Evaluation of the impact of quorum sensing transcriptional regulator SdiA on long-term persistence and fecal shedding of Escherichia coli O157:H7 in weaned calves

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**A R T I C L E  I N F O**

**Abstract**

*Escherichia coli* O157:H7 (O157) colonization of bovine intestine is mediated through the locus of enterocyte effacement (LEE)-encoded type III secretion system and secreted virulence proteins that promote colonization of the recto-anal junction (RAJ) of the large intestine of cattle. The quorum sensing transcriptional regulator SdiA, a homolog of LuxR, has been shown in vitro to repress LEE strongly when overexpressed from a multi-copy recombinant plasmid or when its activity is enhanced by the binding of N-acyl-L-homoserine lactones (AHLs), the quorum sensing signals that are detected by SdiA. Since LEE has been shown to be essential for colonization and persistence of O157 in bovine intestine, we examined whether a mutation in sdiA, which normally represses LEE in vitro, would also exert negative effect on colonization and long-term persistence of O157 in weaned calves. Ten-week-old weaned calves (n = 4) group) were inoculated orally with 10^10 cfu of either the wild-type or sdiA mutant strain. Initial fecal shedding of the sdiA mutant and the wild-type strain were similar in magnitude and declined during the first 2 weeks post-inoculation. The sdiA mutant was detected in feces of only one of the four calves at low levels (≥10^2 cfu/g feces) from days 19 – 27 post-inoculation, whereas, the fecal shedding of the wild-type strain persisted at approximately 4-logs in all four calves from days 19 – 27. We also confirmed that SdiA represses ler, which encodes a positive transcriptional regulator of LEE, in response to AHLs, and reduces adherence of O157 to HEP-2 cells. In conclusion, this study demonstrates that although in vitro the sdiA gene represses LEE and LEE-mediated adherence to cultured cells, the presence of sdiA is necessary for colonization of bovine large intestine that in turn promotes persistent fecal shedding of O157 by these animals.

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1. Introduction

*Enterohemorrhagic Escherichia coli* (EHEC) O157:H7 (referred to as O157) bacteria cause a broad spectrum of diarrheal illnesses, including uncomplicated diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome [1]. O157 colonizes the large intestines of cattle, which are considered the primary reservoir for this important human bacterial pathogen [2]. Colonization requires intimate adherence of O157 bacteria to intestinal epithelial cells, a process that culminates in the formation of characteristic histopathological lesions called attaching and effacing (A/E) [3,4]. The A/E lesions are produced as a result of rearrangements of host cell cytoskeletal elements and degeneration of microvilli of these cells to small pedestals that cup attached bacterial cells. The genes responsible for the production of A/E lesions are encoded on a pathogenicity island termed the locus of enterocyte effacement (LEE) [5]. LEE encodes for a type III secretion system for secretion of several LEE and non-LEE-encoded proteins for adherence to intestinal epithelial cells and various cell lines used for in vitro adherence assays. Expression of LEE is activated by Ler, encoded by the first gene of LEE1 operon [6].

Several transcriptional regulators affect the expression of ler directly or indirectly in response to a variety of physiological, environmental, and quorum sensing signals [7]. Quorum-sensing signaling, which is dependent on the bacterial population reaching a threshold that usually occurs as bacteria enter stationary phase, plays an important role in controlling LEE expression by activating or repressing ler expression [8]. For example,
bacterial-encoded quorum sensing signaling molecules AI (autoinducer)-2 and AI-3, as well as, the mammalian hormones epinephrine and norepinephrine have all been shown to regulate LEE and flagellar gene expression [8–10].

