<table>
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<th>NOMBRE BECARIO</th>
<th>ACOSTA LÓPEZ CAMILA</th>
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<td>UNIVERSIDAD</td>
<td>IMPERIAL COLLEGE LONDON</td>
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<td>TITULO OBTENIDO</td>
<td>MSC IN APPLIED BIOSCIENCES AND BIOTECHNOLOGY</td>
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<td>TEMA DE TESIS</td>
<td>Caracterización de dos proteínas efectoras putativas de <em>Citrobacter rodentium</em>: NleN y NleO.</td>
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Characterisation of *Citrobacter rodentium* putative effectors NleN and NleO

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Acknowledgments

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Abstract

*Citrobacter rodentium* is a Gram-negative, mouse-restricted pathogen, commonly used as a murine infection model for clinically important human pathogenic strains of *Escherichia coli*, such as enterohaemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC). These pathogens primary infection strategy is the intimate attachment to the host’s gut epithelial cells, in order to translocate toxins, termed effectors. Intimate attachment is achieved through the formation of A/E (Attaching/Effacing) lesions, which depends on the Type 3 Secretion System (T3SS). The T3SS is the ‘injectosome’ through which effectors are translocated. Effectors target host cellular processes and signalling pathways in order to subvert immune responses, alter the cell cycle and allow bacterial proliferation. Despite the fact that many effectors have been characterised, the number of signalling pathways targeted and infection strategies displayed cannot be linked to all of them. This highlights the importance of characterising novel effectors and their relevance in the pathogenesis of these bacteria. Through translocation assays two putative novel effectors were found, termed NleN and NleO. Initial characterisation using bioinformatics analyses indicate that NleN and NleO share identities with hypothetical, uncharacterised proteins from *E. coli* pathogenic strains and are predicted to be non-cytoplasmic. Assays to visualise sub-cellular localisation showed that NleN exhibits perinuclear localisation while NleO gives rise to either a vesicular phenotype or cell death. Further analyses are needed for establishing the interaction partners of NleN and NleO, as well as their function and relevance in the pathogenesis of *C. rodentium*. 
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Introduction

*Citrofacter rodentium* is a Gram-negative, mouse-restricted pathogen that causes colitis and in some cases overgrowth of the gut epithelium (Petty *et al*., 2010). *C. rodentium* shares 67% of its genome, including several virulence genes, with pathogenic strains of *Escherichia coli*, such as enterohaemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC), which are clinically important human pathogens (Petty *et al*., 2010; Collins *et al*., 2014). EHEC causes enterohaemorrhagic colitis, haemolytic uraemic syndrome and can lead to kidney failure (Gruenheid *et al*., 2004), while EPEC accounts for high rates of infantile diarrhoea in developing countries, resulting in fatality in several cases (Collins *et al*., 2014). The primary infection strategy of *C. rodentium* and its human equivalents relies on the generation of attaching and effacing (A/E) lesions to colonize the gastrointestinal tract of the host, through which bacteria adhere closely to the host epithelium in order to translocate toxins, termed effector proteins (Clements *et al*., 2012; Mundy *et al*., 2005). These pathogens share the important structures and molecules needed to perform their infection strategies, including the Type 3 Secretion System (T3SS), the Type VI Secretion System (T6SS) and a number of effector proteins (Collins *et al*., 2014). As a result, *C. rodentium* is commonly used as a murine infection model for human pathogenic *E. coli* strains.

A/E lesions require the intimate attachment of the bacteria to the plasma membrane in pedestal-like structures together with the destruction of the microvilli (Clements *et al*., 2012). Formation of A/E lesions depends on proteins encoded on a pathogenicity island (PI) called the locus of enterocyte effacement (LEE), such as: EspA, which forms the needle portion of the T3SS, EspB and EspD, which form the translocation pore, the remaining components of the T3SS, together with intimin, gene regulators, chaperones and a number of translocated effectors (Clements *et al*., 2012; Collins *et al*., 2014; Petty *et al*., 2010). Effector proteins are translocated by the T3SS directly into the cytoplasm of the host cells (Figure 1), in order to modulate or subvert host immune responses and cellular functions (Deng *et al*., 2009). Effectors subvert several host signalling pathways, modify cellular structures and
interfere with protein translation (Teper et al., 2016). Some of the better characterised examples include; Cif, which induces detention of the cell cycle; EspB related to the inhibition of phagocytosis; EspF, which induces apoptosis and EspG, which disrupts cellular trafficking (Clements et al., 2011). The T3SS inserts the translocated intimin receptor (Tir) into the host cell. In this manner Tir forms a hairpin structure that interacts with the intimin protein in the bacterial outer membrane and initiates actin polymerisation in the site of attachment to form pedestal structures (Gruenheid et al., 2004), generating A/E lesions.

**Figure 1. Basic model of a Type III secretion system (T3SS).** Simplified representation of the T3SS. The T3SS is composed of over 20 proteins. The basal body is formed by the inner and outer membrane rings and periplasmic shaft, followed by EspA subunits that form the extracellular needle-conduct protein and finishing in the tip which is inserted in the host cell. Effectors are transported through the needle with the help of chaperone proteins and an ATPase located at the base of the complex (Burkinshaw & Strynadka 2014).

Effector proteins have also been identified on other genomic islands besides the LEE, which are also translocated by the T3SS (Collins et al., 2012). Bioinformatics analyses have suggested there are a great number of putative effector genes encoded outside the LEE; at present, 17 genomic islands (GI1-GI14) carrying several T3SS effectors have been identified (Petty et al., 2010). These non-LEE-encoded
effectors (Nles) are accountable for the variety of host processes that are modulated by A/E pathogens during infection (Echentkamp et al., 2008). Non-LEE encoded effectors are not required for the generation of A/E lesions but enhance colonisation efficiency (Collins et al., 2012). Most LEE-encoded effectors found in C. rodentium present a homologue in its E. coli counterparts, however each species or serovar can present a unique repertoire of effectors (Deng et al., 2009) and a great diversity has been seen for Nles. This could expand host range or contribute to an enhanced colonisation efficiency (Collins et al., 2012). The molecular architecture of effectors includes an N-terminal translocation signal and one or more functional domains (Teper et al., 2016), as well as a chaperone-binding domain to enable translocation (Mills et al., 2007).

