Quorum-Sensing *Escherichia coli* Regulator A: a Regulator of the LysR Family Involved in the Regulation of the Locus of Enteroocyte Effacement Pathogenicity Island in Enterohemorrhagic *E. coli*

Vanessa Sperandio,† Caiyi C. Li, and James B. Kaper*

Center for Vaccine Development and Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland 21201

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The locus of enteroocyte effacement (LEE) is a chromosomal pathogenicity island that encodes the proteins involved in the formation of the attaching and effacing lesions by enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC). The LEE comprises 41 open reading frames organized in five major operons, LEE1, LEE2, LEE3, tir (LEE5), and LEE4, which encode a type III secretion system, the intimin adhesin, the translocated intimin receptor (Tir), and other effector proteins. The first gene of LEE1 encodes the Ler regulator, which activates all the other genes within the LEE. We previously reported that the LEE genes were activated by quorum sensing through Ler (V. Sperandio, J. L. Mellies, W. Nguyen, S. Shin, and J. B. Kaper, Proc. Natl. Acad. Sci. USA 96:15196-15201, 1999). In this study we report that a putative regulator in the *E. coli* genome is itself activated by quorum sensing. This regulator is encoded by open reading frame b3243; belongs to the LysR family of regulators; is present in EHEC, EPEC, and *E. coli* K-12; and shares homology with the AphB and PtxR regulators of *Vibrio cholerae* and *Pseudomonas aeruginosa*, respectively. We confirmed the activation of b3243 by quorum sensing by using transcriptional fusions and renamed this regulator quorum-sensing *E. coli* regulator A (QseA). We observed that QseA activated transcription of ler and therefore of the other LEE genes. An EHEC qseA mutant had a striking reduction of type III secretion activity, which was complemented when qseA was provided in trans. Similar results were also observed with a qseA mutant of EPEC. The QseA regulator is part of the regulatory cascade that regulates EHEC and EPEC virulence genes by quorum sensing.

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is responsible for outbreaks of bloody diarrhea in several countries. It has a very low infectious dose (as low as 50 CFU in one outbreak) and colonizes the large intestine, where it causes attaching and effacing (AE) lesions (reviewed in reference 28). The AE lesions are characterized by effacement of the intestinal epithelial cell microvilli and the rearrangement of the cytoskeleton to form pedestal-like structures that cup the bacterial individual. Similar AE lesions are also seen with enteropathogenic *E. coli* (EPEC), which is a leading cause of infant diarrhea in developing countries (28). The genes involved in the formation of these AE lesions are encoded within a chromosomal pathogenicity island named the locus of enteroocyte effacement (LEE) (25). The LEE comprises 41 open reading frames organized in five major operons, LEE1, LEE2, LEE3, tir (LEE5), and LEE4, and contains (i) genes encoding a type III secretion system (13); (ii) the eae gene, encoding the intimin adhesin (14); (iii) tir, which encodes the translocated intimin receptor (15); (iv) the *espA/B* genes, which encode proteins that are secreted by the type III secretion system (6, 16, 23) (including EspA, which forms filamentous-like structures [18] involved in the translocation of EspB and Tir; and (v) ler (for LEE-encoded regulator), which encodes an H-NS-like protein that activates the expression of the LEE genes (26).

We previously reported that the first gene of the LEE1 operon encodes an activator of the LEE genes, which we named Ler (26). Friedberg et al. (10) and Elliott et al. (7) reported that mutations in ler abolished the ability of EPEC and EHEC to produce AE lesions. Sperandio et al. (37) demonstrated that Ler directly binds to the upstream regulatory region of LEE2, which is downstream of LEE3, to activate transcription of both the LEE2 and LEE3 operons. Bustamante et al. (2) reported that Ler acts as an antirepressor for H-NS, probably by competing with H-NS for binding to the regulatory regions of LEE2 and LEE3. These studies demonstrated that Ler directly activates transcription of the LEE genes by competing for binding with H-NS, which represses their expression in the absence of Ler. We previously reported that the LEE genes were regulated by quorum sensing through autoinducer-2 (AI-2), which is synthesized by the product of the luxS gene. Transcription of ler was activated in the presence of AI-2, leading to transcription of the other LEE genes in a cascade fashion (36).

