Secreted indole serves as a signal for expression of type III secretion system translocators in enterohaemorrhagic *Escherichia coli* O157:H7

Hidetada Hirakawa,^{1,4} Toshio Kodama,³ Asuka Takumi-Kobayashi,^{1,2} Takeshi Honda³ and Akihito Yamaguchi^{1,2}

Correspondence

Akihito Yamaguchi

akihiro@sanken.osaka-u.ac.jp

¹Department of Cell Membrane Biology, Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567-0047, Japan

²Graduate School of Pharmaceutical Science, Osaka University, Suita, Osaka 565-0871, Japan

³Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan

⁴Department of Microbiology, University of Washington, WA 98195, USA

Indole is produced by tryptophanase during growth of enteric bacteria and accumulates in the culture medium. The physiological role of indole production is poorly understood. We discovered that enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 with a *tnaA* deletion has decreased secretion of EspA and EspB via the type III secretion system and as a result there is reduced formation of attaching and effacing (A/E) lesions in HeLa cells. Addition of indole restored and enhanced secretion of EspA and EspB and formation of A/E lesions by the *tnaA* deletion mutant EHEC. Indole addition moderately increased the promoter activity of *LEE4* genes, including *espA* and *espB*, in the locus of enterocyte effacement. Thus in EHEC indole can serve to signal EspA and EspB expression and secretion and stimulate the ability of EHEC to form A/E lesions on human cells.

Received 8 May 2008

Revised 29 August 2008

Accepted 2 September 2008

INTRODUCTION

Many enteric bacteria including *Escherichia coli* produce indole from tryptophan, a reaction that is catalysed by tryptophanase (TnaA). Indole is exported by the AcrEF-TolC multidrug efflux system, and accumulates in culture media during the stationary phase (DeMoss & Moser, 1969; Kawamura-Sato *et al.*, 1999; Wang *et al.*, 2001). TnaA is strongly induced under alkaline conditions (Blankenhorn *et al.*, 1999) and is regulated by catabolite repression (Deeley & Yanofsky, 1982). Therefore, enterobacteria may be exposed to high concentrations of indole when they infect alkaline regions in the body such as the pancreatic duct. To elucidate the behaviour of enteric bacteria at their site of infection and in their habitat, it is therefore important to understand the regulatory mechanism and the roles of indole signalling.

We previously reported that indole induces multidrug resistance as a result of its ability to stimulate transcription of *E. coli* drug exporter genes (*mdtEF*, *acrD*, *mdtABC*, *acrEF*, *cusABC*, *emrKY* and *yceL*) through multiple regulatory pathways [BaeS-BaeR and CpxA-CpxR (the bacterial two-component signal transduction pathway) and

RpoS-Hfq[GadY]-GadX (a two-component signal transduction system-independent regulatory cascade)] during stationary phase (Hirakawa *et al.*, 2005; Kobayashi *et al.*, 2006). Other studies have shown that indole controls biofilm formation and the expression of genes (*gabT*, *astD* and *tnaAB*) for amino acid metabolism in *E. coli* K-12 (Di Martino *et al.*, 2003; Lee *et al.*, 2007b; Wang *et al.*, 2001).

Enterohaemorrhagic *E. coli* (EHEC) causes severe infectious diseases, non-bloody diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome, which can lead to the death of the patient (Karmali, 1989; Nataro & Kaper, 1998). EHEC adheres to epithelial cells, triggering the formation of hallmark lesions called attaching and effacing (A/E) lesions at an early stage of infection (Nataro & Kaper, 1998). A/E lesions are characterized by the localized destruction of brush border microvilli, intimate attachment of the organism to the host cell membrane and formation of an underlying actin-rich structure in the host cell. The capacity to form A/E lesions is conferred by a type III secretion system (T3SS) and the proteins that it secretes (translocators and effectors), which are gene products of the locus of enterocyte effacement (LEE), a 36 kbp chromosomal pathogenicity island carrying 41 ORFs clustered in five major operons (*LEE1* to *LEE5*) (McDaniel & Kaper, 1997). To counter EHEC pathogenesis, it is therefore

Abbreviations: A/E, attaching and effacing; EHEC, enterohaemorrhagic *Escherichia coli*; LEE, locus of enterocyte effacement; T3SS, type III secretion system.

important to identify the signals that induce secretion and/or expression of the secreted proteins and to elucidate the mechanism of regulation of *LEE* expression and type III secretion-related protein secretion under conditions similar to those at sites of infection.

When EHEC adhere to epithelial cells, secreted Tir (an *LEE5* gene product) is translocated into the host cell membranes, where it serves as the receptor for intimin, an extracellular bacterial adhesin. EHEC contact host cells via the interaction of intimin and Tir receptor. EspA and EspB (*LEE4* gene products) are translocators required for the targeting of Tir. Therefore, these proteins are essential for T3SS-dependent pathogenicity of EHEC.

The recent sequencing of the complete EHEC O157:H7 genome has revealed an ORF homologous to *tnaA* that encodes tryptophanase in *E. coli* K-12. To investigate roles of indole in the T3SS-dependent pathogenicity of EHEC, we examined effects of *tnaA* deletion and of adding indole to a *tnaA* deletion mutant on the secretion and production of EspA, EspB and Tir and on A/E lesion production. We found that indole enhances secretion and production of EspA/B and also production of type III-dependent A/E lesions.

