Microbiology (2009), **155**, 3214–3225 **DOI** 10.1099/mic.0.030759-0

Enterohaemorrhagic *Escherichia coli* serogroup O111 inhibits NF-κB-dependent innate responses in a manner independent of a type III secreted OspG orthologue

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Received 11 May 2009
Revised 17 July 2009
Accepted 20 July 2009

Enterohaemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC) inject a repertoire of effector proteins into host cells via a type III secretion system (T3SS) encoded by the locus of enterocyte effacement (LEE). OspG is an effector protein initially identified in *Shigella* that was shown to inhibit the host innate immune response. In this study, we found *ospG* homologues in EHEC (mainly of serogroup O111) and in *Yersinia enterocolitica*. The T3SS encoded by the LEE was able to inject these different OspG homologues into host cells. Infection of HeLa cells with EHEC O111 inhibited the NF-κB-dependent innate immune response via a T3SS-dependent mechanism. However, an EHEC O111 *ospG* mutant was still able to inhibit NF-κB p65 transfer to the nucleus in infected cells stimulated by tumour necrosis factor α (TNF-α). In addition, no difference in the inflammatory response was observed between wild-type EHEC O111 and the isogenic *ospG* mutant in the rabbit ligated intestinal loop model. These results suggest that OspG is not the sole effector protein involved in the inactivation of the host innate immune system during EHEC O111 infection.

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) are foodborne pathogens that cause severe diarrhoea and haemorrhagic colitis that may be complicated by thrombotic thrombocytopenic purpura or haemolytic uraemic syndrome (Kaper *et al.*, 2004). EHEC O157 is the predominant serogroup associated with large outbreaks, but the clinical importance of EHEC O111, O26 and O103 strains is increasingly recognized (Ogura *et al.*, 2007). Shiga toxins (Stx) are the major determinants of EHEC pathogenicity (Karmali, 1989). In addition, a repertoire of effector proteins secreted via a type III secretion system (T3SS) is recognized as essential for EHEC virulence as for enteropathogenic *E. coli* (EPEC) (Garmendia *et al.*, 2005). These effector proteins are coded not only by a pathogenicity island that codes for the T3SS and is called the locus of enterocyte effacement (LEE), but also by other chromosomal loci, mainly exchangeable effector loci (EEL)

Abbreviations: EEL, exchangeable effector loci; EHEC, enterohaemorrhagic *Escherichia coli*; EIEC, enteroinvasive *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; FCS, fetal calf serum; FRT, Flp recombination target; IL, interleukin; IS, insertion sequence; LEE, locus of enterocyte effacement; TNF-α, tumour necrosis factor α; T3SS, type III secretion system.

The GenBank/EMBL/DDBJ accession numbers for the sequences (between the *lom* and *yciE* genes) reported in this paper are AB438937 (strain 12009), and AB438936 (strain 11128).

within lambdoid prophages (Loukiadis *et al.*, 2008; Ogura *et al.*, 2007; Tobe *et al.*, 2006).

Homeostasis of the mammalian intestinal epithelium is maintained by keeping a balance between tolerance and response to luminal micro-organisms. Infection with enteric pathogens disrupts this balance, leading to intestinal inflammation (Sansonetti, 2004). The family of NF-κB transcription factors essentially regulates immune-related gene expression. Without stimulation, NF-κB is in an inactive state, bound to its inhibitor IκB in the cytoplasm. Following activation, IκB undergoes phosphorylation, ubiquitinylation and subsequent degradation by the proteasome, thus allowing NF-κB to translocate to the nucleus and to regulate the transcription of genes involved in the inflammatory response, including interleukin (IL)-8 (Li & Verma, 2002). The host inflammatory response against infections with EHEC or EPEC is the result of a balance between bacterial extracellular pro-inflammatory components and intracellular anti-inflammatory effectors that are injected into host cells (Sharma *et al.*, 2006). Several studies indicate that EHEC and EPEC activate the NF-κB signalling pathway (Dahan *et al.*, 2002; Savkovic *et al.*, 1997) and it was reported that flagellin is a major pro-inflammatory mediator (Berin *et al.*, 2002; Zhou *et al.*, 2003). It was also reported that infection of HeLa cells with EHEC and EPEC suppresses NF-κB activation in a T3SS-dependent manner (Hauf & Chakraborty, 2003). Another study using human colonic epithelial cells confirms a role for the T3SS in the ability of EHEC to modulate the host innate response (Gobert *et al.*, 2005). Ruchaud-Sparagano *et al.* (2007) reported that infection of differentiated Caco-2 cells with EPEC prevents the activation and translocation of NF-κB upon tumour necrosis factor α (TNF-α) stimulation in a T3SS-dependent manner, but this mechanism is independent of all LEE-encoded effectors and at least four non-LEE-encoded effectors (Orf3, NleA, NleF and NleH). These findings suggest that unknown EHEC/EPEC effector(s) is/are involved in the modulation of inflammatory response.

Several T3SS effectors from animal and plant pathogens have been reported to inhibit the NF-κB signalling pathway (Bhavsar *et al.*, 2007; Espinosa & Alfano, 2004). Among these T3SS effectors, *Shigella flexneri* OspG binds various ubiquitinylated ubiquitin-conjugating enzymes and negatively controls the host innate response induced by *Shigella* upon invasion of the epithelium (Kim *et al.*, 2005). YopP/J, produced by *Yersinia* spp., acetylates and inhibits kinase activity of IKK and consequently blocks the NF-κB signalling pathway (Mukherjee *et al.*, 2006).

In this study, we found that *ospG* homologues are present not only in *Shigella* spp. but also in non-O157 EHEC and in *Yersinia enterocolitica*. We observed that these OspG homologues could be translocated into host cells through the T3SS encoded by the LEE. However, the deletion of the *ospG* homologue did not affect the inflammatory response to EHEC infection *in vitro* or *in vivo* in the rabbit ligated intestinal loop model.

METHODS

Bacterial strains, plasmids and cell lines. Bacterial strains and plasmids used in this study are shown in Table 1. HeLa (ATCC CCL-2) and RK13 actin-GFP cells (Peralta-Ramirez *et al.*, 2008) were maintained in D-MEM (Invitrogen) supplemented with 10 % (v/v) fetal calf serum (FCS, Eurobio) and 80 μg gentamicin ml^{-1} at 37 °C in 5 % CO_2. G418 (Invivogen) was added at 400 μg ml^{-1} to RK13 actin-GFP cells.

Sequencing and *in silico* analysis. The sequences of EELs encoding OspG homologues within prophages in EHEC O111 : H− strain 11128 and EHEC O103 : H2 strain 12009 were determined as a part of the genome sequencing project that is in progress in the laboratory of one of the authors (T. H.). The sequence assembly for each OspG homologue encoding EELs was confirmed by PCR using primers targeting the *lom* family gene and the *yciE* gene (Table 1). *In silico* analyses were done with the ORF Finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and BLAST programs (Altschul *et al.*, 1990) The Mac Vector software (Mac Vector, Inc.) was used to create a phylogenetic tree for OspG homologues using the Kimura two-parameter neighbour-joining method.

