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DOCTORAL SCHOOL IN BIOLOGY

Biology Applied to Human Health

XXVII DOCTORAL PROGRAM

Functional characterization of SslE, a novel protective antigen involved in *Escherichia coli* translocation of mucosal surfaces

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ABBREVIATIONS

AIDS: acquired immune deficiency syndrome
BBB: blood-brain barrier
BFP: bundle forming pili
BSA: bovine serum albumin
CAECAM: carcinoembryonic antigenrelated cell adhesion molecule
cDNA: complementary deoxyribonucleic acid
CDT: cytolethal distending toxin
CF: Colonization factors
CFU: colony forming unit
CNF-1: cytotoxic necrotizing factor 1
CpG: cytosine phosphate guanine
DAEC: diffusely adherent *Escherichia coli*
DAF: decay-accelerating factor
DNA: deoxyribonucleic acid
EAEC: enteroaggregative *Escherichia coli*
EAF : EPEC adherence factor
EDTA: ethylenediaminetetraacetic acid
Efb: extracellular fibrinogen-binding protein
EGTA: ethylene glycol tetraacetic acid
EHEC: enterohemorrhagic *Escherichia coli*
Ehly: enterohemolysin
EIEC: enteroinvasive *Escherichia coli*
EL: erythrocyte lysis
ELISA: enzyme-linked immunosorbent assay
EPEC: enteropathogenic *Escherichia coli*
ETEC: enterotoxigenic *Escherichia coli*
ExPEC: extraintestinal pathogenic *Escherichia coli*
FACS: fluorescence-activated cell sorting
FdeC: Factor adherence *E. coli*
FH: factor H
FITC: fluorescein isothiocyanate
FMLP: formyl-methionyl-leucyl-phenylalanine
GBS: group B streptococcus
GEMS: Global Enteric Multi-Center Study
GTP: guanosine triphosphate
HBSS: Hank's balanced salt solution
HC: hemorrhagic colitis
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV: human immunodeficiency virus

HSA: human serum albumin

HUS: hemolytic uremic syndrome

IBC: intracellular bacterial community

Ig: immunoglobulin

IgA-BP: immunoglobulin A-binding protein

IL: interleukin

InPEC: intestinal pathogenic *Escherichia coli*

IVIS: *In Vivo* Imaging System

LB: Luria-Bertani

LBSS: luminol-balanced salt solution

LEE: locus of enterocyte effacement

LP: lectin pathway

LPS: lipopolysaccharide

LT: heat-labile enterotoxin

MGL : mucus gel layer

mRNA: messenger ribonucleic acid

MUC: mucin

NAC: N-acetyl cysteine

NHS: normal human serum

NMEC: neonatal meningitis *Escherichia coli*

OD: optical density

OMP: outer membrane protein

ORF: open reading frame

PAI: pathogenicity island

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PE: protective efficacy

pEAF: enteropathogenic *Escherichia coli* adhesion-factor plasmid

pENT: enterotoxin-encoding plasmid

PLC: phospholipase C

PMN: polymorphonuclear leukocyte

RNA: ribonucleic acid

RPMI: Roswell Park Memorial Institute

RT-PCR: reverse transcription-polymerase chain reaction

Sat: secreted autotransporter toxin

SC: secretory component

SD: standard deviation

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM: standard error of the mean

SslE: secreted and surface associated lipoprotein for *E. coli*

ST : heat-stable enterotoxin

STEC: Shiga-toxin producing *E. coli*

Stx: Shiga toxin

TEER: Trans Epithelial Electrical Resistance

THB: Todd-Hewitt broth

TIR: translocated initmin receptor

TMD: transmembrane domain

TNF-α: tumor necrosis factor α

tRNA: transfer ribonucleic acid

T2SS: type II secretion system

TTP: thrombocytopenic purpura

UPEC: uropathogenic *Escherichia coli*

UTI: urinary tract infection

VF: virulence factor

Abstract

Pathogenic *Escherichia coli* are responsible for a heterogeneous group of disorders, including diarrhoea, urinary tract infections, sepsis and neonatal meningitis, that collectively cause significant morbidity, lost productivity and high healthcare costs. Considering the incidence and also the increasing antibiotic resistance of *E. coli* strains, the prevention of infections is of pressing concern from both the public health and economic perspectives. Since conventional attempts to develop a highly immunogenic, safe and polyvalent vaccine had failed, we decided to apply the reverse vaccinology approach for the identification of protective and broadly conserved vaccine antigens. Although some of the protective candidates have been previously described, most of them have just putative or hypothetical functions assigned and, therefore, their characterization could contribute to the understanding of *E. coli* pathogenesis. In this study, vaccine antigen EKO_K1 3385, formally known as SslE (Secreted and surface-associated lipoprotein from *Escherichia coli*) has been characterized.

By applying a number of *in vitro* bioassays and comparing wild type, knockout mutant and complemented strains, we have demonstrated that SslE specifically contributes to degradation of mucin substrates, typically present in the intestine and bladder. Mutation of the zinc metallopeptidase motif of SslE dramatically impaired *E. coli* mucinase activity, confirming the specificity of the phenotype observed.

SslE can be divided into two main variants, we proved that antibodies raised against SslE variant I are able to inhibit translocation of *E. coli* strains expressing a different variant through a mucin-based matrix, suggesting that SslE induces cross-reactive functional antibodies that affect the metallopeptidase activity. To test this hypothesis, we used well established animal models and demonstrated that immunization with SslE variant I significantly reduced gut, kidney and spleen colonization by strains producing variant II SslE and belonging to different pathotypes.

Furthermore by exploiting a human *in vitro* model of mucus-secreting cells, we demonstrated that bacteria expressing SslE have a metabolic benefit which results in an increased growth rate postulating the importance of this antigen in enhancing *E. coli* fitness.

The results presented in this work conclusively designate SslE as an important colonization factor favouring *E. coli* access to both metabolic substrates and target cells and reinforce the use of this antigen as a component of a protective vaccine against pathogenic *E. coli* species.

