Analysis of the expression, regulation and export of NleA–E in *Escherichia coli* O157:H7

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Previous work has shown that locus of enterocyte effacement (LEE)-encoded effector proteins such as Tir and Map can be exported via the type III secretion system (T3SS) of *Escherichia coli* O157:H7. Additionally, a family of non-LEE-encoded (Nle) effector proteins has been shown to be secreted from *Citrobacter rodentium*, homologues of which are located on the *E. coli* O157 chromosome. While NleA has been shown to be secreted from pathogenic *E. coli*, the secretion of other Nle effector proteins has only been detected under induced conditions, or using a mutated T3SS. This study aimed to determine: (1) which *nle* genes are expressed in *E. coli* O157:H7 under secretion-permissive conditions; (2) if Nle proteins are secreted from wild-type *E. coli* O157:H7 under secretion-permissive conditions; and (3) if *nle* gene expression is regulated co-ordinately with other LEE-encoded effectors. Using data generated from a combination of transcriptome arrays, reporter fusions and proteomics, it was demonstrated that only *nleA* is expressed co-ordinately with the LEE. Secretion and expression of NleA were regulated directly or indirectly by *ler*, a key activator of the LEE. MS confirmed the secretion of NleA into the culture supernatant, while NleB–F were not detected.

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INTRODUCTION

Many Gram-negative enteric pathogens export effector proteins into eukaryotic cells via a type III secretion system (T3SS). These include *Salmonella* species, *Yersinia* species, *Shigella* species, and enteropathogenic and enterohaemorrhagic *Escherichia coli* (EPEC and EHEC, respectively) (Buttner & Bonas, 2002; Coombes & Finlay, 2005; Cornelis, 2002; Galan, 2001; Journet *et al.*, 2005; Stebbins & Galan,

2003; Troisfontaines & Cornelis, 2005). The effectors have numerous functions, including inhibition of phagocytosis, invasion, cytotoxicity and bacterial attachment, mostly through effects on signal-transduction pathways (Bruckner *et al.*, 2005; Crane *et al.*, 2001; Garmendia *et al.*, 2005; Hapfelmeier *et al.*, 2005; Kenny *et al.*, 1997; Kim *et al.*, 2005; Knodler *et al.*, 2005; Nataro & Kaper, 1998; Patel & Galan, 2005; Yuk *et al.*, 2000; Zhang & Bliska, 2005). Our understanding of EPEC and EHEC pathogenesis has benefited from the study of the mouse pathogen *Citrobacter rodentium*, which also expresses a T3SS encoded from a homologous locus of enterocyte effacement (LEE), and this pathogen forms characteristic attaching and effacing lesions *in vivo* and in eukaryotic HeLa cells (Deng *et al.*, 2004; Gruenheid *et al.*, 2004; Mundy *et al.*, 2004b). Through an understanding of the regulation of LEE expression in *C. rodentium*, a number of putative type III (T3)-secreted effector proteins have been identified (Deng *et al.*, 2005). As the genes for these proteins are not encoded

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Abbreviations: eGFP, enhanced green fluorescent protein; EHEC, enterohaemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; GFP, green fluorescent protein; LEE, locus of enterocyte effacement; RFP, red fluorescent protein; T3, type III; T3SS, type III secretion system.

The array data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO series accession number GSE6296.

on the LEE, they are termed non-LEE-encoded effector (Nle) proteins. Seven putative effector proteins have been identified from *C. rodentium* and designated NleA–G. Subsequent research has established that NleA is conserved in many pathogens containing an LEE, including EHEC O157:H7 from which it is expressed, exported and localized to the Golgi apparatus (Gruenheid *et al.*, 2004; Mundy *et al.*, 2004a, b). Less is known about the other putative effector proteins, although the T3-dependent export of NleC and NleD can be induced from EPEC, and deletion of these genes in *E. coli* O157:H7, has no obvious impact on pathology in lambs (Marches *et al.*, 2005). More recently, an *nleB* mutant has been shown to be important for the colonization of mice by *C. rodentium*, and induced export of an NleB::Bla fusion from *C. rodentium* and EPEC has been shown to be T3-dependent (Kelly *et al.*, 2006). A recent study of proteins exported from an *sepL* mutant of *E. coli* O157:H7 has identified 39 potential effectors in the bacterial supernatant, and induced plasmid-based expression of these effectors from EPEC results in their delivery into host cells (Tobe *et al.*, 2006). Therefore, apart from NleA, the expression and secretion of the different Nle proteins from wild-type *E. coli* O157:H7 are unknown.

There is considerable variation in the levels of T3-associated proteins secreted from EHEC O157:H7 strains. This has been shown to be due, in part, to heterogeneous expression of the EspADB translocon apparatus (Roe *et al.*, 2003, 2004). The expression of the translocated intimin receptor (Tir) is co-ordinated with production of EspA filaments at the level of the single cell (Roe *et al.*, 2004). The molecular basis of this heterogeneity and whether it governs expression of effector proteins located outside the LEE are not known. The aim of the current study was to analyse NleA–F expression and secretion from *E. coli* O157:H7. To address this, we screened for Nle proteins in the supernatant of an *E. coli* O157:H7 strain capable of high-level secretion. This was carried out by tandem MS of bacterial supernatants, as well as the construction of full-length translational fusions of the Nle proteins to β -lactamase to allow detection by Western blotting. Through the construction of promoter fusions to both green fluorescent protein (GFP) and red fluorescent protein (RFP), and an analysis of global transcription, expression was analysed, as was co-ordination with EspA filament production. Expression of *nleA–F* in different genetic backgrounds and on contact with eukaryotic cells was also determined.

