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The possible influence of LuxS in the in vivo virulence of rabbit enteropathogenic *Escherichia coli*

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Abstract

Attaching and effacing (A/E) organisms, such as rabbit enteropathogenic *Escherichia coli* (EPEC), human EPEC or enterohemorrhagic *E. coli* (EHEC) share attaching and effacing phenotype and LEE pathogenicity island responsible for A/E. The present study was undertaken to investigate the impact of the LuxS quorum sensing (QS) signaling system in vitro and in vivo pathogenicity of A/E organisms using rabbit EPEC (rEPEC) strain E22 (O103:H2). Analysis of the bioluminescence indicated abolished production of the QS signal AI-2 by *luxS* mutant (E22Δ*luxS*). Strain E22Δ*luxS* also exhibited impaired expression of several normally secreted proteins and reduced adherence to cultured HeLa cells. Complementation of the intact *luxS* gene to E22Δ*luxS* restored secreted protein expression comparable to the WT type but not adherence to HeLa cells. In experimentally infected rabbits, the isogenic *luxS* mutant induced clinical illness and intimate adherence to the intestinal mucosa, albeit to a less extent, comparable to that seen with the parent virulent strain. It is worth noting that reduced fecal bacterial shedding, mucosal adherence and improved cumulative weight gain were seen for the mutant strain complemented with *luxS* when compared to the WT. It appears that the *luxS* gene is not essential for in vivo pathogenicity by rEPEC where exogenous QS signals are present in the gut. The impact of AI-2 provided by multicopy plasmid on bacterial virulence is discussed.

Keywords

Quorum sensing; Autoinducer; LEE; Regulation

1. Introduction

The rabbit enteropathogenic *Escherichia coli* (rEPEC) share with human EPEC, enterohemorrhagic *E. coli* (EHEC) or *Citrobacter rodentium* associated with colonic hyperplasia in mice the attaching and effacing (A/E) phenotypes, characterized by close bacterial attachment to the epithelial cells and effacement of microvilli (Nataro and Kaper, 1998). The A/E phenotype is encoded on a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) (McDaniel et al., 1995). The LEEs from rabbit EPEC (rEPEC)

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O15:H- (Zhu et al., 2001), human EPEC (strain E2348/69, O127:H6) (Elliott et al., 1998), EHEC (O157:H7) (Perna et al., 1998) and *C. rodentium* (Deng et al., 2001) share a conserved region clustering into five major operons, LEE1-5. The LEE encodes a type III secretion system (T3SS), the LEE-encoded regulator (*ler*), bacterial adhesin intimin, translocated intimin receptor (Tir) and various secreted effector proteins (Elliott et al., 1998).

Ler is a central regulator for the expression of the LEE genes in A/E organisms (Mellies et al., 1999). It has been shown that the quorum sensing (QS) controls the expression of T3SS and protein secretion via the activation of Ler in EPEC and EHEC (Sperandio et al., 1999, 2000, 2001). QS confers the ability to communicate and to alter (regulate) behavior in response to fluctuations in cell population density (Miller and Bassler, 2001). QS-mediated signaling can be achieved through the secretion of low-molecular-weight hormone-like compounds called autoinducers (AIs) into the extracellular milieu (Sperandio et al., 2003). When these AIs reach a designated threshold concentration, they interact with bacterial transcriptional regulators, thereby regulating gene expression. While AI-1 (AHL, acyl-homoserine lactones) appears to be the predominant intra-species quorum signal in gram-negative bacteria, an alternate AI-2, discovered in *Vibrio harveyi* (Miller and Bassler, 2001) is proposed to act as an inter-species cellular communication molecule among both gram-negative and gram-positive species (Vendeville et al., 2005; Xavier and Bassler, 2005). The AI-2 signaling pathway has been linked in diverse bacteria to many phenotypes including bioluminescence, antibiotic resistance, biofilms, plasmid conjugal transfer and pathogenicity (DeLisa et al., 2001; Gonzalez et al., 2006).