Riassunto

I ceppi patogeni di *Escherichia coli* sono causa di un ampio spettro di malattie, quali diarrea, infezioni del tratto urinario, sepsi e meningite neonatale, che, complessivamente, sono responsabili di elevati tassi di morbilità, perdita di produttività e ingenti costi sanitari. A causa della crescente incidenza delle infezioni provocate da questi ceppi e dell'aumento della resistenza antibiotica, la prevenzione delle infezioni riveste un'importanza sempre maggiore sia in ambito sanitario sia in ambito economico. Poiché gli approcci convenzionali si sono dimostrati inadeguati allo sviluppo di un vaccino polivalente, sicuro e altamente immunogenico, è stato scelto di applicare la "reverse vaccinology" per l'identificazione di antigeni protettivi e ampiamente conservati. Sebbene alcuni dei candidati selezionati siano stati in precedenza descritti in letteratura, alla maggior parte di essi è stata assegnata soltanto una funzione putativa o ipotetica, pertanto la loro caratterizzazione può costituire uno strumento utile all'elucidazione dei meccanismi di patogenesi. In questo studio, è stato caratterizzato l'antigene vaccinico EKO_K1 3385, noto formalmente come SslE (Secreted and surface-associated lipoprotein from *Escherichia coli*).

Utilizzando differenti test biologici e confrontando ceppi wild type, knockout e complementanti, abbiamo dimostrato che SslE contribuisce alla degradazione di substrati mucosi, tipicamente presenti nell' intestino e nella vescica. Mutazioni del motivo metallopeptidasico compromettono l'attività enzimatica di questa proteina, confermando il fenotipo osservato.

Esistono due diverse varianti di SslE, nel presente studio abbiamo dimostrato che anticorpi diretti contro la variante I possono inibire la traslocazione attraverso una matrice mucosa di ceppi di *E. coli* che esprimono la variante II, suggerendo che SslE induce anticorpi funzionali cross-reattivi che interferiscono con l' attività metalloproteasica. Per testare questa ipotesi, abbiamo utilizzato modelli animali ben validati e abbiamo dimostrato che l'immunizzazione con SslE variante I riduce in modo statisticamente significativo la colonizzazione del cieco, dei reni e della milza da parte di ceppi che producono la variante II e appartenenti a differenti patotipi.

Inoltre, utilizzando un modello *in vitro* di cellule umane che producono muco, abbiamo provato che SslE conferisce un beneficio metabolico ai batteri che la esprimono che porta a un aumentato tasso di crescita, postulando l'importanza di questo antigene nell' aumentare la fitness di *E. coli*.

I risultati presentati in questo lavoro designano in modo conclusivo SslE come un importante fattore di colonizzazione che favorisce l'accesso di *E. coli* sia ai substrati metabolici che alle cellule target, rinforzando l' uso di questo antigene come componente di un vaccino protettivo contro i ceppi patogenici di *E. coli*.

Chapter 1

Introduction and aims

Chapter 1

Introduction and aims

Escherichia coli is a gram-negative bacterium commonly found in the intestine of humans and other mammals. Most *E. coli* strains are harmless commensals, however, pathogenic *E. coli* strains also exist and these isolates are typically categorized based on their mechanisms of disease and clinical outcomes [1].

For many years, *E. coli* pathotypes have been studied in isolation instead of addressing *E. coli* as a single microorganism responsible for human and animal infections. Considering the increasing antibiotic resistance present among pathogenic strains, which is derived from an uncontrolled use of antibiotics in humans and in the veterinary field, vaccination is the most promising approach to control disease. Comparative genome analysis and reverse vaccinology led to the identification of nine antigens capable of inducing protective immune responses against ExPEC strains, several of which are also prevalent in different intestinal *E. coli* pathotypes [2].

The aim of my PhD stems from this previous research and by functionally characterize SslE (EKO_K1 3385), the most protective vaccine candidate identified during the screening, try to define the molecular mechanisms of *in vivo* protection.

1.1. *E. coli*: A Versatile Species

Theodor Escherich first reported the isolation and characterization of slender short rods from infant stool, which he named *Bacterium coli* commune, in his 1885 publication [3]. Over 125 years later, *Escherichia coli* is known as a harmless commensal of the gastrointestinal tract in warm-blooded animals and is used as the colloquial laboratory workhorse. The bacterial species *E. coli* is a member of the family Enterobacteriaceae, located taxonomically within the gamma subdivision of the phylum Proteobacteria [1,4].

Normally, *E. coli* colonizes the infant gut within hours of birth and establishes itself as the most abundant facultative anaerobe of the human intestinal microflora for the remainder of life, equipped with the abilities to grow in the ever-changing environment in the gut and cope with the mammalian host interaction. Nevertheless, *E. coli* can survive in many different ecological habitats, including abiotic environments, and is considered a highly versatile species. Known habitats of *E. coli* include soil, water, sediment, and food. However, there are several highly adapted *E. coli* clones that have acquired specific virulence attributes, which confers an increased ability to adapt to new niches and allows them to cause a broad spectrum of disease [1,5-9].

Pathogenic *E. coli* strains can be divided into intestinal pathogenic *E. coli* (InPEC) and extraintestinal pathogenic *E. coli* (ExPEC), depending on the site of infection. Both are further subcategorized into distinct pathotypes, defined as a group of strains of a single species with certain pathogenic traits. Pathotype classification is based on the clinical manifestation of disease, the virulence factors (VFs) involved, and the phylogenetic background. Among the intestinal pathogens there are six well-described categories: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC),

enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) [10]. UTIs are the most common extraintestinal *E. coli* infections and are caused by uropathogenic *E. coli* (UPEC). An increasingly common cause of extraintestinal infections is the pathotype responsible for meningitis and sepsis — meningitis-associated *E. coli* (MNEC) [11].