METHODS

Minimal media. Two defined media were used in this study: Dulbecco's Modified Eagle's Medium (D-MEM; Sigma D5671) and MEM-HEPES (Sigma M7278). The formulation of both media is available from the Sigma website (www.sigmaaldrich.com). Glucose was added to MEM-HEPES to give a final concentration of 0.2%. Antibiotics were included when required at the following concentrations: 12.5 μ g chloramphenicol ml⁻¹ and 25 μ g kanamycin ml⁻¹.

Western analysis. Bacteria were cultured at 37 °C in MEM-HEPES medium to OD₆₀₀ 0.8. Secreted proteins were extracted by TCA

precipitation as described previously (Roe *et al.*, 2003). For protein-localization experiments, whole-cell fractions were prepared by centrifugation (20 min, 4000 g), washing twice in 20 ml PBS, and final suspension in 400 μ l protein A buffer (Roe *et al.*, 2003). Proteins were analysed by SDS-PAGE, and Western blotting for β -lactamase was carried out as described by Karavolos *et al.* (2005).

MS analysis of secreted proteins. *E. coli* strain TUV93-0 was grown statically to mid-exponential phase (OD₆₀₀ 0.4) in MEM-HEPES supplemented with 2 mM L-glutamine at 37 °C in a 5% CO₂ atmosphere. Bacteria were pelleted by centrifugation in a swing-out rotor at 4000 g for 40 min at 4 °C. The clarified supernatants were then filter-sterilized by passing through 0.2 μ m low-protein-binding filters (Millipore) under a vacuum. Secreted proteins were then precipitated from the supernatant by the addition of TCA to 10% (v/v) final volume, and incubated at 4 °C overnight. Precipitates were pelleted via centrifugation in a swing-out rotor at 4000 g for 40 min at 4 °C. Pellets were then washed twice in 20 ml ice-cold acetone and pelleted before being allowed to briefly air dry.

Protein pellets were resuspended in Laemmli sample buffer before separation on a 12% SDS-PAGE Mini-PROTEAN III (Bio-Rad) gel. Resolved proteins were visualized using colloidal Coomassie blue stain (Genomics Solutions). The lane containing resolved secreted protein was excised from the gel and sliced horizontally into 25 equal slices of ~2.5 mm. These slices were then processed for MS, essentially as described by Batycka *et al.* (2006).

Liquid chromatography was performed using an Ultimate 3000 nano-HPLC system (Dionex; LC-Packings), comprising a WPS-3000 well-plate micro autosampler, an FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micropump, and an SRD-3600 solvent rack controlled by Chromeleon software. The monolithic column (200 μ m internal diameter $\times$ 5 cm; LC-Packings) was maintained at a constant 50 °C and was run at a final flow rate of 3 μ l min⁻¹. Samples of 4 μ l were applied to the column by direct injection. Peptides were eluted by the application of a 15 min linear gradient from 8 to 45% solvent B (80% acetonitrile, 0.1% formic acid) and directed through a 3 nl UV detector flow cell. The LC system was interfaced directly with a 3D high-capacity ion trap mass spectrometer (Esquire HCTplus; Bruker Daltonics) utilizing a low-volume (50 μ l min⁻¹ maximum) stainless steel nebulizer (Agilent), and electrospray ionization (ESI)-MS/MS analysis was initiated on a contact closure signal triggered by the Chromeleon software. *m/z* data were processed, and MASCOT-compatible files were created using DataAnalysis 3.2 software (Bruker Daltonics) with the following parameters: compounds (autoMS) threshold, 1000; number of compounds, 500; retention time window, 0.8 min. Searches were performed using MASCOT software (Perkins *et al.*, 1999; Matrix Science) and an in-house *E. coli* O157:H7 EDL933 database. The interpretation and presentation of MS/MS data were performed according to published guidelines (Taylor, 2005). The peptide and fragment mass tolerances were 2.5 and 0.8, respectively. Individual MS/MS spectra for peptides with a Mascot MOWSE (molecular weight search) score lower than 40 were inspected manually, and only included in the statistics if a series of at least four continuous y or b ions were observed.

Plasmid-based promoter fusion construction. Promoters for *nleA–E* were amplified from strain ZAP193 and cloned into pAJR70 to create pTD-1A-pTD7El (Table 1). Fig. 1 shows the putative promoter regions that were amplified and cloned to create the constructs. For *nleC* and *nleE*, only small intergenic regions are present 5' of the ATG codons. The close proximity to the upstream gene presented the possibility of these genes being co-transcribed as part of an operon. To address this, two constructs were created that utilized primers spanning both putative promoter locations (Fig. 1). As an additional reporter, the enhanced RFP gene (*rfp*) was cloned into

