Evaluation of the effects of *sdiA*, a *luxR* homologue, on adherence and motility of *Escherichia coli* O157:H7

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Quorum-sensing (QS) signalling pathways are important regulatory networks for controlling the expression of genes promoting adherence of enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 to epithelial cells. A recent study has shown that EHEC O157:H7 encodes a *luxR* homologue, called *sdiA*, which upon overexpression reduces the expression of genes encoding flagellar and locus of enterocyte effacement (LEE) proteins, thus negatively impacting on the motility and intimate adherence phenotypes, respectively. Here, we show that the deletion of *sdiA* from EHEC O157:H7 strain 86-24, and from a *hha* (a negative regulator of *ler*) mutant of this strain, enhanced bacterial adherence to HEp-2 epithelial cells of the *sdiA* mutant strains relative to the strains containing a wild-type copy of *sdiA*. Quantitative reverse transcription PCR showed that the expression of LEE-encoded genes *ler*, *espA* and *eae* in strains with the *sdiA* deletions was not significantly different from that of the strains wild-type for *sdiA*. Similarly, no additional increases in the expression of LEE genes were observed in a *sdiA hha* double mutant strain relative to that observed in the *hha* deletion mutant. While the expression of *fliC*, which encodes flagellin, was enhanced in the *sdiA* mutant strain, the expression of *fliC* was reduced by several fold in the *hha* mutant strain, irrespective of the presence or absence of *sdiA*, indicating that the genes *sdiA* and *hha* exert opposing effects on the expression of *fliC*. The strains with deletions in *sdiA* or *hha* showed enhanced expression of *csgA*, encoding curli of the curli fimbriae, with the expression of *csgA* highest in the *sdiA hha* double mutant, suggesting an additive effect of these two gene deletions on the expression of *csgA*. No significant differences were observed in the expression of the genes *lpfA* and *fimA* of the operons encoding long polar and type 1 fimbriae in the *sdiA* mutant strain. These data indicate that SdiA has no significant effect on the expression of LEE genes, but that it appears to act as a strong repressor of genes encoding flagella and curli fimbriae, and the alleviation of the SdiA-mediated repression of these genes in an EHEC O157:H7 *sdiA* mutant strain contributes to enhanced bacterial motility and increased adherence to HEp-2 epithelial cells.

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INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is responsible for outbreaks of bloody diarrhoea, haemorrhagic colitis and haemolytic-uraemic syndrome (HUS) in humans (Nataro & Kaper, 1998). In addition to Shiga toxins that act on vascular endothelial cells to produce HUS (Karmali, 1989), EHEC O157:H7 produces characteristic attaching and effacing (A/E) lesions on infected

host epithelial cells (Francis *et al.*, 1986). The genes required for the formation of A/E lesions are encoded by the locus of enterocyte effacement (LEE), which contains 41 ORFs organized into operons *LEE1*–*LEE5* (Elliott *et al.*, 2000). The expression of the LEE operons is regulated by the LEE-encoded positive regulator Ler. Several positive and negative regulators have been shown to regulate *ler* expression. In previous studies, we have reported that Hha represses *ler* transcription, and that the deletion of *hha* increases expression of *ler* and *ler*-regulated *LEE1*–*LEE5* operons, thereby enhancing the adherence of the mutant strain to HEp-2 cells (Sharma & Zuerner, 2004; Sharma

Abbreviations: EHEC, enterohaemorrhagic *Escherichia coli*; LEE, locus of enterocyte effacement; QRT-PCR, quantitative real-time polymerase chain reaction; QS, quorum sensing.

et al., 2005). In addition to LEE-mediated intimate adherence to epithelial cells, EHEC O157:H7 presumably also uses surface appendages, such as flagella and fimbriae, in the initial stages of the adherence process. For example, recent studies have shown that flagella of EHEC O157:H7 bind bovine intestinal mucos to promote intestinal colonization, and *fliC* deletion mutants exhibit reduced adherence to bovine intestinal tissues (Erdem *et al.*, 2007). Although EHEC O157:H7 does not express type 1 fimbriae because of a 16 bp deletion in the *fim* switch (Roe *et al.*, 2001), it contains several other fimbrial operons, including the operons encoding curli and long polar fimbriae. Recent studies have implicated long polar fimbriae in adherence of EHEC O157:H7 to epithelial cells, and intestinal colonization of ruminants (Torres *et al.*, 2007). Similarly, the expression of curli fimbriae has been shown to enhance adherence of EHEC O157:H7 and O157:NM strains to HEP-2 and Caco-2 cells, respectively (Rosser *et al.*, 2008; Uhlich *et al.*, 2002).