METHODS

Bacterial strains, host cells and plasmids. EHEC O157:H7 Sakai (RIMD 0509952) (Hayashi *et al.*, 2001), its derivative strains, and the plasmids used in this study are described in Table 1. Bacteria were grown in LB broth. Gene-deletion mutants of EHEC except for the *hly* single-deletion mutant were constructed by a previously described pKO3-based gene-replacement method (Link *et al.*, 1997). Basically, all the genes targeted for inactivation were replaced by a 33 bp linker sequence that retained the start and stop codons. The No and Co primers described in Table 2 anneal at about 500 bp upstream of the start codon and about 500 bp downstream of the stop codon, respectively. To verify the gene-deletion mutations, we performed PCR with No and Co primers, and the length of the respective PCR fragments was compared in the wild-type and the gene-deletion mutant. For construction of the *hly* single-deletion mutant, the chloramphenicol-resistance cassette was inserted into the internal region of the *hly* gene. A DNA fragment including the 300 bp upstream and 48 bp of coding region of *hly* was cloned upstream of the chloramphenicol resistance gene in the suicide vector pYAK1 (Kodama *et al.*, 2002). After homologous recombination, the inactivation of *hly* was established. HeLa cells (RIKEN) were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS, Sigma) and 100 mg gentamicin l⁻¹ (Sigma) at 37 °C under 5% CO₂ in air. Before infection, the HeLa cells were washed with PBS before the FBS and gentamicin-free DMEM were added.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i> strains		
O157:H7 Sakai	Wild-type EHEC O157:H7 (RIMD 0509952)	Hayashi <i>et al.</i> (2001)
O157Δ <i>tnaA</i>		This study
O157Δ <i>lacZ</i> I		This study
O157Δ <i>lacZ</i> IΔ <i>tnaA</i>		This study
O157Δ <i>lacZ</i> IΔ <i>tnaA</i> Δ <i>baeR</i>		This study
O157Δ <i>lacZ</i> IΔ <i>tnaA</i> Δ <i>cpxR</i>		This study
O157Δ <i>lacZ</i> IΔ <i>tnaA</i> Δ <i>gadX</i>		This study
O157Δ <i>lacZ</i> IΔ <i>tnaA</i> Δ <i>hly</i>		This study
O157Δ <i>lacZ</i> IΔ <i>tnaA</i> Δ <i>ler</i>		This study
O157Δ <i>escN</i>		Cantarelli <i>et al.</i> (2007)
O157Δ <i>escN</i> Δ <i>tnaA</i>		This study
O157Δ <i>hly</i> :Cm ^r		This study
Plasmids		
pKO3	<i>rep</i> (Ts), Cm ^r , <i>sacB</i> ⁺	Link <i>et al.</i> (1997)
pNN387	Single-copy vector, Cm ^r , <i>NotI</i> – <i>HindIII</i> cloning site upstream of promoterless <i>lacZ</i>	Elledge & Davis (1989)
pNNLEE1-P	pNN387 (<i>LEE1</i> gene promoter- <i>lacZ</i>)	This study
pNNLEE2-P	pNN387 (<i>LEE2</i> gene promoter- <i>lacZ</i>)	This study
pNNLEE3-P	pNN387 (<i>LEE3</i> gene promoter- <i>lacZ</i>)	This study
pNNLEE4-P	pNN387 (<i>LEE4</i> gene promoter- <i>lacZ</i>)	This study
pNNLEE5-P	pNN387 (<i>LEE5</i> gene promoter- <i>lacZ</i>)	This study
pTrc99A	Expression vector, Ap ^r , multiple cloning site downstream of <i>trc</i> promoter	Amersham Pharmacia Biotech
pTrc99 <i>tnaA</i>	Expresses TnaA from <i>trc</i> promoter	This study
pYAK1	R6Kori, Cm ^r , <i>sacB</i> ⁺	Kodama <i>et al.</i> (2002)
pUC19 <i>hly</i>	Expresses Hly from native promoter	This study

Table 2. Oligonucleotides used for plasmid construction, and verification of gene replacement

Bold type indicates the restriction enzyme sites.

