma^{54}\)-dependent response regulator, which then binds to DNA to activate transcription of genes encoding metabolic enzymes and permeases in response to carbon and nitrogen status in \textit{E. coli} (Ninfa \& Atkinson, 2000; Ninfa \textit{et al.}, 2000). The kinase and phosphatase activities of NtrB are regulated by PII, which upon binding to NtrB inhibits the kinase activity and activates the phosphatase activity (Ninfa \& Atkinson, 2000; Ninfa \textit{et al.}, 2000).

In enterohaemorrhagic \textit{Escherichia coli} (EHEC) O157: H7, QseE, F and G are involved in virulence. EHEC is an enteric pathogen that causes disease worldwide, leading to cases of haemorrhagic colitis and life-threatening haemolytic uraemic syndrome (Kaper \textit{et al.}, 2004). EHEC colonizes the large intestine and is able to tightly adhere to intestinal epithelial cells. Among the virulence factors of EHEC is the ability to form attaching and effacing (AE) lesions on intestinal epithelial cells, which efface the microvilli and reorganize the host cell cytoskeleton into a pedestal-like structure (Kaper \textit{et al.}, 2004). Most of the genes necessary for EHEC to form AE lesions are encoded in a region known as the locus of enteroceytic effacement (LEE) (McDaniel \textit{et al.}, 1995). This region is composed of 41 genes, and encodes all the components of a type III secretion system, an outer membrane adhesin, intimin, and an effector named translocated intimin receptor (Tir) (Elliott \textit{et al.}, 1998; Jarvis \textit{et al.}, 1995; Jerse \textit{et al.}, 1990; Kenny \textit{et al.}, 1997). Tir is translocated through the type III secretion machinery into eukaryotic host cells. This protein embeds itself in the eukaryotic membrane and acts as a receptor for intimin. In addition, Tir initiates a signalling cascade that leads to actin nucleation and the formation of the AE lesion (Kenny \textit{et al.}, 1997). EHEC also harbours numerous type III-secreted effectors encoded outside the LEE region that are also translocated via the LEE-encoded type III secretion system (Deng \textit{et al.}, 2004; Tobe \textit{et al.}, 2006). One such effector molecule is EspFu (TccP), which acts with Tir to promote AE lesion formation (Campellone \textit{et al.}, 2004; Cheng \textit{et al.}, 2008; Garmendia \textit{et al.}, 2004).

QseE autophosphorylates and then transfers a phosphate to QseF (Yamamoto \textit{et al.}, 2005), which in turn activates transcription of \textit{espFu/tccP} in EHEC (Reading \textit{et al.}, 2007). The QseEQseF outer membrane protein is involved in Tir translocation by the type III secretion system (Reading \textit{et al.}, 2009). The concerted action of these three proteins facilitates AE lesion by EHEC on host epithelial cells. The aim of this study was to elucidate the global role of the QseEFQ, by examining the transcriptional profiles of cells with non-polar mutations in \textit{qseE}, \textit{qseF} or \textit{qseG}, compared with the wild-type (WT). Here we show that QseEFQ also regulate metabolism, iron uptake and other two-component systems, suggesting other roles for this three-component system beyond AE lesion formation in EHEC.

**METHODS**

**Strains and plasmids.** All bacterial strains and plasmids used in this study are listed in Table 1. \textit{E. coli} strains were grown in aerobic conditions in low-glucose Dulbecco’s modified Eagle’s medium (DMEM) at 37 °C for microarray studies and real-time PCR. All overnight cultures were grown in Luria–Bertani (LB) broth at 37 °C unless otherwise noted. Antibiotics for culture growth were added at the following concentrations: ampicillin, 100 \(\mu\)g ml\(^{-1}\); chloramphenicol, 30 \(\mu\)g ml\(^{-1}\); kanamycin, 50 \(\mu\)g ml\(^{-1}\); and tetracycline, 25 \(\mu\)g ml\(^{-1}\).