EPEC/EHEC use an AI-2 system, a furanosyl borate diester, whose synthesis depends on LuxS (Sperandio et al., 2003). Previous studies demonstrated that QS signaling is an important regulatory mechanism for in vitro LEE gene expression by EPEC and EHEC (Sircili et al., 2004; Sperandio et al., 1999). However, the role of LuxS-mediated QS in the in vivo pathogenicity of these organisms has not been fully elucidated. In the current study, we constructed a defined deletion mutation in the *luxS* gene of wild-type (WT) rEPEC strain and determined the role of LuxS by in vitro and in vivo assays. The possibility that the QS signals are being supplied by the resident flora is discussed.

2. Materials and methods

2.1. Strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The EHEC O157:H7 strain 86-24 and *V. harveyi* BB170 were used as controls (Sperandio et al., 1999). The cloning vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA) was used to clone intermediate PCR products. The positive selection suicide vector plasmid pCVD442 was used for site-directed mutagenesis (Donnenberg and Kaper, 1991). All constructed plasmids were maintained in Top10 One Shot competent cells (Invitrogen) except for pCVD442 and derivatives which were maintained in strains SY327 λ pir or SM10 λ pir (Donnenberg and Kaper, 1991). Bacterial strains were grown overnight on Luria-Bertani (LB) agar, or LB broth, or otherwise stated. Appropriate antibiotics were added at the following concentrations: carbenicillin (Car), 50 μ g/ml; kanamycin (Kan), 50 μ g/ml, nalidixic acid (Nal), 50 μ g/ml; tetracycline (Tet), 25 μ g/ml.

2.2. Construction of the defined *luxS* deletion mutation

The defined deletion mutation in the *luxS* gene (the *ygaG* in *E. coli* K-12, GenBank accession #AE000353) was generated by single-overlap extension PCR (SOE-PCR) as previously described (Zhu et al., 2006). Briefly, the 5' and 3' regions of the *luxS* gene including the flanking sequences were amplified using two pairs of primers, B621/B657 and B656/B620, respectively

(Table 1). Primers B657 and B656 contain 18 nucleotides complementary to each other. The resulting PCR reactions were separated by agarose gel electrophoresis and the amplified bands were excised and purified with QIAquick gel extraction kit (QIAGEN, Valencia, CA). These purified PCR amplicons were used as DNA templates in subsequent PCR amplification using primers B621 and B620 for assembly. The resultant 954 bp PCR product containing the deletion-mutated *luxS* was purified as described above and cloned into a pCR2.1-TOPO vector to generate pCR2.1:: $\Delta luxS$. The nucleotide sequence of the insert was determined and aligned to the *ygaG* gene for verification. The plasmid pCR2.1:: $\Delta luxS$ was digested with *Sac* I and *Xba* I endonucleases to obtain mutated *luxS* fragments which were subsequently cloned into pCVD442 (digested with *Sac* I and *Xba* I). The resultant plasmids were maintained within strain SY327 (an intermediate strain for pCVD442). The suicide plasmid was subsequently prepared and transformed into SM10 to obtain the donor strain (SM10::pCVD442).

Conjugation between the recipient (strain E22) and the donor (SM10::pCVD442) strains and subsequent homologous recombination were achieved as described (Donnenberg and Kaper, 1991; Zhu et al., 2006). Following selection on sucrose media, the Amp^S, NaI^R organisms were screened for *luxS* mutation by PCR using primers B621/B620 (Table 1). Further confirmation of deletion-mutation in the *luxS* was made by PCR as above with an additional primer B651 (Table 1), which is internal to the deletion fragment. Thus, PCR amplifications using primers B621/B620/B651 will yield two bands (1235 or 720 bp) for the intact *luxS* but only a single 954 bp band for the mutated *luxS*. The plasmid pVS68 (Table 1) was transformed into E22 $\Delta luxS$ to complement the *luxS* mutation, obtaining strain E22 $\Delta luxS$ (pVS68) which was grown in the presence of Car (Sperandio et al., 1999).