Quorum-sensing (QS) systems have emerged as important regulatory pathways in controlling the expression of multiple cellular functions in *E. coli* O157:H7 such as flagellation, motility, and intimate adherence to epithelial cells (Reading *et al.*, 2007; Sperandio *et al.*, 2002, 2003). In some Gram-negative bacterial species, QS requires LuxR, a transcriptional factor that recognizes and binds to specific QS signalling molecules called autoinducers (Fuqua *et al.*, 1994). These autoinducers, which belong to a family of secreted molecules called acylhomoserine lactones, are synthesized via the pathways requiring LuxI-encoded autoinducer synthase (Fuqua *et al.*, 1994).

Like *E. coli* K-12 and *Salmonella*, EHEC O157:H7 contains a LuxR homologue called SdiA, but lacks its cognate autoinducer-synthase-encoding gene (Henikoff *et al.*, 1990). Therefore, only a few studies have focused on the understanding of the biological significance of SdiA in controlling specific cellular functions in *E. coli* strains. For example, Kanamaru *et al.* (2000a) reported that overproduction of SdiA from a high-copy-number plasmid reduces motility and the expression of some of the LEE-encoded genes of EHEC O157:H7. Other studies have implicated *sdiA* as an important component of the regulatory cascade that governs the formation of biofilms by *E. coli* K-12 by regulating the expression of motility and chemotaxis genes in response to indole, which is a by-product of tryptophan metabolism (Lee *et al.*, 2007). However, types of regulatory controls exerted by the chromosomal copy of *sdiA* on the expression of genes, such as those encoded by the flagellar, fimbrial and LEE operons, which are essential for motility and adherence of EHEC O157:H7 to epithelial cells, are not completely understood.

In this study, we describe the effects of *sdiA* deletion on the adherence of EHEC O157:H7, with and without the gene *hha*, to HEP-2 cells, and correlate the degree of adherence of these mutants to the relative expression of LEE-encoded

genes and the genes involved in the biosynthesis of flagella and fimbriae.

METHODS

Media and growth conditions. Bacterial strains were grown in either Luria–Bertani broth (LB; 10 g tryptone, 5 g yeast extract, 10 g NaCl per litre of medium) or Dulbecco's modified Eagle's medium (DMEM), at 37 °C, with or without shaking (200 r.p.m.). Media were supplemented with appropriate antibiotics.

Construction of *sdiA* and *sdiA hha* mutants. EHEC O157:H7 strain 86-24 Δ *stx2* Δ *lac* (Sharma & Zuerner, 2004) was used for construction of the mutants by using the lambda-Red-mediated recombineering method (Murphy & Campellone, 2003). Briefly, a 1.539 kb DNA fragment containing a 1.1 kb fragment encoding kanamycin resistance, flanked at its 5' and 3' ends with 254 bp and 175 bp sequences representing nucleotide regions immediately upstream and downstream of *sdiA*, respectively, was isolated from a recombinant plasmid using primers *sdiA*-F and *sdiA*-R (Table 1). This 1.539 kb fragment was purified from an agarose gel using a gel extraction kit, according to the manufacturer's instructions (Qiagen). The gel-purified fragment was electroporated into electrocompetent cells of EHEC O157:H7 strain 86-24, and its *hha* mutant (Sharma & Zuerner, 2004) carrying plasmid pKM208; kanamycin-resistant colonies were isolated, and screened by PCR to confirm that *sdiA* had been deleted from the chromosome of the electroporated strain.

RNA isolation. An overnight culture of a bacterial strain grown in LB broth plus 100 µg streptomycin ml⁻¹ was diluted 1:100 in DMEM, and incubated at 37 °C with shaking (200 r.p.m.). At an OD₆₀₀ of approximately 1.0, the culture was mixed with 2 vols RNeasy Protect (Qiagen) by vortexing for 5 s, then incubated for 5 min at room temperature, and centrifuged at 5000 g for 10 min at 4 °C. Total RNA was isolated from cell pellets by using an RNeasy Mini kit, according to the manufacturer's instructions (Qiagen). RNA was electrophoresed in 1 % standard agarose gels using Tris/borate/EDTA buffer. Following electrophoresis, the gel was stained with ethidium bromide to make sure that the RNA appeared as a smear containing two prominent rRNA bands. Any contaminating DNA present in the purified RNA preparation was removed by DNase treatment using the TURBO RNase-free DNase kit, according to the manufacturer's instructions (Ambion). Purified RNA was checked for its concentration using a spectrophotometer (NanoDrop Technologies), and it was used as a template in a standard PCR (PE Biosystems) containing primers for *rpoA* amplification to verify that it was free of DNA contamination.