Quorum sensing is a mechanism through which bacteria regulate gene expression by cell density. The bacteria produce hormone-like compounds called autoinducers that interact with regulatory proteins after they reach a certain threshold concentration. Quorum sensing was first described in the regulation of bioluminescence in *Vibrio fischeri* and *Vibrio harveyi* (29, 30). The luciferase operon in *V. fischeri* is regulated by two
One of the most widely distributed systems is the and peptide autoinducers (reviewed in references 1 and 3).

Gram-positive bacteria have other quorum-sensing systems which employ different components, such as two-component regulatory systems (reviewed in references 1 and 31). Gram-positive bacteria have other quorum-sensing systems which employ different components, such as two-component regulatory systems (reviewed in references 1 and 31). Gram-positive bacteria have other quorum-sensing systems which employ different components, such as two-component regulatory systems (reviewed in references 1 and 31). Gram-positive bacteria have other quorum-sensing systems which employ different components, such as two-component regulatory systems (reviewed in references 1 and 31). Gram-positive bacteria have other quorum-sensing systems which employ different components, such as two-component regulatory systems (reviewed in references 1 and 31). Gram-positive bacteria have other quorum-sensing systems which employ different components, such as two-component regulatory systems (reviewed in references 1 and 31). Gram-positive bacteria have other quorum-sensing systems which employ different components, such as two-component regulatory systems (reviewed in references 1 and 31). Gram-positive bacteria have other quorum-sensing systems which employ different components, such as two-component regulatory systems (reviewed in references 1 and 31).

We proposed that activation of the LEE genes by the AI-2–luxS quorum-sensing system would occur in response to the AI-2 produced by the intestinal flora and that this intraintestinal signaling could be one explanation for the low infectious dose of EHEC (36). The regulatory network of this quorum-sensing system in V. harveyi has been extensively described (reviewed in reference 33); however, very little is known about it in E. coli. In an effort to examine how global quorum-sensing regulation is for E. coli, we hybridized an E. coli gene array with cDNA synthesized from RNA extracted from wild-type EHEC strain 86-24 and its isogenic luxS mutant VS94 (38). The results of that recent study showed that in addition to Shiga toxin and the LEE gene products, quorum sensing regulated ca. 10% of the EHEC genome shared by K-12 and EHEC, including phenotypes such as flagellation and motility that could also play a role in pathogenesis. In the array study we observed 19 putative E. coli transcriptional regulators regulated by the AI-2–luxS quorum-sensing system that might be part of this intricate regulatory network (V. Sperandio and J. B. Kaper, unpublished data).

In the present paper, we describe one of these putative regulators, which is a member of the LysR family of regulators, and demonstrate that it activates transcription of the LEE genes by quorum sensing. This regulator shares homology with AphB from Vibrio cholerae and PtxR from Pseudomonas

TABLE 1. Strains and plasmids used in this study

<table>
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<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>
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<td>EPEC wild-type strain</td>
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<td>DH5α</td>
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<td>86-24 luxS</td>
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<td>VS94 with plasmid pVS84</td>
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<td>pVS195</td>
<td>ler-1[LEE1]:lacZ in pJRlacZins</td>
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proteins, LuxI, which is responsible for the production of the acylhomoserine lactone autoinducer, and LuxR, which is activated by this autoinducer to increase transcription of the lux operon (8, 9). Since that initial description, homologs of LuxR-LuxI have been identified in other bacteria, and in all of these LuxR-LuxI systems, the bacteria produce an acylhomoserine lactone autoinducer that binds to the LuxR protein and regulates the transcription of several genes involved in a variety of phenotypes, including production of extracellular matrix, antimicrobial agents, flagellation, motility, and other phenotypes (reviewed in references 1 and 31). Gram-positive bacteria have other quorum-sensing systems which employ different components, such as two-component regulatory systems and peptide autoinducers (reviewed in references 1 and 3).