Construction of mutants and plasmids. To remove the kanamycin cassette from E22 escN : : kmlox (Marches *et al.*, 2003), plasmid pJW168 was used (Wild *et al.*, 1998). To delete the *escN* gene in EHEC strain 13369, a DNA fragment including 500 bp up- and downstream of *escN*, a kanamycin cassette and flanking FRT (Flp recombination target) sites was prepared by PCR using primers Kmcassette-F and Kmcassette-R (Table 1) and plasmid pKD4 as the template. The fragment was cloned into suicide vector pKNG101 to generate pKNG101-escN : : kmFRT. To delete the *ospG* homologue in EHEC strain 13369, a DNA fragment was amplified by the overlap extension PCR method (Ho *et al.*, 1989), using primers mutospG1-F, mutospG1-R, mutospG2-F, mutospG2-R, mutospGKm-F and mutospGKm-R (Table 1), with the genomic DNA and pKD4 plasmid DNA as templates. This fragment was cloned into pKNG101 to generate pKNG101-kmospG : : kmFRT. The resulting plasmids were electroporated in SM10*λpir*, and then transferred into EHEC strain 13369 by conjugation. EHEC mutants were selected at 30 °C on MinA plates (Miller, 1992) and the FRT-kanamycin cassette was removed using plasmid pCP20 (Cherepanov & Wackernagel, 1995). The *ospG* genes (excluding their stop codons) were amplified by PCR with primers ospGgateway-F and ospGgateway-R (Table 1) using the genomic DNA of EHEC strain 11128, *S. flexneri* strain SF620 and *Y. enterocolitica* strain 8081 (a kind gift from Dr Elisabeth Carniel, Institut Pasteur, Paris, France). The genes were cloned into pDONR201 vector (Invitrogen), and derivatives of the pKTEM vector encoding OspG proteins fused to the TEM reporter enzyme, were named pKospGTEM (OspG from EHEC O111), pKshiospGTEM (*S. flexneri*) and pKyerospGTEM (*Y. enterocolitica*), respectively. These plasmids were electroporated into EPEC strains E22 and E22Δ*escN*, and EHEC strains 13369 and 13369Δ*escN*. For complementation, plasmid pABBospG, encoding the EHEC OspG homologue, was constructed by using the Gateway cloning system with pDONR201, encoding OspG, and pABB-99ccdB.V5His6 (a kind gift from Dr Akio Abe, Kitasato University, Tokyo, Japan). The pABBospG and the pUC18-OspG plasmid encoding OspG from *S. flexneri* (a kind gift from Dr Claude Parsot, Institut Pasteur, Paris, France) were electroporated into the EHEC *ospG*-deletion mutant.

Translocation assay. The production and secretion of TEM fusion proteins were carried out as described previously (Charpentier & Oswald, 2004; Jubelin *et al.*, 2009), except that bacteria were grown in D-MEM/HEPES (Invitrogen) with kanamycin at 37 °C for 6.5 h without adding IPTG. The data presented (normalized 450/520 nm emission ratio) are the mean values of the results from triplicate wells

Table 1. Strains, plasmids and primers used in this study

Strain, plasmid or primer	Description	Reference/source
E. coli		
E22	Rabbit EPEC of serotype O103:H2	Nougayrede *et al.* (1999)
E22 *escN*	E22 *escN*::*aphT*, EscN$^-$	Marches *et al.* (2003)
E22Δ*escN*	E22 *escN*::*loxP*, *escN*, *aphT* deletion mutant	This study
13369	Human EHEC of serotype O111:H–	Ogura *et al.* (2007)
13369Δ*escN*	13369 *escN*::FRT, *escN* deletion mutant	This study
13369Δ*ospG*	13369 *ospG*::FRT, *ospG* deletion mutant	This study
DH5α	*E. coli* for cloning	Invitrogen
DB3.1	*E. coli* for maintenance of plasmid encoded *ccdB* gene	Invitrogen
SM10λ*pir*	*E. coli* for conjugation	Laboratory strain
S. flexneri		
SF620	M90T *ipaB* deletion mutant	Menard *et al.* (1993)
Plasmids		
pKD4	Template for PCR amplification of kanamycin cassette for Red recombinase-mediated recombination	Datsenko & Wanner (2000)
pKNG101	Suicide vector	Kaniga *et al.* (1991)
pDONR201	Gateway system donor vector	Invitrogen
pKTEM	Vector for TEM translocation assay	Jubelin *et al.* (2009)
pABB-99ccdB.V5His6	Vector for V5 and His tag C-terminal fusion	Dr Akio Abe, Kitasato University, Tokyo, Japan
pJW168	Plasmid for Lox cassette elimination	Wild *et al.* (1998)
pCP20	Plasmid for FRT cassette elimination	Cherepanov & Wackernagel (1995)
pUC18-OspG	Plasmid encoding *ospG* from *S. flexneri*	Kim *et al.* (2005)
Primers		
attB1	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′	Invitrogen
attB2	5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′	Invitrogen
Kmcassette-F	5′-GTGTAGGCTGGAGCTGCTTC-3′	This study
Kmcassette-R	5′-CATATGAATATCCTCCTTAG-3′	This study
lom-sense	5′-ATGCGTAAACTTTATGCCGC-3′	This study
mutospGKm-F	5′-TAACAAGGGCATGTCGTGTAGGCTGGAGCTGCTTC-3′	This study
mutospGKm-R	5′-ATGTACGCATATAGCCATATGAATATCCTCCTTAGTTCC-3′	This study
mutospG1-F	5′-GGTTGTATGTTTAGCATAAGAACCC-3′	This study
mutospG1-R	5′-AGCTCCAGCCTACACGACATGCCCTTGTTATGAAATTTA-3′	This study
mutospG2-F	5′-GAGGATATTCATATGGCTATATGCGTACATTCTCAACC-3′	This study
mutospG2-R	5′-ACATAAACACCCACGGATCGAG-3′	This study
ospG-F	5′-CCATTTGAGAATAATAATTCTCATGCTG-3′	This study
ospG-R	5′-GCATTTGTAATCGTCGGTCGATAATC-3′	This study
ospGgateway-F	5′-AAAAAGCAGGCTCATAACAAGGGCATGTCATGAAAATA-3′	This study
ospGgateway-R	5′-AGAAAGCTGGGTCTAAATATTTTCTGTTTAATAATGAAT-3′	This study
whole-ospG-F	5′-TTCATAACAAGGGCATGTCATGA-3′	This study
whole-ospG-F2	5′-GATCATGGCGGTTATCTATG-3′	This study
whole-ospG-R	5′-AGTGATTCAACCTGCTACGTG-3′	This study
yciE-sense	5′-ATGAATCGTATTGAACATTA-3′	This study
16S rRNA-F	5′-ACTCAAATGAATTGACGGGGGC-3′	Viswanathan *et al.* (2004)
16S rRNA-R	5′-AGGCCCGGGAACGTATTCAC-3′	Viswanathan *et al.* (2004)

from three independent experiments and bars indicate standard error of the mean (SEM). For statistical analyses, two-sided Student's *t*-test was used with independent samples. *P*-values <0.05 were considered statistically significant.