Quantification of gene expression by QRT-PCR. Quantitative real-time RT-PCR (QRT-PCR) was performed in 15 µl reaction volumes containing 2 µl (25 ng RNA) DNA-free RNA, 7.5 µl 2 × QRT-PCR master mix, 0.6 µl RT/RNase block mixture (Stratagene), 0.225 µl 0.066 M reference dye ROX, 1.5 µl 1 × SYBR Green (Molecular Probes), appropriate volumes of forward and reverse primers (Table 1), and RNase-free water. QRT-PCR was carried out in a single-step reaction format using Mx3005P (Stratagene) programmed to generate cDNA in one cycle of 30 min at 50 °C, followed by a single 10 min cycle at 95 °C, and 40 cycles of cDNA amplification (95 °C for 30 s, 55 °C for 60 s, and 72 °C for 30 s). A comparative quantification option provided in the Mx3005P system set-up procedure was used for QRT-PCR data acquisition and analysis. The sample containing RNA from the parent strain was used as a calibrator to which all other RNA samples were compared, with respect to the expression of the target gene. To account for any variations in the amounts of RNA across samples, QRT-PCR

Table 1. Primers used in this study

Primer	Nucleotide sequence (5'–3')	Location*
<i>espA</i> -F	ACGCTCTGAGGAAGTTTGGC	4662381–4662400
<i>espA</i> -R	ATCTAAAGCGTCAACCACGG	4662491–4662472
<i>ler</i> -F	GTAAACACCTTTTCGATGAGTTCC	4688913–4688935
<i>ler</i> -R	GAGTCGATTGAGAAGCAGATTAC	4689001–4689023
<i>rpoA</i> -F	GGCTTGACGATTTTCGACATC	4242887–4242906
<i>rpoA</i> -R	GGTGAGAGTTTCAGGGCAAAG	4242997–4242978
<i>fliC</i> -F	TCGTCAAAGTTGCCTGCATC	2700385–2700403
<i>fliC</i> -R	TTAGCTGCCACCCTTCATG	2700535–2700517
<i>eae</i> -F	TCTGTGTGGATGGTAATAAATTTTG	4665647–4665671
<i>eae</i> -R	GTAAGTTACACTATAAAAGCACCGTCG	4665752–4665726
<i>lpfA</i> -F	CACCGTTAAGAGCGACCAAGG	4524871–4524891
<i>lpfA</i> -R	GAAGATTGCGATACCACCACG	4525036–4525016
<i>csgA</i> -F	GATGTTGGTCAGGGCTCAG	1548912–1548930
<i>csgA</i> -R	CCACCGAATTGTTAACTGTC	1549033–1549013
<i>fimA</i> -F	TGTCCCTCAGTTCTACAGCG	5428049–5428068
<i>fimA</i> -R	TCCTAACTGAACGGTTTGATC	5428182–5428162
<i>sdiA</i> -F	CGCGTGATGTTAAAGATGGAAC	2693417–2693438
<i>sdiA</i> -R	CCTCGAGAAGTTTCTGCTGC	2694568–2694549
<i>sdiA</i> -F915	GTACGTCGACGACCAGGGCGGATAGTC	2694628–2694646
<i>sdiA</i> -R864	GTACGTCGACTCAAATTAAGCCAGTAGCGG	2693592–2693611
<i>lac</i> -F824	GATCTCTAGAAGCATGATCTCGACTCGCTG	426579–426599
<i>lac</i> -R881	GATCTCTAGAGCGTTTATGCAGGGAATCGG	417736–417755

*The *E. coli* EDL933 nucleotide sequence (AE005174) was used for selection of these primer sequences.

amplification of each sample was normalized to samples containing primers for *rpoA* (Table 1). The passive reference dye ROX allowed compensation for non-PCR related variations in the fluorescence data acquisition. QRT-PCR data are presented as fold change in the expression level of the target gene relative to the expression level for the same gene in the calibrator, where the expression level of the calibrator is set to 1.0. Final data represent expression levels as means ($\pm$ SEM) of the data collected from three independent experiments.

Determination of adherence to HEp-2 cells. Bacterial adherence to HEp-2 cells was performed by a previously described procedure (Sharma *et al.*, 2005). Briefly, fresh cultures (0.5 ml) of HEp-2 cells (5.0×10^4) were added to a well of a chamber slide (Nalge Nunc International), and incubated at 37 °C overnight in an atmosphere of 5% CO₂ in air. The wells were washed three times in Dulbecco's PBS, followed by addition of 0.5 ml RPMI 1640 containing 1% fetal bovine serum (Invitrogen), and 50 μ l of an overnight bacterial culture prepared as follows: a few colonies of a bacterial strain grown overnight on LB agar were inoculated into LB broth, incubated at 37 °C on a shaker (200 r.p.m.) for 8 h, diluted 1:500 in fresh LB broth, and incubated overnight at 37 °C, without shaking.

The chamber slides were incubated at 37 °C for 3 h in an atmosphere of 5% CO₂ in air. The chamber portion of chamber slide was removed, and the slide was washed four times in PBS, fixed for 1 min in 95% ethanol, stained in toluidine blue for 15 s, washed in distilled H₂O, dipped in 95% ethanol to fix the stain, washed briefly in water, and air-dried. A coverslip was mounted on the air-dried slide using VectaMount, and the slide was visualized under a microscope at $\times 100$ magnification. Ten visual fields containing confluent HEp-2 cells were randomly selected for enumerating the number of bacterial cells adhered per HEp-2 cell. The adherence data are represented as the mean number of bacteria per HEp-2 cell in a given visual field obtained from three independent assays.