E. coli O157:H7 also encodes a chromosomal LuxR homolog called SdiA, which is a receptor for the AI-1 class of quorum sensing signaling molecules, such as N-acyl-L-homoserine lactones (AHLs) [11,12]. However, O157, like other E. coli and Salmonella strains does not synthesize AHLs; instead these bacterial species utilize SdiA to detect AHLs produced by other bacterial species [12,13]. The first reported function of SdiA in E. coli was transcriptional activation of the bacterial cell division gene cluster ftsQAZ [14]. Subsequent in vitro studies showed that while the expression of SdiA from a high-copy-number plasmid reduced motility and expression of LEE-encoded genes of O157, the deletion of sdiA enhanced motility, curli gene expression, and biofilm formation [15,16]. Interestingly, however, SdiA was demonstrated in a later study to be essential in O157 colonization of the bovine intestine since sdiA transposon insertion mutants were not recovered or recovered at low levels in the feces of 10–14 days old Friesian bull calves inoculated with a library of signature-tagged mutants of O157 [17]. A recent report has provided a greater insight into the mechanism by which SdiA promotes colonization of O157 in the large intestine of adult cattle [18]. According to this study, AHLs produced by the resident rumen microbial species presumably activate SdiA and the activated SdiA increases survival of O157 in the acidic compartment of the rumen by enhancing its acid resistance through the activation of gad-encoded acid-resistance pathway [18]. In addition, AHL-activated SdiA that represses LEE in vitro presumably also causes repression of LEE in the rumen, and since O157 does not colonize ruminant, repression of LEE by SdiA in the ruminant represents an energy-conserving adaptation to O157 [18]. Thus, increased survival of O157 in the acidic rumen compartment facilitates entry of “healthy” O157 bacteria into the large intestine. Since AHLs are reportedly absent in the large intestine, LEE is no longer repressed by SdiA and increased expression of LEE due to the presence of other signaling molecules enables O157 to colonize large intestine of cattle. The colonized animals continue to shed these bacterial pathogens of humans in their feces at variable magnitudes and duration.

The major objective of the current study was to determine if sdiA, which has already been shown to enhance survival of O157 in the acidic rumin compartment of adult cattle through the activation of gad-encoded acid-resistance pathway so that O157 exiting the rumen could colonize large intestine [18], would also be required for colonization and persistence of O157 in weaned younger calves that were fed an adult cattle diet. Relative importance of sdiA in colonization and persistence of O157 was determined by comparing the duration and magnitude of fecal shedding of the sdiA mutant to that of the wild-type O157 in calves of this age group over a period of four weeks.

### 2. Materials and methods

#### 2.1. Bacterial strains, culture media, and growth conditions

Table 1 lists bacterial strains, plasmids, and plasmid vectors used in this study. E. coli O157:H7 strain 86-24 was used as the parent for constructing an isogenic sdiA deletion mutant strain [19]. Commercially available vectors pCR2.1 TOPo or pC2.1 XL were used as cloning vectors and E. coli TOP10 cells were used as a bacterial host for these vectors (Invitrogen, Carlsbad, CA). Plasmid DNA from bacterial cells was isolated using mini or midi kits according to the manufacturer’s instructions (Qiagen, Valencia, CA). Luria–Bertani broth (LB) or LB containing 1.5% agar (Becton, Dickinson and Company, Sparks, MD) were used for growth of bacterial strains. For RNA isolation, bacterial strains were grown in Dulbecco’s Modified Eagle Medium (DMEM catalog # 11054). Media were supplemented with antibiotics at the following final concentrations: streptomycin, 100 mg L⁻¹; kanamycin 50 mg L⁻¹; and ampicillin 100 mg L⁻¹.