Table 1. Strains and plasmid constructs used in this study

Strain or plasmid	Description	Reference
Strains		
ZAP193	<i>Stx</i> −; NCTC 12900	Roe <i>et al.</i> (2003)
TUV93-0	<i>stx</i> − derivative of EDL933	Campellone & Leong (2003)
ZAP193Δ <i>escN</i>	Δ <i>escN stx</i> −	This work
ZAP1004	Δ <i>ler stx</i> −	Low <i>et al.</i> (2006)
Plasmids		
pAJR70	pACYC eGFP	Roe <i>et al.</i> (2003)
pAJR104	pACYC Bla	Karavolos <i>et al.</i> (2005)
pAJR145	pACYC rpsm::GFP +	Roe <i>et al.</i> (2004)
pDW16	pACYC RFP	This work
pTD-1A	<i>nleA</i> :: <i>gfp</i> /pAJR70	This work
pTD-2B	<i>nleB</i> :: <i>gfp</i> /pAJR70	This work
pTD-3CS	<i>nleC</i> :: <i>gfp</i> /pAJR70	This work
pTD-4CL	<i>nleC</i> :: <i>gfp</i> /pAJR70	This work
pTD-5D	<i>nleD</i> :: <i>gfp</i> /pAJR70	This work
pTD-6Es	<i>nleE</i> :: <i>gfp</i> /pAJR70	This work
pTD-7El	<i>nleE</i> :: <i>gfp</i> /pAJR70	This work
pTD-8AT	<i>nleA</i> :: <i>bla</i> /pAJR104	This work
pTD-9BT	<i>nleB</i> :: <i>bla</i> /pAJR104	This work
pTD-10CT	<i>nleC</i> :: <i>bla</i> /pAJR104	This work
pTD-11CLT	<i>nleC</i> :: <i>bla</i> /pAJR104	This work
pTD-12DT	<i>nleD</i> :: <i>bla</i> /pAJR104	This work
pTD-13ET	<i>nleE</i> :: <i>bla</i> /pAJR104	This work
pTD-14ELT	<i>nleE</i> :: <i>bla</i> /pAJR104	This work
pTD-15A	<i>nleA</i> :: <i>rfp</i> /pDW16	This work
pTD-16B	<i>nleB</i> :: <i>rfp</i> /pDW16	This work

pACYC184. Primer pair RFP5'/RFP3' (Table 2) was used to amplify the DsRed T3_S4T gene (Sorensen *et al.*, 2003). The resultant plasmid (pDW16) had single *Bam*HI and *Kpn*I sites 5' of the *rfp*+ gene, allowing promoters of interest to be cloned in-frame with the reporter gene. The promoters from *nleA* and *nleB* were cloned into pDW16 to create pTD-15A and pTD-16B (Table 1).

Analysis of bacterial fluorescence. To measure population fluorescence, triplicate 100 µl aliquots of bacteria were dispensed into 96-well black microtitre plates and analysed using a BMG Fluorstar plate reader at 37 °C. Cultures were also monitored at OD₆₀₀. For any combination of strain and medium, control cultures containing a promoterless plasmid were also assayed. At least three biological replicates were carried out for each experiment. Fluorescence was plotted against optical density using Microsoft Excel software and the line of best fit obtained. Using this method, data were corrected for background fluorescence. To measure single-cell expression by fluorescence microscopy, a Z-stack of 10 images was captured at a spacing of 0.1 µm on a Leica DMLB fluorescence microscope, using OpenLab software (Improvision). These images were used to create a composite image that reduced the spatial effects of bacteria in different focal planes. Bacteria were stained for EspA filaments following fixation with 4% paraformaldehyde for 5 min, as described previously (Roe *et al.*, 2003). The slides were then examined by fluorescence microscopy using appropriate filter sets, and the images were captured as above.

Expression on contact with EBL cell lines. Embryonic bovine cells (German Collection of Microorganisms and Cell Cultures, no. ACC192) were prepared and cultured as described previously (Roe *et al.*, 2004). The ZAP193 strain transformed with the appropriate

RFP reporter plasmids was cultured in MEM-HEPES to OD₆₀₀ 0.6 at 37 °C, added to the multichamber slide, and centrifuged onto the EBL cells (1000 g) for 5 min. The cells were fixed at intervals by removal of the culture and addition of 4% paraformaldehyde. Time points analysed were 0, 5, 30 and 180 min after addition. Fluorescence analysis using Openlab and Leica software was then performed as described above and previously (Roe *et al.*, 2004).

Plasmid-based translational fusions to β-lactamase. To allow the export of the Nle proteins to be assayed, translational fusions were created to β-lactamase. The region amplified for these fusions consisted of the promoter regions described in Fig. 1, but also covered the entire coding sequence. The PCR products were cloned into pAJR104 (Karavolos *et al.*, 2005) to create pTD-8AT–pTD-14ELT (Table 1).

Whole-genome array analysis. Preparation of mRNA, labelling, hybridization and analysis of array data were carried out as described by Zhang *et al.* (2004), with the following amendments. To provide the biological replicates, triplicate cultures of strain TUV93-0 were grown with shaking at 37 °C in MEM-HEPES or DMEM to OD₆₀₀ 0.6. Culture (15 ml) was mixed with 30 ml RNAProtect bacterial reagent (Qiagen), and an RNeasy minikit was used to prepare total RNA according to the manufacturer's instructions (Qiagen). Any contaminating DNA was removed using a DNase column kit (Qiagen). Total RNA was transcribed to Cy3- and Cy5-labelled cDNA, hybridized onto Corning GAPSII glass slides with the 6112 70-mer oligonucleotides of the Operon Array Ready *E. coli* set 1.0, as described by Zhang *et al.* (2004). The slides were washed and scanned, and data were analysed using Genepix and Genespring software as described by Zhang *et al.* (2004).