Determination of cellular morphology and motility. Bacterial wet mounts prepared from cultures grown in DMEM were examined, using a phase-contrast microscope at $\times 400$ magnification, to determine any gross changes in cellular morphology, such as the appearance of filamentous cells as opposed to small rod-shaped bacterial cells typical of *E. coli* strains. For determination of bacterial motility, overnight cultures were centrifuged at 5000 g for 5 min, washed in PBS, and spotted (2 μ l per spot) on motility agar plates, which were prepared by adding 0.32% agarose to DMEM medium. The diameters of motility haloes were measured after incubation for 18 h at 37 °C.

Construction of *sdiA* complementation strains. The gene encoding *sdiA* was amplified by PCR from EHEC O157:H7 strain 86-24 Δ stx2 Δ lac using primers *sdiA*-F915 and *sdiA*-R864, which contain *SalI* restriction sites at their 5' ends (Table 1). These primers were used to amplify a 1.1 kb fragment containing the promoter and the ORF encoding SdiA. This 1.1 kb fragment was cloned into the *SalI* site of a previously described temperature-sensitive plasmid (pSM80) used for deleting the *lac* operon in strain 86-24 (Sharma & Zuerner, 2004). The plasmid pSM80 containing the 1.1 kb *sdiA* gene fragment was introduced into *sdiA* and *sdiA hha* mutant strains by electroporation, and clones containing the *sdiA* gene in the *lac* region were selected by using a previously described allelic replacement procedure (Sharma & Zuerner, 2004). The insertion of the 1.1 kb fragment in the *lac* regions of *sdiA* and *sdiA hha* mutant strains was confirmed by PCR using primers *lac*-F824 and *lac*-R881 (Table 1).

Congo red binding and biofilm assays. The abilities of bacterial cells to bind the dye Congo red, and produce biofilms on plastic surfaces, were determined by the minor modifications of previously described procedures (Hammar *et al.*, 1996; Uhlich *et al.*, 2006a). For Congo red binding assays, Congo red and Coomassie brilliant blue dyes were used at 50 μ g ml⁻¹ and 6.25 μ g ml⁻¹, respectively, in

YESCA agar medium. Bacterial strains grown overnight in LB broth were streaked onto this medium, and, following incubation at 28 °C for 48 h, the plates were checked for growth, and photographed under identical settings for exposure, background light and contrast to eliminate any digital biasing of the resulting images. For biofilm assays, 96-well polystyrene plates were inoculated with 200 µl of 1:100-fold diluted, overnight bacterial cultures grown in YESCA broth at 37 °C with shaking (175 r.p.m.). After incubation at 28 °C for 48 h, the plates were inverted on paper towels to drain the wells of the culture. The plates were heat-fixed, stained with 0.1% crystal violet solution, and scanned at 590 nm to assess the amount of biofilm production. Biofilm production is given as the mean ($\pm$ SEM) of three independent experiments.

Statistics. One-way ANOVA with Tukey–Kramer multiple comparisons test was performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software).

RESULTS

Deletion of *sdia* enhances cellular adherence

As shown in Fig. 1, deletion of *sdia* from EHEC O157:H7 strain 86-24 enhanced adherence to HEp-2 cells twofold. Furthermore, deletion of *sdia* from the *hha* mutant strain resulted in an additional twofold increase in bacterial adherence of the *sdia hha* double mutant relative to the hyperadherent *hha* mutant strain, indicating that SdiA inhibits the adherence of EHEC O157:H7 to epithelial cells.

Fig. 1. Effect of *sdia* on adherence of EHEC O157:H7. Adherence of *sdia* deletion mutants of EHEC O157:H7 with or without *hha* was monitored by exposing HEp-2 cells to overnight bacterial cultures for 3 h at 37 °C. After washing and fixing, the HEp-2 cells were stained with toluidine blue and adherent bacteria were counted at $\times 100$ magnification. Assays were performed in triplicate in which adherent bacteria were enumerated from 10 HEp-2 cells for each replicate. Error bars indicate SEM. The *P*-values, indicated as ** (<0.01) and *** (<0.001), are reported in relation to the adherence level exhibited by the parent strain.

Effect of *sdia* on expression of LEE-encoded genes

In order to understand the genetic basis for the increased adherence of the *sdia* deletion mutant strains to HEp-2 cells, we first analysed total RNA by QRT-PCR to determine expression levels for several LEE-encoded genes, as their increased expression has been shown to facilitate increased adherence to epithelial cells (Sharma & Zuerner, 2004; Sharma *et al.*, 2005). As shown in Table 2, *sdia* deletion resulted in very low-level increases, which were determined to be statistically insignificant, in the expression of LEE-encoded *ler* (1.3-fold), *espA* (1.2-fold) and *eae* (1.6-fold), relative to the parent strain. However, in comparison, high levels of increase in expression observed for *ler* (15-fold), *espA* (22-fold), and *eae* (166-fold) in the *hha* mutant strain were independent of the presence or absence of the *sdia* deletion, indicating that SdiA has either no or a very weak effect on the expression of LEE-encoded genes, in comparison with the stronger repression exerted by Hha on the same genes.