#### 2.2. Construction of the sdiA deletion mutant

A previously described allelic replacement procedure was used for constructing an in-frame sdiA deletion mutant of O157 strain 86-24 [20]. Briefly, a 1.5 kb DNA fragment upstream (US) and 1.5 kb DNA fragment downstream (DS) of sdiA ORF were isolated by PCR from strain 86-24 using primers sdiA-US/sdiA-US and sdiA-DS/sdiA-DS (Table 2). After agarose gel purification using a Gel Extraction Kit (Qiagen, Valencia, CA), these fragments were ligated to each other to generate a 3 kb fragment containing DNA fragments 1.5 kb US–1.5 kb DS of sdiA. This 3 kb DNA fragment was cloned into plasmid pAM450, which is temperature sensitive for its replication as it is unable to replicate at temperatures above 37 °C [20]. The pAM450 plasmid containing the 3 kb (1.5 kb US–1.5 kb DS) fragment was named pSM498. The recombinant pSM498 was electroporated into strain 86-24 for deleting the sdiA gene according to a previously described procedure [20]. A 1.1 kb DNA fragment containing the sdiA gene was also isolated from strain 86-24 by PCR using primers sdiAR and sdiAF (Table 2) and cloned in a low-copy vector pSMART-LC according to the manufacturer’s instructions (Lucigen Corp., Middleton, WI). The

### Table 1: Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and description</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli O157 strain</td>
<td>stx2² and Streptomycin-resistant</td>
<td>[19,24]</td>
</tr>
<tr>
<td>Strain 86-24 and/or TOP10</td>
<td>endA1 recA1 hsdR17(fcr mcr) supE44</td>
<td>This study InTrovigen</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pSM498</td>
<td>Plasmid used for sdiA deletion</td>
<td>This study (Lucigen, Corp., Middleton, WI)</td>
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<tr>
<td>pSMART-LCamp</td>
<td>Low-copy cloning vector</td>
<td></td>
</tr>
<tr>
<td>pSM606</td>
<td>pSMART-LC carrying cloned copy of sdiA</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Detailed description of bacterial strains and plasmids listed in this table is provided under material and methods section.

### Table 2: Primers used for PCR and QRT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
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<th>Location</th>
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<tr>
<td>sdiA-US</td>
<td>CAGTCTAGACCTTTCCGCGACATGATAACC</td>
<td>2692801-2692102</td>
</tr>
<tr>
<td>sdiA-US</td>
<td>CAGTGTCGACTCTTTCTTCTTGCTGCGCC</td>
<td>2693591-2693570</td>
</tr>
<tr>
<td>sdiA-DS</td>
<td>CAGTCTAGAACCCTTTCCGCGACATGATAACC</td>
<td>2695859-2695839</td>
</tr>
<tr>
<td>sdiA-DS</td>
<td>CAGTGTCGACTCTTTCTTCTTGCTGCGCC</td>
<td>2694315-2694334</td>
</tr>
<tr>
<td>sdiAR</td>
<td>GTACGTGCGACACCCGGCCAAAG</td>
<td>2694645-2694628</td>
</tr>
<tr>
<td>sdiAR</td>
<td>GTACGTGCGACACCCGGCCAAAG</td>
<td>2693592-2693611</td>
</tr>
<tr>
<td>lcrF-US</td>
<td>GTAACACCTTTCCGCGACATTCC</td>
<td>4688913-4688935</td>
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<td>4242977-4242978</td>
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<td>lcrF-DS</td>
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<td>4242877-4242900</td>
</tr>
<tr>
<td>lcrF-DS</td>
<td>CAGTGTCGACTCTTTCTTCTTGCTGCGCC</td>
<td>4242977-4242976</td>
</tr>
</tbody>
</table>

* Nucleotide sequence of E. coli primers used in this study were selected from the published genome sequence of E. coli O157:H7 strain EDL933 with the accession number AE005174.2. Location refers to the position of primer sequence in the genome of EDL933.

Subscripts F, R and P indicates forward primer, reverse primer, and probe.
recombination of sdiA-pSMART-LC (pSM606) and pSMART-LC were used for complementation of the sdiA deletion mutant strains.