1.2. Evolution of pathogenic *E. coli*

As a population, *E. coli* strains can be assigned phylogenetically to 5 main clusters, i.e., A, B1, B2, D, and E, with *Shigella* forming a different group [12]. Commensal isolates mostly group in phylogroup A; however, not all *E. coli* pathotypes group together. Intestinal pathogenic *E. coli* strains derive from phylogenetic groups A, B1, D or from ungrouped lineages and are seldom found in the fecal flora of healthy individuals. These obligate pathogens are generally unable to cause extraintestinal disease and have evolved a special ability to induce colitis or gastroenteritis if ingested with contaminated food or water. Usually, mere acquisition of these bacteria by the naïve host is sufficient for disease to ensue. Each intestinal pathotype possesses a characteristic combination of virulence traits which allows the colonization of specific niches and results in a unique diarrheal syndrome (Figure 1-1) [13,14].

Unlike commensal and intestinal pathogenic *E. coli*, ExPEC strains belong predominantly to phylogenetic group B2 and, to a lesser extent, group D and have acquired various virulence genes that allow them to induce extraintestinal infections in both normal and compromised hosts. ExPEC are incapable of causing gastrointestinal disease, but they can asymptomatically colonize the human intestinal tract and may represent the predominant strain in approximately 20% of normal individuals. Therefore, although they are best known for their virulence behavior, ExPEC actually associate with the host primarily in a commensal fashion, causing disease only when they exit the gut and enter a normally sterile body site (Figure 1-1) [14,15].

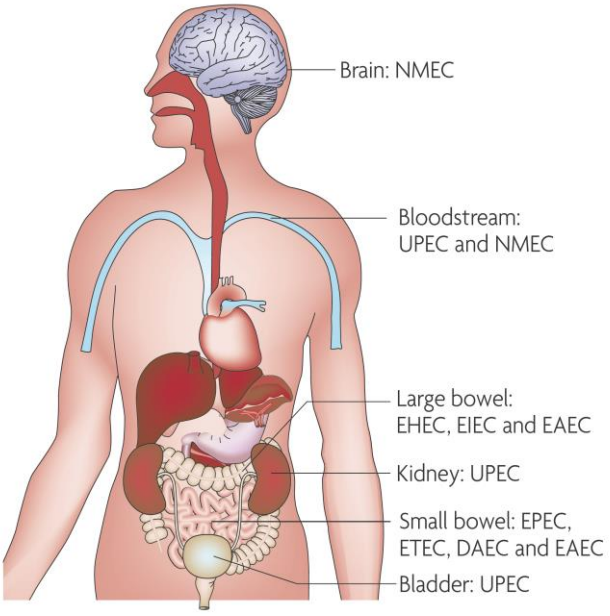

Figure 1-1: Sites of pathogenic *E. coli* colonization (Croxen and Finlay, 2010). Pathogenic *E. coli* colonize various sites in the human body. Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and diffusely adherent *E. coli* (DAEC) colonize the small bowel and cause diarrhea, whereas enterohemorrhagic *E. coli* (EHEC) and enteroinvasive *E. coli* (EIEC) cause disease in the large bowel; enteroaggregative *E. coli* (EAEC) can colonize both the small and large bowels. Uropathogenic *E. coli* (UPEC) enter the urinary tract and travel to the bladder to cause cystitis and, if left untreated, can ascend further into the kidneys to cause pyelonephritis. Septicemia can occur with both UPEC and neonatal meningitis *E. coli* (NMEC), and NMEC can cross the blood–brain barrier into the central nervous system, causing meningitis.

Genome sizes of *E. coli* can differ by a million base pairs between commensals and pathogenic variants, and this extra genetic content can contain virulence and fitness genes. Comparative genomics have shown that *E. coli* genomes are split between a shared, conserved set of genes, called the core genome, and a flexible gene pool. The pathogenic ability of *E. coli* is therefore largely afforded by the flexible gene pool through the gain and loss of genetic material at a number of hot spots throughout the genome [12]. DNA can be moved between prokaryotic hosts through mechanisms such as conjugation, transformation, and transduction, encoded by mobile genetic elements, resulting in horizontal gene transfer (HGT). Mobile genetic elements, such as transposons, insertion sequences, bacteriophages, and plasmids, can exist either integrated into the chromosome or through self-replication within the new host to provide new traits and fitness advantages[16]. A recent comparison of 186 *E. coli* genomes found approximately 1,700 homolog gene clusters shared in all genomes and a pangenome of about 16,400 gene clusters [17]. The pathogenic ability of *E. coli* is therefore largely afforded by the flexible gene pool through the gain and loss of genetic material at a number of hot spots throughout the genome (Figure 1-12) [12].

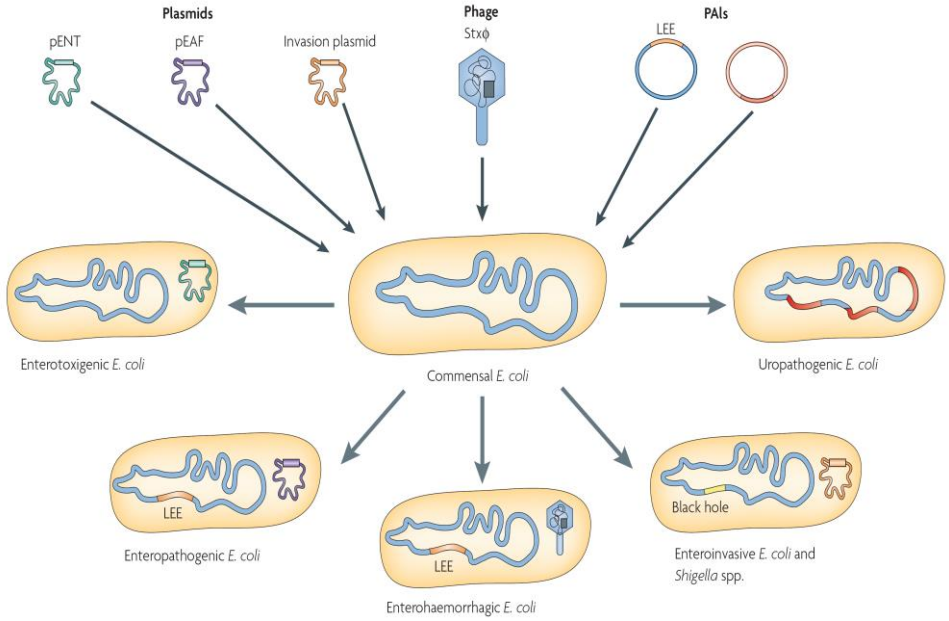

Figure 1-2: Horizontal gene transfer contribution to the evolution of *E. coli* pathotypes (Ahmed et al., 2008). The uptake of mobile genetic elements (phages, virulence plasmids and pathogenicity islands), as well as the loss of chromosomal DNA regions in different *E. coli* lineages, has enabled the evolution of separate clones, which belong to different *E. coli* pathotypes and are associated with specific disease symptoms. LEE, locus of enterocyte effacement; PAI, pathogenicity island; pEAF, enteropathogenic *E. coli* adhesion-factor plasmid; pENT, enterotoxin-encoding plasmids; Stx, Shiga-toxin-encoding bacteriophage.