Characterisation of putative effectors could help clarify the number of signalling pathways and infection strategies that A/E pathogens display, as well as the coordinate activities of these. Through collaborations, the Frankel group assessed 10 putative proteins for C. rodentium through translocation assays (Unpublished data, Figure 2). To analyse translocation, the effectors were fused to a β-lactamase (BlaM) reporter and introduced into C. rodentium strains to infect HeLa cells. CCF2, a fluorescent substrate of BlaM was added to the media. By monitoring infection in a plate reader, hydrolysis of CCF2 could be observed and directly related to the concentration of translocated effector in infected cells (Mills et al., 2007). Through this analysis, 2 proteins were found to be translocated: NleN and NleO (previously annotated as ROD_48841 and ROD_40891 respectively). NleN begins with an alternative start codon to methionine, hence a construct with the native start codon and a construct with a methionine start codon were analysed. NleN was shown to be translocated regardless of the start codon being used. NleN and NleO are located in the genomic islands described by Petty et al. (2010), GI14 and GI11 respectively, close to other known effectors such as: EspJ, NleH, NleG7, NleG8, EspO (Figure 3).
Figure 2. Translocation assays for putative effector proteins. (A and B) β-lactamase activity in HeLa cells was measured by the amount of CCF2 cleavage product. C. rodentium strains expressing tagged EspH was included as a positive control for translocation. A deletion mutant for EspA was used as negative control. ROD_48841 (NleN) and ROD_40941 (NleO) were found to be translocated via the T3SS (Frankel Group, Unpublished Data).

Figure 3. Diagram of the genomic islands GI11 and GI14. GI11 starts at position 4,316,630 and finishes at position 4,333,192. GI14 starts at position 5,146,469 and finishes at position 5,158,968 of the C. rodentium chromosome (Petty et al., 2010).
Aims

Owing to the important role of effectors in pathogenicity, identifying and characterising the functions of effector proteins and how their activity is coordinated is mandatory to understanding the molecular basis of pathogen-host interactions. The goal of this study was to begin molecular characterisation of putative effectors NleN and NleO through in vitro techniques. Bioinformatics analyses will be conducted with the nucleotide and amino-acid sequence of the effectors in search for homologies and conserved domains. Sub-cellular localisation of the proteins will be analysed through transfection and infections of HeLa cells with C. rodentium expressing tagged versions of the effectors. Analysis of possible interaction partners will be evaluated through two assays: yeast-2-hybrid (Y2H) and Magnetic Streptactin®XT pull down.
Materials and methods

Bioinformatics analysis

DNA sequences for NleN and NleO were analysed using bioinformatics tools to enable their initial characterisation. Homology searches and conserved domain identification was performed using Basic Local Alignment Search Tool (BLAST), Position Specific Iterated (PSI) BLAST, TBLASTX (Translated nucleotide) (Mount, 2004). Prediction of possible transmembrane regions was performed using Phobius (Käll et al., 2004) and MEMSAT3/MEMSAT-SVM (Nugent & Jones, 2012). Possible important domains or signatures were analysed through InterPro. 3D structure prediction was conducted using Phyre2 (Kelley et al., 2015) and PSIPRED (Buchan et al., 2013).

Molecular cloning

Genes encoding the putative proteins NleN and NleO were cloned into a number of plasmids to allow their initial characterization through transfection and infection. NleN and NleO sequences, as well as their flanking regions were amplified through PCR (The primers used in this study can be found in Appendix 1). The obtained plasmids were transformed into chemically competent E. coli Top 10 and BL21 cells. Clones were selected by growth in antibiotic resistant agar, according to each plasmid used. The positive clones were evaluated through colony PCR using OneTaq® DNA polymerase and sequenced for confirmation (GATC Biotech, Konstanz, Germany). Constructs for infection (Table 2) were transformed into electrically competent Citrobacter rodentium ICC169. Constructs for the Y2H assay were transformed into Saccharomyces cerevisiae AH109.
Table 1: Bacterial strains used for molecular cloning in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference/Source</th>
</tr>
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<tbody>
<tr>
<td>E. coli Top10</td>
<td>F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ⁺</td>
<td>ThermoFisher Scientific</td>
</tr>
<tr>
<td>E. coli BL21</td>
<td>B⁻ F⁻ ompT gal dcm ion hsdSa(rS⁻mS⁻) [malB¹]K⁻12(λ⁺)</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>C. rodentium</td>
<td>Spontaneous Nal⁺ derivative of ICC168 (wild type)</td>
<td>Petty et al. 2010</td>
</tr>
</tbody>
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Table 2: Constructs generated in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGBK7-NleN/pGBK7-NleO</td>
<td>Encodes the putative effector next to a GAL4 DNA binding domain</td>
<td>Yeast-2-Hybrid system for analysis of interacting partners</td>
</tr>
<tr>
<td>pGBT9-NleN/pGBT9-NleO</td>
<td>Encodes the putative effector myc-tagged</td>
<td>Transfection for observing sub-cellular localisation</td>
</tr>
<tr>
<td>pRK5-myc-NleN/prK5-myc-NleO</td>
<td>Encodes the putative effector 4xHA-tagged</td>
<td>Infection for confirming transfection data</td>
</tr>
<tr>
<td>pSA10:4xHA-NleN/pSA10:4xHA-NleO</td>
<td>Encodes the putative effector Strep-tagged</td>
<td>Infection for confirming transfection data and pull down</td>
</tr>
<tr>
<td>pGEMt-NleN+0.2kb flanking regions/ pGEMt-NleO+0.2kb flanking regions</td>
<td>Includes the putative effector with 200 bp up and downstream</td>
<td>Posterior replacement of the putative protein with a Kan⁺ cassette</td>
</tr>
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Cell culture

HeLa cells were grown in 75 cm cell culture flasks with 30 mL of Dulbecco’s Modified Eagle Medium (DMEM) (4500 mg/L glucose) (Sigma-aldrich, MI, USA) supplemented with 10% fetal calf serum (FCS), 1% GLUTAMAX (Thermo Fisher Scientific, MA, USA) and 1% non-essential amino acids (Sigma-Aldrich), at 37 °C, 5% CO₂ in a static incubator.