2.3. Bioluminescence assay

The *V. harveyi* reporter strain BB170 (sensor 1⁻, sensor 2⁺) and the pre-conditioned media (PM) of the parent strain E22 or its isogenic derivatives, E22 $\Delta luxS$ and E22 $\Delta luxS$ (pVS68) were prepared and the bioluminescence assay was performed as previously described (Sperandio et al., 1999; Surette and Bassler, 1998). PM of *V. harveyi* strain BB152 and EHEC O157:H7 strain 86-24 and LB medium were used as positive and negative controls, respectively.

2.4. Examination of secreted proteins

Strain E22 and its derivatives were grown in Dulbecco's modified Eagle's medium (DMEM) and the secreted proteins were prepared as described (Zhu et al., 2006). After removal of bacterial cells by centrifugation, a protease inhibitor cocktail was immediately added and the culture supernatant was concentrated by trichloroacetic acid precipitation. The protein content was determined using a Micro Protein Determination kit (Sigma Chemical Co. Dorset, England). A total of 10 μ l of the preparation was separated by SDS-PAGE on a 12% resolving gel and the protein bands visualized by Coomassie Blue staining.

2.5. Growth curves and HeLa cell adherence assay

Bacterial growth in DMEM was examined as described (Sperandio et al., 2001). The cell adherence assay for WT strain E22 and its derivative mutants was performed on HeLa cell monolayers as described (Torres et al., 2002). Briefly, HeLa cells were seeded at 1×10^5 cells/ml and grown overnight to yield a semi-confluent cell monolayer. Overnight bacterial cultures were added to tissue culture cells replenished with fresh DMEM at a concentration of 10^7 bacteria per well and grown at 37 °C for 6 h. The cell monolayers were then treated with 0.1% Triton X-100 in PBS (phosphate-buffered saline) to lyse the cells and disperse the adherent bacteria, which were plated on MacConkey agar plates for viable bacteria counting. Data were expressed as the number of attached bacteria per cell.

2.6. Rabbit ileal loop studies

All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee protocols. Ligated ileal loops were made in three 12-week-old New Zealand White (NZW) male rabbits (Covance Research Products) (Bloom and Boedeker, 1996). Briefly, rabbits were fasted overnight and fed only water ad libitum. The rabbits were anesthetized, a laparotomy was performed and the small intestine was tied off into 10 cm loops with 3 cm interloops between each main loop. Six loops were prepared in each rabbit containing two PBS control loops and four loops for bacteria. By using a 25-gauge needle, 1 ml bacterial suspension containing 1×10^8 CFU of rEPEC E22 and its derivatives or PBS were inoculated into each loop. The intestine was then returned to the peritoneal cavity, the incision was closed, and the rabbits were returned to their cage and given water ad libitum. The rabbits were sacrificed 18 h post-operation. After the removal of the small intestine, the length of the loops and the fluid in each loop were measured. For loops without liquid accumulation, 1 ml sterile PBS was used to flush the intestinal lumen. The fluid accumulation ratio was expressed as ml/cm by measuring the fluid (ml) in the loops and divided by the length (cm) of the loop. An aliquot from each loop contents was diluted and plated onto LB agar containing appropriate antibiotics to obtain viable counts (CFU/ml). Bacteria recovered from the loop contents were expressed as percentage of inoculated CFU. The loops were examined for bacterial attachment and the severity of microvillus effacement as described (Agin et al., 2005).

2.7. Experimental inoculation of rabbits

Eighteen 2-month-old NZW rabbits were divided into three groups [E22, E22*ΔluxS*, or E22*ΔluxS* (pVS68)] with six in each group. The bacterial inocula were prepared as described to obtain approximately 1×10^5 CFU/ml for inoculation (Zhu et al., 2006). Rabbits were inoculated intragastrically via a pediatric feeding tube with 10 ml 10% bicarbonate solution to neutralize gastric contents followed by 3 ml inoculum. The feeding tube was then flushed with 5 ml sterile PBS to ensure complete delivery of the inocula.