Oligonucleotide	Sequence (5'–3')	Restriction enzyme
Gene deletion and verification		
tnaA-No	gc ggatcc atcaatatcatcatcttcaac	<i>Bam</i> HI
tnaA-Ni	cacgcaataaccttcacactccaaattataacggtttccattacataatccttc	
tnaA-Ci	gttataaattggagtggtgaaggttattgcgtggtttaataactactgagtggc	
tnaA-Co	gc gtcgac aagtgctgtgcagatcccgac	<i>Sal</i> I
lacZI-No	gc cgccg ccgccaacacagccaacatcc	<i>Not</i> I
lacZI-Ni	cacgcaataaccttcacactccaaattataactaacgctaccattaccagttggtctgg	
lacZI-Ci	gttataaattggagtggtgaaggttattgcgtgcattcaccaccctgaattg	
lacZI-Co	gc gtcgac gtttattgagcaaccggtgcc	<i>Sal</i> I
baeR-No	gc gtcgac gttgacgatcttcatcagttg	<i>Sal</i> I
baeR-Ni	cacgcaataaccttcacactccaaattataactaacggtcactacttctctcg	
baeR-Ci	gttataaattggagtggtgaaggttattgcgtggttttagcgacattac	
baeR-Co	gc ggatcc tacgtattgtctttcaccg	<i>Bam</i> HI
cpxR-No	c ggtcgac cgggcggtcaaacagtaag	<i>Sal</i> I
cpxR-Ni	cacgcaataaccttcacactccaaattataacgtaagatggtcaccggtgg	
cpxR-Ci	gttataaattggagtggtgaaggttattgcgtgcattgtttaatacctccgaggcag	
cpxR-Co	cg ggatcc taaacgctgtgctgctcggg	<i>Bam</i> HI
gadX-No	gc ggatcc ctgctgcgtatccggtgaac	<i>Bam</i> HI
gadX-Ni	cacgcaataaccttcacactccaaattataacttatagagttttactcagac	
gadX-Ci	gttataaattggagtggtgaaggttattgcgtgtgattgcatagttgacttaa	
gadX-Co	gc gtcgac tatgtgctgcggtctcg	<i>Sal</i> I
hfq-No	gc ggatcc gtttccgggcactggaag	<i>Bam</i> HI
hfq-Ni	cacgcaataaccttcacactccaaattataacttgccccttagccattctctc	
hfq-Ci	gttataaattggagtggtgaaggttattgcgtgaccgaataaggtttcgggctg	
hfq-Co	gc gtcgac caaggtgggtccagccacgc	<i>Sal</i> I
ler-No	gc ggatcc ttttctattatttctattccgttg	<i>Bam</i> HI
ler-Ni	cacgcaataaccttcacactccaaattataacgaagattttctgtgaaggac	
ler-Ci	gttataaattggagtggtgaaggttattgcgtgcataataataatctccgcac	
ler-Co	gc gtcgac ctttttataatgcaccggttc	<i>Sal</i> I
IM-Hfq(H)-F	ggatcccggttgctgggttatcgccagatg	<i>Bam</i> HI
IM-Hfq(H)-R	ctgcagttaacgcagtgctgttcaggaacggatc	<i>Pst</i> I
Promoterless <i>lacZ</i>-fused reporter plasmid construction		
LEE1-PF	gc cgccg ccgctccgggaacagctaataag	<i>Not</i> I
LEE1-PR	gcaagcttccgcatgcttaatatatttaagc	<i>Hind</i> III
LEE2-PF	gc cgccg ccgctcaataatctgcacatgctg	<i>Not</i> I
LEE2-PR	gcaagctttgtcttcattgatctttctcc	<i>Hind</i> III
LEE3-PF	gc cgccg ccgctttattatgcatggggc	<i>Not</i> I
LEE3-PR	gcaagcttaagattcatctgcaggctctg	<i>Hind</i> III
LEE4-PF	gc cgccg ccgacgctcgccagataaggcg	<i>Not</i> I
LEE4-PR	gcaagcttattagccattggaactcacg	<i>Hind</i> III
LEE5-PF	gc cgccg ccgcttctgtgtatagcatggc	<i>Not</i> I
LEE5-PR	gcaagcttaccataggcataaataatctcc	<i>Hind</i> III
Expression cloning		
tnaA-F	gc ggatcc tgatttgccctctctagacc	<i>Bam</i> HI
tnaA-R	gc gtcgac ttaaacttcttcagttttgc	<i>Sal</i> I

A *tnaA* expression plasmid was constructed as follows. The *tnaA* gene including a sequence 220 bp upstream of the start codon was cloned into pTrc99A by using the primers listed in Table 2. This plasmid was named pTrc99tnaA. We expected that TnaA would be expressed under control of the native promoter and IPTG-inducible *trc* promoter. In the presence of excess IPTG, the growth rate was reduced, but addition of 10 μ M IPTG did not affect the growth. Therefore, TnaA expression studies were done on cells grown with

10 μ M IPTG. In the β -galactosidase assay, Δ *lacZI* and derivative strains lacking the chromosomal *lacZ* gene were used to eliminate the effects of endogenous β -galactosidase.

For construction of a *hfq* expression plasmid, the *hfq* gene including native promoter was amplified by PCR with hfq-F (gccgatccactgttagtggg) and hfq-R (gccagcttacagccgaacatttctcg) primers. The fragment was ligated into pUC19 vector.

Indole production assay. The extracellular indole concentration was determined by a previously described HPLC method (Kobayashi *et al.*, 2006). EHEC and its derivative strains were grown at 37 °C in LB broth and pelleted by centrifugation at 20 000 g. The resulting supernatants were extracted twice with ethyl acetate and the extracts were loaded on to a Symmetry C₁₈ column (5 µm, 4.6 × 150 mm; Waters) attached to an L2130 HPLC system (Hitachi). The samples were eluted with acetonitrile/H₂O (1:1, v/v) at a flow rate of 0.8 ml min⁻¹; the indole peak was detected by its absorbance at 276 nm and its identity was confirmed by correspondence to the elution time for pure indole. The indole concentration was calculated from the ratio of the area of the detected peak to that of a standard peak.