Transfection

HeLa cells were seeded at a density of 3.4 x 10⁴ cells/ml in 1 mL of HeLa growth media in a 24 well plate and incubated for 16 h at 37°C with 5% CO₂ (v/v). Cells were transfected at 60 – 70% confluency. After removing 0.6 mL of the culture media, cells received 0.25 µg of the respective DNA in 0.75 µl of GeneJuice (Novagen, WI, USA) and 20 µl of OptiMEM serum free medium (Life Technologies, NY, USA). Cells were incubated at 37°C for 3 h, 6 h or 19 h. Transfection efficiency was calculated by performing
a cell count of the cells stained with the different phenotypes observed for the effector versus the cell with a stained nucleus; a minimum of 100 cells were counted in each condition.

Infection for confirming transfection results

HeLa cells were seeded at a density of 2 x 10⁴ cells/well and the C. rodentium ICC169 (WT, pSA10-Strep-Tir, pSA10-Strep-NleN, pSA10-Strep-NleO, pSA10:4xHA-NleN, pSA10:4xHA-NleO) strains were grown for 8 h throughout the day in 3 mL LB (100 µg/mL Amp) at 37°C, shaking. After 8 h, 5 mL of DMEM (1000 mg/L L-glucose, no additives) were inoculated with 10 µl of culture and incubated overnight at 37°C, static. Bacterial culture was induced with 1 mL of 0.2 mM IPTG in DMEM (1000 mg/L L-glucose, no additives) for 1 h. Cells were washed once with warm PBS, medium was replaced with 1 mL of warm DMEM (1000 mg/L L-glucose, no additives) and 200 µl of induced bacterial culture was added. Plates were centrifuged at 500 x g for 5 mins to synchronise infection and incubated at 37°C, 5% CO₂, static for 2.5 h. The percentage of cell death was calculated by performing a life/death cell count and is presented in comparison with the WT strain.

Immunofluorescence and Imaging

The following protocol was conducted for transfection and infection coverslips. Coverslips were washed twice in warmed PBS and fixed for 20 mins in 4% paraformaldehyde (PFA) in PBS at room temperature. Coverslips were washed 3 x in PBS and PFA was quenched with 50 mM NH₄Cl for 15 mins. Coverslips were washed 3 x in PBS before adding 0.1% Triton-X-100 for 8 mins in order to permeabilise the cells. Coverslips were washed again 3 x in PBS before blocking for 30 mins in 3% bovine serum albumin (BSA) in a dark humid chamber. Cells were stained for 1 h with primary antibodies (listed in Table 3), washed 3 x in PBS and further stained with secondary antibodies for 1 h. Finally, coverslips were washed 3 x in PBS and once in water and mounted using ProLong Gold antifade mountant (Life Technologies, CA, USA). Images were acquired on an Axio Observer Z1. Image analysis was performed with FIJI (v 2.0.0).
Table 3: Antibodies used for immunofluorescence and western blot assays.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>1st/2nd</th>
<th>Origin</th>
<th>Species Specificity</th>
<th>Dilution</th>
<th>Target</th>
<th>Source</th>
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<tr>
<td>Phalloidin-iFluor 647 Conjugate</td>
<td></td>
<td></td>
<td>-</td>
<td>1:100</td>
<td>Actin</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>Myc Tag, clone 4A6 Primary</td>
<td></td>
<td>Mouse</td>
<td>-</td>
<td>1:200</td>
<td>Myc Tagged NleN and NleO Myc Tag</td>
<td>Merck Millipore, MA, USA</td>
</tr>
<tr>
<td>Mouse IgG Secondary Donkey Mouse</td>
<td></td>
<td></td>
<td>-</td>
<td>1:1000</td>
<td>Myc Tag</td>
<td>Jackson Immunoresearch, UK</td>
</tr>
<tr>
<td>Hoechst Dye</td>
<td>Dye</td>
<td>Mouse</td>
<td>-</td>
<td>1:1000</td>
<td>DNA Strep Tagged effectors Strep tag</td>
<td>GenScript, USA</td>
</tr>
<tr>
<td>Anti-Strep Primary Mouse</td>
<td></td>
<td></td>
<td>-</td>
<td>1:500 (IF)/1:1000 (WB)</td>
<td>DNA Strep Tagged effectors Strep tag</td>
<td>GenScript, USA</td>
</tr>
<tr>
<td>Anti-Mouse HRP Secondary Goat Mouse</td>
<td>Secondary</td>
<td>Goat</td>
<td>Mouse</td>
<td>1:100000</td>
<td>Strep tag</td>
<td>Jackson Immunoresearch, UK</td>
</tr>
</tbody>
</table>

**Magnetic Streptactin®XT Pull Down**

HeLa cells were seeded at a density of 4 x 10⁵ cells/mL, 3 mL/well in a 6 well plate and the different strains of *C. rodentium* ICC169 (WT, pSA10-Strep-Tir, pSA10-Strep-NleN, pSA10-Strep-NleO) were grown for 8 h throughout the day in 3 mL LB (100 µg/mL) at 37°C, shaking. After 8 h, 5 mL of DMEM (1000 mg/L L-glucose, no additives) was inoculated with 10 µl of bacterial culture and incubated overnight at 37°C, static at 5% CO₂. Bacterial culture was induced with 1 mL of 0.2mM IPTG in DMEM (1000 mg/L L-glucose, no additives) for 1 h. Cells were washed once with warm PBS, medium was replaced with 1 mL of warm DMEM (1000 mg/L L-glucose, no additives) and 500 µl of induced bacterial culture were added. Plates were centrifuged at 500 x g for 5 mins to synchronise infection and incubated at 37°C, 5% CO₂, static for 2.5 h.