2.3. Quantitative reverse transcriptase PCR (QRT-PCR)

Bacterial strains were grown in DMEM with or without 10 μM of N-3-Oxooctanoyl-α-homoserine lactone (AHL) (Sigma Chemical Co. St. Louis, MO) to an A600 of 1.3. Total bacterial RNA was isolated using RNeasy Mini Kit according to the manufacturer’s instructions (Qiagen Inc., Valencia, CA). RNA was treated with TURBO DNase for removal of contaminating DNA (Applied Biosystems/Ambion, Austin, TX). QRT-PCR was performed by adding 50 ng of RNA, 0.75 μM each of antisense and sense primers (Table 2), 0.25 μM of TaqMan probe (labeled at the 5’ and 3’ ends with FAM reporter and TAMRA quencher dyes, respectively) to a QRT-PCR Master Mix (Agilent Technologies, Inc., Santa Clara, CA). The reaction mixtures were incubated in MX3005P (Agilent Technologies, Inc., Santa Clara, CA) for cDNA synthesis (50 °C for 30 min), amplification, and real-time detection of amplified products (95 °C for 10 min; 35 cycles of 95 °C for 30 s, 55 °C for 60 s, 72 °C for 30 s) specific for the LEE-encoded ler of O157. A quantification program of the MX3005 system allowed calibration of the target gene expression in the mutant strains to the expression of the corresponding genes in the parent strain. In addition, expression data for ler was normalized to the expression of rpoA, a gene whose expression is not affected by sdiA in order to account for minor variations in the amounts of RNA between samples. The change in target gene expression in the mutant strains was plotted as a fold change by adjusting the difference in the ler expression and in vitro adherence of the sdiA mutant in comparison to the wild-type O157 (Prism 6 for Windows, GraphPad Software, Inc., La Jolla, CA). The two sample t-test was also used to assess the significance of the difference in the duration of fecal shedding between the mutant and the wild-type strain on specific days. The differences were considered significant at p < 0.05. The significance of the difference in the magnitude of fecal shedding between the wild-type and the sdiA mutant for each sampling day was determined by using One-Way Analysis of Variance for each day of the fecal shedding (JMP 10.0.0, SAS Institute, Inc., Cary, NC). The differences were considered significant at p < 0.05.

3. Results

3.1. Deletion of the sdiA gene reduces the magnitude and duration of O157 fecal shedding in weaned calves

A previous study demonstrated that adult cows (1.5-year-old) inoculated directly into the rumen with a mixture of a sdiA deletion mutant and wild-type strain showed reduced fecal shedding of the sdiA deletion mutant relative to the wild type strain both in the rumen digesta and feces over 3 and 8 days of sampling, respectively [18]. However, we wanted to determine the effects of sdiA deletion on fecal shedding patterns over a longer time period by using younger calves that were inoculated orally with either the sdiA mutant or the wild-type strain. The highest magnitude of fecal shedding, expressed as log10 cfu/g feces, for both the sdiA mutant and the wild-type strain was 6–logs at 1 day post-inoculation (Fig. 1A). The fecal shedding of calves inoculated with the sdiA mutant declined to about 4–logs by day 12 post-inoculation, 2–logs by day 15, became undetectable at day 18, and increased to and maintained at 2–logs (detection limit of the spread plate counting) from days 21 through 27. Fecal shedding in calves inoculated with the wild-type strain decreased to about 5–logs by day 12, decreased to approximately 3–logs on day 15, and persisted at ≥ 4 logs from day 18 through 27 (the last day of fecal sampling).Thus, similar O157 fecal shedding patterns were observed between calves inoculated with the sdiA mutant or the wild-type strain for the first two weeks following challenge, but there was greater decline in fecal shedding of the sdiA mutant compared to the wild-type strain starting at day 15 post-inoculation until the end of the study (27 days).

Analysis of fecal shedding data showed that the calves (n = 4) inoculated with the sdiA deletion mutant had a shorter mean duration (15–days) of fecal shedding compared to the group (n = 4) of calves inoculated with the wild-type (26–days) strain (p < 0.05) (Fig. 1B). The duration of fecal shedding in calves inoculated with the sdiA mutant ranged from 5 to 27 days. In this group, three calves were positive for fecal shedding for ≤16 days and only one of four calves shed the sdiA mutant for 27–days. In the four calves inoculated with the wild-type strain, duration of fecal shedding lasted for 25–days in two calves and 27–days in two calves. The fecal shedding was also significantly (p < 0.05) higher at days 18 and 27 in calves inoculated with the wild-type strain.