Detection of EspA and EspB. Cells were grown at 37 °C for 8 h (stationary phase) and separated by centrifugation and filtration. Secreted proteins were precipitated from the supernatants with 10 % TCA and dissolved in SDS sample buffer. Cell pellets were dissolved in SDS sample buffer and the solutions were boiled. The samples were analysed by 10 % SDS-PAGE. EspA and EspB were detected by Western blotting with an EspA-specific polyclonal antibody and EspB-specific antiserum as described previously (Cantarelli *et al.*, 2007; Kodama *et al.*, 2002). Protein bands of EspA and EspB were visualized on a LAS-3000 Luminescent Image Analyser (Fujifilm) or X-ray film, and they were quantified by Image J software (National Institutes of Health).

Detection of A/E lesions by fluorescence microscopy. HeLa cells were grown on glass coverslips and infected for 4 h with 1 µl (3.2–3.4 × 10⁶ bacterial cells) EHEC from an 8 h shaking culture (stationary phase) in LB broth. After infection, the cells were washed five times with PBS, fixed with 3 % paraformaldehyde for 15 min, and permeabilized with 0.1 % Triton X-100 in PBS. F-actin was stained with rhodamine/phalloidin (1 U ml⁻¹, Molecular Probes) and the HeLa cell nuclei were visualized with Hoechst 33258 (1 µg ml⁻¹, Molecular Probes). The mounted samples were observed by fluorescence microscopy, and the number of A/E lesions formed on 100 randomly selected cells was counted. HeLa cells were counted in three microscopic fields; ± values indicate the range of A/E lesion numbers in three microscopic fields.

Construction of LEE gene reporter plasmids. The reporter plasmids were constructed as follows. DNA fragments containing the promoter region [513 bp upstream of the start codon for the first gene (*ler* of *LEE1* and *sepZ* of *LEE2*) of the *LEE1* and *LEE2* operons and 506, 516 and 395 bp upstream of the start codon for the first gene (*Ecs4570* of *LEE3*, *sepL* of *LEE4* and *tir* of *LEE5*) of the *LEE3*, *LEE4* and *LEE5* operons, respectively] were amplified by PCR by using the primers listed in Table 2. These DNA fragments were cloned in front of the promoterless *lacZ* reporter gene in the single-copy pNN387 vector (Elledge & Davis, 1989). The resulting plasmids were introduced into EHEC and its derivative strains for β-galactosidase assays.

Reporter gene assays. To determine the effects of indole and TnaA on the transcription of various reporter constructs, each bacterial strain was grown for 8 h (stationary phase) or 2 h (exponential phase) at 37 °C in LB broth containing indole and chloramphenicol. β-Galactosidase activity was assayed in cell lysates using ONPG as the substrate (Miller, 1992).

RESULTS

Indole production by EHEC O157:H7 Sakai and the *tnaA* deletion mutant

To examine indole production by EHEC O157:H7 Sakai and the *tnaA* deletion mutant (Ecs4645), extracellular

Fig. 1. Indole production (bars) and growth (triangles) of wild-type EHEC (open symbols) and the *tnaA* deletion mutant (solid symbols) cultured in LB medium. The extracellular indole concentrations were measured by HPLC, as described in Methods, after 2, 4, 8, and 24 h of culture. The EHEC cell density was determined as OD₆₀₀. At least three independent experiments were performed in each case.

concentrations were measured as described in Methods. When wild-type cells were grown for 8 h (stationary phase) in LB medium, the extracellular concentration of indole reached a maximum value of 465 µM and did not increase on further incubation (Fig. 1). In contrast, the *tnaA* deletion mutant produced no detectable indole. Growth of the Δ *tnaA* strain was the same as that of the wild-type (Fig. 1). We also examined the indole productivity of EHEC grown in tissue cell culture medium (DMEM). However, wild-type EHEC hardly produced indole (extracellular concentration was less than 10 µM; data not shown). Therefore, to investigate the effect of *tnaA* deletion and indole addition on EHEC pathogenicity, EHEC and its *tnaA* mutant were cultured in LB medium for 8 h in this study.

Effects of indole on the secretion and production of T3SS-dependent EspA and EspB

When EHEC infect epithelial cells, Tir secreted by EHEC is translocated to the plasma membrane of the host cell by EspA and EspB. Therefore, these proteins play central roles in T3SS-mediated EHEC pathogenicity. To examine the relationship between EHEC pathogenicity and indole, we assessed the effects of the *tnaA* deletion and indole addition on the secretion and production of EspA, EspB and Tir by immunoblotting with anti-EspA antibody, anti-EspB antiserum and anti-Tir antibody. The secretion of EspA and EspB was greatly decreased in the *tnaA* deletion mutant (Fig. 2) whereas that of Tir was hardly affected (data not shown). The *tnaA* expression plasmid pTrc99tnaA restored most of the EspA and EspB secretion in the Δ *tnaA* strain (Fig. 2). To determine whether the reduction of EspA/B proteins caused by *tnaA* deletion is due to the loss of indole, Δ *tnaA* was cultured in the presence and absence of indole. The secretion of EspA and EspB was increased to the wild-type level by addition of 125 or 250 µM indole (Fig. 2). Similarly, the amount of

Fig. 4. (a, b) Effect of indole and *tnaA* gene deletion on *LEE4* promoter activity. (a) Wild-type ($\Delta lacZl$) and $\Delta tnaA$ ($\Delta lacZl\Delta tnaA$) EHEC were grown to stationary phase in LB medium with (solid bars) or without (open bars) indole. (b) Wild-type ($\Delta lacZl$) and $\Delta tnaA$ ($\Delta lacZl\Delta tnaA$) EHEC cells were grown to exponential phase with (solid bars) or without (open bars) indole. (c) Effects of deletion of *ler*, *baeR*, *cpxR*, *gadX* and *hfq* on indole-induced expression of *LEE4*. The respective mutant strains were grown to stationary phase with (solid bars) or without (open bars) 2 mM indole. The β -galactosidase activities of the *lacZ* fusion of the *LEE4* promoter were measured. At least three independent experiments were performed in each case.

was also decreased by the *tnaA* deletion, and increased by the addition of 500 μ M indole. However, these effects were modest (data not shown). The activities of the *LEE2* and *LEE5* promoters were not affected by either *tnaA* deletion or the addition of indole (data not shown).