Fig. 1. Representation of the physical arrangement of the *nleA–E* genes in EHEC O157. The regions used to amplify putative promoters are indicated for each gene analysed, as well as the size of the PCR product. Both *nleA* and *nleF* were located on the same cryptic prophage (CP-933P). Furthermore, *nleB* (Z4328) and *nleE* (Z4329) were consecutive genes on the chromosome. For both *nleC* and *nleE*, two putative promoter regions were identified and amplified, as indicated.

RESULTS

Identification of secreted proteins by tandem MS

Shotgun proteome analysis of secreted proteins was employed as a sensitive means to identify proteins secreted from *E. coli* O157:H7 TUV93-0 in MEM-HEPES, a medium known to induce secretion via the EHEC T3SS (Roe *et al.*, 2003). Table 3 summarizes the highest MOWSE scores obtained for each of 10 recognized secreted proteins of *E. coli* O157:H7. Proteins detected in supernatants included seven proteins known to be secreted via T3SS (EspA, EspB, EspD, Tir, Map, EspF and NleA), as well as proteins secreted independently of T3SS (EspP, StcE and EhxA). For all proteins except NleA, multiple tryptic peptides (at least three) were detected, giving confidence in the identification from the tandem MS data (Table 3). Although the MOWSE score for NleA was below the significance threshold, closer examination of the ion data from tandem MS analysis indicated a continuous stretch of 7 aa represented by y or b

ions. This heptapeptide (GETPLTP) was searched against the entire NCBI database using BLAST with default settings. Despite the high probability of a random match, NleA/EspI represented top hits along with putative and hypothetical proteins from other bacterial genera, although the latter could be disregarded, since the sample material was a pure culture of *E. coli* O157:H7.

Identification of genes that are co-ordinately regulated with the LEE

In order to assess if the *nle* genes were expressed co-ordinately with the LEE, whole transcript array analysis was performed. Strain TUV93-0 was cultured in two minimal media, DMEM and MEM-HEPES, total RNA was extracted, and hybridization was performed as described in Methods. *E. coli* O157 strains ZAP193 and TUV93-0 were shown to secrete high levels of EspD and Tir effector proteins when cultured in MEM-HEPES, typically 5–10-fold higher than when the same strains were cultured in DMEM (data not shown).

Table 4 shows a selection of genes on the *E. coli* chromosome that are differentially regulated when cultured in MEM-HEPES compared with DMEM. The majority of genes are unaffected in their level of transcription, with 88% showing less than a twofold increase or decrease in transcription. Under the conditions used, 166 genes (~3% of the genome) showed significant ($P \leq 0.05$) upregulation, with the O157 LEE pathogenicity island showing marked changes. For the LEE, genes encoding basal apparatus (*escJ/escN*), translocon (*espA/espD/espB*) and effector proteins (*tir*) showed significant changes in transcription when cultured in MEM-HEPES (Table 4). Analysis of *nle* genes showed that *nleA–E* gave a hybridization signal when cultured in DMEM or MEM-HEPES (Table 4). *nleF* gave no hybridization signal and was not investigated further in this study. The data showed that *nleA* displayed the most marked (ninefold) and significant increase ($P = 0.001$) in transcript level when cultured in MEM-HEPES. Slight increases were detected for *nleB* (twofold), *nleD* (1.8-fold) and *nleE* (2.6-fold) (Table 4).

Analysis of *nleA–E* promoter expression in MEM-HEPES

In order to study the regulation of the *nle* genes in more detail, translational fusions were constructed by amplification and cloning of the relevant promoter region, native Shine–Dalgarno sequence and initial coding region into pAJR70. This produced in-frame protein fusions to enhanced GFP (eGFP) (Tables 1 and 2). These fusion constructs were used to analyse the expression profiles of the *nle* genes in liquid culture and during contact with bovine epithelial cells. *E. coli* O157:H7 strain ZAP193 was used for this work, as our previous research has characterized the expression of LEE-encoded factors in this background. When cultured in MEM-HEPES, both *nleA::gfp* (pTD-1A) and *nleB::gfp* (pTD-2B) fusions gave a high level of