Rabbits were weighed daily and observed for stool characteristics and clinical signs of illness as described previously (Agin et al., 2005). Briefly, stools were graded on a scale between 1 (normal, hard pellets), 2 (soft), 3 (mixed soft and liquid) or 4 (completely liquid). For fecal bacterial excretion, rectal swabs were rolled onto MacConkey plates supplemented with Nal (for E22 and E22*ΔluxS*) or Nal and Car [for E22*ΔluxS*(pVS68)], grown overnight at 37 °C, and graded as 0 (no CFU), 1+ (1–50 CFU), 2+ (50–200 CFU), 3+ (>200 CFU) or 4+ (confluent growth). Rabbits were sacrificed 7 days post-inoculation for histopathologic evaluation of bacterial mucosal adherence.

At necropsy, the intestinal tract was inspected for serosal hemorrhage, bowel edema and the degree of liquidity of cecal contents was observed and recorded. For histopathology, transmural sections from the distal ileum, cecum, proximal colon and distal colon were excised and fixed in 10% buffered formalin for sectioning and staining (hematoxylin and eosin or Giemsa). Microscopic examination of histopathology was based on 10 sequential, well-oriented, 400× fields from each sample. Bacterial enteroadherence was graded as the percentage of surface area in the field that is covered by closely adherent bacteria (Agin et al., 2005).

2.8. Statistical analysis

Values for differences in rabbit weight gain and bacterial adherence between experimental groups were compared by the Student's *t*-test. A nonparametric test (Wilcoxon Rank Sum) was used to compare scores for fecal bacterial shedding and stool consistency between experimental groups.

3. Results and discussion

We generated a defined 282 bp deletion mutation internal to the *luxS* gene (from 120 to 401 nt of *luxS* coding sequence) in the WT rEPEC strain. PCR amplification using primers B621/B620/B651 yielded a single 0.95 kb band for strain E22 Δ *luxS* but two 1.2 or 0.7 kb bands for the WT E22 (data not shown). As a result, a deletion spanning 94 amino acids (from 41 to 134aa) was made in the LuxS central region removing the active binding site of the protein.

The *luxS* gene encodes an enzyme involved in the metabolism of *S*-adenosylmethionine, ultimately leading to the production of the AI-2 molecule (Schauder et al., 2001). LuxS is a homodimeric enzyme with a novel fold that incorporates two identical tetrahedral metal-binding sites (Hilgers and Ludwig, 2001). A metal ion identified as zinc was seen bound to a Cys-His-His triad located in the middle of the molecule. Thus, elimination of residues between 41 and 134aa removes the active binding sites of LuxS thereby inactivating the LuxS functions. When tested by bioluminescence assay, the WT E22 produced levels of the AI-2 signal comparable to those by EHEC 86-24 or *V. harveyi* strain BB152 (Fig. 1). In contrast, the AI-2 production by E22 Δ *luxS* was reduced to the background levels seen in medium control. Complementation of Δ *luxS* with pVS68 restored the capacity of E22 Δ *luxS* to produce AI-2 at a level comparable to the parent strain E22.

We observed an altered secreted protein profile by the isogenic mutant E22 Δ *luxS* (Fig. 2). At least four protein bands, with respective apparent molecule mass of 55, 48, 30 and 17 kDa, were missing from E22 Δ *luxS* when compared to the WT E22. Production of these protein bands was restored when E22 Δ *luxS* was complemented with pVS68. Strain E22 Δ *luxS*(pVS68) expressed an additional unidentified protein band with apparent MW of 15 kDa. Previous in vitro studies, together with the current study, have shown that the LuxS QS system is essential for the expression of type III-secreted proteins in EHEC, EPEC, and rEPEC (Sircili et al., 2004; Sperandio et al., 1999, 2001).

We observed similar growth curves by WT E22 and its isogenic *luxS* mutant (Fig. 3A). However, complementation of *luxS* gene resulted in slower growth compared to that of the wild-type strain E22. Our results are in agreement with the previous studies indicating the potential role of the presence of the *luxS* gene on a multicopy plasmid on the over production of AI-2 (Sperandio et al., 2001). HeLa cell adherence indicated reduced in vitro adherence by the LuxS mutant strains (Fig. 3B). While an average of 97 bacterial cells per cell was observed for WT E22, adherent E22 Δ *luxS* was seen significantly less (average 50/cell, $p < 0.05$). Complementation of pVS68 to E22 Δ *luxS* did not restore adherence phenotype [$p > 0.05$, E22 Δ *luxS* (pVS68) versus E22 Δ *luxS*].