We also examined whether the *LEE4* promoter is stimulated by indole in the wild-type background. *TnaA* is induced in stationary phase. Therefore, the wild-type and $\Delta tnaA$ strains were cultured to exponential phase (for 2 h) in the presence or absence of indole to avoid the effect of intrinsic indole produced by wild-type (Fig. 1), and the activities of the *LEE4* promoter were measured. As expected, deletion of *tnaA* did not reduce activity of the *LEE4* promoter, and addition of 500 μ M or 2 mM indole efficiently enhanced it (Fig. 4b). We believe the primary effect of indole on *LEE* gene transcription is at the *LEE4* promoter.

Next, we investigated the mechanism of regulation of *LEE4* expression by indole. We previously reported that the induction of drug exporter genes by indole depends on BaeSR/CpxAR (a two-component signal transduction system in *E. coli*), GadX (an acid-induced regulatory protein) and Hfq (sRNA chaperone protein) (Kobayashi *et al.*, 2006; Hirakawa *et al.*, 2005). It has also been reported that Ler is a positive regulator of *LEE* genes in EHEC (Elliott *et al.*, 2000). Therefore, BaeSR, CpxAR, GadX, Hfq and Ler are possible indole signal-transducing mediators in the induction of *LEE4* expression. Appropriate deletion mutants were constructed in the $\Delta tnaA$ background, and the effects of indole on *LEE4* promoter activity were examined. As shown in Fig. 4(c), *LEE4* expression was higher in the *hfq* deletion mutant ($\Delta lacZl\Delta tnaA\Delta hfq$) than in the *hfq* intact strain ($\Delta lacZl\Delta tnaA$), and it was not significantly induced by indole (1.1-fold induction). The levels of induction by indole in the other mutants were almost the same as in the parent mutants (Fig. 4c). To examine the effect of *hfq* deletion on EspA/B (gene products of *LEE4*) production and the sensitivity to indole, levels of secreted and intracellular EspA/B were determined when these mutant strains were cultured in the presence and absence of exogenous indole. The *hfq* deletion mutant ($\Delta lacZl\Delta tnaA\Delta hfq$) produced large amounts of EspA/B even in the absence of exogenous indole. However, levels of neither secreted EspA/B nor intracellular EspA/B were increased by the addition of indole (Fig. 5). These observations are consistent with the results of the promoter activity measurements. The results indicate that Hfq represses the expression of *LEE4* while it is responsible for the induction of *LEE4* by indole.

Since the effect of *hfq* deletion on EspA/B secretion and production had been examined in the *tnaA* deletion background, we tested whether a single *hfq* mutant also increases EspA/B secretion and production. A large amount of intracellular and extracellular EspA and EspB was detected in the Δhfq :Cm strain carrying pUC19 vector. However, when the *hfq* expression plasmid (pUC19hfq) was introduced, the amount of EspA and EspB was decreased to about the wild-type level (Fig. 6).

Effects of indole on the formation of A/E lesions in HeLa cells

Because the secretion and production of EspA/B are stimulated by indole, T3SS-dependent EHEC pathogenicity,

Fig. 5. Secretion and production of EspA and EspB in $\Delta lacZ$, $\Delta lacZ/\Delta tnaA$ and $\Delta lacZ/\Delta tnaA\Delta hfq$ EHEC. Strains were cultured in LB medium for 8 h (stationary phase) in the presence or absence of indole, and levels of secreted and intracellular EspA and EspB were detected. As a negative control, intracellular DnaK was detected by means of a DnaK-specific antibody (Calbiochem). The respective protein bands were visualized and quantified as described for Fig. 2.

Fig. 6. Secretion and production of EspA and EspB in wild-type and *hfq* single-mutant ($\Delta hfq : Cm^r$) EHEC strains cultured in LB medium for 8 h (stationary phase). Wild-type and $\Delta hfq : Cm^r$ harbouring the pUC19 vector (mock), and $\Delta hfq : Cm^r$ harbouring the pUC19*hfq* (*hfq* expression) plasmid were grown for 8 h and secreted and intracellular EspA/B were measured. The respective protein bands were visualized on X-ray film, and quantified by using Image J software; values indicate ratio against the parent strain.