After, 2.5 h, cells were washed 4 times in 2 mL PBS and 200 µl HEPES/Phosphate lysis buffer (HEPES 50 mM, NaCL 150 mM, 1% Triton X 100 and EDTA 5mM) per well were added and incubated for 30 mins on ice. Cells were scraped into 1.5 mL Eppendorf tubes and spun for 20 mins at 20,000 x g at 4°C. HEPES/Phosphate lysis buffer was used to equilibrate 20 µl of magnetic Streptactin®XT beads (IBA
GmbH, Göttingen, Germany) with 3 x 1 mL washes. After spinning, the sample was added and incubated end-to-end for 2 h at 4°C, 40 µl were kept for input control. After 2 h the samples were spun down and the supernatant was kept for flow through control. Streptactin XT beads were washed in 1 mL lysis buffer 4 times, for each wash beads were incubated for 1 min on ice followed by 1 min on the magnetic rack. The first wash was kept for control. Finally, the beads were boiled for 5 mins and the eluant was retained (final sample).

**Western blotting**

Expression test and pull down samples were run at 180 V for 1 h in 4-15% SDS-PAGE gels, before semi-dry transfer to PVDF membranes for 45 mins at 25 V. Membranes were then blocked in 3% BSA in 0.1% PBS-T at room temperature for 1 h then washed 3 x with 0.1% PBS-T. Membranes were incubated with anti-Strep antibody (Table 3) for 1 h, followed by anti-mouse HRP (Table 3) for 1 h. Antibodies were diluted in 3% BSA in 0.1% PBS-T and the membranes were washed 3 x with 0.1% PBS-T after each antibody. Western Blotting Substrate (Thermo Fisher Scientific) was applied to the membranes for 30 seconds before images were acquired on a FUJIFILM LAS-3000 imager.

**Expression/Induction assay**

Overnight cultures of *C. rodentium* strains (WT, pSA10-Strep-Tir, pSA10-Strep-NleN, pSA10-Strep-NleO) in 5 mL of LB, were induced for 1 h with IPTG (0.05 mM, 0.2 mM and 0.5 mM); an uninduced ('leaky' expression) sample was also retained. A 500 µl volume of each sample was pelleted, the supernatant was removed and pellets were re-suspended in 50 µl of Sodium Dodecyl Sulphate – Laemmli buffer (SDS-LB) (0.1% 2-mercaptoethanol, 0.0005% bromophenol blue, 10% glycerol, 2% SDS, 53 mM Tris-HCl (pH 6.8)) and boiled for 5 mins. Samples were kept at -20°C until SDS-PAGE electrophoresis.
Yeast-2-Hybrid

NleN and NleO sequences were cloned into bait plasmids pGBK7 (and later pGBT9) and resultant constructs were transformed in *S. cerevisiae* AH109. Autoactivation controls were performed by restreaking the bait strains transformants (pGBK7-NleN, pGBK7-NleO, pGBT9-NleN, pGBT9-NleO) onto Single Dropout (SDO) (Sabouraud dextrose (SD) – trp) and Double Dropout (DDO) (SD-trp-leu) plates by themselves and co-transformants (bait strains + pGADT7 empty) onto DDO and Quadruple Dropout (QDO) (SD-trp-leu-ade-leu). Evaluation of possible toxicity of the expression of NleO and NleN was conducted by recording a growth curve. Concentrated overnight culture of bait strains were prepared by inoculating 50 mL of SD – trp broth. Cultures were incubated at 30°C, shaking at 200 rpm for 16 to 20 h. The library strain was diluted up to 1 in 10⁶ for calculating the number of clones screened. Library and bait strains were combined (1 mL of library strain for 5 mL of bait strain) in 45 mL Yeast Peptone Dextrose Adenine (YPDA) media and incubated at 30°C for 25 h, shaking at 30 rpm. Using 40 µl from the mated culture, dilutions up to 1 in 10⁶ were plated, incubated at 30°C for 3 days and counted to determine mating efficiency. 200 µl of remaining culture was plated per QDO SD plate for up to 3 weeks.
Results

Bioinformatics analysis of NleN and NleO.

Bioinformatics analyses are a useful tool in the characterisation of proteins; homologues in close species can be found and functional domains and 3D structure can be predicted through them (Mount, 2004). BLAST analysis based on a BLOSUM-62 scoring matrix for pairwise alignment (Mount, 2004) provided the following results. BLAST analysis for the NleN translated nucleotide sequence yielded matches with hypothetical, uncharacterised proteins from *E. coli*, with sequence identities ranging from 52 to 58%. The top 20 hits included sequences isolated from human faecal samples, such as serovars O111:H8 from USA and O26:H11 from Australia. BLAST analysis for the translated NleO nucleotide sequence top 10 hits found matches with more than 90% coverage in sequence, hosting between 78% and 89% of identity and reflecting E-values from 3e-102 to 9e-129. The top 20 hits included sequences extracted from EPEC isolates from human faecal samples collected in Kenya, Mozambique, Gambia and Tanzania. Most of the hits identified for NleO, including hits with higher E-values and lower percentage of identity were hypothetical, uncharacterised proteins in *E. coli* or *Escherichia albertii*, which is an enterobacteriaceae species described after being isolated from diarrhoea in Bangladeshi children in 2003 (Abbot *et al.*, 2003). Interestingly, a match with an E-value of 7e-44 and 44% identities to an RTX toxin (RtxA) from *E. coli* TA280 was found. *E. coli* TA280 is another *E. coli* pathogenic strain (Broad Institute, USA). Results indicate that NleN and NleO effectors could have homologues in pathogenic strains of *E. coli*, highlighting the relevance of characterising novel effectors.