SdiA reduces flagellar and fimbrial gene expression

Several reports have described that the expression of flagellar and various fimbrial operons affects adherence of EHEC O157:H7 to epithelial cells (Kim & Kim, 2004; Rosser *et al.*, 2008; Saldana *et al.*, 2009; Uhlich *et al.*, 2002). Therefore, we used QRT-PCR to examine the effects of *sdia* deletion on the expression of *fliC*, *lpfA*, *csgA* and *fimA*, genes representing operons encoding flagella, long polar fimbriae, curli fimbriae and type 1 fimbriae, respectively. Since type 1 fimbriae are not expressed in EHEC O157:H7 strains because of a 16 bp deletion in the *fimA* regulatory region (Roe *et al.*, 2001), the expression of *fimA* in *sdia*, *hha* and *sdia hha* mutant strains was expected to be identical to that of the parent strain. As shown in Table 2, *fliC* expression in the *sdia* deletion mutant was significantly increased by 2.7-fold relative to the parent strain 86-24. On the other hand, *fliC* expression in the *hha* mutant strain was reduced by 5-fold compared with that in the parent strain. It was also apparent that the deletion of *sdia* did not alter the expression of *fliC* in the *hha* deletion strain, suggesting that Hha is necessary for the expression of *fliC*, and the positive effect of *sdia* deletion on *fliC* expression is evident in a *hha*⁺ strain only. However, the expression of *lpfA*, the structural gene for the long polar fimbriae, in the *sdia* (1.124-fold), *hha* (0.81-fold) and *sdia hha* (0.934-fold) mutant strains was not significantly different from that of the parent (1.00-fold) strain. However, the expression of *csgA*, encoding the structural protein curli of the curli fimbriae, was increased by approximately three- to fourfold in *sdia* and *hha* deletion mutants relative to the parent strain. In the *sdia hha* double mutant strain, the expression of *csgA* was increased by ninefold relative to the parent strain, and by two- to threefold relative to the *csgA* expression observed in either

Table 2. Effect of *sdiA* on the expression of adherence and motility genes

Strain	Relative change in gene expression*						
	LEE-encoded genes				Flagellar and fimbrial genes		
	<i>ler</i>	<i>espA</i>	<i>eae</i>	<i>fliC</i>	<i>lpfA</i>	<i>csgA</i>	<i>fimA</i>
Wild-type†	1.0 ± 0.06	1.0 ± 0.07	1.0 ± 0.11	1.0 ± 0.13	1.0 ± 0.08	1.0 ± 0.08	1.0 ± 0.15
<i>sdiA</i> mutant	1.3 ± 0.1	1.2 ± 0.07	1.6 ± 0.15	2.7 ± 0.29‡	1.12 ± 0.11	3.2 ± 0.33‡	0.98 ± 0.14
<i>hha</i> mutant	15.7 ± 0.9‡	21.8 ± 3.0‡	166.8 ± 12.8‡	0.14 ± 0.018‡	0.81 ± 0.16	4.5 ± 0.37‡	0.69 ± 0.13
<i>sdiA hha</i> mutant	16.0 ± 0.18‡	28.0 ± 2.3‡	120 ± 10.0‡	0.16 ± 0.02‡	0.93 ± 0.13	8.85 ± 0.67‡	0.86 ± 0.11

*The relative change in expression of each gene was computed from three independent experiments, and by testing each gene for its expression in duplicate. Values are means ± SEM.

†Expression values for each gene in the wild-type strain were set to 1 in order to determine the relative change in the expression of the same genes in the mutant strains.

‡Significant values ($P < 0.05$).

sdiA or *hha* deletion mutants. The increased expression of *csgA* in *sdiA* and *hha* deletion mutants indicates that both SdiA and Hha exert a negative effect on the expression of *csgA*. As expected, the expression of *fimA*, encoding the major structural protein FimA of type 1 fimbriae, was not significantly different between the parent strain (1.00-fold) and *sdiA* (0.98-fold), *hha* (0.68-fold) and *sdiA hha* (0.86-fold) deletion mutant strains.

***sdiA* deletion affects bacterial motility, but has no effect on cellular morphology**

Kanamaru *et al.* (2000a) have shown that the expression of SdiA from a high-copy-number plasmid not only results in the appearance of elongated cells due to abnormal cell division, but also reduces the motility of EHEC O157:H7. However, examination of the wet mounts of *sdiA*, *hha* and *sdiA hha* mutants under a phase-contrast microscope showed the presence of small, rod-shaped cells that are considered typical of *E. coli* strains (data not shown). On the other hand, visual examination of soft-agar motility plates revealed that motility of the *sdiA* mutant was increased by approximately 30 % relative to the parent strain, indicating that SdiA exerts a negative effect on the motility of EHEC O157:H7 (Fig. 2). It was also apparent that the positive effect of *sdiA* deletion on motility was dependent on the presence of *hha*, since the motility of the *sdiA hha* double mutant was reduced to the levels that were observed for the *hha* mutant strain (approximately 35 % lower than the parent strain).