ExPEC differ from IPEC, because these facultative pathogens were traditionally already regarded as derived from different phylogenetic groups, illustrated for instance by their diversity of serotypes. Additionally, they do not host an unambiguous distinctive repertoire of VFs characteristic for a specific type of disease. Many ExPEC virulence-associated features are also present in commensal *E. coli*. Genome sequencing projects revealed extensive genome diversity among ExPEC, but also identified some pathotype-specific genes including toxins, iron acquisition systems, adhesins, lipopolysaccharides (LPS), polysaccharide capsules, proteases, and invasins. Again, these factors are frequently encoded on mobile elements [18,19].

1.3. Extraintestinal pathogenic *Escherichia coli*

Extraintestinal pathogenic *E. coli* represent a major but little-appreciated health threat. Although ExPEC strains have not captured the public's attention as have intestinal pathogenic *E. coli*, probably because extraintestinal infections do not occur in a sensational food-borne epidemic fashion, their medical importance cannot be ignored. By virtue of their numerous virulence traits, ExPEC clearly possess a unique ability to cause disease outside the host intestinal tract and are responsible for a heterogeneous group of disorders that collectively cause considerable morbidity, lost productivity and increased healthcare costs [15]. Indeed, ExPEC is the most common cause of urinary tract infections (UTIs) in ambulatory and hospital settings. It is responsible for 85 to 95% of the cases of uncomplicated cystitis and for over 90% of the episodes of uncomplicated pyelonephritis in premenopausal women. An estimated 40 to 50% of women will experience at least one case of UTI due to *E. coli* during the lifetime and one fourth will experience a recurrent infection within 6 months of initial infection. ExPEC is also responsible for 25 to 35% of the episodes of catheter-associated UTIs. Furthermore, along with group B *Streptococcus* (GBS), *E. coli* is one of the leading causes of neonatal meningitis, accounting for an estimated 20 to 40% of the cases, with a fatality rate ranging from 25 to 40% and with neurological sequelae affecting 33 to 50% of survivors. *E. coli* also accounts for 17% of the cases of severe sepsis, with a mortality rate of approximately 30%. In addition, ExPEC can be associated with intra-abdominal infections and nosocomial pneumonia and occasionally participates in other extraintestinal infections, such as osteomyelitis, cellulitis and wound infections [13,20,21].

ExPEC strains possess a broad range of virulence factors that are distinct from those found in the intestinal pathotypes and that allow them to colonize host mucosal surfaces, avoid or subvert local and systemic host defense mechanisms, scavenge essential nutrients such as iron, injure or invade the host, and stimulate a noxious inflammatory response [15]. Most of these virulence factors have been acquired by mobile genetic elements.

PAIs carry many of the virulence factors characteristic of ExPEC strains. PAIs are large clusters (10-200 kb) of virulence genes that are present in the genomes of pathogenic strains but absent from the genomes of non-pathogenic members of the same or related species. They are typically associated with t-RNA genes, have a different G+C content and a different codon usage compared to the core genome and often carry cryptic or functional genes that encode mobile elements [1].

Among the ExPEC virulence factors frequently harboured by PAIs, a fundamental role is played by adhesins, which allow the strict interaction of the pathogen with the host, facilitating the colonization and invasion processes and avoiding clearance by the host immune defences. Despite the similarity in their tertiary structure, each adhesin recognizes a specific host receptor [22]. For example, type 1 fimbriae of UPEC strains recognize manno-oligosaccharides naturally present on glycoprotein molecules in the human urinary tract and participate in bacterial adhesion, invasion and formation of biofilms. P fimbriae recognize a digalactoside component of the P blood group antigen abundantly present on the surface of uroepithelial cells and are strictly related to bacteria ascending to the kidneys and causing acute pyelonephritis [23,24].

The presence of group 2 and 3 capsules confers additional selective advantages to ExPEC strains. Indeed, their molecular mimicry to host tissue components helps the bacteria to evade the immune response, providing protection against phagocytic engulfment and complement-mediated bactericidal activity [25,26].

Some virulence factors do not remain attached to the bacterial outer membrane, but are released into the extracellular milieu. The most important secreted factor of ExPEC strains is α-hemolysin (HlyA), a toxin with a promiscuous spectrum of target cells, including not only erythrocytes, but also leucocytes, endothelial cells and renal epithelial cells. It is intracellularly activated by fatty acylation and extracellularly activated by calcium, allowing the insertion into the cell membrane followed by pore formation and disruption of the phospholipid bilayer [27-30]. Other secreted proteins also play important roles in ExPEC pathogenesis, for example: cytotoxic necrotizing factor 1 (CNF-1), which interferes with polymorphonuclear phagocytosis and evokes apoptotic death of bladder epithelial cells [31]; secreted autotransporter toxin (Sat), a serine protease autotransporter with vacuolating activity on bladder and kidney cells [32,33]; Pic and PicU, other type V secreted proteins with serine protease activity [34,35]; cytolethal distending toxin (CDT), responsible for the arrest of cell cycle by inducing DNA double-strand breaks and preventing the transition between G2 and M phases [36-39].

Growth of ExPEC strains in iron-limited conditions, such as urine, requires successful mechanisms for the scavenging of iron, which rely on siderophores and iron-complex receptors [40,41]. Several iron and siderophore receptors, which are highly expressed during infection of the urinary tract, have already been described in *E. coli*, for example the salmochelin siderophore receptor IroN [42], the enterochelin siderophore receptor FepA [43], the hemoglobin and hemin receptor ChuA [44], the ferric yersiniabactin receptor FyuA [45], FitA [46] and IreA [47].