RT-PCR. RNA was isolated from bacteria grown in D-MEM/HEPES supplemented with 5 % (v/v) fetal calf serum (FCS) at 37 °C, 5 % CO_2 to OD_{600} ~0.5, using TRIZOL (Invitrogen) and then treated with the TURBO DNA-free kit (Ambion). One microgram of total RNA and random primers were used for reverse transcription (RT) using the SuperScript III First-Strand Synthesis System (Invitrogen). The PCR amplification was done with RT reactant and primers ospG-F and ospG-R or primers 16S rRNA-F and 16S rRNA-R (Table 1).

Fluorescent-actin staining (FAS) assay. RK13 actin-GFP cells were inoculated with the bacteria (m.o.i. 50) and incubated for 6 h, then F-actin was visualized as described previously (Peralta-Ramirez *et al.*, 2008).

Analysis of NF-κB (p65) translocation and IκB-α degradation. Two days before the infection, 2×10^6 HeLa cells were seeded on 100 mm dishes with 10 ml D-MEM 10 % (v/v) FCS 0.1 mM non-essential amino acids (NEAA, Invitrogen). Twenty hours before infection, the culture medium was replaced with D-MEM 1 % (v/v) FCS and NEAA. Overnight bacterial LB cultures were inoculated (1 : 100) in D-MEM/HEPES and grown at 37 °C for 3 h. The bacteria were added to the HeLa cells (m.o.i. 50) and incubated at 37 °C, 5 % CO_2 for 1–6 h. The cells were washed three times with Hanks' balanced salt solution (HBSS), and 10 ml D-MEM containing TNF-α (20 ng ml^{-1}, Peprotech) was added to the cells and incubated for 30 min. Cells were lysed and fractionated into cytoplasmic and nuclear fractions using a Nuclear Extract kit (Active motif). Ten micrograms of nuclear proteins was used for detection of NF-κB (p65) nuclear translocation using the TransAM NFκB (p65) kit (Active motif). The cytoplasmic fractions were subjected to Western blot analysis with anti-IκB-α antibody.

Western blot analyses. Proteins were separated on NuPAGE gels and blotted on PVDF membranes (Invitrogen). Western blot analyses were performed using anti-TEM antibody (Gentaur), anti-IκB-α antibody (Upstate) and anti-actin antibody (Sigma), combined with peroxidase-conjugated secondary antibodies. To strip and reprobe the membranes, the Restore Western Blot Stripping Buffer (Pierce) was used. Proteins were detected by chemiluminescence using ECL plus (GE Healthcare) and the ChemiDoc XRS system (Bio-Rad).

Ligated intestinal loop assay. The assay was performed as previously described (Goffaux *et al.*, 1997), with some modifications. All strains (EHEC 13369, 13369Δ*ospG*, 13369Δ*ospG* hosting the pABBospG plasmid, 13369Δ*escN*, and the non-EPEC/EHEC negative control strain T282) were tested in four 12-week-old rabbits (Charles River Laboratories). In each rabbit five loops were injected with the bacteria and one additional loop was injected with sterile culture medium. The rabbits were anaesthetized with isoflurane and euthanized after 9.5 h (two rabbits) or 12.5 h (two rabbits). The rabbits received one injection of buprenorphone (0.02 mg kg^{-1}) at the end of the surgery. After euthanasia the intestines were immediately removed and fixed with 4 % formalin/1 % glutaraldehyde, then trimmed and embedded in paraffin wax according to standard laboratory procedures. Transverse intestinal sections of 4 μm thickness were stained with haematoxylin and eosin and examined by light microscopy. All tissue sections were examined for congestion, oedema and infiltration of mononucleated cells and of heterophils in the mucosa and submucosa and for the height of intestinal villi with adherent bacteria. Eight microscopic fields were scored in a blinded manner by an experienced histopathologist. The protocol was approved by the Ethical Committee of the University of Liège (21 November 2006, N 563).

RESULTS

ospG homologues are present in *Enterobacteriaceae* species with different T3SSs

OspG was originally identified in *Shigella* spp. The *ospG* gene resides on the large virulence plasmids of *Shigella* spp. and codes for a T3SS effector protein that negatively controls the host innate immune response (Kim *et al.*, 2005). We identified *ospG* homologues in the draft genomic sequences of EHEC strain 11128 of serogroup O111 and EHEC strain 12009 of serogroup O103. While the *ospG* homologue of strain 11128 of serogroup O111 encodes a full-length protein, that of strain 12009 is likely a pseudogene with a 9 bp deletion at the 5′ end of the coding sequence. The presence of *ospG* homologues in EHEC strains suggested a wide distribution of *ospG* homologues in pathogenic bacteria. By searching in the DNA databases, we found additional *ospG* homologues in the genome of *Y. enterocolitica* strain 8081 and the plasmid of EIEC strain 53638 (Table 2). The deduced amino acid sequences of OspG homologues from *E. coli*, *Shigella* and *Yersinia* strains are highly conserved (>90 % identity to each other) and the three catalytic motifs involved in the autophosphorylation activity of OspG from *Shigella* (Kim *et al.*, 2005) are conserved (Fig. 1a). These OspGs are distantly related (about 30 % identity) to another T3SS effector family (Fig. 1b), which comprises NleH of *Citrobacter rodentium* (Garcia-Angulo *et al.*, 2008) and NleH1-1 and/or NleH1-2 of EHEC O157 strain Sakai, EHEC O111, rabbit EPEC

Table 2. *ospG* loci in some *ospG*-positive strains

Strain	Plasmid or genome	Full-length or pseudogene	Accession no.	Reference
E. coli				
EHEC 11128 (O111 : H–)	Genome	Full-length	AB438936	This study
EHEC 12009 (O103 : H2)	Genome	Pseudogene	AB438937	This study
EIEC 53638 plasmid p53638_226	Plasmid	Full-length	CP001064	–
Shigella				
S. flexneri virulence plasmid pWR100	Plasmid	Full-length	AL391753	Buchrieser *et al.* (2000)
S. flexneri 5a plasmid virulence plasmid pWR501	Plasmid	Full-length	AF348706	Venkatesan *et al.* (2001)
S. flexneri 2a str. 301 plasmid pCP301	Plasmid	Full-length	AF386526	Jin *et al.* (2002)
S. flexneri plasmid pSF5	Plasmid	Full-length	AY879342	–
S. sonnei Ss046 plasmid pSS_046	Plasmid	Full-length	CP000039	Jiang *et al.* (2005)
S. dysenteriae Sd197 plasmid pSD1_197	Plasmid	Full-length	CP000035	Yang *et al.* (2005)
S. boydii BS512 plasmid pBS512_211	Plasmid	Pseudogene	CP001062	–
Yersinia				
Y. enterocolitica 8081	Genome	Full-length	AM286415	Thomson *et al.* (2006)