which is characterized by accumulation of actin in the host cells (A/E lesions), may also be stimulated by indole. Therefore, the effects of *tnaA* deletion and the addition of indole on the formation of A/E lesions in HeLa cells were examined. HeLa cells were infected for 4 h with stationary-phase wild-type EHEC or with indole-treated or untreated $\Delta tnaA$ mutant. The infected cells were fixed and stained with rhodamine-labelled phalloidin and Hoechst 33258 to identify actin and nuclei, respectively. The A/E lesions, which are characterized by accumulated F-actin, were then counted. When the HeLa cells were infected with wild-type EHEC, 14 ± 3 A/E lesions per 100 HeLa cells were observed 4 h post-infection, whereas A/E lesions were rarely observed in uninfected cells (<1 A/E lesion per 100 HeLa cells). When HeLa cells were infected with $\Delta tnaA$ EHEC, the number of A/E lesions produced was about half the wild-type value (6 ± 2 A/E lesions per 100 HeLa cells). However, when $\Delta tnaA$ EHEC was cultured for 8 h (stationary phase) with 125 μM indole before infection, the number of A/E lesions reached about 18 ± 3 per 100 HeLa cells, indicating that indole restores and promotes the induction of A/E lesions by $\Delta tnaA$ EHEC in HeLa cells.

DISCUSSION

Homologues of *E. coli* K-12 *tnaA* are found in many bacteria, and indole-producing bacteria are found in the human intestinal tract (DeMoss & Moser, 1969). It was previously reported that *tnaA* deletion in EPEC abrogates the ability to paralyse or kill *Caenorhabditis elegans* (Anyanful *et al.*, 2005). Although exogenous indole induces *LEE1* expression, the reduction of virulence in the *tnaA* mutant was not restored by indole. On the other hand, it was recently reported that indole represses several EHEC virulence-related phenotypes (motility, biofilm formation and attachment to HeLa cells) (Bansal *et al.*, 2007). However, the relationship between indole and type III secretion, and the formation of A/E lesions, has not been investigated previously.

We show here that indole increases the production and secretion of EspA/B, which are T3SS-mediated translocators. This results in an increase in the formation of A/E lesions in HeLa cells. Indole also elicits a modest induction of *LEE4* transcription. We found that deletion of *tnaA* reduced the secretion and production of EspA/B and the formation of A/E lesions in HeLa cells (Fig. 2). All of these effects were overcome by addition of 125 μM indole to O157 $\Delta tnaA$ cultures. This is a physiologically relevant indole concentration: in wild-type EHEC culture medium in the stationary phase, the indole concentration is about 465 μM (Fig. 1). Therefore, the effect of *tnaA* deletion can be explained by the loss of indole (Fig. 2).

Experiments using a T3SS mutant, $\Delta escN$, showed that indole stimulates the production of EspA and EspB without secretion and the deletion of *tnaA* decreases cellular EspA and EspB levels (Fig. 3). However, the

influence of indole addition and the *tnaA* deletion in Δ *escN* was less than that in the parent strain. We also showed that the promoter activity of *espA* and *espB* (located in the *LEE4* operon) was moderately decreased by the deletion of *tnaA*, and induced by indole (Fig. 4a). The change of promoter activity was less than that of EspA and EspB protein levels. Thus, indole participates in three kinds of regulatory process of EspA/B. Among them, indole primarily controls the secretion of EspA/B. Indole also appears to control EspA and EspB translation and at the level of transcription.

We also found that deletion of *hfq* abolishes the influence of indole on the induction of *LEE4*, and fully induces it even when indole is absent. These results suggest that Hfq tightly represses the expression of *LEE4*. Hfq acts as a regulator in complex with sRNAs (Gottesman, 2004), which determine the specificity of regulation. In addition, we note that the effect of *hfq* deletion on the increase of EspA/B production is greater than that on the increase of the promoter activity (Figs 4c, 5 and 6). We interpret this to mean that the *LEE4* operon including *espA* and *espB* may be transcriptionally and post-transcriptionally regulated by Hfq-sRNA. It will be of interest to identify the hypothetical sRNA for regulation of EspA and EspB.

It was previously reported that motility-related genes and *LEE* operons are inversely regulated by GrlA and GrlR (Iyoda *et al.*, 2006). Therefore, GrlA and GrlR could be considered as candidates mediating the induction of *LEE* genes and the repression of motility by indole. However, this seems unlikely because the *LEE* gene induction by indole is independent of Ler (Fig. 4c) whereas the regulation of *LEE* promoters by GrlA and GrlR depends on Ler (Iyoda *et al.*, 2006).

In EHEC, effector proteins are delivered into host cells through EspA filaments and pores in the plasma membrane formed by EspB. Intimin, an outer-membrane protein of EHEC, then binds to Tir on the host-cell plasma membrane (Garmendia *et al.*, 2005; Kenny *et al.*, 1997). Although indole obviously enhances the secretion of EspA and EspB, the production of Tir and intimin (the *eae* gene product) is not affected (data not shown). This suggests that indole probably assists the efficient delivery of effector proteins, promoting A/E lesion formation.

If indole is produced in stationary phase in LB medium, it is anticipated that the indole-regulated *LEE4* promoter should be induced in stationary phase. The activity of this promoter in exponential phase is clearly lower than in stationary phase. However, *LEE4* promoter activity of a *tnaA* deletion mutant in stationary phase and *LEE4* in exponential phase is reasonably high (Fig. 4a, b). Therefore, we believe that indole partly contributes to the stimulation of *LEE* gene expression in the stationary phase.