**Recombinant DNA techniques.** PCR, restriction digestions, plasmid preparations, transformations, gel electrophoresis and ligations were all performed according to standard methods (Sambrook \textit{et al.}, 1989). Primers used in real-time PCR are listed in Table 2.

**RNA extraction.** Cultures of 86-24, NR01, NR02 and NR03 were grown aerobically in LB medium at 37 °C for microarray studies and real-time PCR. All overnight cultures were grown in LB medium at 37 °C and then were diluted 1 : 100 in low-glucose DMEM and allowed to grow at 37 °C until they reached OD\textsubscript{600} 1.0. RNA was extracted from three replicates of each strain using a RiboPure bacterial RNA isolation kit (Ambion) according to the manufacturer’s instructions.

**Microarrays.** The GeneChip \textit{E. coli} Genome 2.0 array of the Affymetrix system contains 10 000 probe sets directed towards genes from four different strains of \textit{E. coli}: K-12 laboratory strain MG1655, uropathogenic strain CFT073, O157:H7 enterohaemorrhagic strain EDL933, and O157:H7 enterohaemorrhagic strain Sakai (http://www.affymetrix.com). These GeneChips were used in order to compare the transcriptome of 86-24 with strains NR01, NR02 and NR03. Processing of extracted RNA, cDNA labelling, hybridization and slide-scanning procedures were performed according to manufacturer’s instructions found in the ‘Affymetrix Gene Expression Technical Manual’ (http://www.affymetrix.com).

**Microarray data analysis.** The data from scanning a single replicate of the Affymetrix GeneChip \textit{E. coli} Genome 2.0 array for each strain were gathered using GCOS v1.4 as per the manufacturer’s instructions. Normalization of data was conducted using Robust Multiarray analysis (Bolstad \textit{et al.}, 2003; Irizarry \textit{et al.}, 2003) at the RMAExpress website (http://www.rmaexpress.bmbolstad.com). Output data were analysed for differences in gene expression resulting from the removal of \textit{qseE}, \textit{qseF} or \textit{qseG}.

N. C. Reading and others
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>86-24</td>
<td>Stx2+ EHEC strain (serotype O157:H7)</td>
<td>Griffin et al. (1988)</td>
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<td>NR01</td>
<td>86-24 qseE non-polar mutant</td>
<td>Reading et al. (2007)</td>
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<td>86-24 qseF non-polar mutant</td>
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<td>NR03</td>
<td>86-24 qseG non-polar mutant</td>
<td>Reading et al. (2009)</td>
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<tr>
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<tr>
<td>NR05</td>
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<tr>
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<td>Reading et al. (2007)</td>
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<td><strong>Plasmids</strong></td>
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<td>pNR02</td>
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<td>pNR03</td>
<td>qseG in pBadMycHis</td>
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Real-time RT-PCR. Primers used in real-time RT-PCR analysis were designed using Primer Express v1.5 (Applied Biosystems) and are listed in Table 2. Real-time RT-PCR analysis was conducted using an Applied Biosystems ABI 7500 sequence detection system using a one-step reaction. Each primer set was checked for amplification efficiency by standard curves resulting from using varying concentrations of RNA template. To ensure template specificity, products were heated to 95°C for 15 s, cooled to 60°C, and heated to 95°C while fluorescence was monitored. To analyse gene expression in NR01, NR02 and NR03 compared with 86-24 E. coli, relative quantification analysis was used. Parameters for cDNA generation and amplification were as follows: 48°C for 30 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. The RNA polymerase subunit Z, rpoZ, was used as an endogenous control. In each reaction of 20 μl, 10 μl 2X SYBR master mix, 0.1 μl Multiscribe reverse transcriptase (Applied Biosystems) and 0.1 μl RNase inhibitor (Applied Biosystems) were added.

Detection, quantification and statistical analysis. Applied Biosystems ABI Sequence Detection 1.3 software was used for initial collection of data. Values were normalized to rpoZ and analysed using the comparative critical threshold (Ct) value as previously described (Anonymous, 1997). Expression is shown in graphs as n-fold change in expression level compared with WT levels at late exponential growth. Error bars represent the standard deviations of the ΔΔCt value (Anonymous, 1997). Student’s t test was performed to assess statistical significance. A P value of <0.05 was considered significant.