Transmembrane domain predictions characterise the physicochemical properties of a protein, indicating at the same time possible localisations and functions (Mount, 2004). Phobius predicted a 70% probability that NleN is non-cytoplasmic, as well as an 80% probability that NleO is non-cytoplasmic (Appendix 2). MEMSAT3/MEMSAT-SVM analysis predicted a transmembrane pore-lining helix formed by 15 amino acids, together with a cytoplasmic region in the N-terminus and an extracellular region in the C-terminus of NleN. MEMSAT3/MEMSAT-SVM analysis predicted 1 transmembrane helix together
with an extracellular region in the C-terminus and a cytoplasmic region in the N-terminus of NleO (MEMSAT/MEMSAT-SVM is part of PSIPRED protein structural analysis, see below). Both results suggest that both proteins are likely to be localised to plasma membranes.

Analysis of the 3D structure could lead more strongly the predictions for localisation and function of proteins by finding conserved domains, homologues and key features (Mount, 2004). PSIPRED analysis predicted 4 short β-strands and 3 coiled regions with high confidence (p < 0.001) for NleN. On the other hand, PSIPRED predicted 2 short β-strands and 3 α-helices with high confidence (p < 0.001) for NleO. PSIPRED analysis is based on PSI-BLAST and confidence is established by p-value calculations ranging from <1 to <0.0001 (Bioinformatics Group, UCL) (Appendix 3) (Buchan et al., 2013). Phyre2 could only model 16% of the sequence with 67.2% confidence for NleN, using an RNA polymerase subunit as a template, to yield a predicted helix and a coiled region (Figure 4). Other models obtained had lower confidence estimations. Phyre2 predicted a model with 45% confidence and 46% coverage of the sequence for NleO, using the crystal structure of ATP-dependant Clp protease subunit P from Francisella tularensis as a template, representing 2 β-strands and 3 α-helices (Figure 4). InterPro analysis could not predict any conserved domains, biological processes, molecular function or cellular component for either NleN or NleO.
Figure 4. Predicted 3D structure for NleN and NleO from Phyre2. Image is coloured by rainbow from the N to C terminus. A. NleN predicted structure has 67.2% confidence and covers 16% of the sequence (14 residues), the template used corresponds to a DNA-directed RNA polymerase subunit A, which is part of the crystal structure of RNA polymerase from *Thermococcus kodakarensis*. B. NleO predicted structure has 45% confidence and covers 46% of the sequence (89 residues), the used template corresponds to the crystal structure of ATP-dependent Clp protease subunit P from *Francisella tularensis*.

Sub-cellular localisation of ectopically expressed NleN and NleO.

Transfection allows us to introduce foreign DNA into a cell, to study gene expression, however transfection may not mimic the exact conditions in which such gene is expressed naturally. Transfection was carried out with pRK5-myc-effector constructs in HeLa cells for 3 different time points: 3 h, 6 h and 19 h. The phenotype from transfected effectors was compared against the phenotype shown by untransfected cells. Transfection of NleN showed no clear phenotype after 3 or 6 h (Figure 6 and 7). However, after 19 h, NleN showed a perinuclear localisation (Figure 8). The transfection efficiency for pRK5-Myc-NleN was 12.7%. NleO transfection showed 2 phenotypes in all of the time points: either staining could be observed in dead transfected cells (Figure 6), or staining was punctate and possibly vesicular (Figures 7 and 8). Transfection efficiency for pRK5-Myc-NleO was 17.6% from which 5.6%
presented vesicular staining and the remaining 12% of transfected cells appeared rounded and apoptotic.

**Figure 6. NleN and NleO transfection into HeLa cells after 3 h.** HeLa cells were transfected with pRK5-myc-NleN, pRK5-myc-NleO or untransfected as a control. Cells were labelled with immunofluorescent antibodies or dyes specific for DNA (blue), Actin (yellow) and the Myc-Tag (Red), channels are shown separately to visualise localisation of the effector, followed by an overlay.
Figure 7. NleN and NleO transfection into HeLa cells after 6 h. HeLa cells were transfected with pRK5-myc-NleN, pRK5-myc-NleO or untransfected as a control. Cells were stained for DNA (blue), Actin (yellow) and Myc-Tag (red), channels are shown separately to evidence localisation of the effector, followed by an overlay. Arrows indicate the vesicular staining for NleO.
Figure 8. NleN and NleO transfection into HeLa cells after 19 h. HeLa cells were transfected with pRK5-myc-NleN, pRK5-myc-NleO or not transfected as a control. Cells were stained for DNA (blue), Actin (yellow) and Myc-Tag (red), channels are shown separately to evidence localisation of the effector, followed by an overlay.

Analysis of translocation of NleN-Strep and NleO-Strep during infection.

Infection of HeLa cells was conducted to assess expression of the effector proteins in a setting that resembles closely the conditions in which they are naturally expressed. HeLa cells were infected with C. rodentium ICC169 wild type (WT), C. rodentium ICC169 Tir-Strep, C. rodentium ICC169 carrying pSA10:4xHA-NleN-Strep, C. rodentium ICC169 carrying pSA10:4xHA-NleO-Strep, C. rodentium carrying pSA10:4xHA-NleN and C. rodentium carrying pSA10:4xHA-NleO after 1 h of induction with 0.2mM IPTG, for 3 different time points: 1 h, 3 h and 6 h. C. rodentium ICC169 Tir-Strep was used as a positive control, since its translocation, as well as the observed phenotype through IF in culture cells has been well characterised (Frankel & Phillips, 2008). However, only DNA and actin were clearly visible under epifluorescence and no staining for the effector-Strep constructs or effector-Hemaglutinin (HA) could be visualised. Cell death was 50% higher after infection with the strains carrying the Effector-Strep.
constructs than with the WT strain, indicating that induction enhanced expression of the effectors. Cell death shows the effect of the *C. rodentium* infection in HeLa cells and its effectors. Lack of visualization of the Strep-tagged proteins could be due to the effectiveness of the antibody, for which the optimal experimental conditions have not yet been found.