Table 2. Oligonucleotide primers used in this study

Primer	Sequence (5'–3')	Plasmid constructs		
		GFP	RFP	TEM-1 β -lactamase
RFP5'	ccggatccccagatctccggtaccatggcctccaccgaggacgtc		pDW16	
RFP3'	ccggatccctacaggaacaggtggtg		pDW16	
AG3'	gcggtaccggtgattccagattgatggt	pTD-1A	pTD-15A	
AG5'	gcggtaccagagcattatgaagataacat	pTD-1A	pTD-15A	pTD-8AT
BG3'	gcggtaccgacatttaataagataacat	pTD-2B	pTD-16B	
BG5'	gcggtaccggccggagaaacgcagacagg	pTD-2B; pTD-7El	pTD-16B	pTD-9BT
CG3'	gcggtaccatgtttatatctaataacct	pTD-3Cs; pTD-4Cl		
CG5's	gcggtaccgcaggagagataattcaggtgc	pTD-3Cs		pTD-10CT
CG5'l	gcggtaccgcttatgcagagtctgtgtcg	pTD-4Cl		pTD-11CLT
DG3'	gcggtaccggacgtaggcgcatcaggac	pTD-5D		
DG5'	gcggtaccgaccgcgaagaatgagtt	pTD-5D		pTD-12DT
EG3'	gcggtaccgccctgagtattagtaacagg	pTD-6Es; pTD-7El		
EG5'	gcggtaccggtgcgatatgttcaatcgt	pTD-6Es		pTD-13ET
EGL5'	gcggtaccggccggagaaacgcagacagg	pTD-7El		pTD-14ELT
TEMA 3'	gcggtacctaccacatcctctgatttgg			pTD-8AT
TEMB 3'	gcggtaccctgaactgcaggtatacatac			pTD-9BT
TEMC 3'	gcggtaccctgattgtgtttgtccacatc			pTD-10CT
TEMD 3'	gcggtaccgaatggatgcagtcttacctg			pTD-12DT
TEME 3'	gcggtaccactgtctatttcccaggcat			pTD-13ET

expression in exponential phase and into stationary phase (Fig. 2a). In comparison, *nleC::gfp* (pTD-4l) and *nleD::gfp* (pTD-5D) were expressed at a 20-fold lower level (determined at OD₆₀₀ 0.6) than *nleA::gfp* (Fig. 2b). The reporter pTD-3Cs, which consisted of a 484 bp putative promoter region amplified immediately 5' of the *nleC* ATG was measured at <50 relative fluorescence units (RFU) throughout the growth curve. In addition, expression of either *nleE::gfp* construct (pTD-6Es and pTD-7El) was <50 RFU through the growth curve in this medium (Fig. 2b). To validate the microarray results for MEM-HEPES versus DMEM expression of *nleA* and *nleB*, the expression of the *nleA::gfp* and *nleB::gfp* fusions was measured in the two media. At OD₆₀₀ 0.6 (the same optical

density used for the microarray experiments), there was a 5.1-fold increase in *nleA* expression in MEM-HEPES compared to that in DMEM, and a 2.5-fold increase for *nleB*. These data are similar to the nine- and twofold differences, respectively, measured by microarray analysis (Table 4).

Previous work has shown that EspA filaments are produced only by a subset of cells cultured in MEM-HEPES, and that this expression is controlled post-transcriptionally (Roe *et al.*, 2003). This production of EspA filaments is also co-ordinated with the expression of the LEE5 genes *tir* and *eae* (intimin). As both *nleA* and *nleB* showed a high level of expression when cultured in MEM-HEPES by both

Table 3. Proteins detected in *E. coli* O157:H7 supernatants using tandem MS

Common name	Gene locus	Coverage (%)	No. peptides detected	MOWSE score
T3SS proteins				
EspB	Z5105	65	17	936
Tir	Z5112	52	18	833
EspA	Z5107	73	11	568
EspD	Z5106	34	7	406
Map	Z5111	19	3	126
EspF	Z5100	25	4	123
NleA	Z6024	2	1	26
T3SS-independent proteins				
EspP	L7020	39	35	1902
StcE	L7031	45	29	1606
EhxA	L7048	3	3	48

Table 4. Selected EHEC O157 genes differentially regulated in MEM-HEPES

The complete dataset is available on GEO (accession no. GSE6296). NA, Not applicable.

Common name	Gene locus	Putative protein function	Fold induction	P (t test)
<i>escJ</i>	Z5124	T3SS basal protein	6.4	0.07
<i>escN</i>	Z5119	T3SS ATP synthase	8.6	0.04
<i>tir</i>	Z5112	Translocated intimin receptor protein	9.7	0.001
<i>eaeA</i>	Z5110	Intimin adherence protein	12.2	0.06
<i>espA</i>	Z5107	Secreted translocon protein	11.2	0.02
<i>espB</i>	Z5105	Secreted protein	16.7	0.03
<i>espD</i>	Z5106	Secreted protein	16.3	0.01
<i>nleA</i>	Z6024	Secreted protein	9.0	0.001
<i>nleB</i>	Z4328	Secreted protein	2.0	0.1
<i>nleC</i>	Z0986	Secreted protein	0.7	0.09
<i>nleD</i>	Z0990	Secreted protein	1.8	0.01
<i>nleE</i>	Z4329	Secreted protein	2.6	0.05
<i>nleF</i>	Z6020	Secreted protein	Not detected	NA

Fig. 2. Expression of the *nle::gfp* constructs (pTD-1A–pTD-7El) in strain ZAP193 cultured in MEM-HEPES. The marked differences in expression levels were shown by plotting two separate graphs with different scales. (a) Expression of *nleA::gfp* (pTD-1A, ■) and *nleB::gfp* (pTD-2B, ●); (b) expression of *nleC::gfp* (pTD-3C, ○ and pTD-4C, ◆), *nleD::gfp* (pTD-5D, ▲), and *nleE::gfp* (pTD-6Es, □ and pTD-7El, ◇). All values were corrected for background by deducting the fluorescence value for ZAP193 transformed with pAJR70 (promoterless *gfp*) measured at the same optical density. Experiments were repeated at least three times with the data plotted being representative of the results obtained.

microarray and reporter gene assays, the possibility of co-ordinate regulation with the T3SS was addressed. Single-cell imaging of the fusions showed that *nleB::rfp+* was expressed in all the cells in the population (Fig. 3a), with no correlation to the expression of heterogeneous *EspA* filaments (data not shown). In contrast, *nleA::rfp+* expression was clearly heterogeneous, with only a subset of cells expressing *rfp+* when cultured in MEM-HEPES (Fig. 3b). Co-staining for *EspA* filaments showed that the subset of cells actively transcribing *nleA::rfp+* was also producing the T3SS filament required for protein export (Fig. 3c). These data indicate that *nleA* is co-ordinately regulated with the translocon of *E. coli* O157 at the single-cell level.