Since the type III secretion system is crucial for A/E phenotype, a logical assumption is that virulence of these pathogens would be attenuated in vivo by mutation of *luxS*. To determine the importance of LuxS in in vivo virulence, we did infection experiments in rabbits. Rabbits are natural hosts of rEPEC (Marches et al., 2000), and rabbits are well-established models for the study of rEPEC pathogenesis.

When tested in ileal loops, fluid accumulation and enteroadherence were most severe among ileal loops inoculated with the WT E22 or its isogenic LuxS mutant (Fig. 4). Loops inoculated with WT E22 had a range of 4–10 ml liquid per loop whereas loops inoculated with E22 Δ *luxS* had a range of 1.5–5.5 ml liquid per loop. No statistical significance was observed for liquid volumes or ratios (ml liquid/cm loop) between E22 and E22 Δ *luxS* groups although there was a trend of less severity in the mutant groups. However, liquid in the ileal loops inoculated with E22 Δ *luxS*(pVS68) was less than 1.5 ml, significantly less than WT E22 ($p < 0.01$). None of the loops inoculated with PBS showed any apparent fluid accumulation, detectable bacterial adherence or edema (Fig. 4) except one loop that had 2.5 ml liquid (8 cm

loop) accumulation and showed focal bacterial adherence and slight edema. The bacteria seen in this loop may have come from the adjacent loop inoculated with WT E22.

Bacterial adherence by E22 Δ luxS (6% surface adherent) was lower than WT E22 (12% surface adherent), although the difference was not statistically significant (Fig. 4B). Similar to fluid accumulation, bacterial adherence by E22 Δ luxS(pVS68) (1% surface adherent) was significantly ($p < 0.017$) less than WT E22 (Fig. 4B). Cecal edema, moderate or severe, was observed at comparable levels among loops inoculated with all three strains (Fig. 4C).

Following orogastric inoculation with WT E22, E22 Δ luxS or E22 Δ luxS(pVS68) we observed bacterial colonization in all experimental groups. All rabbits consistently discharged normal stools within 2 days post-inoculation (p.i.) but started developing diarrhea thereafter in all rabbit groups (Fig. 5A) except one rabbit from each E22 Δ luxS and E22 Δ luxS(pVS68) which appeared normal. The Wilcoxon Rank Sum test showed that the mean measure of abnormal stool consistency was comparable in all three groups. Heavy fecal bacterial shedding was observed among rabbits inoculated with E22 or E22 Δ luxS, with a trend towards lower shedding in the mutant. Notably, the shedding was significantly decreased in rabbits inoculated with E22 Δ luxS(pVS68) ($p < 0.003$, two-sample Wilcoxon Rank Sum test) (Fig. 5B). Rabbits inoculated with E22 or E22 Δ luxS demonstrated an average weight loss of 177 or 208 g at day 7 p.i. (Fig. 5C). However, rabbits receiving E22 Δ luxS (pVS68) maintained their body weight during the same period (Fig. 5C). No statistical significance was observed between the groups.

Gross examination of intestinal organs revealed varying degrees of gross pathological changes, including watery intestinal contents, and edematous and thickened cecal walls in rabbits inoculated with WT E22 or its derivatives. Microscopy examination revealed extensive bacterial attachment covering nearly 24% mucosal area by WT E22 whereas 5.5 or 4.2% mucosal area by E22 Δ luxS or E22 Δ luxS (pVS68), respectively (Fig. 6A). In all groups of animals, similar degrees of submucosal edema were observed (Fig. 6B). Statistical analysis showed no significant difference between groups.