1.4. Pathogenesis of ExPEC

Among ExPEC strains, uropathogenic *E. coli* and neonatal meningitis *E. coli* are characterized by different molecular mechanisms of pathogenicity.

Urinary tract infection usually begins with the colonization of the bowel with an uropathogenic strain in addition to the commensal flora. This strain, by virtue of its virulence factors, is able to colonize the periurethral area and to ascend the urethra to the bladder. Between 4 and 24 hours after infection, the new environmental conditions in the bladder select for the expression of type 1 fimbriae that allow the adhesion to the uroepithelium [1]. This attachment is mediated by fimbrial adhesin H (FimH), which is located at the tip of type 1 pili. FimH binds to mannose moieties of the receptors uroplakin Ia and IIIa that coat terminally differentiated superficial facet cells in the bladder, stimulating also unknown signalling pathways that induce invasion and apoptosis . Bacteria internalization is also mediated by FimH binding to α3 and β1 integrins that are clustered with actin at the sites of invasion, as well as by microtubule destabilization. These interactions trigger local actin rearrangement by stimulating kinases and Rho-family GTPases, which results in the envelopment and internalization of the attached bacteria. Once internalized, UPEC can rapidly replicate and form biofilm-like complexes called intracellular bacterial communities (IBCs), which act as transient, protective environments. UPEC can also leave the IBCs through a fluxing mechanism and enter again the lumen of the bladder. Filamentous UPEC has also been observed fluxing out of an infected cell, looping and invading surrounding superficial cells in response to innate immune responses. During infection, the influx of polymorphonuclear leukocytes (PMNs) causes tissue damage, while apoptosis and exfoliation of bladder cells can be induced by UPEC attachment and invasion, as well as by sublytic concentrations of the pore-forming toxin HlyA. This breach of the superficial facet cells

temporarily exposes the underlying transitional cells to UPEC invasion and dissemination. Invading bacteria are trafficked in endocytic vesicles enmeshed with actin fibers, where replication is restricted. Disruption of host actin allows rapid replication, which can lead to IBC formation in the cytosol or fluxing out to the cell. This quiescent state may act as a reservoir that is protected from host immunity and may, therefore, permit long-term persistence in the bladder, as well as recurrent infections [9].

In strains causing cystitis, type 1 fimbriae are continuously expressed and the infection is confined to the bladder. In strains that are able to cause pyelonephritis, the invertible element that controls type 1 fimbriae expression turns to the "off" position and type 1 pili are less well expressed. This releases the UPEC strain from bladder epithelial cell receptors and allows the microorganism to ascend through the ureters to the kidneys, where it can attach by P fimbriae to digalactoside receptors that are expressed on the kidney epithelium. At this stage, hemolysin could damage the renal epithelium inducing an acute inflammatory response with the recruitment of PMNs to the infection site. Hemolysin has also been shown to cause calcium oscillations in renal epithelial cells, resulting in increased production of interleukin-6 (IL-6) and -8 (IL-8). Secretion of the vacuolating cytotoxin Sat damages glomeruli and is cytopathic for the surrounding epithelium. In some cases, bacteria can cross the tubular epithelial cell barrier and penetrate the endothelium to enter the bloodstream, leading to bacteremia [1].

The pathogenesis of NMEC strains is a complex mechanism, as the bacteria must enter the bloodstream through the intestine and ultimately cross the blood-brain barrier (BBB) into the central nervous system, which leads to meningeal inflammation and pleocytosis, that means presence of a higher number of cells than normal, in the cerebrospinal fluid. Bacteria can be acquired perinatally from the mother and, after the initial colonization of the gut, they can translocate to the bloodstream by transcytosis through enterocytes. The progression of disease is dependent on high bacteremia ($>10^3$ colony forming units per ml of blood), therefore survival in the blood is crucial. NMEC is protected from the host immune responses by its K1 antiphagocytic capsule, made up of a homopolymer of polysialic acid, and by outer membrane protein A (OmpA), which confers serum resistance through manipulation of the classical complement pathway. NMEC has also been shown to interact with immune cells: invasion of macrophages and monocytes prevents apoptosis and chemokine release, providing a niche for replication before dissemination back into the blood. Bacterial attachment to the BBB is mediated by FimH binding to CD48 and by OmpA binding to its receptor, ECGP96. Invasion of brain microvascular endothelial cells involves CNF-1 binding to the 67 kDa laminin receptor (67LR), which leads to myosin rearrangement, as well as OmpA and FimH binding to their receptors, which results in actin rearrangement. The K1 capsule, which is found in approximately 80% of NMEC isolates, also has a role in invasion by preventing lysosomal fusion and thus allowing delivery of live bacteria across the BBB. Collectively, these mechanisms allow NMEC to penetrate the BBB and gain access to the central nervous system, where they cause edema, inflammation and neuronal damage [9].

1.5. Intestinal pathogenic *E. coli*

Diarrheal illness causes much mortality worldwide, particularly in children under the age of 5 [48] and particularly in countries in sub-Saharan Africa and South Asia, whose children suffer many diarrhea-related deaths. Recent data from the Global Enteric Multi-Center Study (GEMS) illustrate that

enterotoxigenic *E. coli* and *Shigella* are among two of the four main causative agents of moderate to severe diarrhea among children in these areas [49]. The pathogenic *E. coli* isolates share many virulence strategies. Adhesion to host cells is a requirement for all pathovars and is frequently achieved through long appendages called fimbriae or pili. Following attachment, *E. coli* must subvert host cell processes, often using secreted proteins. Hijacking and manipulating host cell signalling pathways can result in the coordinated invasion of host cells, evasion of host immune responses and efficient colonization, and ultimately leads to disease. Each pathovar has its own characteristic mechanisms of attaching to and exploiting host cells although they often target the same host machinery [10].