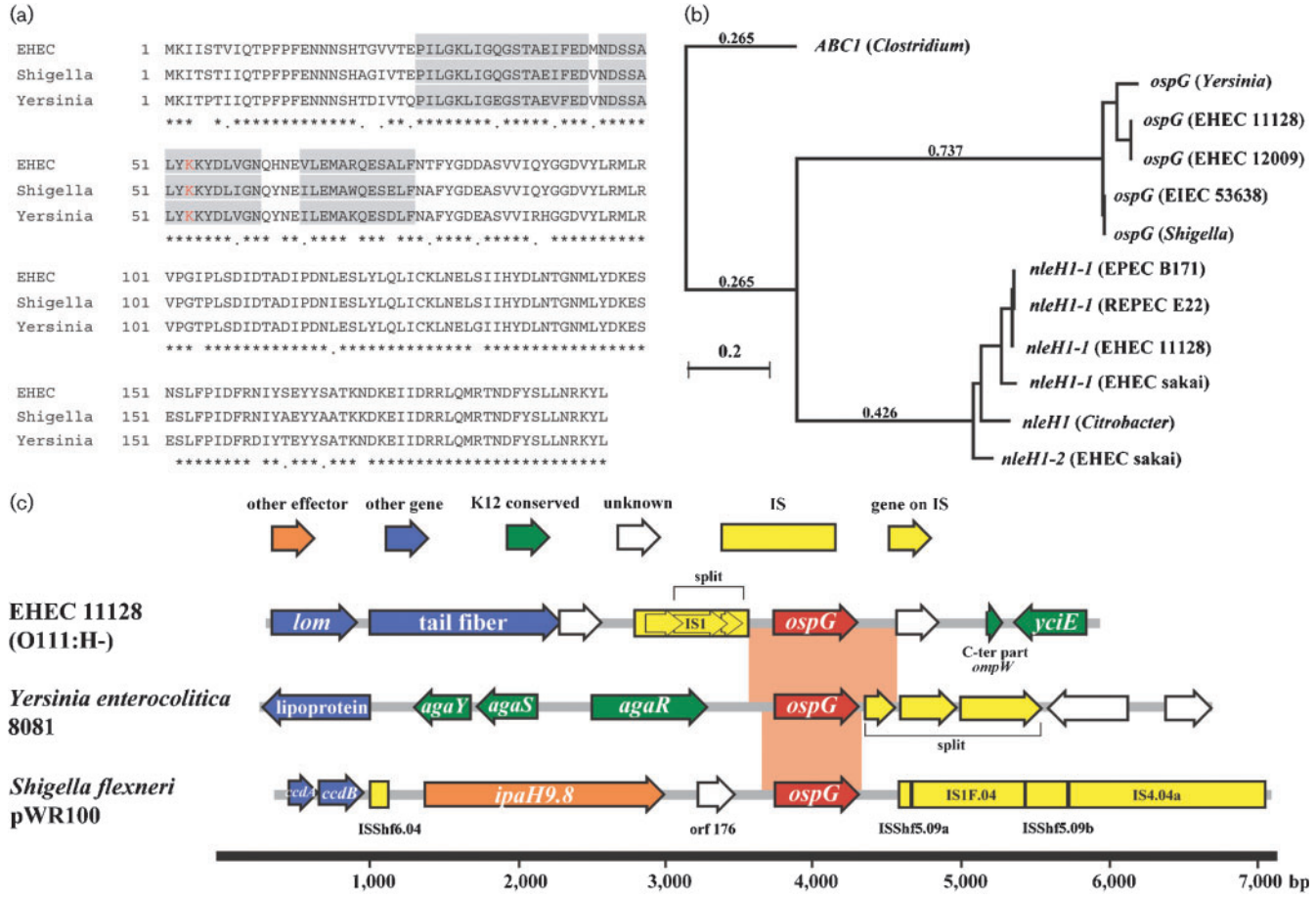

Fig. 1. *In silico* analysis of OspG effector gene distribution among EHEC O111, *Shigella* and *Yersinia*. (a) Alignment of deduced amino acid sequence of EHEC O111, *Shigella* and *Yersinia* OspGs. Asterisks and dots indicate identical and similar amino acids, respectively. Three conserved catalytic motifs are indicated by grey boxes. The residue K53 (shown in red) of *Shigella* OspG is involved in autophosphorylation activity of OspG (Kim *et al.*, 2005). (b) Phylogenetic analysis of *ospG* homologues. The following *ospG* homologues were analysed: *ospG* genes in *Y. enterocolitica* strain 8081 (accession no. AM286415), *S. flexneri* plasmid pWR100 (AL391753), EIEC strain 53638 plasmid p53638_226 (CP001064), EHEC O103 strain 12009 and EHEC O111 strain 11128 (both sequenced in this study); *nleH1* gene in *Citrobacter rodentium* (AY559032); *nleH1-1* genes in EHEC O111 strain 11128 (AB303061), EHEC O103 strain 12009 (AB303062), EHEC O157 Sakai (BA000007, ECs 0804), EPEC O111 strain B171 (AAJX01000049) and rabbit EPEC O103 strain E22 (AAJV01000058); *nleH1-2* genes in EHEC O157 Sakai (BA000007, ECs 1814). The *ABC1* gene of *Clostridium acetobutylicum* strain ATCC 824 (NC_003030), which shares a serine/threonine protein kinase catalytic domain, was used as the outgroup. (c) Organization of OspG-encoding region in EHEC O111, *Y. enterocolitica* and *S. flexneri*. The sequences of *Y. enterocolitica* strain 8081 (AM286415) and *S. flexneri* plasmid pWR100 (AL391753) were used for comparison. The regions indicated by orange shading showed over 80 % nucleotide-sequence identity among the three strains.

strain E22, and human EPEC strain B171-8 (Loukiadis *et al.*, 2008; Tobe *et al.*, 2006).