One of the most widely distributed systems is the luxS system, first described for V. harveyi. Surette and Bassler (39) showed that V. harveyi produced a second autoinducer, which is not an acylhomoserine lactone, that they named AI-2. AI-2 seems to be a furanone (34), and the enzyme responsible for synthesizing it is encoded by the luxS gene (40). Many bacterial species produce AI-2 and possess the luxS gene, and this quorum-sensing system has been proposed to be involved in interspecies communication. Among these diverse bacterial species that contain the luxS quorum sensing system is E. coli, including EHEC serotype O157:H7 (39).
aeruginosa and was renamed quorum-sensing *E. coli* regulator A (QseA).

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. All *E. coli* strains were grown aerobically at 37°C in Luria-Bertani medium (LB) or Dulbecco modified Eagle medium (DMEM) (catalog no. 11054; Gibco-BRL). Selective antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 30 μg/ml; and streptomycin, 100 μg/ml.

**Recombinant DNA techniques.** Plasmid purification, PCR, restriction digestion, ligation, transformation, and DNA gel electrophoresis were performed by standard methods (32). DNA sequence analysis was performed at the University of Maryland Biopolymer Facility on an ABI automated sequencer with DNA purified by Qiagen mid-columns. All oligonucleotide primers used are listed in Table 2.

Plasmid pVS130 was constructed by amplifying the b3243 gene from *E. coli* K-12 strain MG1655 with *Pwo* polymerase (Boehringer Mannheim), using primers K1977 and K1978, and cloning into ZERO BLUNT TOPO PCR vector (Invitrogen). Plasmid pVS150 was constructed by cloning b3243 digested with *Xho*I and *Hind*III from pVS130 into vector pACYC177 (*Xho*I/*Hind*III). Plasmid pVS208 was generated by digesting plasmid pVS150 with *Sca*I (site within the *bla* gene) and cloning a blunt tetracycline resistance gene from pBR322. Plasmid pVS139 was constructed by cloning the blunt-ended b3243 PCR product into the *Eco*RI/*Sma*I site of vector pBluescript KSII (Stratagene). Plasmid pVS139 was digested with *Eco*RI (there is a single *Eco*RI site in the middle of b3243 gene) and blunt ended with the Klenow fragment of DNA polymerase of *E. coli*. A chloramphenicol resistance cassette (*cat*) amplified from pACYC184 with *Pwo* polymerase (Boehringer Mannheim) and primers CmF and CmR was cloned into the blunt-ended *Eco*RI site of pVS139, generating plasmid pVS141, which has the cloned b3243 gene interrupted by *cat*. Plasmid pVS141 was digested with *Eco*RI (there is a single *Eco*RI site in the middle of b3243 gene) and blunt ended with the Klenow fragment of DNA polymerase of *E. coli*. A chloramphenicol resistance cassette (*cat*) amplified from pACYC184 with *Pwo* polymerase (Boehringer Mannheim) and primers CmF and CmR was cloned into the blunt-ended b3243 PCR product into the *Eco*RI/*Sma*I site of vector pBluescript KSII (Stratagene).

**FIG. 1.** Activation of b3243 transcription by quorum sensing. (A) Portion of *E. coli* K-12 gene array (Sigma-Genosys) containing the b3243 open reading frame hybridized with cDNA synthesized from RNAs extracted from strains 86-24 (wild-type) and VS94 (*luxS* mutant). (B) β-Galactosidase activities of a *qseA*:lacZ fusion (pVS160) in 86-24 (wild type [WT]), VS94 (luxS mutant), and VS95 (luxS complemented) strain backgrounds in DMEM at an OD 600 of 1.0. (C) β-Galactosidase activity of a *qseA*:lacZ fusion (pVS160) in VS94 (luxS mutant) in fresh DMEM, DMEM preconditioned by growth of wild-type strain 86-24 (PC-WT), DMEM preconditioned by growth of strain VS94 (PC-luxS −), and DMEM preconditioned by growth of strain VS95 (PC-luxS +). Error bars indicate standard deviations.
digested with XbaI and SalI, generating plasmid pVS143. The EHEC b3243 mutant (named VS145) was generated by allelic exchange using vector pVS143, and the mutants were selected on plates containing chloramphenicol and 5% sucrose as previously described (5). The b3243 mutant (VS145) was complemented with plasmid pVS150, generating strain VS151. The EPEC b3243 mutant (named VS193) was also generated by allelic exchange using vector pVS143 as described above.