Given the co-ordinate expression of *nleA* and *EspA*, the effect of the LEE-encoded regulator on *nleA::gfp* expression was determined. There was a consistent twofold reduction in *nleA::gfp* expression at OD₆₀₀ 0.2–0.8 in a *ler* deletion mutant when cultured under T3 secretion-permissive conditions in MEM-HEPES (data not shown). This indicates either direct or indirect regulation of *nleA* by *ler*.

Expression of *nleA–E* on contact with EBL cell lines

The expression of the *nleC*, *nleD* and *nleE* reporter constructs in MEM-HEPES gave rise to the hypothesis that the culture conditions being tested did not contain the environmental signals required for the activation of their respective promoters. In order to test this hypothesis, the interaction of the promoter fusions was analysed during contact with eukaryotic cells. *E. coli* O157:H7 ZAP193 was transformed with the *nle::gfp* fusion plasmids (pTD1A–7El, Table 1), cultured in MEM-HEPES, and added to EBL cells. At different time points 0, 30 and 180 min, the supernatant was removed, the samples were fixed, and bacteria were

Fig. 3. Single-cell microscopy of strain ZAP193 containing pTD-15A (*nleA::rfp+*) and pTD-16B (*nleB::rfp+*). Bacteria were cultured in MEM-HEPES to OD₆₀₀ 0.6 and fixed in 4% paraformaldehyde, and slides were prepared (see Methods). For pTD-16B (*nleB::rfp+*; a), expression was equivalent in all bacteria within the population, in contrast to pTD-15A (*nleA::rfp+*; b) which showed a heterogeneous pattern of expression. Co-staining for EspA filaments (FITC, green; c) showed co-expression of EspA and *nleA* at the level of individual bacteria. The white boxes highlight a number of bacteria that could be identified by phase-contrast microscopy, but which did not express *rfp+* (b) or EspA filaments (c).

stained with fluorescently labelled anti-O157 antibodies to allow identification by fluorescence microscopy. The expression of the six *nle::gfp* fusions was quantified over the time course as described in Methods. During the initial culture to OD₆₀₀ 0.6, *nleA::gfp* and *nleB::gfp* fusions gave the highest level of expression, with lower but detectable levels from *nleC* and *nleD* fusions. As described above,

nleA::gfp gave a heterogeneous expression pattern (Fig. 4a, b). On contact with EBL cells, the expression of *nleA::gfp* was markedly reduced, even within 30 min of contact with host cells (Fig. 4a). At 180 min, expression had fallen to <30 % of the initial level before cell contact. The *nleB::gfp* fusion showed a reduction in expression to 18 % of the starting value over the same time course. A control plasmid consisting of the *rpsM* transcriptional fusion to GFP+ (Roe *et al.*, 2004) gave consistent levels of expression throughout the course of the experiment (Fig. 4a). Constructs for *nleC*, *nleD* and *nleE* showed no stimulation on contact with EBL cells, indicating that the environmental signals from the EBL cells, or physical contact with eukaryotic cell surfaces, were not stimuli for their expression (data not shown).

nleA-E::β-lactamase fusion analysis

To allow investigation of the production and potential export of the Nle proteins, β -lactamase fusions to the whole proteins were constructed. These fusion proteins allowed the localization of the proteins to be investigated, including whether any export was dependent on T3 secretion. When cultured in MEM-HEPES, Western blotting for β -lactamase detected NleA::Bla, NleB::Bla, NleC::Bla, NleD::Bla and NleE::Bla in the bacterial whole-cell preparations (Fig. 5a). NleA::Bla and NleD::Bla were also detected in the supernatant fraction (Fig. 5b). Using an *escN* (T3SS ATPase) mutant, the export of NleA::Bla and NleD::Bla was shown to be dependent on the presence of a functional T3SS (Fig. 5c, e).

The regulatory cross-talk between the production of the LEE and the Nle proteins was analysed by examining the expression and export of NleA::Bla and NleD::Bla in a *ler* mutant. While the export of NleA::Bla and NleD::Bla was found to be markedly reduced in this background (Fig. 5f), the production of NleA::Bla and NleD::Bla in the whole-cell fractions was also reduced in the *ler* mutant when compared with that in the wild-type strain (Fig. 5d).