3.2. The sdiA gene product represses expression of ler in response to AHL or when expressed at high levels

Since SdiA has been shown to repress LEE expression in the presence of AHLS, we compared the relative expression of ler by QRT-PCR using RNA prepared from bacterial cultures of the sdiA

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mutant, the sdiA (pSM606)-complemented mutant, and the wild-type strain grown in the presence or absence of a commercial preparation of AHL (N-(3-oxooctanoyl-homoserine lactone). As shown in Fig. 2A, expression of ler in the wild-type strain was reduced by 73% ($p < 0.05$) in the presence of AHL. In the sdiA mutant, expression of ler was increased by 3.5-fold ($p < 0.0001$) in the absence and 4.11-fold ($p < 0.0001$) in the presence of AHL relative to the wild-type strain without AHL. As compared to the wild-type strain, expression of ler decreased by 2.7-fold ($p < 0.0001$) and 3.0-fold ($p < 0.0001$) in the sdiA (pSM606)-complemented mutant strains grown in media without or with AHL, respectively (Fig. 2A).

3.3. Inhibition of O157 adherence to HEp-2 cells correlates with the levels of SdiA expression

Microscopic examination and enumeration of bacterial cells adherent to HEp-2 cells showed a 2.3-fold increase ($p < 0.001$) in the number of adherent bacteria for the sdiA mutant compared to the wild-type strain (Fig. 2B). However, the numbers of adherent bacterial cells were 6.7 fold lower ($p < 0.0001$) for the sdiA (pSM606)-complemented mutant relative to the wild-type strain (Fig. 2B).

4. Discussion

The data presented in this report demonstrate that SdiA promotes colonization and persistence of O157 in 10-week old weaned calves that were inoculated orally with either the sdiA mutant or the wild-type strain. Our results are in agreement with an earlier...
report demonstrating that a transposon insertion in the sdiA gene resulted in the lower recovery of this mutant strain in 2-week old calves inoculated orally with a pool of signature-tagged mutants [17]. Our results also support a recent study in which 18-month old heifers inoculated with a mixed inoculum of an sdiA mutant and a wild-type strain showed reduced recovery of the sdiA mutant relative to the wild-type strain in samples taken from the recto-anal junction (RAJ) on day 8 post-inoculation [18]. However, due to the short duration of this study [18], it was not possible to determine if sdiA deletion affected both the long-term persistence as well as the magnitude of fecal shedding of the sdiA mutant relative to the wild-type strain. In our study in which fecal samples were collected for 4 weeks, we were able to demonstrate that there were no differences in the fecal shedding of the sdiA mutant and the wild-type strain for the first 2 weeks. It was only during week 3 and 4 that a decrease in the magnitude of fecal shedding of the sdiA mutant was observed with no detectable shedding in 3 of the 4 calves after day 16. Overall, mean duration of fecal shedding was significantly reduced in calves inoculated with the sdiA mutant (p < 0.05). Thus, it is possible that during the first two weeks post-inoculation the majority of sdiA mutant and wild-type bacterial cells were continually excreted in feces but a larger fraction of uninjured wild-type bacterial cells, because of their acid resistance [18], were able to transit from rumen to establish colonization in the large intestine of calves. This increased colonization of the large intestine during the first two weeks was then followed by an increased shedding of the wild-type bacteria during weeks 3–4 in feces of calves inoculated with the wild-type strain. The reduced fecal shedding of the sdiA mutant, on the other hand, could be attributed to its reduced acid resistance [18], which presumably contributed to its poor survival in the rumen resulting in the reduced colonization of the large intestine and reduced magnitude of fecal shedding of the sdiA mutant during week 3 and 4 by animals inoculated with the sdiA mutant strain.