RESULTS

Microarray analysis of ΔqseE, ΔqseF and ΔqseG compared with WT EHEC

We have previously shown that QseEFG in EHEC is involved in AE lesion formation (Reading et al., 2007, 2009). To identify additional targets of QseEFG, we performed transcriptome studies utilizing the Affymetrix GeneChip E. coli Genome 2.0 arrays to compare the transcripts of WT EHEC with those of ΔqseE, ΔqseF and ΔqseG single mutants. The GeneChip E. coli Genome 2.0 Array includes approximately 10 000 probe sets for all 20 366
genes and several intergenic regions present in four strains of *E. coli*: K-12 (MG1655 laboratory strain), CFT073 (uropathogenic strain), O157:H7 EDL933 and Sakai (enterohaemorrhagic strains) (http://www.affymetrix.com). The results of these analyses revealed that across the three mutant strains, 445 probe sets showed increased expression and 130 probe sets showed decreased expression (Fig. 1), while different sets of probes were differentially expressed in each of the mutants. A total of 3057 probe sets were differentially expressed in the *qseE* mutant as compared with the WT strain (Fig. 1), with 1726 showing increased, and 1331 showing decreased expression. Comparisons between the WT and the *qseF* mutants showed that 1405 probe sets were differentially expressed between these strains, and a greater proportion of these genes showed increased expression than decreased expression (1101 increased versus 304 decreased).

A similar scenario can be observed comparing the transcriptomes of the WT and the *qseG* mutant, where 1661 probe sets were differentially expressed between these strains, and a greater proportion of these genes showed increased expression than decreased expression (1101 increased versus 304 decreased). The majority of the genes with an altered profile were derived from the *E. coli* MG1655 strain. These features represent a common *E. coli* backbone conserved among all *E. coli* pathovars, and many of the features are associated with central metabolism and core biological processes (Table 3). More similarity was seen in the effect of knocking out these genes, when *DqseE* and *DqseG* were compared; however, there was a high degree of overlap between all three mutants, as shown in the Venn diagram in Fig. 1.

Several notable areas of regulation were identified through this screen, including many metabolic genes and numerous outer membrane proteins and transport systems. Of particular interest, genes involved in both iron and nitrogen utilization were regulated by this three-component system (Figs 2 and 3).

**QseEFG does not regulate nitrogen metabolism**

Given the abundance of metabolic genes that appeared to be differentially regulated in the array, we would expect to see a difference in growth ability between the WT and each of the mutants. To follow up the microarray results that showed that the genes involved in nitrogen regulation (Fig. 2a) were differentially regulated in the Δ*qseE*, Δ*qseF* and Δ*qseG* mutants, we conducted a series of growth experiments. Because *glnB* is in the same operon as *qseEGF*, it is not surprising that nitrogen metabolism gene regulation was altered in the mutant strains. The *glnB* gene encodes the α-ketoglutarate sensor PII. This protein negatively regulates the two-component system NtrB/NtrC, which activates glutamine synthetase when the cell is growing in low-nitrogen media (Ninfa & Jiang, 2005). However, we found no defect in nitrogen utilization in any of the mutants compared with the WT, given that all strains had similar growth rates in minimal media with or without glutamine supplementation (Fig. 2).

We then tested the ability of each mutant to grow on plates containing arginine as the sole nitrogen source. Again, each one of the mutant strains was able to grow on this minimal medium at a rate comparable to that of the WT (data not shown). These data indicate that although this three-component system modulates expression of genes involved in nitrogen metabolism, the Δ*qseE*, Δ*qseF* and Δ*qseG* mutants are not defective in nitrogen utilization. This suggests that QseEFG does not overcome the primary nitrogen signalling system, NtrB and NtrC.