DISCUSSION

T3 secretory proteins play a critical role in the pathogenesis of many bacteria, with well-characterized pathogens such as *Salmonella typhimurium* being capable of secreting at least 19 distinct effectors via the *Salmonella* pathogenicity island (SPI)-1 system alone. In comparison, the suite of proteins known to be secreted by *E. coli* O157 has been somewhat limited, with only five LEE-encoded translocated effectors identified to date. However, a novel family, Nle (non LEE-encoded proteins), has been identified in studies of a *C. rodentium* Δ *sepL* mutant, with six clear homologues in *E. coli* O157 (Deng *et al.*, 2004). Using a Δ *sepL* mutant of *E. coli* O157, Tobe *et al.* (2006) have recently identified some 39 proteins, falling into over 20 separate families. These advances in identification of novel effector proteins raise the question of how the Nle family of effector proteins is regulated. If the effectors are secreted through the T3SS, the

Fig. 4. Expression level of *nleA* and *nleB* following bacterial binding to bovine epithelial cells. (a) Fluorescence of individual bacteria transformed with the *nleA::gfp* (pTD-1A, ■), *nleB::gfp* (pTD-2B, ▲) and *rpsM::GFP* + (pAJR145, ●) plasmids. A subpopulation of bacteria expressed *nleA::gfp*, and only these bacteria were analysed for fluorescence output. This proportion of *nleA::gfp*-expressing bacteria was determined over the time course of the assay (□). Slides were prepared at 30 min following cell contact, and stained using anti-O157 antibodies to allow rapid identification of bacteria on the EBL cell surface. A heterogeneous population of *nleA::gfp*-expressing bacteria at time 0 (b) showed marked down-regulation of expression 180 min after cell contact (c).

expectation would be that the expression of the effectors would be co-ordinated with that of the Esp translocation complex, as this is the physical apparatus required for

Fig. 5. Western analysis of the production and secretion of NleA–E β -lactamase fusions in strain ZAP193. Bacteria were cultured in MEM-HEPES, and whole-cell (a) and supernatant (b) fractions were prepared. Lanes: 1, NleA::Bla (pTD-8AT); 2, NleB::Bla (pTD-9BT); 3, NleC₁::Bla (pTD-11CLT); 4, NleC₈::Bla (pTD-10CT); 5, NleD::Bla (pTD-12DT); 6, NleE₁::Bla (pTD-14ELT); 7, NleE₈::Bla (pTD-13ET); 8, pUC18 positive control. In addition, the fusions for NleA::Bla and NleD::Bla were transformed into ZAP193 ΔescN and Δler backgrounds to examine cross-regulation with the LEE and the requirement for a functional T3SS in their export. (c) (ΔescN) and (d) (Δler) show the whole-cell fractions for pUC18 (positive control, lane 1), NleA::Bla (lane 2) and NleD::Bla (lane 3). (e) (ΔescN) and (f) (Δler) show the corresponding secreted proteins.

injection of the proteins into eukaryotic cells. In this work, we characterized the secretome of an *sepL* wild-type strain to see which Nle effectors (from the originally described NleA–F) were secreted in co-ordination with the complete T3SS. Using tandem MS, we observed many of the established proteins that have been shown to be secreted by EHEC O157. These included the Esp family of translocon proteins (EspA, B, D and F) (Abe *et al.*, 1998; Viswanathan *et al.*, 2004), the translocated intimin receptor, Tir (DeVinney *et al.*, 1999), the mitochondrion-associated protein, Map (Kenny & Jepson, 2000), secreted proteases EspP (Brunner *et al.*, 1997) and StcE (Lathem *et al.*, 2002) (both encoded on pO157), and enterohaemolysin (Schmidt *et al.*, 1995) (also on pO157). Using this wild-type strain, only NleA of the Nle

proteins was identified in the supernatants of *E. coli* O157 cultured in MEM-HEPES. Shotgun proteomics methods do not provide quantification of proteins, although levels may be inferred from data parameters such as the number of peptides detected for proteins; on that basis, NleA was secreted at very low levels compared to the LEE-encoded translocon (EspA, B, D) or effector (Tir, Map, EspF) proteins. These data suggested that either secretion of the other Nle proteins was at a very low level, or the culture conditions did not support expression or secretion of the effectors. We therefore analysed expression of the *nleA*-*F* genes using a combination of arrays and fusions in liquid media, and (for the fusions) during interaction with a bovine-derived eukaryotic cell line.

To analyse the expression of the *nleA*-*F* genes on a DNA microarray, expression conditions were used that were highly selective for upregulation of the LEE. The hypothesis was that non-LEE-encoded effector proteins would be upregulated in a co-ordinated manner with the LEE operon, as the T3SS is the mechanism required for effector protein delivery. The *E. coli* O157:H7 transcriptome of bacteria cultured in either MEM-HEPES or DMEM was compared. Transcript levels of LEE-encoded genes increased in MEM-HEPES, e.g. *escJ* (6.4-fold) and *espB* (16.7-fold). For the *nle* genes examined, *nleA* was markedly upregulated (nine-fold), which correlated well with its detection in the bacterial supernatant and likely cross-regulation with the LEE. Expression of *nleA* was then examined using an *nleA*::*rfp* promoter fusion that demonstrated co-ordinate expression with EspA filaments at the single-cell level. This result showed that a gene encoding an effector protein located outside the LEE locus is regulated co-ordinately with the physical apparatus for its delivery. In addition, we showed that NleA production and expression are dependent on the LEE-encoded regulator (*ler*). Therefore, NleA expression appears to be regulated by a two-stage process involving both LEE-encoded and non-LEE-encoded regulators.