The *ospG* genes have likely spread in *Enterobacteriaceae* by horizontal gene transfers because the *ospG* genes of *Shigella* spp. reside on plasmids, those of EHEC on lambdoid prophages, and that of *Yersinia* on a genomic island. The EHEC prophages carrying *ospG* genes are both integrated in the *ompW* gene. Like other non-LEE-encoded effector genes, the *ospG* genes are localized in the EEL of the prophages (Fig. 1c). The *ospG* gene of *Y. enterocolitica* strain 8081 resides on a genomic island specific to *Y. enterocolitica*. Interestingly EHEC, *Shigella* and *Yersinia* *ospG* genes are always associated with IS elements, suggesting that IS-mediated mechanisms have been involved in the acquisition of the *ospG* genes by each mobile genetic element. The 5′ non-coding regions show >80 % nucleotide-sequence identity (68 bp sequence between EHEC, *Shigella* and *Yersinia* strains, and 130 bp sequence between EHEC and *Yersinia* strains) and the 3′ non-coding regions also show 80 % identity (240 bp sequences between EHEC and *Yersinia* strains) (Fig. 1c).

EHEC, *Shigella* and *Yersinia* OspGs are recognized and injected into mammalian cells by the EPEC T3SS

To test the T3SS-dependent secretion and translocation of EHEC, *Shigella* and *Yersinia* OspGs, each protein was cloned and fused to the reporter TEM (β-lactamase) enzyme. The plasmids encoding OspG-TEM fusions were introduced into rabbit EPEC strain E22 and its isogenic T3SS mutant (EPECΔ*escN*). Each OspG-TEM fusion protein was detected in the supernatants of the wild-type EPEC by Western blotting analysis, but not in the supernatants of EPECΔ*escN* (Fig. 2a), indicating that OspGs from EHEC, *Shigella* and *Yersinia* were recognized by the T3SS encoded by the EPEC LEE. In addition, the translocation of these OspG-TEM fusion proteins into infected HeLa cells was determined by monitoring the intracellular cleavage of a fluorescent β-lactamase substrate (Charpentier & Oswald, 2004). In this experiment, Tir and Cif were used as EPEC LEE-encoded and non-LEE-encoded effector controls, respectively. As expected, the TEM protein (negative control) was not translocated into infected HeLa cells whereas Tir-TEM and Cif-TEM translocation were readily detected (Fig. 2b). Each OspG effector protein fused to TEM was efficiently translocated into infected HeLa cells, as cleavage of the substrate was detectable 30 min after the onset of infection, reaching a plateau after 1.5 h (Fig. 2b and data not shown). The percentage of translocation of OspGs from EHEC and *Yersinia* was ~75 % compared to the Tir-TEM translocation level, whereas the translocation of *Shigella* OspG was significantly lower (~60 %) (Fig. 2b). These results indicated that OspGs from EHEC, *Shigella* and *Yersinia* can be secreted and translocated into host cells by the LEE-encoded T3SS.

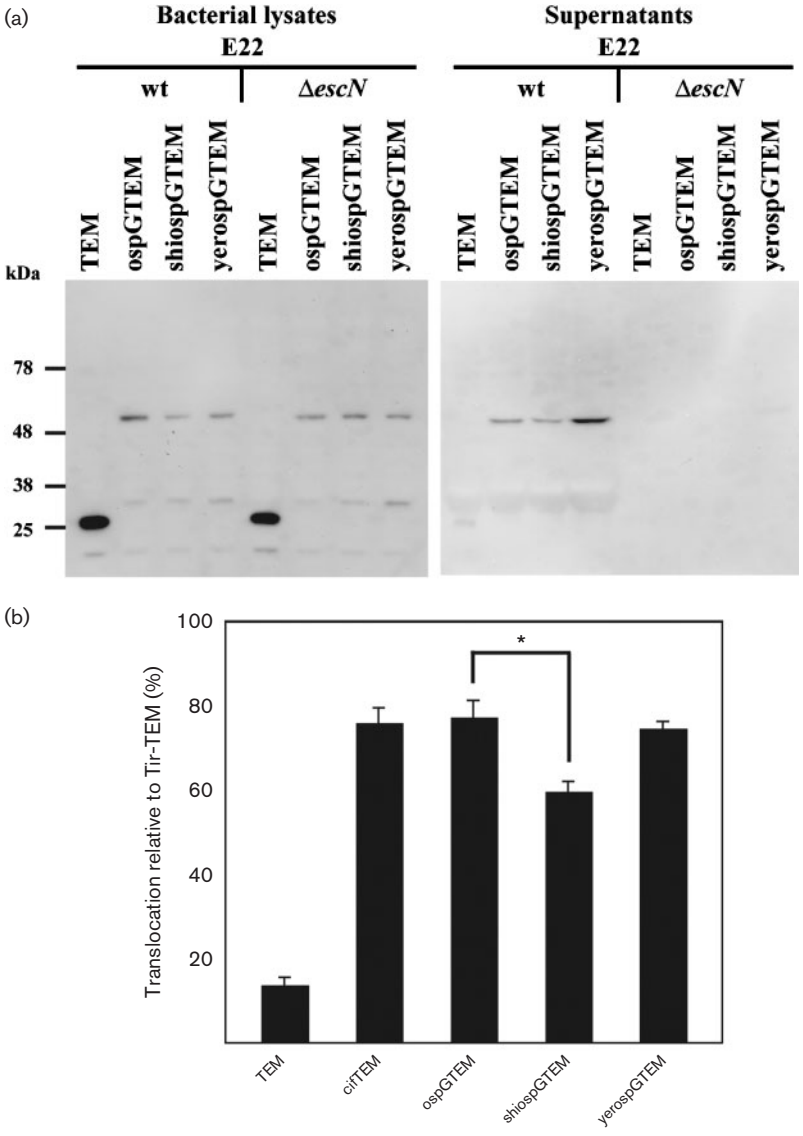

Fig. 2. OspG-TEM fusion proteins derived from EHEC O111, *Shigella* and *Yersinia* expressed in EPEC are efficiently secreted and translocated into cultured cells. (a) Plasmid-encoded OspGs-TEM fusion proteins were expressed in rabbit EPEC strain E22 (wt) or in the *escN* mutant. Bacterial lysates and supernatants were analysed by Western blotting with anti-TEM antibody. OspGTEM, shiospGTEM and yerospGTEM indicate OspGs-TEM fusion proteins derived from EHEC O111, *S. flexneri*, and *Y. enterocolitica*, respectively. (b) HeLa cells were infected for 3 h (m.o.i. 100) with E22 hosting the indicated plasmids. Plasmids encoding Tir-TEM or Cif-TEM fusion proteins were used as controls. The translocation levels are shown relative to that of Tir-TEM. The bars show the standard error of the mean (n=3). Asterisk indicates $P<0.05$.