**QseEFG has a mild effect on iron utilization**

The gene array studies suggested that QseEFG modulate expression of several iron-uptake systems. To confirm the array studies, we performed real-time quantitative RT-PCR. Our real-time experiments confirmed that transcription of *entB* was decreased in the *qseG* mutant, while it was unaltered in the *qseE* and *qseF* mutants. Expression of *fepE* was decreased in each of the mutants, while expression of *tonB* was unaltered (Fig. 3a). EntB is involved in the synthesis of the enterobactin siderophore (Hantash & Earhart, 2000), while FepE is involved in enterobactin

![Fig. 1.](image-url)
synthesis and transport (Ozenberger et al., 1987) and has been implicated in modification of LPS in *Salmonella* (Murray et al., 2003). TonB confers energy for several iron-transport systems.

To investigate differential iron uptake and utilization in each of the mutant strains, we performed several iron-utilization and uptake assays. As shown in Fig. 3(b), when the mutants were grown on LB plates containing either the iron chelator 2,2'-dipyridyl (DPD) or DPD and haemin (for recovery) for 48 h, there was no difference in the effect of these compounds on any of the mutants compared with the WT. The growth of all strains was inhibited by the addition of 350 μM DPD and all strains were able to recover to an equal extent with the addition of 8 μM haemin. When the strains were grown in minimal media (Fig. 3c), no difference in growth was seen. When the strains were grown in minimal media containing DPD, the *qseE* and *qseG* mutants grew slightly more slowly in the presence of 350 μM DPD (Fig. 3d). This slight defect in growth was complemented when a copy of any one of the genes was expressed in the mutant strains (Fig. 3d). These data indicate that while numerous iron-uptake and -utilization genes are differentially regulated in the mutants, the effect on iron utilization or growth is minimal. However, there did seem to be a small defect in mutant growth under iron-limiting conditions in minimal media, which were the media utilized in the transcriptional studies.

Cross-talk between QseEFG and other two-component signalling systems

Cross-talk has been shown to exist between two-component systems at the protein level by non-cognate phosphorylation (Yamamoto et al., 2005). The gene array studies indicated that numerous two-component signalling systems are regulated by QseEFG at the transcriptional level. To validate these findings, we performed real-time RT-PCR and found that *phoP* expression was downregulated in all three mutant strains, while *phoQ* expression was downregulated in both the *qseF* and the *qseG* mutants (Fig. 4). *PhoPQ* is a two-component system known to be involved in the sensing of antimicrobial peptides (Bader et al., 2005). In addition, the RcsBC system, which has been shown to modulate expression of the LEE region (Tobe et al., 2005), was regulated by QseEFG. Expression of *rcsB* was upregulated in *qseE, qseF* and *qseG* mutants, while *rcsC* was only upregulated in the *qseG* mutant (Fig. 4). These data indicate that QseEFG transcriptionally regulate other two-component systems, and provide a link between QseEFG regulation of *espFu* expression, Tir translocation and expression of the LEE region.

DISCUSSION

Two-component signalling systems are involved in the recognition and transduction of environmental signals by bacteria, so they can efficiently and effectively adapt to
certain niches. These systems have been shown to be important in bacterial pathogenesis in both Gram-positive and Gram-negative organisms (Laub & Goulian, 2007). In this study, we report a global transcription analysis of the recently described QseEF two-component system and the QseG membrane protein. Through microarray analysis, it was confirmed that many genes are equally affected transcriptionally compared with the WT by knocking out qseE, qseF and qseG (Fig. 1, Table 3). This trend was observed in several sets of genes, including many metabolism genes. However, within these datasets, independent regulation of some genes by each of the three proteins was also seen.

The genes encoding QseEFG constitute an operon with glnB. The glnB gene encodes the PII protein, involved in the modulation of the kinase and phosphatase activities of the E. coli nitrogen sensor kinase NtrB (Ninfa & Atkinson, 2000; Ninfa et al., 2000). This genetic organization combined with the microarray studies suggested that several nitrogen-utilization genes were regulated by QseEFG and led us to investigate whether qseE, qseF or qseG mutants had any defects in nitrogen utilization. As shown in Fig. 2, several nitrogen-utilization tests suggested that mutants for these genes are indistinguishable from the WT. Altogether, these data suggest that QseEFG play a minor role in regulation of nitrogen-utilization genes, which does not translate into defects in nitrogen utilization, a metabolic phenotype primarily regulated by the NtrBC system.