**Analysis of possible interacting partners for NleN and NleO through a Y2H system.**

The Y2H system is based on the modularity shown by transcriptional factors, the ‘bait’ plasmid contains the GAL4 DNA binding domain followed by the gene of interest (pGBK7 and pGBT9) while the ‘prey’ plasmid contains the GAL4 activating domain followed by the interactor proteins (library clones) (pGADT7). When both proteins are produced and interact, transcription of the reporter gene is initiated (Crieke & Beyaert, 1999; Tucker et al., 2009). In order to include a selection method ‘bait’ plasmids are able to synthesize tryptophan while ‘prey’ plasmids are able to synthesize leucine. Simple transformants should grow only on (SDO) (SD–trp) plates by themselves and co-transformants (bait strains + pGADT7 empty), while co-transformants should grow only on DDO (SD-trp-leu). When performing the preliminary controls, we found that pGBK7-NleN caused autoactivation, allowing the yeast to grow in the absence of an interacting partner (Figure 9C), while pGBK7-NleO exhibited a growth defect (Figure 9B). Due to this drawbacks, effectors were then cloned in pGBT9, a different, low expression, bait plasmid for the yeast-2-hybrid system, and these constructs were then transformed into *S. cerevisiae* AH109. When performing the controls, pGBT9-NleN caused autoactivation of the system once more. On the other hand, pGBT9-NleO did not cause autoactivation and a growth curve was performed to ensure expression of the effector was no longer toxic upon expression (Figure 10). pGBT9-NleO was then used for mating with the yeast library strain, a mating efficiency of 6.15% was achieved screening 10⁷ clones from the library. Nonetheless, following 3 weeks after plating no colonies were obtained.
Figure 9. *S. cerevisiae* transformed strains plated onto selective SD agar plates for autoactivation controls. (A and B) *S. cerevisiae* strain carrying pGBK7 constructs was streaked onto SDO SD-agar, strain carrying pGBK7-NleO shows a growth defect. (C and D) *S. cerevisiae* strains carrying pGBK7 constructs together with pGADT7 empty plasmid, streaked onto QDO SD-agar. Growth on the plate labelled C indicates autoactivation of the system, in absence of an interacting partner.
Figure 10. *Growth curve for the different strains of S. cerevisiae generated.* A growth curve was recorded on the plate reader to evaluate possible toxicity of the expression of the effector protein for the yeast. Growth was measured through OD600, for 30 hours, in 60 (30 min) cycles.

**Analysis of possible interacting partners through a magnetic Streptactin®XT pull down.**

HeLa cells were infected with *C. rodentium* ICC169 wild type (WT), *C. rodentium* ICC169 Tir-Strep, *C. rodentium* ICC169 carrying pSA10:4xHA-NleN-Strep or *C. rodentium* ICC169 carrying pSA10:4xHA-NleO-Strep after induction of tagged effector protein expression for 1 h with 0.2mM IPTG. The Tir-Strep strain was used as a positive control for evaluating the antibody experimental conditions and as a well characterised effector protein. After a 2.5 h incubation, cells were lysed and whole cell lysate samples were pulled down using magnetic Streptactin®XT beads and analysed through western blots. We were not able to pull down tagged Tir for any of the samples. No specific bands were visible for the strains carrying the Strep-tagged effectors in any of the different samples taken throughout the pull down process: input, flow through, wash and elution. Lack of presence for Tir-strep bands in the input contradicts the results found for the expression assay (Appendix 4) and indicates flaws in the experimental parameters of the pull down.
Since the conducted infections with the different *C. rodentium* strains for both immunofluorescence and pull-down assays yielded little information, optimisation experiments to troubleshoot this problem were conducted. Expression conditions for tagged effectors in *C. rodentium* ICC169 wild type (WT), *C. rodentium* ICC169 Tir-Strep, *C. rodentium* ICC169 carrying pSA10:4xHA-NleO-Strep or *C. rodentium* ICC169 carrying pSA10:4xHA-NleO-Strep were assessed, post induction with different concentrations of IPTG (0.05mM, 0.2mM, 0.5mM) via western blot analysis. Expression of the tagged proteins was visible for the strains expressing the Tir and NleO constructs (Appendix 4).
Discussion

Several biochemical, bioinformatics and genetic screenings have been conducted to identify Type III secreted proteins (Burstein et al., 2015; Petty et al., 2010; Teper et al., 2016; Deng et al., 2010). Among studies with the same goal, translocation assays of putative effector proteins fused to β-lactamase were conducted via C. rodentium infection of HeLa cells and two novel effectors NleN and NleO were found to be translocated (Frankel Group, Unpublished data). Translocation indicates that the novel effectors contain the type III secretion and translocation signals required for transfer of the protein into the host cell during infection (Deng et al., 2010). \textit{nleO} encodes a 22 kDa protein, while \textit{nleN} encodes a 9.45 kDa protein. BLAST analysis showed that both effectors have homology to uncharacterised, hypothetical proteins in pathogenic \textit{E. coli} strains. These results, together with the location of both effectors in previously defined pathogenicity islands containing other known effectors (Petty et al., 2010), allows us to propose that the proteins are in fact related to the pathogenicity of \textit{C. rodentium}. Further studies could indicate how these effectors fit in infection strategies of this A/E pathogen.