The binding of AHLs to SdiA is considered essential for conversion of inactive SdiA into an active SdiA that interacts with the promoters of target genes to affect their transcription [12,21,22]. A recent study showed that AHLs are present in the adult bovine rumen and O157 uses these AHLs to activate SdiA for increasing expression of the gad operon encoding an acid–resistance pathway that enhances survival of O157 in the acidic rumen compartment thereby enabling transit of larger number of uninjured O157 bacterial cells to enter large intestine to establish colonization of the RAJ [18]. The same study also showed that transcriptional levels of ler and the ler-regulated espA were significantly lower in the wild-type strain compared to the sdiA mutant grown in the presence of oxo-C6-HSL. We also observed reduced transcription of ler in the wild-type strain when grown in the presence of oxo-C8-HSL but the magnitude of this reduction was much lower. One possible reason that we observed less pronounced repression of ler could be that we used oxo-C8-HSL in the growth medium. It has been suggested that SdiA can bind to a wide range of AHLs but with varying sensitivities that would presumably affect the expression of target genes with varying magnitudes [23].

Our data from animal inoculation studies with the sdiA mutant and the wild-type strain demonstrate that sdiA is important for colonization potential of O157 in younger calves and therefore supports similar conclusions reached in a competitive index study using an sdiA mutant and the wild-type strain in older animals [18]. It appears that the in vivo effects of SdiA on the expression of LEE in response to AHLs are different than those observed in vitro without AHL. For example, it has been shown that in response to rumen AHLs, SdiA increases the expression of gad-encoded acid resistance and represses LEE via ler repression. This opposing effect of SdiA on expression of acid resistance and LEE is advantageous since O157 does not colonize rumen, and therefore LEE expression is not required at this site. The LEE is turned on in the large intestine where LEE-encoded effectors are needed during colonization of the RAJ [18]. In earlier studies, the effects of SdiA on LEE expression in the absence of AHLs did not show appreciable increases in the expression of ler and the Ler-regulated LEE genes [16,18]. However in both these studies, RNA was isolated from bacterial cultures grown in DMEM at mid- to late-log phase corresponding to an A600 of 1.0. However, when we used RNA from bacterial cultures grown to an A600 of 1.3–1.4, which according to our previous study corresponds to the maximal expression of the LEE-encoded genes [20], we observed significant increases in the expression of ler in the sdiA deletion mutant and several-fold reduction in the expression of ler in the complemented mutant compared to the wild-type strain. It is possible that maximal LEE expression during late-log to early-stationary phases might be accomplished either by the reduced expression of SdiA or increased expression of positive regulators of ler that could potentially interfere with the binding of SdiA to the ler promoter. Similarly, determination of in vitro adherence to HEp-2 cells also showed a positive correlation between ler expression and number of HEp-2-adherent bacterial cells in the sdiA mutant and sdiA-complemented strain.

In conclusion, this study provides strong evidence that the transcriptional regulator encoded by the sdiA gene is important for O157 to colonize bovine intestine and to persist at this site for extended periods of time. Since a previous study has demonstrated that the AHLs are present in the bovine rumen and SdiA can sense these AHLs to enhance survival of O157 by enhancing its acid resistance so that wild-type bacterial cells can survive the rumen environment and subsequently colonize bovine large intestine [18], we could presume that SdiA works through this same mechanism in enhancing O157 colonization of the large intestine of younger animals. However, in light of another study demonstrating that normal gastrointestinal microbiota in most animals including cattle do not produce sufficient AHLs to activate SdiA [23], it would be interesting to determine if animals shedding O157 in their feces contain AHLs in their rumen. This information would be important in identifying dietary, biological, and chemical or combinations of one or more of these strategies to suppress the production of AHLs in bovine rumen in order to reduce colonization of O157 in these important food animals.

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