There are many recent reports that intercellular signal molecules contribute to the pathogenicity of bacteria

(Bassler, 2002). In Gram-negative bacteria, three kinds of quorum-sensing molecules (or autoinducers) (AI-1, AI-2 and AI-3) have been reported (Fuqua *et al.*, 2001; Sperandio *et al.*, 2001, 2003). In addition, there is evidence that indole also acts as an intercellular signal molecule during the stationary phase in *E. coli* and other enteric species (Kobayashi *et al.*, 2006; Lee *et al.*, 2007a; Wang *et al.*, 2001). Here we show that indole enhances expression of specific virulence factors in EHEC. Previously, the relationship between intercellular signal transduction and the production of type III secretion-related proteins was described in EHEC. A LuxR-like quorum-sensing regulator, SdiA, AI-3 and related regulatory factors (*luxS*, *qseA* and *qseBC*) affect the production of type III secretion-related proteins (Kanamaru *et al.*, 2000; Sharp & Sperandio, 2007; Sperandio *et al.*, 1999, 2003). However, the addition of indole did not affect the transcription of the related genes (*sdiA*, *luxS*, *qseA* and *qseBC*) (data not shown) although indole activates the transcription of *sdiA*, and results in reduction of biofilm formation in *E. coli* K-12 (Lee *et al.*, 2007b). In addition, indole is produced later in the stationary phase than AI-1, AI-2 and AI-3, which are active in the early stationary phase. Therefore, the indole-sensing system probably differs from these intercellular signal transduction systems.

As mentioned above, it was previously reported that indole represses several virulence-related phenotypes in EHEC (motility, biofilm formation and attachment to HeLa cells) (Bansal *et al.*, 2007). Addition of 500–600 μ M indole decreased these phenotypes whereas we showed that type III secretion-related protein production and virulence phenotypes are stimulated by indole concentrations of 125 μ M or more. These results may indicate that indole has dual roles in the virulence of EHEC. Judging from these observations, the virulence of EHEC seems to be tightly regulated by the concentration of indole. Indole concentration in the enteric site may change intermittently with the amount of indole-producing enteric bacteria and enteric environmental conditions. According to an *in vivo* study with mice, tryptophan in the food markedly affects tryptophanase, and hence presumably indole production, by enteric bacteria (Botsford & Demoss, 1972).

To counteract bacterial infections, it is important to understand the behaviour of bacteria at their site of infection and in their habitat. Although indole is secreted by many bacteria and is abundant in the intestinal tract, its significance in bacterial physiology has remained unclear. We therefore believe that the characterization of the indole signal and the identification of an indole regulon have great interest. Moreover, interestingly, serotonin, melatonin and indole-3-acetic acid, which are typical signal molecules secreted by animal and plant cells, have an indole-like chemical structure. Therefore, it will be intriguing to investigate how these indole-like signals act on bacteria, or

how indole affects the signal transduction in eukaryotic hosts.

ACKNOWLEDGEMENTS

We wish to thank E. Peter Greenberg for constructive comments, George M. Church for the plasmid pKO3, and Ronald W. Davis for the plasmid pNN387. We are grateful for the technical assistance in HPLC analysis provided by Nobuo Kato and Hajime Nitta of the Institute of Scientific and Industrial Research, Osaka University. H. H. was supported by a research fellowship from the Japan Society for the Promotion of Science for Young Scientists. This research was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and CREST, Japan Science and Technology Agency, Japan.