***sdiA* deletion increases Congo red binding by bacterial cells**

It has been reported that the ability of *E. coli* strains to bind Congo red is affected by the levels of curli fibres expressed at the bacterial cell surface (Kim & Kim, 2004; Rosser *et al.*, 2008; Uhlich *et al.*, 2006b; Vidal *et al.*, 1998). Since the deletion of *sdiA* enhanced the expression of *csgA* (the gene

encoding major structural component of curli fimbriae) in a *hha*-independent manner, we wanted to determine if an increased expression of *csgA* in strains deleted of *sdiA* would enable them to bind increased amounts of Congo red. In addition, we inserted a single copy of the wild-type *sdiA* gene in the *lac* region of the chromosome of *sdiA* and *sdiA hha* mutant strains to determine whether SdiA would complement the abilities of these strains to bind Congo red

Fig. 2. Effect of *sdiA* deletion on the motility of EHEC O157:H7. Each strain was spot inoculated onto soft DMEM agar. The diameters of the motility haloes (means ± SEM of three independent experiments) were as follows: A (parent), 9.38 ± 1.21 mm; B (*hha* mutant), 6.33 ± 0.73 mm; C (*sdiA* mutant), 12.28 ± 1.25 mm; D (*sdiA hha* mutant), 6.04 ± 0.82 mm. The difference in the motility diameter for each mutant strain was determined to be significant ($P < 0.05$) relative to the parent strain.

in amounts comparable with those bound by the *sdia*⁺ or *sdia*⁺ *hha* strains, respectively. Visual examination, comparing growth of *sdia* (Fig. 3a, panel C) and *sdia hha* (Fig. 3b, panel C) mutants on Congo red medium, revealed the presence of dark-pink colonies with dark-red centres (fish-eye appearance) produced by the *sdia*⁺ (Fig. 3a, panel A) and *sdia*⁺ *hha* (Fig. 3b, panel A) strains. Complementation of the *sdia* (Fig. 3a, panel B) and *sdia hha* (Fig. 3b, panel B) mutant strains with *SdiA* decreased Congo red binding to a level similar to that observed for colonies of the *sdia*⁺ (Fig. 3a, panel A) and *sdia*⁺ *hha* (Fig. 3b, panel A) strains.

Reduction of biofilm formation by *sdia* in EHEC O157:H7

The formation of biofilms in *E. coli* is influenced by motility and the expression of fimbrial structures, which are required for reversible and irreversible phases of adherence during the genesis of biofilms, respectively (Kim & Kim, 2004; Pratt & Kolter, 1998; Ryu & Beuchat, 2005; Wood *et al.*, 2006). Since we determined that the *sdia* deletion caused increased expression of *fliC* and *csgA*, which correlated positively with enhanced motility and increased Congo red binding, we hypothesized that the *sdia* deletion mutant might show increased biofilm pro-

duction. As shown in Fig. 4, the amount of biofilm produced by the *sdia* deletion mutant was only 1.56-fold higher than that produced by the *sdia*⁺ parent strain. On the other hand, the amount of biofilm produced by the *hha* mutant was 3.56-fold higher than the *sdia*⁺ parent and 2.3-fold higher than the *sdia* deletion mutant strains. The deletion of *sdia* in the *hha* deletion mutant caused only a small increase (1.22-fold) in biofilm formation over the *hha* mutant strain, suggesting that *SdiA* exerts effects of lower magnitude than *Hha* on the production of biofilms in EHEC O157:H7. The fact that the *hha* deletion mutant displayed reduced expression of *fliC* (Table 2), reduced motility (Fig. 2), and increased *csgA* expression (Table 2) suggests that the repression of motility and increased expression of curli fimbriae are necessary for increased biofilm formation by EHEC O157:H7. Thus, the increased motility caused by deletion of *sdia* might be responsible for preventing enhanced biofilm formation by the *sdia* mutant strain, despite its ability to express higher levels of *csgA* and increased Congo red binding compared with the *sdia*⁺ parent strain.

DISCUSSION

In this study, we have demonstrated that the deletion of a single chromosomal copy of *sdia* significantly increases the

Fig. 3. Phenotypic production of curli on medium containing Congo red. Overnight bacterial cultures were streaked on YESCA agar containing Congo red. After incubation for 48 h at 28 °C, the plates were photographed to capture coloured phenotypes of colonies that grew on these plates. (a) Coloured phenotypes of the colonies produced by the parent strain (panel A), *sdia* deletion mutant complemented with a wild-type copy of *sdia* (panel B), and *sdia* deletion mutant (panel C). (b) Coloured phenotypes expressed by the parent strain (panel A), *sdia* complemented *sdia hha* deletion mutant (panel B), and the *sdia hha* deletion mutant (panel C).