We have demonstrated heterogeneous expression of a number of EHEC virulence factors, including EspA (Roe *et al.*, 2003), intimin, *tir* and *map* (Roe *et al.*, 2004), and now *nleA*, using fluorescent gene fusions. The molecular mechanism that controls this single-cell 'cross-talk' between multiple T3SS effectors and the translocon is known to be controlled by factors not encoded by the LEE (Roe *et al.*, 2003, 2004). The likely biological function of heterogeneous expression is to restrict expression of antigenic factors such as type 1 fimbriae or T3SS needle EspA filaments, and also to prevent co-expression and therefore physical interference between surface factors on individual cells. Furthermore, co-ordination of EspA expression with effector proteins including NleA and Tir is logical, as this is the physical delivery system into eukaryotic cells. Indeed, a previous study has demonstrated the co-ordinated down-regulation of non-LEE-encoded factors on contact with eukaryotic membranes (Dahan *et al.*, 2004).

In contrast to *nleA*, the expression and export of *nleB*-*E* are less clear. The *gfp* promoter fusion to *nleB* showed high-level expression throughout the growth curve, but no exported protein was detected by MS. The β -lactamase fusion to NleB confirmed that no secretion was detectable and indicated that very little full-length protein was being produced inside the bacterial cell. Therefore, expression of NleB appears to be tightly controlled by a post-transcriptional mechanism under the conditions tested. While NleB has been shown to be required for the colonization of mice by *C. rodentium* (Kelly *et al.*, 2006), it has yet to be identified as a colonization factor for EHEC.

The expression and regulation of *nleC* and *nleE* can be summarized relatively easily; very-low-level expression was observed by the use of *gfp* and *bla* fusions, and no protein was detectable by MS. As gene expression was low, coupled with no detectable protein, we can conclude that under the conditions tested, these factors were produced either at a low level or not at all. It is interesting to note that a deletion of *nleC* in EHEC O157 has shown no difference in the colonization of a lamb animal model (Marches *et al.*, 2005).

NleD expression and export were the most complex, as MS analysis failed to detect this protein in the bacterial supernatant, even though a full-length NleD::Bla fusion was detected in the whole-cell and supernatant fractions. The difference is likely to be a result of the high sensitivity of Western blot analysis. The secretion of the β -lactamase fusion was shown to be dependent on a functional T3SS, and expression in the cell was reduced (twofold) in a *ler* mutant. One explanation for the low-level expression of *nleC*, *nleD* and *nleE* is a lack of appropriate signals in the liquid media used in this study. However, the finding that no additional activation of *nleA*::*gfp* was observed after contact with eukaryotic cells suggests that neither T3-permissive liquid media nor contact with EBL eukaryotic cells over a 3 h time period provides signals for activation. The same applies to *nleB*-*E*, as no further expression of the promoters was measured following contact of the bacteria with eukaryotic cells, compared to culture in MEM-HEPES. This lack of activation must be considered in the context of previous research that has demonstrated down-regulation of LEE-encoded factors on contact with eukaryotic cells (Dahan *et al.*, 2004; Roe *et al.*, 2004). In our study, expression of both *espJ* and *tccP* was shown to be increased by culturing *E. coli* O157:H7 in secretion-permissive conditions [*espJ* expression was increased threefold in MEM-HEPES ($P=0.16$) and *tccP* 2.4-fold ($P=0.007$)]. This co-ordinate expression agrees with earlier published data (Dahan *et al.*, 2004). It is not possible to rule out the activation of promoters by additional signals *in vivo*.

Work to date has focused on the localization of the NleA-D proteins and their effects on eukaryotic cells. These questions have been addressed in studies that have relied on overexpression of the proteins by exogenous promoters, coupled with creation of constructs to reporters such as

β -lactamase and haemagglutinin. Such systems ensure high-level expression of the protein coupled with sensitive detection by Western analysis, and have proved very valuable: for example, the subcellular localization of NleA in eukaryotic cells has been demonstrated using such technology (Gruenheid *et al.*, 2004). More recently, the repertoire of T3-secreted proteins has been expanded in EHEC O157 by analysis of an *sepL* deletion strain. SepL, along with SepD, is considered to act as a gating switch between translocon and effector-protein secretion. Mutation of *sepL* results in higher levels of effector-protein secretion, possibly by reducing competition for the export apparatus of EspA, EspB and EspD. In theory, this mutation then allows easier detection in the bacterial supernatant of effector proteins otherwise secreted at lower levels. A caveat with the *sepL* mutation approach is that the mechanism of SepL/SepD control of translocon secretion is unknown, and it is not possible to know whether the secretion profile of this mutant reflects that of the wild-type strain. Many of the targets identified have been overexpressed and detected in the bacterial supernatant, and furthermore can be exported into host cells using induced expression from EPEC as the delivery vehicle. Therefore, these proteins all have the potential to be effectors of *E. coli* O157 infecting host cells, but for the majority of these proteins, whether they are in fact expressed or secreted by wild-type *E. coli* O157:H7 remains to be determined.

In conclusion, we have confirmed that NleA is exported from EHEC O157, and that this is dependent on a functional T3SS. Furthermore, we have demonstrated that expression of *nleA* is heterogeneous under the conditions tested. Only a proportion of bacteria expressed *nleA*, as determined by a *gfp* fusion, and this subpopulation correlated with bacteria that produced EspA filaments. The evidence from this work implies that NleD is produced at a low level, and that this protein is exported in a T3-dependent manner. No evidence for production of NleB, NleC and NleE was obtained, but specific environmental stimuli may be required for their expression.

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