One main feature of the different intestinal *E. coli* pathotypes is the presence of pathotype-specific plasmids that often encode toxins [50]. Concentrations of important intracellular messengers, such as cyclic AMP, cyclic GMP and Ca^{2+}, can be increased, which leads to ion secretion by the actions of the heat-labile enterotoxin (LT), heat-stable enterotoxin (STa) and heat-stable enterotoxin b (STb), respectively — all of which are produced by different strains of ETEC [51]. The Shiga toxin (Stx) of EHEC cleaves ribosomal RNA, thereby disrupting protein synthesis and killing the intoxicated epithelial or endothelial cells [52]. The cytolethal distending toxin (CDT) has DNaseI activity that ultimately blocks cell division in the G2/M phase of the cell cycle [36]. The Map protein of EPEC and EHEC has at least two independent activities — stimulating Cdc42-dependent filopodia formation and targeting mitochondria to disrupt membrane potential in these organelles [53].

Additionally, large so-called colicin plasmids seem to contain several gene clusters, including the salmochelin determinant, that can also be found within chromosomal PAIs in *E. coli* and closely related species. Nearly one-quarter of the EAEC strain 042 genomic content is made up of genomic islands [54], similar to the percentage of unique genomic islands found in STEC O157:H7 strain EDL933 [55].

1.6. Pathogenesis of InPec

Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* is a leading cause of potentially fatal infant diarrhoea in developing countries and also an important cause of intestinal infection in industrialized countries. EPEC infection is primarily a disease in infants younger than 2 years. Sporadic disease also occurred in some adults with compromising conditions. Once defined only on O and H serotyping, they are currently defined by pathogenic features as those diarrheagenic *E. coli* that induce attaching and effacing (A/E) lesions on intestinal cells and do not produce Shiga toxins. They could be divided into typical EPEC and atypical EPEC based on the presence of EPEC adherence factors (EAF) plasmid. Molecular detection and differentiation of EPEC could be based on the *eae* gene (A/E lesions) and *bfp* gene (which resides on EAF plasmids and encodes bundle- forming pilus). Typical EPEC contain both *eae* and *bfp* genes, while atypical EPEC contain only *eae* gene. Typical EPEC infections are common in developing countries while atypical *E. coli* seems to predominate in the industrialized countries. Not like typical EPEC which are found only in human so far, atypical EPEC have been isolated from a variety of animal species such as cattle, goats, sheep, chickens, pigeons and gulls [56,57].

Pathogenesis of EPEC is currently considered to include four stages: expression of adhesion factors, initial localized adherence, signal transduction and intimate contact, cytoskeletal rearrangement and pedestal formation. Initially the bacteria attach to intestinal epithelial cells by adhesive fimbriae called bundle forming pili (BFP) or EPEC adherence factor (EAF) [58]. EPEC also adhere to epithelial cells by expressing intimin (encoded by *eae* gene) and surface–associated filament EspA. After initial binding, EPEC utilize type III secretion system to inject into host cells translocated initmin receptor (TIR) and several effector molecules, which activate cell signaling pathways and actin polymerization and depolymerization to alter cytoskeletal structure. TIR is then phosphorylated and inserted into the host cell membrane for later intimate contact. Activated host cell signal transduction pathway causes extensive rearrangement of actin, which results in the formation of the characteristic attaching and effacing lesions [59]. The membrane under the bacteria forms a pedestal due to host cell cytoskeletal rearrangement. The microvilli are lost due to depolymerization of actin filament in microvilli. The effector proteins also influence membrance permeability and cause diarrhea-associated symptoms. Virulence genes in EPEC are mostly located in a pathogenic island called locus of enterocyte effacement (LEE).

Enterohaemorrhagic *E. coli* (EHEC)

The EHEC group is also referred to Shiga-toxin producing *E. coli* (STEC) because its pathogenicity is largely attributed to the production of Shiga toxins. It should be noted that some researchers prefer to use EHEC only for those STEC containing LEE pathogenic island, while others use them exchangeably. Among six categories of diarrheagenic strains, EHEC strains distinguish themselves by their ability to cause severe life-threatening complications, such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). Other symptoms of EHEC infection include bloody diarrhea and hemorrhagic colitis (HC). Children and the elderly are more susceptible to severe STEC infections than healthy adults. Outbreaks and sporadic cases of EHEC infections are frequently reported worldwide, indicating the great threat that EHEC could pose for human health [60-62]

EHEC can be disseminated through a variety of means. Most human infections are caused by consumption of contaminated foods. Domestic and wild ruminant animals, in particular cattle, are considered as the main reservoir of EHEC and the main source for contamination of the food supply [63-65]. Food products derived from these animals can be contaminated with EHEC during slaughter and further processing. In addition, vegetables contaminated with cattle manure have been also implicated in many cases and outbreaks of EHEC infections [66]. EHEC have been also isolated from other food animals such as pigs and poultry, but whether these animals represent real hosts or are just temporarily colonized with EHEC is not clear [67,68].

The mechanisms of EHEC infection in humans are not fully understood. The major virulence factors implicated are potent Shiga toxins, which are classified into two groups: Stx1 and Stx2. In each group, variants that differ in toxicity, toxin receptor, and amino acid sequences have been described. Few variants (Stx1c, Stx1d) were found for Stx1, whereas Stx2 contains several variants including Stx2c, Stx2d, Stx2e, Stx2f, Stx2g [69-74]. Shiga toxin types were suggested to correlate with the clinical symptoms of EHEC infection. Some Stx types, such as Stx2, Stx2c and Stx2d-activatable, have been associated with high virulence and ability to cause HUS, while Stx1, Stx1c, Stx2e occurred mainly in milder diarrhea patients or

asymptomatic carriers [75]. In addition to Stx production, other (putative) virulence factors that could contribute to the pathogenicity have been discovered. The *eae* gene, which is located in a pathogenic island in the chromosome called the locus of enterocyte effacement (LEE), is the best characterized virulence loci other than *stx*. The *eae* gene encodes the adherence factor intimin, an outer membrane protein involved in the attachment of *E. coli* to the enterocyte. In addition, many pathogenic EHEC possess a large plasmid that harbors several putative virulence factors such as EHEC-hlyA, which encode for a cytolytic EHEC-hemolysin. EHEC hemolysin is strongly associated with EHEC isolates causing severe infections in human, but its exact role in pathogenesis is still yet to be known.