The observed in vivo virulence of the LuxS mutant is suggestive that the LuxS function had been restored in vivo. The LuxS autoinducer synthase is present in a wide variety of gram-negative and gram-positive bacteria including common members of the commensal intestinal flora as non-pathogenic *E. coli*, and members of the *Enterobacter*, *Enterococcus*, *Clostridium* and *Streptococcus* genera (Schauder et al., 2001; Keller and Surette, 2006). This suggests that the AI-2 provided by members of the commensal intestinal flora could be complementing the rEPEC luxS mutation to up-regulate LEE-encoded virulence genes. Other AIs, such as AI-3 may also play a role in restoring the in vivo virulence of E22 Δ luxS. The AI-3 molecule is also produced by commensal intestinal bacteria such as *E. coli*, *Enterococcus*, *Clostridium* and *Bacteroides* (Kaper and Sperandio, 2005). The AI-3 is used for interkingdom signaling between prokaryotic and eukaryotic cells (Sperandio et al., 2003; Clarke et al., 2006). It has been demonstrated that human gastrointestinal flora produces both AI-3 and AI-2 as fecal filtrates were able to activate transcription of the *LEE1* promoter, as well as *V. harveyi* light production (Sperandio et al., 2003). Furthermore, there is growing evidence that eukaryotes produce compounds that can interfere with QS systems in bacteria by acting as agonists or antagonists (McDougald et al., 2007). A luxS mutant of EHEC O157:H7 was able to respond to a eukaryotic cell signal to activate expression of the LEE-encoded type III secretion system (Sperandio et al., 2003). In another study in gnotobiotic piglets (Jordan et al., 2005), a luxS mutant of EHEC O157:H7 induced clinical symptoms comparable to the parent strain. Thus, these exogenous signals, or naturally occurring biomimics in the gut may act in concert in vivo to regulate the gene expression of an E22 Δ luxS deficient in AI-2 production.

We observed reduced intimate bacterial adherence to the intestinal mucosa among rabbits receiving E22Δ*luxS* as shown in Fig. 6B. This may be due to metabolic deficiencies caused by impaired function of LuxS. A *luxS* mutation not only disrupts production of AI-2 but also several amino acid synthesis and catabolic pathways within the cell, thereby changing the entire metabolism of the organism (Schauder et al., 2001). A microarray study found that transcription of 10% of the EHEC genome was affected by a *luxS* mutation including genes involved in such fundamental physiological properties as growth rate and motility (Sperandio et al., 2001). Phenotype microarrays revealed such effectors in a LuxS mutant in human EHEC (Walters et al., 2006; Walters and Sperandio, 2006).

The *luxS* mutation was complemented by addition of the wild-type *luxS* gene on a multicopy plasmid, thereby restoring in vitro protein secretion. However, it appears that complementation of *luxS* in-trans reduced bacterial virulence as shown by reduced fecal bacterial shedding and reduced enterobacterial adherence in both ileal loop and oral inoculation experiments (Figs. 4 and 6). As QS provides a mechanism for bacteria to modulate gene expression in response to changes in population density, the constitutive expression of LuxS initiates a signal transduction cascade that culminates in a population-wide alteration of gene expression. That is, the upregulation of LEE-encoded virulence factors. In a real scenario, the initial adherence of these A/E organisms requires genes encoded outside of LEE, which were expressed prior to activation of Ler. Thus, the over production of LuxS in the complemented strain may cause the upregulation of LEE genes while downregulated the gene required for initial attachment. In this circumstance, these bacteria became less virulent than the parent WT strain. Indeed, complementation of an EHEC *luxS* mutant using a multicopy plasmid vector led to a substantial decrease in growth rate compared to the wild-type (Sperandio et al., 2001).

Cell-to-cell signaling can have a profound impact on microbial community structure and host-microbe interactions (Vendeville et al., 2005). The role of QS in the pathogenesis of disease has been investigated for a wide range of bacterial pathogens, however, no universal conclusions have been drawn. The multiplicity of QS systems in the gut having multiple different systems further complicates efforts to make broad sweeping conclusions. The *luxS*/AI-2 system has been shown to be important in regulating expression of virulence factors in *V. cholerae*, *V. parahaemolyticus*, EPEC and EHEC (Kaper and Sperandio, 2005) but has not yet been shown to be important in regulating virulence factors in other pathogens such as *Shigella* and *H. pylori* (Day and Maurelli, 2001; Joyce et al., 2000). Because of the numbers of commensal flora capable of producing one or more QS signaling molecules in the intestinal tract, obtaining definitive answers on the specific significance in vivo of quorum sensing of intestinal pathogens will be more challenging than studying this phenomenon in sites with little or no commensal flora.