**Preconditioned media.** To prepare preconditioned media, an initial inoculum of strain 86-24, VS145, or VS151 was grown at 250 rpm in LB for 18 h at 37°C. This culture was diluted 1:1,000 in fresh LB or DMEM and grown to an optical density at 600 nm (OD600) of ca. 1.0. Following centrifugation (12,000×g, 4 min, 25°C) and filtration (0.2-μm-pore-size filter), the pH was adjusted to 7.0.

**V. harveyi luminescence assay.** The presence of AI-2 in the preconditioned media was assayed using the V. harveyi BB170 (luxN::Tn5) reporter strain, which responds only to AI-2 (39). The luminescence assays were performed as described (39) and the assays were read in a Wallac 1420 multilabel counter.

**Constructions of lacZ operon fusions.** Operon fusions with lacZ were constructed by amplifying the regulatory region of b3243 with Pwo polymerase, using primers K2180 and K2179, and cloning into plasmid pRS551, which contains a promoterless lac operon (35), thereby generating plasmid pVS160. This fusion was transformed into host strains 86-24 (wild-type EHEC), VS94 (luxS mutant of 86-24), and VS95 (VS94 complemented with luxS on a plasmid) (Tables 1 and 2).

We constructed ler-1 [LEE1::lacZ] and sepZ1 [LEE2::lacZ] fusions in single copy in the chromosome of EPEC strain E2348/69, but for unknown technical reasons we were unable to construct these integrated fusions in EHEC strain 86-24. Plasmids pVS232Z (ler-1 [LEE1::lacZ] in pRS551) and pVS21 (sepZ1 [LEE2::lacZ] in pRS551) (36) were digested with EcoRV and SalI, which cut in the middle of the bla and lacZ genes of pRS551, respectively. This digestion will release the LEE::lacZ fusion together with the kanamycin resistance gene. The Km::LEE::lacZ fragment was cloned into plasmid pRLacZins (21), which is a pCVD442 (5) derivative containing an added lacZ gene, yielding plasmids pVS194 (sepZ1 [LEE2::lacZ]) and pVS195 (ler-1 [LEE1::lacZ]). During the construction process, we observed that the lacZ gene was not efficiently expressed in EHEC strain 86-24, which might be due to the presence of the EHEC specific leuS gene, which could interfere with the expression of lacZ. To overcome this issue, we constructed the lacZ fusion in a pCVD442 derivative containing an additional lacZ gene, pRLacZins, which was able to efficiently express lacZ in EHEC strain 86-24.

**FIG. 2.** Alignments of EHEC and K-12 QseA with AphB from V. cholerae and PtxR from P. aeruginosa. Underlined amino acid residues comprise the predicted helix-turn-helix of QseA.
jugative transfer of pVS194 and pVS195 by SM10/p pir strain to E2348/69 and VS193, the ler-1[LEE1]:lacZ and sepZ1[LEE2]:lacZ fusions were recom-
combined into the lacZ sites of the E2348/69 and VS193 chromosomes, generat-
ing the following strains: VS196 (sepZ1[LEE2]: lacZ in E2348/69), VS197 (ler-1[LEE1]: lacZ in E2348/69), VS198 (sepZ1[LEE2]: lacZ in VS193), and VS199 (ler-1[LEE1]: lacZ in VS193). Strains VS198 and VS199 were com-
plemented with the wild-type qseA gene cloned on plasmid pVS208 to gen-
erate strains VS210 and VS211, respectively.

- Galactosidase assays. The strains containing the operon::lacZ fusions were
grown in DMEM at 37°C to an OD600 of 1.0. These cultures were diluted 1:10 in
Z buffer (Na2HPO4, 0.06 M; NaH2PO4, 0.04 M; KCl, 0.01 M; MgSO4, 0.001 M;
mercaptoethanol, 0.05 M) and assayed for -galactosidase activity by using
o-nitrophenyl- -D-galactopyranoside as a substrate as previously described (27).

Primer extension. DNA was extracted from strains grown to an OD600 of 1.0 in
DMEM at 37°C according to standard protocols (32). Primer extension was also performed according to standard protocols (32), using primer K983 (36).