Fig. 4. Evaluation of biofilm formation in polystyrene plates. Overnight bacterial cultures were diluted 1 : 100 in YESCA broth and pipetted into the wells of a 96-well polystyrene plate. Following 48 h of incubation at 28 °C, the amount of biofilm produced was determined by crystal violet staining as described in Methods. Biofilm production is shown as the mean $\pm$ SEM of three independent experiments.

adherence of EHEC O157:H7 and its isogenic *hha* mutant strain to HEp-2 cells, suggesting that SdiA exerts a negative effect on the adherence phenotype. These findings are in agreement with the results reported in a previous study showing that the expression of *sdiA* from a high-copy-number plasmid causes a dramatic reduction in the ability of EHEC O157:H7 to adhere to epithelial cells (Kanamaru *et al.*, 2000a). In addition, Kanamaru *et al.* (2000a) demonstrated that an increased expression of *sdiA* from a high-copy-number plasmid results in abnormal cell division. However, deletion of *sdiA* from the EHEC O157:H7 chromosome, as we have described in this study, had no effect on cellular morphology.

Unlike most of the known bacterial QS systems that activate virulence gene expression in EHEC O157:H7 (Sperandio *et al.*, 1999, 2002), SdiA-mediated QS had been shown to repress transcription of the flagellar gene *fliC*, and the virulence genes *espD* and *eae* encoded by the *LEE4* and *LEE5* operons, respectively (Kanamaru *et al.*, 2000b). However, these negative effects of SdiA were demonstrated upon increased expression of *sdiA* from a high-copy-number plasmid. By using QRT-PCR, we demonstrated very low levels of increase in the expression of LEE-encoded genes *ler*, *espA* and *eae* in the *sdiA* deletion strain compared with the high levels of increase observed for these genes in the *hha* mutant strain. These low levels of increase in the expression of LEE-encoded genes observed in our study were statistically insignificant compared with the effect of SdiA reported for the repression of LEE-encoded genes in the study by Kanamaru *et al.* (2000a). Thus, the lack of a significant effect on the expression of LEE-encoded genes in the *sdiA* mutant in our study may be due to the reduced intracellular amounts of SdiA produced

from a single chromosomal copy of *sdiA*. For example, it has been postulated that the majority of SdiA expressed from the chromosomal copy of *sdiA* is a monomer, and that expression of SdiA from a high-copy-number plasmid increases the levels of SdiA dimers, which are required for repressing transcriptional activities of the target gene promoters (Henikoff *et al.*, 1990; Michael *et al.*, 2001). The proposal of a repressor function of SdiA is supported by the presence of a helix–turn–helix DNA-binding motif, and, like most repressors, SdiA dimerizes or oligomerizes to affect the repression of their target gene promoters (Burz & Ackers, 1994; Burz *et al.*, 1994; Harrison & Aggarwal, 1990; Sharma *et al.*, 1998). Thus, the highly impaired adherence of EHEC O157:H7 reported by Kanamaru *et al.*, (2000a) could very well have resulted from an increased intracellular availability of SdiA, so that concentrations of dimers reached levels that were high enough to inhibit transcriptional activities initiated from the affected promoters of the *LEE4* and *LEE5* operons.

Several studies have shown that the ability of EHEC O157:H7 to produce flagella and certain types of fimbriae enhances its adherence to epithelial cells in animal models and in tissue culture assays (Erdem *et al.*, 2007; Torres *et al.*, 2007; Uhlich *et al.*, 2002). In the current study, we found that the strain deleted of *sdiA* showed increased transcriptional levels of *fliC* and enhanced motility compared with the parent strain. The overall smaller sizes of motility haloes produced in our studies could be attributed to the use of a minimal medium containing a higher percentage of agar (0.32 %). For example, strain 86-24 has been used by other investigators to examine the impact of QS signalling molecules (epinephrine and norepinephrine) on its motility (Sperandio *et al.*, 2003). In those studies, the sizes of motility haloes produced by strain 86-24 in the absence of epinephrine or norepinephrine were not substantially different from the ones observed in our studies, despite the fact that the motility haloes in those studies were determined on media containing a lower percentage of agar (0.25 %). Although the negative effects of SdiA on *fliC* expression and/or on motility were qualitatively similar to those reported for a strain of EHEC O157:H7 producing high levels of SdiA (Kanamaru *et al.*, 2000a; Wei *et al.*, 2001), the negative effect of SdiA on *fliC* expression was only apparent in the strain wild-type for *hha*, because strains deleted of *hha* showed reduced expression of *fliC*, irrespective of the presence or absence of *sdiA*. These findings suggest that Hha plays a positive role, via an unknown mechanism, in the expression of *fliC*. Deletion of *hha* resulted in reduced *fliC* expression (and reduced bacterial motility), but enhanced expression of LEE-encoded genes, and bacterial adherence to HEp-2 cells; these findings are similar to those reported in a previous study (Sharma *et al.*, 2005), and suggest that the enhanced *in vitro* adherence of *hha* or *sdiA hha* mutants to HEp-2 cells may not require optimal expression of flagella. Since biosynthesis of both flagella and the type III secretion system requires investment of large amounts of cellular

resources, it is reasonable to assume that an increase in the expression of the type III secretion system in the *hha* mutant might compensate for the reduced production of flagella during bacterial adherence to HEp-2 cells.