More than 400 serotypes of EHEC strains have been implicated in human infections. Although *E. coli* O157: H7 is considered the principal EHEC in the U. S., infections due to non-O157 EHEC occur and are thought to be underreported [76,77]. In some other countries, such as Germany, Australia, and the UK, non-O157 EHEC infections predominate [78,79]. Globally, only a limited numbers of serotypes were frequently observed and are responsible for the majority of EHEC infections.

Enterotoxigenic *E. coli* (ETEC)

ETEC are defined as those *E. coli* strains that contain at least one of two defined groups of enterotoxins: heat stable toxin (ST) and heat labile toxin (LT). ETEC is an important cause of childhood diarrhea in developing countries due to poor sanitary conditions. It is also a common cause of diarrhoea in travellers to developing countries. It is estimated that around 650 million cases of ETEC infection occur each year, which include 800,000 deaths mostly in young children [80]. It causes watery diarrhoea ranging from mild form to severe purging disease. The diarrhoea persists for 3-4 days and is usually self-limiting, however, diarrhoea may be fatal in young children and infants. Epidemiologic studies found that contaminated food and water serves as the most common vehicles for ETEC infections [81].

Colonization factors (CF) and one or more enterotoxins that induce a secretory diarrhoea are the major determinants of ETEC virulence. CFs are proteinaceous fimbrial and afimbrial structures that enable bacteria to attach to intestinal mucosa. More than 20 CFs have been identified and characterized in ETEC [82]. Other adhesion factors such as TibA (an afimbrial adhesion) and Tia (an outer membrane protein) are also involved and implicated in the attachment of ETEC[83].

Having established contact with epithelial cells, ETEC can produce one or more ST or LT. ST have been divided into two distinct groups: methanol soluble STI (or STa) and methanol insoluble STII (or STb) [84]. STa toxins have two genetic variants STh and STp, described originally in association with strains isolated from human and pigs, respectively. However, new studies found that both variants could be found in ETEC strains of human origin. STa binds to guanylate cyclase C receptor and activates its guanylate cyclase domain, which results in an increase in intracellular cGMP level. Increase cGMP influences ion pumps, resulting in enhanced salt and water secretion and inhibition of Na+ absorption. STb is most associated with porcine strains of ETEC.

LT is an oligomeric protein composed by a ring of five identical B subunits with one A subunit. The B subunits bind to a GTP binding protein (ganglioside receptor), while the A subunit is responsible for the enzymatic activity of the toxin [85]. Based on type of cell surface receptor to which B subunits bind, LT could be divided into LT-I (bind to ganglioside receptor GM1) and LT-II (bind to ganglioside receptor GD1). A subunit ADP-ribosylates the alpha subunit of the GTP-binding protein Gs, leading to activation of

adenylate cyclase in the enterocyte and accumulation of cyclic AMP. The increase in intracellular cAMP increases Cl- secretion in crypt cells and decreases absorption of Na+ and Cl- by villus tip cells. Other toxins that may contribute to ETEC infection include a novel heat stable enterotoxin EAST1 and a serine protease autotransporter, EatA, and a pore–forming toxin, ClyA.

Enteroinvasive *E. coli* (EIEC)

EIEC strains are genetically and pathogenically related closely to *Shigella spp*.[86]. EIEC and *Shigella* are highly invasive pathogens that use the intracellular milieu of intestinal epithelial cells (IECs) in the large intestine as their replicative niche. These pathogens are readily adaptable to the various environmental challenges they face during the course of infection, including low gastric pH, temperature changes, oxygen availability, and oxidative stress, as well as osmolarity [87]. Successful infection is dependent on essential virulence determinants that are encoded by both chromosomal and plasmid loci. Key plasmid encoded virulence factors include components of the T3SS needle complex (Mxi-Spa proteins), chaperones (IpgA, IpgC, IpgE, and Spa15), transcriptional regulators (VirF, VirB, and MxiE), translocators (IpaB, IpaC, and IpaD), and approximately 25 effector proteins [88]. Bacterial infection is a multistep process involving penetration of the epithelial barrier, induction of macrophage cell death, IEC invasion, suppression of the immune response, intra and intercellular movement, and modulation of epithelial integrity.

In general, EIEC and *Shigella* employ the same strategies to invade host cells. Nevertheless, EIEC exhibits reduced virulence compared to that of *Shigella*, including reduced expression of virulence genes, less efficient macrophage killing, reduced cell-to-cell spread, and decreased induction of a proinflammatory host response which correlates with the less severe disease induced by EIEC [89,90].

Enteroaggregative *E. coli* (EAEC)

EAEC are defined as *E. coli* that do not produce LT or ST and that adhere to HEp-2 cells in a pattern described as autoaggregative. EAEC have been increasingly recognized as an important causative agent of persistent diarrhoea in children and adults in both developing and developed countries. EAEC mostly cause sporadic cases, but several outbreaks have been reported [91]. Prior to 2011, there were only been a few reports of Shiga toxin-producing EAEC causing bloody diarrhoea and haemolytic uremic syndrome (HUS) [92,93], but these are now more appreciated due to the *E. coli* O104:H4 outbreak in Germany [94]. Characterization of isolates from this outbreak identified key virulence features belonging to different pathotypes, such as an aggregative adhesive phenotype *in vitro*, lack of the LEE PAI, and expression of a Shiga toxin [95]. Therefore, these isolates can be considered a hybrid of both EAEC and EHEC (a subset of STEC), and it has been suggested that the STEC O104:H4 strain associated with the 2011 German outbreak be called enteroaggregative haemorrhagic *E. coli* (EAHEC) [96]. Additionally, genome analysis of LEE-negative STEC has uncovered homologs and subunits of ETEC toxins in some isolates [97], further demonstrating the potential for the emergence of novel pathogenic *E. coli* hybrids. For non-Stx variants, EAEC strain 042 has been used as a prototypical strain to study virulence factors and pathogenicity of diarrheagenic EAEC, as it causes diarrhoea in the majority of volunteers. However, the encoding genes for numerous adhesins, toxins, and proteins associated with virulence are highly variable among strains [1,10,98-101]. Even the site of infection in the gastrointestinal tract is not uniform. For