Table 3. Distribution of *ospG* gene in an EPEC/EHEC collection

Serotype	No. of *ospG*-positive strains/total
Total	39/178
Full-length *ospG* gene	
O111	16/20
O26	1/63
O157	1/28
O103	1/32
Others	0/35
Total	19/178
***ospG* pseudogene (O103-type mutation)**	
O103	13/32
O157	5/28
O26	2/63
Others	0/55
Total	20/178

Intact *ospG* genes are mainly present in EHEC of serogroup O111

By testing a collection of 178 EPEC and EHEC strains of various serogroups with a primer set that amplified an internal *ospG* region, we found 39 *ospG*-positive strains (21.9 %) (Table 3). We further analysed these *ospG*-positive strains by PCR using two sets of primers: one amplifying the full-length gene and the other amplifying the pseudogene containing a 9 bp deletion at the 5′ end of the coding sequence, which was observed in the *ospG* gene of EHEC O103 strain 12009. This analysis revealed that about half the *ospG*-positive strains contain *ospG* pseudogenes. Among the strains bearing a full-length *ospG* gene, 84.2 % (16/19) were of serogroup O111. Most of the O111 strains were EHEC, as they were *stx*-positive. In contrast, *ospG* pseudogenes were mainly distributed in the O103 serotype (65 %, 13/20). Thus full-length *ospG* genes were mainly present in EHEC strains of serogroup O111.

OspG is translocated by EHEC O111 but is not required for attaching and effacing (A/E) lesion formation

We aimed at investigating the role of OspG in EHEC infection. Despite many attempts we were not successful in constructing mutants in the sequenced EHEC O111 strain 11128. Nonetheless we could generate T3SS (Δ*escN*) and OspG (Δ*ospG*) mutants in EHEC O111 strain 13369 (Ogura *et al.*, 2007). We confirmed the expression of the chromosomal *ospG* gene by RT-PCR (Fig. 3a). We next tested whether the EHEC OspG-TEM fusion protein was secreted in EHEC 13369 culture supernatants and translocated into infected host cells. The OspG-TEM was secreted

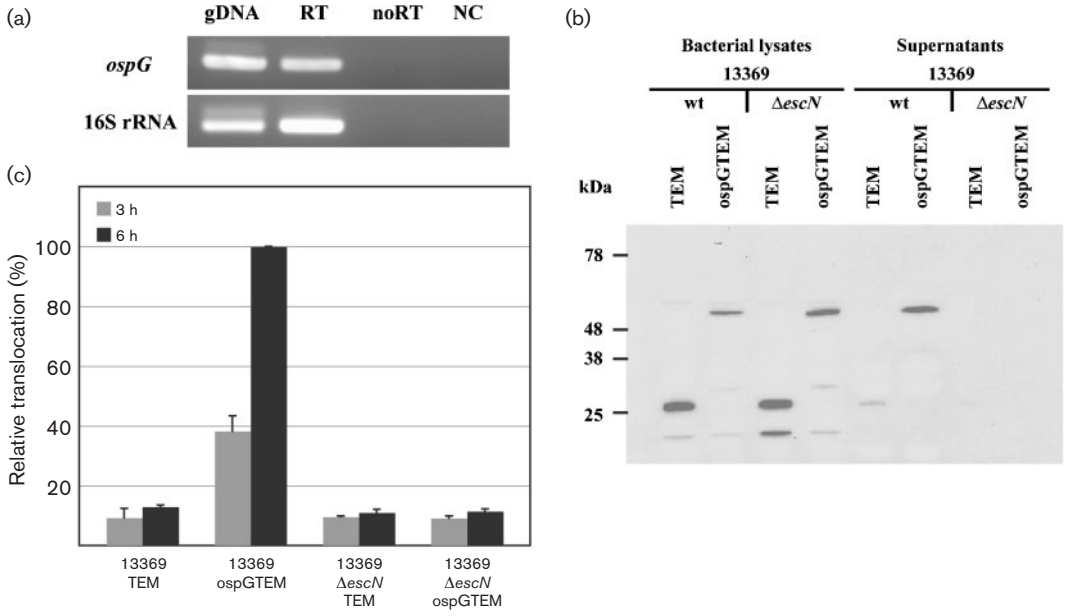

Fig. 3. Expression, secretion and translocation of OspG by EHEC O111. (a) Transcription of *ospG* in EHEC O111 strain 13369 was assayed by RT-PCR. In lane 'noRT' the RT step was omitted and in lane 'NC' (negative control) the template was omitted. 16S rRNA was used as a control. (b) Plasmid encoded OspG (from EHEC O111)-TEM fusion protein was expressed in EHEC O111 strain 13369 (wt) or in the *escN* mutant. Bacterial lysates and supernatants were analysed by Western blotting with anti-TEM antibodies. (c) HeLa cells were infected for 3 or 6 h (m.o.i. 50) with EHEC O111 strain 13369 (wt) or the *escN* mutant hosting plasmids encoding TEM or OspG-TEM. The translocation levels are shown relative to the maximum OspG-TEM translocation level. Bars show the standard error of the mean (n=3).

in a T3SS-dependent manner, but the amount of secreted protein was lower compared to that observed in EPEC E22 (Fig. 3b and data not shown). In infected HeLa cells, translocation of the OspG-TEM was detectable at 3 h after infection, but a 6 h infection was required to reach the full translocation level (Fig. 3c). These results indicated that OspG is a bona fide T3SS effector protein in EHEC. We next tested whether OspG is required for EHEC to form the A/E lesion in infected host cells. RK13 cells expressing the actin-GFP fusion protein were infected with EHEC strains. After a 6 h infection, F-actin rearrangements beneath adherent bacteria were observed with all strains except the Δ*escN* mutant, but no qualitative difference was observed among the wild-type, the Δ*ospG* mutant and the Δ*ospG* mutant complemented by a plasmid-encoded *ospG* (not shown). Thus OspG was not required for formation of the A/E lesion.

Transfer of NF-κB to the nucleus during infection with EHEC O111 and its mutants

It has been reported that *Shigella* OspG binds to host cell ubiquitin E2, prevents the ubiquitinylation and degradation of phosphorylated IκB-α and subsequently reduces the activation of a reporter NF-κB-responding promoter (Kim *et al.*, 2005). The three catalytic motifs involved in the autophosphorylation activity of OspG are conserved in the deduced amino acid sequence of all the OspGs from *E. coli*, *Shigella* and *Yersinia* (Fig. 1a), suggesting that EHEC O111 OspG could have the same function. We infected HeLa cells for 1–6 h with EHEC strain 13369, the *ospG* and *escN* mutants, or the *ospG* mutant complemented with plasmid-encoded *ospG*, and then treated or not the cells with TNF-α for 30 min. Nuclear extracts were prepared from the cells and the p65 subunit of NF-κB was quantified by ELISA. Without TNF-α stimulation, we observed no difference in the levels of nuclear p65 between infected and non-infected cells (data not shown). Following a 3 h infection with the wild-type strain and TNF-α stimulation, the level of nuclear p65 was similar to that in non-infected cells without TNF-α treatment (Fig. 4a). Similar reduced levels of nuclear p65 were observed in cells infected with the *ospG* mutant and the *ospG* mutant complemented with *ospG* from EHEC O111 or from *S. flexneri* (Fig. 4a and data not shown). In contrast, the cells infected with the *escN* mutant were still able to respond to TNF-α stimulation, indicating that the inhibition of p65 nuclear translocation required a functional T3SS but not the OspG effector.