Unlike the negative effect of SdiA on *fliC* gene expression that was apparent only in a *hha*-positive strain, the negative effect of SdiA on the expression of *csgA*, the gene encoding curli of the curli fimbriae, was of a similar magnitude in strains with or without *hha*. However, the *sdiA hha* double mutant had the highest level of *csgA* expression, indicating an additive effect on *csgA* expression due to deletion of *sdiA* and *hha*. In light of a previous report demonstrating that a highly curliated strain of EHEC O157:H7 displays a highly aggregated adherence pattern on HEp-2 cells, as opposed to the more localized adherence pattern observed for the lesser curliated strain (Kim & Kim, 2004), the increased expression of *csgA* might be one of the major factors, considering that the expression of LEE-encoded genes was not significantly affected by *sdiA*, responsible for the enhanced adherence of *sdiA* and *hha* mutant strains to HEp-2 cells. The enhanced expression of *csgA* contributing to an increased adherence of *sdiA* mutant strains to HEp-2 cells was corroborated by the ability of these strains to bind increased amounts of Congo red, and to produce slightly higher levels of biofilm than the strains with a wild-type *sdiA* gene. Since increases in Congo red binding and biofilm production are indicative of enhanced curli production (Kim & Kim, 2004; Uhlich *et al.*, 2006a), a reduction in the levels of Congo red binding and biofilm production by the *sdiA* mutant strains via complementation with a wild-type copy of *sdiA* provided additional evidence for the negative role of SdiA in *csgA* transcription and curli production. These results are corroborated by a study that utilized a whole-transcriptomic approach to show that SdiA represses curli formation in *E. coli* K-12 (Lee *et al.*, 2009).

Interestingly, *sdiA* deletion resulted in increased *fliC* expression and enhanced motility, but only a small increase in the amount of biofilm formation compared with larger increases in the levels of biofilm production in *hha* and *sdiA hha* deletion mutants. These results indicate that while the increased production of curli promotes adherence to HEp-2 cells, increased motility, on the other hand, reduces the ability of the *sdiA* mutant strains to produce biofilms. These findings suggest that in EHEC O157:H7 strain 86-24, unlike other *E. coli* strains (Barrios *et al.*, 2006; Pratt & Kolter, 1998; Wood *et al.*, 2006), motility inhibits biofilm formation on abiotic surfaces, whereas increased production of curli allows increased adherence to both abiotic and biotic surfaces in motility-compromised *hha* mutant strains.

It is also apparent from the data presented in this report that SdiA serves as a strong repressor for the transcriptional regulation of *fliC* and *csgA*, and that it appears to have no effect on the expression of *lpfA* and *fimA*. Moreover, the additive effects of SdiA and Hha on the expression of *csgA*

appears to suggest that these two repressor proteins either bind to independent sites in the *csgA* promoter region, or the binding of the one repressor protein precedes the binding of the other repressor to the same promoter-binding site to cause effective repression of the *csgA* promoter.

In conclusion, our study provides direct genetic evidence that expression of a single chromosomal copy of *sdiA* represses multiple phenotypes, such as flagellation, fimbriation, motility and adherence in EHEC O157:H7. Based on the data provided in this report, SdiA exerts negative effects of varied magnitudes on different sets of genes. For example, the expression of LEE-encoded genes was not significantly affected compared with the expression of *fliC* and *csgA*, which were strongly repressed. Although *in vitro* gel-shift assays have shown that purified SdiA binds to its target promoters with the same affinity (Kanamaru *et al.*, 2000a), the lack of a consensus SdiA-binding sequence in the SdiA-regulated promoters might determine the relative affinities of these promoters for SdiA *in vivo*. Since EHEC O157:H7, like *E. coli* K-12 and *Salmonella* spp., encodes *sdiA*, but lacks the ability to synthesize acylhomoserine lactones, which are the natural inducers of SdiA, understanding the nature of signals influencing expression or repressor activity of SdiA is important for clarifying the role of SdiA in virulence gene expression in these bacterial species. One potential signal could be indole, the metabolic by-product of tryptophan metabolism, which regulates biofilm formation by influencing the activity of SdiA (Lee *et al.*, 2007). Thus, correlating the expression of indole with SdiA effects on virulence gene expression might provide greater insight into the mechanism of SdiA-mediated regulation of gene expression in EHEC O157:H7.

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