\textit{NleO} was interestingly found to have homology with an RTX toxin, RTX refers to the glycine and aspartate rich repeats in the C-terminal of the toxin. Enteric pathogens often export different toxins as part of their pathogenicity. RTX proteins are a family of proteins that encompass a wide range of sizes (40 to 600 kDa) and molecular activities including pore-forming activity as well as secreted proteases and lipases (Linhartová et al., 2010). The most characterised and annotated RTX toxin is a 484 kDa protein from \textit{Vibrio cholerae}. It has been found responsible for cell rounding through the depolymerisation of stress fibres and cross-linking of actin rather than through the generation of a pore in the plasma membrane (Fullner & Mekalanos, 2000). Considering that NleO was found to have 44% homology with an \textit{E. coli} RTX toxin, it is logical to hypothesise that the cell rounding phenotype observed during our transfection experiments may be due to NleO acting in a similar way to an RTX toxin.
MEMSAT3/MEMSAT-SVM predicted a transmembrane helix for both NleN and NleO and Phobius predicted that both proteins are non-cytoplastic. These results point towards the effectors having an interaction with the membranous system of the cell. MEMSAT3/MEMSAT-SVM predicted a pore lining transmembrane helix for NleN. Nugent and Jones incorporated the pore-identification into MEMSAT-SVM, specifying “that pore-lining helices can be detected with an accuracy of 72%” (Nugent & Jones, 2012). However, the authors also mention that the existence of a low number of similar polypeptide sequences for the training set could limit the ability of the software (Nugent & Jones, 2012).

Phyre2 could only model 1 of the α-helices predicted by PSIPRED and a coiled domain with high confidence, representing only 16% of the sequence. On the other hand, for NleO Phyre2 could model 46% of the sequence, reflecting the structures predicted with high confidence by PSIPRED. However, the 3D predicted structures for both effectors exhibited less than 70% confidence. Phyre2, as well as PSIPRED is based on homology, comparing with the existing databases. If homology cannot be found between the query sequence and a sequence of known structure, modelling is unreliable. The existing methods cannot yet predict structure from sequence without any reference (Kelley et al., 2015). This indicates that the obtained models, aside from covering only a portion of the protein sequence, exhibit such confidence percentage due to the lack of similar models. Therefore, these 3D structure predictions reflect the secondary structure predicted by PSIPRED but do not any shed light on the effectors’ function or cellular activity.

While bioinformatics studies are a useful tool for initial characterisation, due to their inherent limitations and being that NleN and NleO sequences do not have a close model, experimental localisation assays can allow us to elucidate possible functions and activities of these effector proteins in eukaryotic cells. Nonetheless, in necessary to keep in mind that NleN and NleO are effector proteins naturally translocated through the T3SS, after a bacterial infection into gut epithelial cells; delivery system and conditions, which are not replicated through transfection assays. Staining for the
transfection of pRK5-Myc-NleN showed perinuclear localisation only after 19 h of incubation. Further differential staining with nuclear proteins such as histones, could indicate co-localisation of the effector and therefore provide an insight into its function. Nle effectors have been seen to target components of inflammatory signalling: NleE blocks translocation of the nuclear factor-kappaB (NF-κB); NleH1 and NleH2 are known to suppress inflammatory signalling and NleC is known to be involved in protein degradation. All of these Nle effectors have a role to prevent the activation of the expression of several cytokines. NleD, on the other hand inactivates the AP-1 transcription factor, which regulates processes like proliferation and apoptosis (Wong et al., 2011). Nuclear localisation of NleN may indicate its function could be related to blocking the transcription of proteins that activate the host immune response, blocking nuclear signalling pathways such as the ones previously mentioned, by interacting with other proteins or its function could be related, similar or coordinated with the other existing Nle effectors.

Staining for pRK5-Myc-NleO presented 2 phenotypes; the first being staining in dying cells, indicating a pro apoptotic effect. The second was vesicular staining in cells, which however, also appeared unhealthy; evidencing deformation of the cytoskeleton and nuclear condensation (Figures 7 and 8). Further analysis of the vesicular staining could be conducted to elucidate the function and origin of such vesicles. For instance, staining of the transfected effector, as well as the Golgi apparatus could indicate if the traffic of the effector through the cell includes this part of the membranous system. Vesicular staining could also be related to compartmentalisation as part of the apoptotic process (Moss et al., 2006; Omran, 2003; Patel et al., 2005). The evidenced phenotype does not show the intrinsic pathway of apoptosis that is triggered. However, late-stage apoptotic features such as cell shrinkage and nuclear condensation are evident in the stained cells. Apoptosis is a regulated pathway that induces cell death without eliciting immune response, pathogens modulation of this pathway could be targeted to eliminate immune cells or evade immune response (Weinrauch & Zychlinsky, 1999). Conversely, we must keep in mind that expression of the effectors from a multicopy plasmid rather than from the single
chromosomal copy of the gene could generate phenotypes different from the ones seen in native infection conditions (Gruenheid et al., 2004). Following a similar line, exogenous introduction of DNA to cells incurs in the effector being translated by host machinery and will be trafficked in the same way. During infection, effectors are translocated at the point of entrance of the T3SS in the host plasma membrane and localised further on, these differences could result in a divergent localisation, as seen for the EHEC effector EspG (Furniss et al., 2016; Clements et al., 2011). In addition, functional redundancy and overlapping functions are common in effectors roles during pathogenesis, some effectors are also able to perform multiple functions (Deng et al., 2010), which could result in a larger challenge to clarify the precise function of each effector.

Infections were conducted in the hope of confirming the phenotypes observed through transfection. However, immunofluorescent staining of infected HeLa cells did not show the expected phenotype. Due to time constraints only one set of growth conditions during infection were tested (DMEM 1000 mg/mL glucose at 37°C, 5% CO₂) and it is possible that NleN and NleO are not stable or properly expressed under the assessed conditions. It is important to bear in mind that culture conditions also do not fully mimic the native conditions in which host infection occurs, which could influence correct expression. Further optimisation of the used protocol is needed; the use of a more relevant cellular lineage could possibly improve the results. 3T3 Swiss albino cells are a murine cellular lineage, which could provide a better reflection of the native C. rodentium infection conditions over a human-derived HeLa cells.

Moreover, it has been demonstrated that the culture media can influence the type III secretion profile of C. rodentium and its homologues EPEC and EHEC. It has been shown previously that WT C. rodentium grown statically in DMEM (1000 mg/mL glucose at 37°C, 5% CO₂ (the conditions used in the present study), secrete translocators preferentially over effectors. Low-calcium concentrations on the other hand, inhibit the secretion of translocators but promote the secretion of effectors (Gruenheid et al.,
2004). Accordingly, different growth conditions and optimisation of the immunofluorescence staining against Strep-tagged effectors is needed to allow the visualisation of the tagged-effectors in cultured cells.