Many iron-utilization genes were also shown to be under QseEFG regulation (Fig. 3). However, we only observed a mild defect in iron utilization when the strains were grown in DMEM, which is the condition in which the RNA was extracted for microarray analyses. These data suggest that although this system regulates iron uptake in EHEC, the presence of additional iron-utilization mechanisms can overcome the defect exerted by downregulation of QseEFG-controlled iron transport systems.

The E. coli genome encodes approximately 32 two-component systems. Although cross-phosphorylation and signalling among non-cognate sensors and response regulators is known to occur, a study by Yamamoto et al. (2005) has shown that phosphorylation of non-cognate response regulators by histidine kinases is rare and occurs in only 22 of 692 combinations in E. coli (Yamamoto et al., 2005). This same study also showed that some two-component systems are more prone to non-cognate signalling than others; among these systems is QseEFG. This point is illustrated by the fact that QseF is phosphorylated by at least four non-cognate histidine kinases in addition to QseE (Yamamoto et al., 2005). QseEFG affect the regulation of other two-component systems, indicating that QseEFG also communicate with other two-component regulatory systems at the transcriptional level. Indeed, transcription of PhoPQ, a two-component system that senses antimicrobial peptides and magnesium (Bader et al., 2005; Groisman & Mouslim, 2006), is activated by QseEFG. Conversely, transcription of rcsBC, a two-component system that regulates flagella (Clemmer & Rather, 2007), and the LEE genes (Tobe et al., 2005) is repressed by QseEFG. RcsBC play a modulatory role in the expression of the LEE genes (Tobe et al., 2005), while QseEFG are involved in activation of espFu/tccP (Reading et al., 2007), transcription, and translocation of type III secretion effectors to host cells (Reading et al., 2009). QseEFG regulation of the RcsBC system ties together type III secretion and effector expression with effector transloca-

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**Fig. 2.** qseEFGglnB affects nitrogen gene regulation. (a) Heat map showing genes involved in nitrogen regulation. These genes are primarily downregulated in qseE, qseG and qseF. (b) Growth curves showing the growth of each mutant in minimal media and media into which glutamine was titrated. In both cases, there was no significant difference in growth between the WT EHEC and the qseE, qseG and qseF mutant strains.
Fig. 3. (a) Real-time RT-PCR showing the regulation of iron-utilization genes in the qseE, qseG and qseF mutant backgrounds. Significant downregulation of fepE and entB expression was seen. (b) Colony growth of mutant and complemented strains on media containing either 350 μM DPD or 350 μM DPD with 8 μM haemin. Each strain was equally affected by DPD addition and able to recover equally to the WT when haemin was added. (c) Growth of mutant strains versus WT EHEC in DMEM. No growth defect was seen in any of the mutant strains. (d) Growth of mutant strains versus WT EHEC in DMEM containing 350 μM DPD. The qseE and qseG mutants grew slightly more slowly than the WT, and this was complemented by the addition of the gene expressed on a plasmid. *P ≤ 0.05 using Student’s t test.

Fig. 4. QseEFG cross-talks with additional signalling systems. Real-time RT-PCR analysis was used to investigate the transcriptional regulation exerted by QseEFG on other two-component systems. Overall, expression of phoP and phoQ was downregulated in qseE, qseF and qseG mutants, while rcsB and rcsC were upregulated in the mutant strains. *P ≤ 0.05; **P ≤ 0.005 using Student’s t test.
tion. This suggests a very finely tuned regulation of AE lesion formation by EHEC.

The gseEFG genes are present in both K-12 and EHEC, and regulate pathogenic phenotypes, such as AE lesion formation (Reading et al., 2007), as well as metabolic genes shared between pathogenic and non-pathogenic strains of E. coli. This study expands on our knowledge of two-component system regulation, and provides yet another example of how pathogens exploit two-component systems to regulate virulence.

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