Secreted proteins. Secreted proteins from EHEC strains 86-24, VS145, and
VS151 were prepared as described by Jarvis et al. (13) after these strains were
grown to an OD600 of 1.0 in DMEM at 37°C.

Western blotting. Total proteins were extracted from strains 86-24, VS94,
VS95, VS145, and VS151 grown in DMEM to an OD600 of 1.0. The protein
concentration was measured using the assay of Lowry et al. (24). Equal concen-
trations of total proteins were electrophoresed in sodium dodecyl sulfate–12%
polyacrylamide gels (22). Western blotting procedures were performed as pre-
viously described (32), and blots were probed with polyclonal antisera directed
against the A subunit of Stx2 (kindly provided by Alison O’Brien [Uniformed
Services University of the Health Sciences]) and anti-H7 flagellin (J. A. Girón
and J. B. Kaper, unpublished data).

FAS test. Fluorescent actin staining (FAS) tests were performed as initially
described by Knutton et al. (17). Briefly, bacterial strains were incubated with
HeLa cells for 6 h at 37°C 5% CO2 after which epithelial cells were permeabil-
ized with 0.1% Triton and treated with fluorescein isothiocyanate-phalloidin to
visualize the accumulation of actin beneath and around the bacteria attached to
the HeLa cells. The bacteria were stained with propidium iodide.

FIG. 4. FAS test with HeLa cells infected for 6 h at 37°C and 5% CO2 with strains 86-24, VS145, and VS151. The upper panels are the cells stained with fluorescein isothiocyanate-phalloidin, and the lower panels are the bacteria stained with propidium iodide. WT, wild type.
RESULTS AND DISCUSSION

*E. coli* putative regulator b3243 is activated by quorum sensing. To expand on our initial report that the LEE genes from EHEC were activated by quorum sensing through the *luxS* system (36), we showed that additional genes in the *E. coli* genome were also regulated by quorum sensing by using gene array technology (38). Through this array analysis, we found that transcription of 19 putative *E. coli* transcriptional regulators was either activated or repressed by quorum sensing (data not shown). One of these putative regulators, whose transcription was up-regulated 23-fold in the wild-type strain compared to the *luxS* mutant, corresponds to the open reading frame (ORF) b3243 in the K-12 and the EHEC genomes (GenBank accession numbers AE000403 and AE005552, respectively) (Fig. 1A). This open reading frame encodes a putative regulator of the LysR family, which is characterized by a helix-turn-helix DNA binding domain in its N terminus. The predicted protein encoded by b3243 shares 28% identity and 53% similarity with AphB from *V. cholerae*, which is involved in regulation of the ToxR regulon, the master virulence regulon of *V. cholerae* (19, 20). This predicted protein also shares 28% identity and 49% similarity with PtxR from *P. aeruginosa*, which is an activator of exotoxin A production (12) (Fig. 2). Since this LysR family *E. coli* regulator shared homology with other virulence regulators and was activated by quorum sensing, we renamed this regulator QseA for quorum-sensing *E. coli* regulator A.

To confirm the regulation of *qseA* by quorum sensing, we generated a *qseA::lacZ* transcriptional fusion by amplifying the regulatory region of this gene (from bp 5491 to 6340 of the sequence under GenBank accession number AE000403) and cloning it into a vector containing a promoterless *lacZ* gene to generate plasmid pVS160 (Table 1). Transcription from the *qseA::lacZ* fusion was down-regulated 3.5-fold in the EHEC *luxS* mutant (VS94) compared to the wild-type (86-24) and the complemented (VS95) strains (Fig. 1B). The discrepancy in fold activations between the gene array data and the promoter fusion data is probably due to copy number effects, since multiple copies of the *qseA* regulatory region in plasmid pVS160 might saturate the effect of other potential regulators involved in the transcriptional activation of *qseA*. To further confirm that *qseA* transcription was up-regulated by quorum sensing and not as a result of some metabolic change associated with a *luxS* mutation, we tested whether transcription of the *qseA::lacZ* fusion in a *luxS* mutant (VS94) was activated by exogenous AI-2. Since the final structure of AI-2 is still not reported and this molecule proved to be very difficult to purify (34), we used media preconditioned with the wild-type (86-24) and complemented (VS95) EHEC strains, both of which produce AI-2. As a negative control we used medium preconditioned with the *luxS* mutant (VS94), which does not produce AI-2. We then observed that transcription of *qseA* was activated by media preconditioned with both the wild-type and complemented strains but not with medium preconditioned with the *luxS* mutant (Fig. 1C), indicating that the activation of *qseA* transcription was due to AI-2 signaling and therefore quorum sensing.