To confirm these results, we monitored the degradation of IκB-α in the cytoplasmic fractions of HeLa cells infected with the wild-type EHEC strain 13369 and the *escN* and *ospG* mutants. A 1 h infection with either strain followed by TNF-α stimulation induced degradation of IκB-α, whereas at 2–6 h after infection with the wild-type EHEC, the *ospG* mutant or the *ospG* mutant hosting the plasmid-encoded *ospG*, IκB-α remained detectable following TNF-α stimulation, indicating that IκB-α degradation was inhibited. In contrast, the *escN* mutant failed to inhibit IκB-α degradation induced by the stimulation with TNF-α (Fig. 4b). Taken together, these results indicate that unknown T3SS effector(s) was/were injected into host cells and inhibited the NF-κB signalling pathway.

Wild-type EHEC O111 and the *ospG* mutant induce similar mild inflammatory responses *in vivo*

We finally examined whether EHEC OspG could modulate the inflammatory response *in vivo*. We performed a rabbit ligated intestinal loop assay as detailed in Methods. In loops inoculated with the wild-type EHEC O111, the *ospG* mutant and the complemented *ospG* mutant, we observed similar mild lesions of congestion, oedema of the submucosa and infiltration of lymphocytes and/or granulocytes in all layers of the wall (especially in the mucosa and submucosa) compared to loops injected with sterile culture medium, or with the *escN* mutant or a control non-EPEC/EHEC strain (Fig. 5). We did not observe differences in the inflammatory response between the loops infected with the wild-type strain, the *ospG* mutant, and the *ospG* mutant expressing plasmid-encoded *ospG*. We also examined in this assay the rabbit EPEC strain E22 (which has no *ospG*) hosting plasmid pABBospG or the plasmid vector, but we did not observe any effect on the inflammatory response (data not shown). These results observed in the rabbit ligated intestinal loop model suggest that EHEC OspG by itself does not modulate the inflammatory response *in vivo*.

DISCUSSION

In this study we observed that OspG is a T3SS effector protein distributed in *Shigella*, *Yersinia* and *E. coli*. In *E. coli*, OspG appeared to be distributed mainly in EHEC of serogroup O111. To our knowledge it is the first T3SS effector that has been found to be shared by *Shigella*, *Yersinia* and EHEC. The *ospG* genes are carried by a lambda-like prophage in EHEC O111, on virulence plasmids in *Shigella* and on a putative pathogenic island in *Yersinia*, suggesting that different types of horizontal gene transfer contributed to the spread of the *ospG* genes in different *Enterobacteriaceae* species.

OspG was expressed by EHEC O111, and OspGs from EHEC, *Yersinia* and *Shigella* were injected into host cells through the EPEC or EHEC T3SS. It has been reported that *Shigella* OspG interferes with the host innate immune responses by binding to ubiquitin-conjugating enzymes. We observed, in HeLa cells infected with EHEC O111, an inhibition of the TNF-α-induced NF-κB subunit p65 nuclear translocation, associated with an inhibition of IκB degradation. This phenotype was clearly T3SS-dependent and similar to that observed in EPEC-infected Caco-2 cells (Ruchaud-Sparagano *et al.*, 2007). However, OspG was not required for EHEC O111 to block the activation of the NF-κB pathway in infected HeLa cells

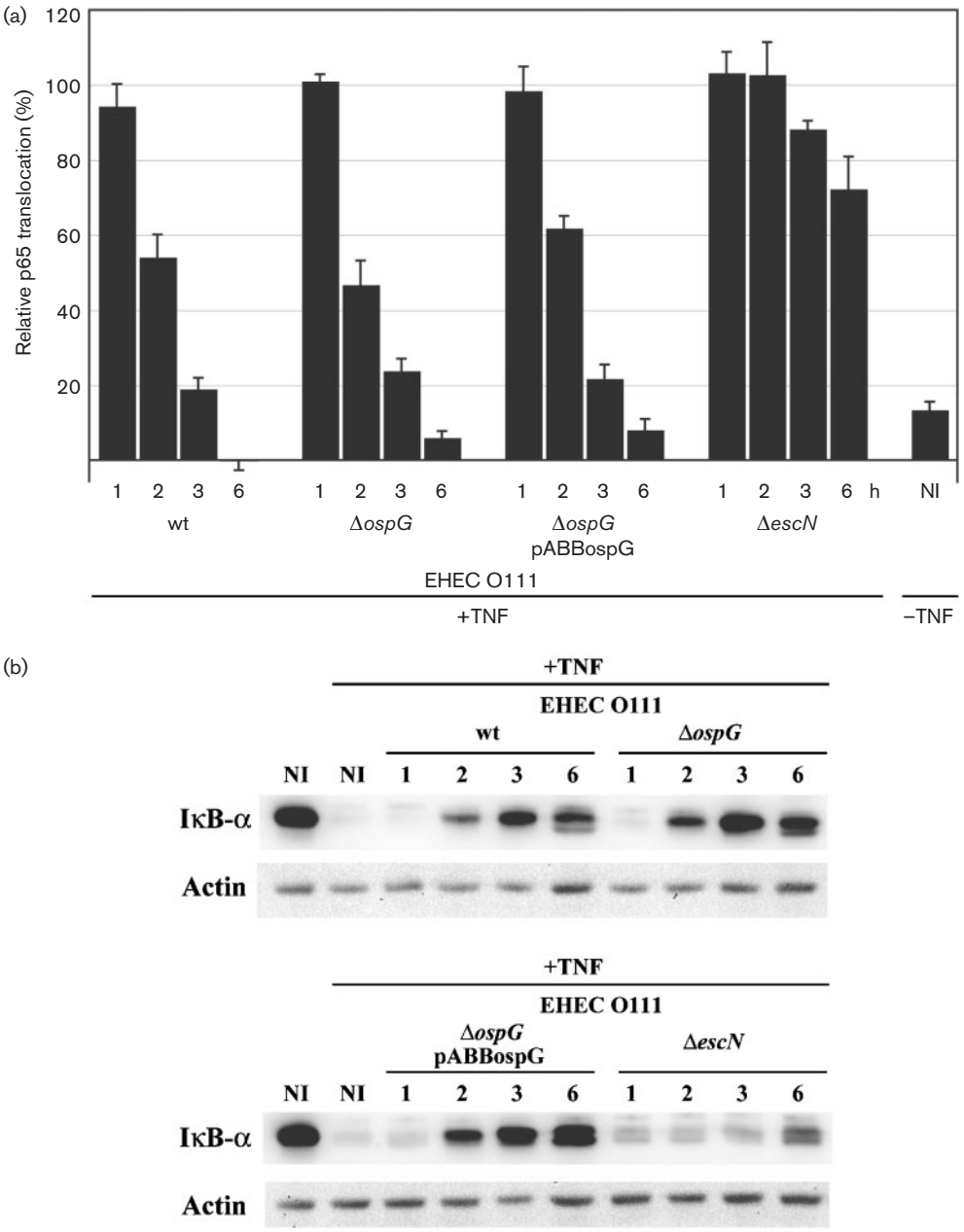

Fig. 4. Infection with EHEC O111 prevents TNF-α-induced NF-κB p65 nuclear translocation and IκB-α degradation. (a) HeLa cells were infected for 1–6 h as indicated with EHEC O111 strain 13369 (wt), the *ospG* mutant, the *ospG* mutant hosting the plasmid pABBospG, or the *escN* mutant. The results are shown relative to the translocation level in non-infected (NI) TNF-α-treated cells. Bars indicate the standard error of the mean (n=3). (b) IκB-α degradation following EHEC O111 infection and TNF-α stimulation. HeLa cells were infected for 1, 2, 3 and 6 h and stimulated with TNF-α for 30 min. NI, not infected.