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Fig. 1. Luminescence assay. The AI-2 signal present in the preconditioned media was examined by using *Vibrio harveyi* reporter strain BB170 (*luxN::Tn5*) as described (Sircili et al., 2004). Bars designate standard errors representing readings from two separate experiments.

Fig. 2.

Secreted protein profiles of WT rEPEC E22 and its derivative mutants. Proteins were separated by 12% SDS-PAGE and visualized by Coomassie Blue staining. Arrows indicate protein band absent from *E22ΔluxS* but present in the WT parent strain and *E22ΔluxS(pVS68)*. The positions of protein standards (in kDa) are indicated on the right.

Fig. 3.
 (A) Growth curves and HeLa cell adherence of rEPECWt E22, E22Δ*luxS* and E22Δ*luxS* (pVS68) in DMEM at 37 °C. Experiments were performed in triplicates. Bars designate standard error.

Fig. 4. Comparisons of fluid accumulation (A), enterobacterial adherence (B) and score of edema (C) in rabbit ileal loops inoculated with WT E22 or its derivatives. Bars designate standard errors.

Fig. 5.

Comparisons of stool consistency (A), fecal bacterial shedding (B) and cumulative weight change (C) of rabbits inoculated with WT E22 (open circles), E22Δ*luxS* (open triangles) and E22Δ*luxS*(pVS68) (cross). Bars designate standard errors. D.P.I.-day post-inoculation.

Fig. 6. Comparisons of percentage of mucosal surface (cecum) with adherent bacteria (A) and score of edema (B) between groups of rabbits inoculated with WT E22 or its derivative *luxS* mutants. Bars designate standard errors.

Table 1

Bacterial strains, plasmids and primers used in this study

Strains and plasmids	Relevant features or primer sequence	Source or primer location
Bacterial strains		
E22	rEPEC O103:H2, NaI ^R	(Marches et al., 2000; Zhu et al., 2006)
E22 $\Delta luxS$	E22 derivative, $\Delta luxS$, NaI ^R	This study
E22 $\Delta luxS$ (pVS68)	E22 $\Delta luxS$ complemented with pVS68, NaI ^R Amp ^R	This study
86-24	EHEC O157:H7	(Sperandio et al., 1999)
BB152	<i>V. harveyi</i> , <i>LuxMN</i> (AI-1 ⁻ ; AI-2 ⁺)	(Sperandio et al., 2001)
BB170	<i>V. harveyi</i> , <i>luxN::Tn5</i> (sensor 1 ⁻ ; sensor 2 ⁺), Kan ^R	(Sperandio et al., 2001)
SM10	SM10 λpir , recipient for suicide vector pCVD442	(Donnenberg and Kaper, 1991)
Plasmids		
pCVD442	Suicide vector, Amp ^R	(Donnenberg and Kaper, 1991)
pCR2.1-TOPO	PCR cloning vector, Amp ^R	Invitrogen
pCR2.1:: $\Delta luxS$	pCR2.1-TOPO:: $\Delta luxS$, Amp ^R	This study
pVS68	pBluescriptII:: <i>luxS</i> , Amp ^R	(Sperandio et al., 1999)
Primers ^a		
B621f	5'-gaccaccattacgctgtc	4236–4252
B657r	5'-atcaccgtgttcgatcag/atgcactcgttcgaggaa	5112–5095/4812–4795
B656f	5'-cagatcgaacacgggtgat	5095–5112
B620r	5'-aagcctaccgtaatctg	5511–5494
B651r	5'-tatgagtctgattgttacg	4945–4927

^aDerived from the *luxS* sequence in GenBank accession #AE000353.