Characterization of a *qseA* mutant. Since transcription of *qseA* was up-regulated by quorum sensing and the predicted QseA protein shared homology with *V. cholerae* and *P. aerugi-
LEE genes by quorum sensing but does not appear to regulate genes involved in flagellation, motility, and Stx production.

**QseA is an activator of ler.** Since the qseA mutant (VS145) was defective for type III secretion, we examined whether QseA might function as an activator for the LEE genes at the transcriptional level. To address this question, we introduced plasmids containing ler-1[LEE1], sepZ1[LEE2], orf12[LEE3], and tir::lacZ transcriptional fusions in three different genetic backgrounds, the wild-type strain (86-24), the qseA mutant (VS145), and the complemented strain (VS151), and assayed these fusions for β-galactosidase activity. There was a fourfold induction in the expression of ler-1[LEE1] in VS151 (VS145 complemented with qseA in pACYC177) compared to the expression of ler-1[LEE1] in 86-24 (data not shown). We could not observe any significant differences in the expression of ler-1[LEE1]::lacZ between 86-24 and VS145. This might be due to copy number effects, since the ler-1[LEE1]::lacZ fusion is in a multicopy plasmid and qseA is in a single copy in the chromosome of 86-24; when both the ler-1[LEE1]::lacZ fusion and qseA were in multicopy plasmids (VS151), we could observe activation of ler-1[LEE1] in the presence of qseA. The first gene in the LEE1 operon is ler, which directly activates the LEE2, LEE3, and tir operons (2, 26, 37; J. L. Mellies, unpublished data). There were no significant differences in the expression of β-galactosidase from plasmids containing sepZ1[LEE2], orf12[LEE3], or tir::lacZ fusions in the wild-type, qseA mutant, and complemented strain backgrounds (data not shown). At the present time, we cannot rule out that these results might also be due to copy number effects, since ler was in a single copy in the chromosome in all of these experiments and QseA acts upstream of Ler.

To address potential copy number effects, we generated...
Quorum sensing

Flagella and motility
SOS (Stx)

QseA

Other genes in E. coli?

Transcriptional factors

ler LEE1

LEE2

LEE3
tir eae LEE4

Concluding remarks. In this study we describe for the first time a regulator that acts upstream of Ler in EHEC and EPEC and is part of the quorum-sensing cascade that regulates the LEE genes through theluxS system. However this regulator is not the sensor for AI-2 in this cascade, i.e., the regulator that binds to AI-2, since aqseA mutant is defective for type III secretion but not for other phenotypes regulated by theluxS quorum-sensing system. We do not know if QseA is directly activatingler transcription or if it is acting through yet another regulator. This question will be addressed in the future by biochemical studies using purified QseA. We also have preliminary data suggesting a role in this quorum-sensing regulatory cascade for three other putative E. coli regulators from the 19 observed through the gene array (data not shown). In Fig. 7 we depict our present model for the quorum-sensing regulatory cascade in EHEC. In the presence of sufficient amounts ofAI-2, qseA transcription is activated, and in turn QseA activates the expression of Ler, which activates the LEE genes. Other regulators are involved in the regulation of flagellar, Shiga toxin production, motility, and other metabolic genes by quorum sensing. One two-component regulatory system, which we have named QseBC, is at least partly responsible for regulation of these factors through quorum sensing (V. Sperandio, A. Torres, and J. B. Kaper, submitted for publication). Quorum-sensing regulatory cascades are usually very complex and involve a variety of regulators (reviewed in reference 3). We are currently conducting additional studies to further elucidate this complex quorum-sensing regulatory cascade.

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