stimulated with TNF-α. In addition, no difference in the inflammatory response was observed between wild-type EHEC strain and the *ospG* mutant in the rabbit ileal loop model. These observations strongly suggest that other unknown EHEC O111 T3SS effector(s) inhibited the NF-κB signalling pathway by suppressing IκB phosphorylation and/or by acting upstream of IκB degradation. Still, we can not exclude the possibility that EHEC OspG has a similar activity that would have been masked by the other putative effector(s). It would be interesting to test a *Shigella ospG* mutant complemented with the allele of *Shigella*, *Yersinia* and EHEC O111.

Tobe *et al.* (2006) described recently the extensive repertoire of T3SS effectors in EHEC O157: 39 of these effectors were confirmed experimentally, including two

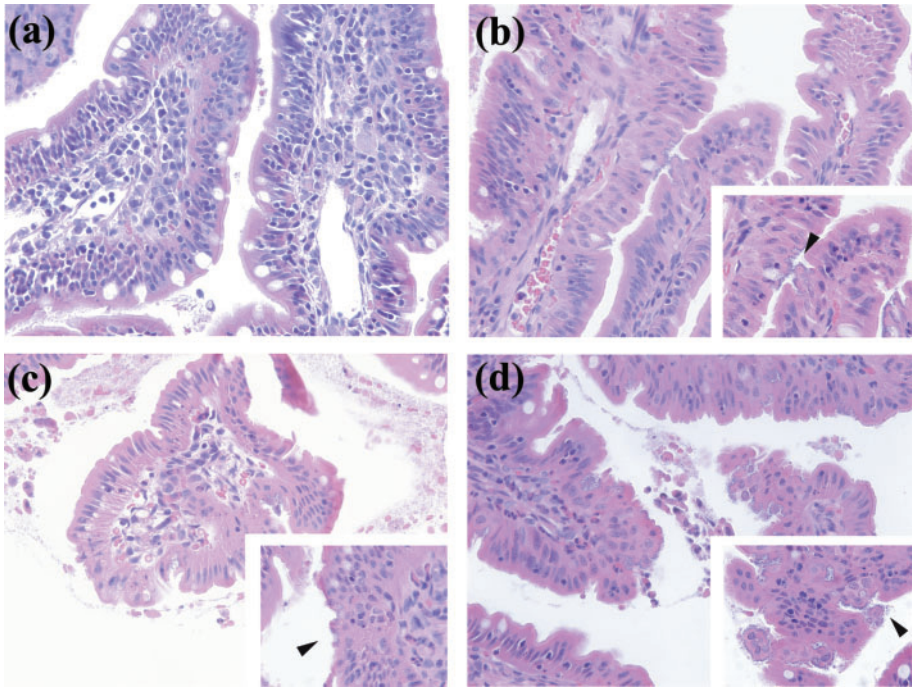

Fig. 5. Wild-type EHEC O111 and *ospG* mutant induce a similar mild inflammatory response in rabbit ileal loops. Rabbit ileal loops were inoculated with (a) non-EPEC/EHEC strain T282 (negative control), (b) EHEC strain 13369, (c) the *ospG* mutant and (d) the *ospG* mutant hosting the pABBospG plasmid. Tissue samples were examined by light microscopy at magnifications ×400 and ×1000 (insets). The arrowheads indicate adherent bacteria and effaced microvilli.

effectors distantly related to OspG, namely NleH1-1 and NleH1-2. The C-terminal part of NleH is 46 % identical to that of the *Shigella* OspG, which contains the three catalytic motifs involved in the OspG function. EHEC O111 also contains a *nleH1-1* homologue (Loukiadis *et al.*, 2008). However, Ruchaud-Sparagano *et al.* (2007) observed that IL-8 secretion by Caco-2 cells was still suppressed when the cells were infected with a CesT-defective mutant that is unable to produce the CesT protein, a chaperone required for NleH translocation. This suggests that NleH either is not involved, or is not the sole T3SS effector involved in the modulation of the host inflammatory response *in vitro*. More systematic analyses based on the complete genome sequence will be required to try to identify the gene(s) encoding the effector protein(s) that could be involved in the inactivation of the host innate system during infection with EHEC O111.

On the other hand, recent *in vivo* experiments indicated that NleHs from *Citrobacter rodentium* and EHEC have a role in the colonization of the gut (Garcia-Angulo *et al.*, 2008; Hemrajani *et al.*, 2008). In contrast with the *in vitro* results, NleH from *C. rodentium* causes an increase in NF-κB activity in the colonic mucosa of NF-κB reporter mice (Hemrajani *et al.*, 2008). The *S. flexneri* effector OspG negatively controls the host innate response induced by *S. flexneri* upon invasion of the epithelium (Kim *et al.*, 2005). However, the physiopathology of EHEC and *Shigella* infections is different. In contrast to *Shigella* and *Yersinia*, which invade and cause inflammatory destruction of the intestinal epithelium (Sansonetti, 2004), EHEC and EPEC colonize and adhere to the epithelium (Kaper *et al.*, 2004). Thus, it may be possible that the OspG effector protein has a role in the colonization of the gut. Further studies will aim at clarifying the role of OspG effector homologues acquired by horizontal transfer in different types of pathogens.

ACKNOWLEDGEMENTS

We thank Drs Yoshitoshi Ogura and Tadasuke Ooka for their help in the sequence analysis of the EHEC *ospG*-encoding regions, Drs Didier Marlier and Ioan Mihai Szalo, Mr Anastasio Poulipoulis of the Clinics for Birds, Rodents and Rabbits, Miss Sabrina Labrozzo, Mr Jean-Noel Duprez for their assistance during the rabbit experiments. Rika Nobe was supported by an INRA in-house fellowship and by the French Ministry of Agriculture. This study was supported by the European project EADGENE CT-2004-506416, and a grant-in-aid for Scientific Research on Priority Areas 'Applied Genomics'.

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Edited by: M. P. Stevens