Subsequent to the experimental localisation assays, the next step in our initial characterisation was the identification of possible interaction partners, to relate the effectors to a specific pathway or molecule. However, no interaction partners were found following a Y2H screening and a magnetic Streptactin®XT pull down. Autoactivation of Y2H system was seen for both assessed constructs for NleN (pGBK7-NleN and pGBT9-NleN). Autoactivation occurs when the protein of interest, NleN in this case, enables the activation of transcription without the need of an interacting partner (Criekinge & Beyaert, 1999). As a result of this NleN constructs were not taken any further in this assay. On the other hand, some proteins become toxic when expressed in yeast, a non-native system, which could also account for the observed phenotype for pGBK7-NleO, for which a growth defect was observed. When pGBT9 effector expressing construct was used, toxicity was no longer evidenced, probably due to pGBT9 being a low copy plasmid resulting in less expression of the effector (Tucker et al., 2009). In spite of obtaining normal growth of the yeast strain, no colonies were retrieved after 3 weeks of incubation.

Following the yeast 2 hybrid, we took a different approach towards the identification of interacting partners. HeLa cells were infected with the C. rodentium strains containing the Strep-tagged effectors (NleN and NleO). As with the infection conducted for immunofluorescence assays, only one set of conditions was assessed and further optimisation of the protocol is needed. Besides the possible different sets of conditions, it is important to keep in mind that the best conditions for expression have not been obtained and in regular conditions small number of proteins are highly prevalent in the secreted proteins and this may obscure the sampling of less abundant proteins (Deng et al., 2010). NleN and NleO are of smaller size and could also be of lower abundance compared with other effectors. Even more so, it has been seen that C. rodentium is less efficient when infecting cultured cells that its
homologues EPEC and EHEC, therefore the infection protocol requires optimisation in order to gain a high enough expression level of NleN and NleO in the C. rodentium strains to be able to efficiently identify potential secondary interactors (Deng et al., 2010).
Conclusion

Our results indicate that NleN and NleO are located in previously defined pathogenicity islands in the *C. rodentium* chromosome and have homology to proteins found in *E. coli* pathogenic strains. Bioinformatics results indicate a possible interaction with the membranous system of the cell through an α-helical transmembrane domain. NleO function could be related to the generation of a rounding and pro-apoptotic phenotype, according to the BLAST results and phenotype observed through transfection. Transfection results suggest NleN may have a nuclear localisation, which could indicate a possible function altering nuclear signalling pathways related to host immune responses, apoptosis or others. NleO on the other hand, shows 2 phenotypes: staining in dead cells or in a vesicular pattern in stressed cells that could be due to compartmentalisation. NleO pro-apoptotic condition was also seen in the Y2H screening assay, when using a high expression plasmid. Further analysis should be conducted to elucidate the function and relevance in the pathogenesis of *C. rodentium* of these two effectors.
Future Work

In order to confirm the phenotype observed by transfection, different sets of conditions should be assessed to ensure the optimisation of the protocol. When found, optimal conditions should be used to find interaction partners by a second magnetic Streptactin®XT pull down, followed by mass spectroscopy analysis of the specific bands confirmed prior by western blot.

Other methods to determine the 3D structure of the effectors should be considered, X-ray crystallography for example, could be used for determining the arrangement of the aminoacids in the polypeptide chain. It could confirm the secondary structures predicted through bioinformatics and modelling the region of the sequences that do not have an existing close template. In this manner, following the determination of structure, analysis of it could clarify the function of these proteins.

The in vivo role of NleN and NleO has not been defined, due to time constraints the generation of the deletion mutant achieved the generation of clones containing the effector sequence with its flanking region. Completion of the generation of a deletion mutant for each effector through lambda red recombination is needed to establish the role of the effectors in vivo, via infection of mice. The deletion mutants should be used to assess changes in the colonisation of the host. Infection of macrophages could also be performed to evaluate the possible role in subverting the host immune responses.
References


Furniss, R.C.D. et al., 2016. *Enterohaemorrhagic E. coli modulates an ARF6:Rab35 signaling axis to prevent recycling endosome maturation during infection,*


### Appendices

#### Appendix 1. Table A.1. Primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Tm°</th>
<th>GC%</th>
<th>Sequence (5'-3')</th>
<th>Application</th>
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<tr>
<td>NleN_upstream_F</td>
<td>63.1</td>
<td>47.6</td>
<td>GACACATCCAGGCAATTTAC</td>
<td>For cloning NleN with its flanking regions into pGEMt</td>
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<tr>
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Appendix 2

Figure A.1. Graphical results for the Phobius predictions of NleN and NleO. (A) Phobius results for NleN indicate the effector is non cytoplasmic. (B) Phobius results for NleO indicate the effector is non cytoplasmic.

Appendix 3

Figure A.3. PSIPRED Secondary structure predictions for NleO. Confidence is based in p-value calculations from p < 1 to < 0.00001.
Figure A.3. PSIPRED Secondary structure predictions for NleO. Confidence is based in p-value calculations from p < 1 to < 0.00001.
Appendix 4

Figure A.4. Western blot obtained to analyze the expression of the effector-strep constructs. L: Color protein Standard. 1: NleO-Strep sample. 2: NleN-Strep sample. 3: Tir-Strep sample. 4: WT sample. A clear band is observed for Tir-Strep of around 80 kDa.

Figure A.5. Western blot obtained for the induction assay of the NleO-Strep constructs. L: Color Protein Standard. 1-4: WT induced samples (uninduced, 0.05 mM IPTG, 0.2 mM IPTG, 0.5 mM IPTG respectively). 5-8: NleO-Strep induced samples (uninduced, 0.05 mM IPTG, 0.2 mM IPTG, 0.5 mM IPTG respectively). Clear bands in lanes 6 to 8 indicate that induction is necessary for the expression of NleO.