REFERENCES

- Anyanful, A., Dolan-Livengood, J. M., Lewis, T., Sheth, S., Dezalia, M. N., Sherman, M. A., Kalman, L. V., Benian, G. M. & Kalman, D. (2005). Paralysis and killing of *Caenorhabditis elegans* by enteropathogenic *Escherichia coli* requires the bacterial tryptophanase gene. *Mol Microbiol* 57, 988–1007.
- Bansal, T., Englert, D., Lee, J., Hegde, M., Wood, T. K. & Jayaraman, A. (2007). Differential effects of epinephrine, norepinephrine, and indole on *Escherichia coli* O157:H7 chemotaxis, colonization and gene expression. *Infect Immun* 75, 4597–4607.
- Bassler, B. L. (2002). Small talk. Cell-to-cell communication in bacteria. *Cell* 109, 421–424.
- Blankenhorn, D., Phillips, J. & Slonczewski, J. (1999). Acid- and base-induced proteins during aerobic and anaerobic growth of *Escherichia coli* revealed by two-dimensional gel electrophoresis. *J Bacteriol* 181, 2209–2216.
- Botsford, J. L. & Demoss, R. D. (1972). *Escherichia coli* tryptophanase in the enteric environment. *J Bacteriol* 109, 74–80.
- Cantarelli, V. V., Kodama, T., Nijstad, N., Abolghait, S. K., Nada, S., Okada, M., Iida, T. & Honda, T. (2007). Tyrosine phosphorylation controls cortactin binding to two enterohaemorrhagic *Escherichia coli* effectors: Tir and EspFu/TccP. *Cell Microbiol* 9, 1782–1795.
- Deeley, M. C. & Yanofsky, C. (1982). Transcription initiation at the tryptophanase promoter of *Escherichia coli* K-12. *J Bacteriol* 151, 942–951.
- DeMoss, R. D. & Moser, K. (1969). Tryptophanase in diverse bacterial species. *J Bacteriol* 98, 167–171.
- Di Martino, P., Fursy, R., Bret, L., Sundararaju, B. & Phillips, R. S. (2003). Indole can act as an extracellular signal to regulate biofilm formation of *Escherichia coli* and other indole-producing bacteria. *Can J Microbiol* 49, 443–449.
- Elledge, S. J. & Davis, R. W. (1989). Position and density effects on repression by stationary and mobile DNA-binding proteins. *Genes Dev* 3, 185–197.
- Elliott, S. J., Sperandio, V., Girón, J., Shin, S., Mellies, J., Wainwright, L., Hutcheson, S., McDaniel, T. & Kaper, J. B. (2000). The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun* 68, 6115–6126.
- Fuqua, C., Parsek, M. R. & Greenberg, E. P. (2001). Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* 35, 439–468.
- Garmendia, J., Frankel, G. & Crepin, V. (2005). Enteropathogenic and enterohemorrhagic *Escherichia coli* infections: translocation, translocation, translocation. *Infect Immun* 73, 2573–2585.
- Gottesman, S. (2004). The small RNA regulators of *Escherichia coli*: roles and mechanisms. *Annu Rev Microbiol* 58, 303–328.
- Hayashi, T., Makino, K., Ohnishi, M., Kurokawa, K., Ishii, K., Yokoyama, K., Han, C. G., Ohtsubo, E., Nakayama, K. & other authors (2001). Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA Res* 8, 11–22.
- Hirakawa, H., Inazumi, Y., Masaki, T., Hirata, T. & Yamaguchi, A. (2005). Indole induces the expression of multidrug exporter genes in *Escherichia coli*. *Mol Microbiol* 55, 1113–1126.
- Iyoda, S., Koizumi, N., Satou, H., Lu, Y., Saitoh, T., Ohnishi, M. & Watanabe, H. (2006). The GrlR-GrlA regulatory system coordinately controls the expression of flagellar and LEE-encoded type III protein secretion systems in enterohemorrhagic *Escherichia coli*. *J Bacteriol* 188, 5682–5692.
- Kanamaru, K., Kanamaru, K., Tatsuno, I., Tobe, T. & Sasakawa, C. (2000). SdiA, an *Escherichia coli* homologue of quorum-sensing regulators, controls the expression of virulence factors in enterohaemorrhagic *Escherichia coli* O157:H7. *Mol Microbiol* 38, 805–816.
- Karmali, M. A. (1989). Infection by verocytotoxin-producing *Escherichia coli*. *Clin Microbiol Rev* 2, 15–38.
- Kawamura-Sato, K., Shibayama, K., Horii, T., Iimura, Y., Arakawa, Y. & Ohta, M. (1999). Role of multiple efflux pumps in *Escherichia coli* in indole expulsion. *FEMS Microbiol Lett* 179, 345–352.
- Kenny, B., DeVinney, R., Stein, M., Reinscheid, D. J., Frey, E. A. & Finlay, B. B. (1997). Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* 91, 511–520.
- Kobayashi, A., Hirakawa, H., Hirata, T., Nishino, K. & Yamaguchi, A. (2006). The growth phase-dependent expression of drug exporters in *Escherichia coli* and its contribution to the drug tolerance. *J Bacteriol* 188, 5693–5703.
- Kodama, T., Akeda, Y., Kono, G., Takahashi, A., Imura, K., Iida, T. & Honda, T. (2002). The EspB protein of enterohaemorrhagic *Escherichia coli* interacts directly with alpha-catenin. *Cell Microbiol* 4, 213–222.
- Lee, J., Bansal, T., Jayaraman, A., Bentley, W. & Wood, T. K. (2007a). Enterohemorrhagic *Escherichia coli* biofilms are inhibited by 7-hydroxyindole and stimulated by isatin. *Appl Environ Microbiol* 73, 4100–4109.
- Lee, J., Jayaraman, A. & Wood, T. K. (2007b). Indole is an inter-species biofilm signal mediated by SdiA. *BMC Microbiol* 7, 42.
- Link, A. J., Phillips, D. & Church, G. M. (1997). Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol* 179, 6228–6237.
- McDaniel, T. K. & Kaper, J. B. (1997). A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. *Mol Microbiol* 23, 399–407.
- Miller, J. H. (1992). *A Short Course in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Nataro, J. P. & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 11, 142–201.
- Sharp, F. C. & Sperandio, V. (2007). QseA directly activates transcription of *LEE1* in enterohemorrhagic *Escherichia coli*. *Infect Immun* 75, 2432–2440.
- Sperandio, V., Mellies, J. L., Nguyen, W., Shin, S. & Kaper, J. B. (1999). Quorum sensing controls expression of the type III secretion

gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proc Natl Acad Sci U S A* **96**, 15196–15201.

Sperandio, V., Torres, A. G., Giron, J. A. & Kaper, J. B. (2001). Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. *J Bacteriol* **183**, 5187–5197.

Sperandio, V., Torres, A. G., Jarvis, B., Nataro, J. P. & Kaper, J. B. (2003). Bacteria–host communication: the language of hormones. *Proc Natl Acad Sci U S A* **100**, 8951–8956.

Wang, D., Ding, X. & Rather, P. N. (2001). Indole can act as an extracellular signal in *Escherichia coli*. *J Bacteriol* **183**, 4210–4216.

Edited by: B. Kenny