example, the EAEC 042 strain has been isolated from the jejunum in infected volunteers, and in tissue culture it adheres strongly to samples of jejunal, ileal, and colonic mucosa [102,103]. In a controlled study looking at five different non-Stx EAEC isolates from children, each strain had a different affinity for the jejunal, ileal, and colonic mucosae [104]. Despite the heterogeneity among the different non-Stx EAEC strains, a general three-part model has emerged for non-Stx EAEC pathogenesis: (i) adherence to the intestinal mucosa, (ii) production of enterotoxins and cytotoxins, and (iii) mucosal inflammation[100]. EAEC express aggregative adherence fimbriae (AAF) I, II and III and outer membrane adhesion proteins. Adherence is described as a stacked-brick shape. EAEC elaborate enteroaggregative heat stable toxin (EAST) and a cytotoxin that is responsible for pathological effects. Infections usually lead to mucoid stool and persistent diarrhoea (often more than 14 days).

Diffusely adherent *E. coli* (DAEC)

The diffusely adherent *E. coli* (DAEC) pathotype describes diarrheagenic *E. coli* strains that attach to cells but do not fall into classical patterns of adherence, such as localized or A/E [10]. They have now emerged as a unique group and are considered distinct from other pathotypes, but because of difficulties in classification and identification, the designation of DAEC as a distinct enteric *E. coli* pathotype [105] requires further epidemiological studies. DAEC has been classically defined by its diffuse adherence (DA) to cultured epithelial HEp-2 cells, where bacterial adherence occurs over the entire surface of the cell in a scattered pattern [106]. The prototypical strain C1845 is a DAEC strain that encodes Afa/Dr adhesins. The Afa/Dr adhesins are a class of adhesins that includes the AfaE-I, AfaE-II, AfaE-III, AfaE-V, Dr, Dr-II, F1845, and NFA-I adhesins[107]. In Afa/Dr DAEC, the Dr and the F1845 adhesins bind to brush border-associated decay-accelerating factor (DAF), a molecule that is highly expressed on the apical surface of polarized epithelial cells. After binding, cytoskeleton rearrangement is induced, destroying or partially rearranging microvilli [107-112]. Some Afa/Dr DAEC strains also bind the human carcinoembryonic antigenrelated cell adhesion molecule (CAECAM) family of receptors in a process that leads to internalization into undifferentiated epithelial cells [113]. In addition to binding DAF and the CAECAM family of receptors, Afa/Dr DAEC has also been shown to bind type IV collagen through the Dr adhesin, an interaction that is important for urinary tract infections caused by Afa/Dr DAEC. After binding DAF, disassembly of F actin and villin results in brush border lesions. This eventually leads to a loss of microvilli due to defective expression of brush border-associated functional intestinal proteins [109]. Rearrangement of the tight-junction- associated proteins ZO-1 and occludin after infection by Afa/Dr DAEC strains leads to increased paracellular permeability but does not affect transepithelial electrical resistance[111]. The proinflammatory cytokine IL-8 is produced through flagellar stimulation of Toll-like receptor 5 (TLR5), resulting in activation of mitogen activated protein kinase, extracellular signal-regulated kinases 1 and 2 (ERK1/2), P38, and Jun-C kinase [114-118].

While the pathogenesis of typical Afa/Dr DAEC is beginning to be characterized, much remains to be discovered for atypical diarrheagenic DAEC strains. There are two different subclasses of atypical DAEC. One subclass contains all the adhesins typical of the Afa/Dr family of adhesins in another *E. coli* background, such as diffusely adherent EPEC. In the other subclass of atypical DAEC, the bacterium does not bind DAF and expresses a different array of adhesins on its surface, including AfaE-VII, AfaE-VIII,

AAF-I, AAF-II, and AAF-III [108]. In this subclass of atypical DAEC, IL-8 is still stimulated by DAEC strains that have been internalized by an uncharacterized mechanism, suggesting that it may elicit pathogenesis mechanisms similar to those of typical Afa/Dr DAEC [119].

1.7. *E. coli* and host interactions in the gut epithelial barrier

The gastrointestinal epithelium is covered by a mucus gel layer (MGL) synthesized and secreted by host goblet cells. The MGL is an integral structural component of the mammal intestine, acting as a medium for protection and transport between the luminal content and the epithelium lining. The major function of the MGL is to lubricate and to protect the intestinal epithelium from damage caused by food and digestive secretions. Moreover, the MGL acts as a trap for microorganisms, including pathogens, preventing their access to the epithelia [120].

Studies by Holm and colleagues showed that there are two mucus layers in the stomach and colon: an outer "loose" layer that was easy to aspirate and an inner mucus layer that was "firmly" attached to the epithelium [121] . These mucus layers are organized around the highly glycosylated MUC2 mucin, forming a large, net-like polymer that is secreted by the goblet cells. The inner mucus layer is dense and does not allow bacteria to penetrate, thus keeping the epithelial cell surface free from bacteria. The inner mucus layer is converted into the outer layer, which is the habitat of the commensal flora. The outer mucus layer has an expanded volume due to proteolytic activities provided by the host but probably also caused by commensal bacterial proteases and glycosidases. This is in contrast to the small intestine, where the mucus is discontinuous and less well defined. The mucus is secreted at the top of the crypts and then moves upward between the villi. Thus, the tips of the villi are not always covered with mucus [122] .

The commensal bacteria in colon live and thrive in the outer loose mucus layer. This is possible after the Muc2 mucin network has expanded in volume, such that it allows the bacteria to penetrate into the mucin network. Once inside the mucus gel, the commensal bacteria can use its large number of glycan-degrading enzymes that release one monosaccharide at a time from the mucin glycans [123].

Pathogenic strains of *E. coli*, without exception, must first colonize the host gastrointestinal tract before causing disease [1]. Freter postulated that successful competition for nutrients allows intestinal bacteria to colonize, which we define as the ability to achieve and maintain a stable population without reintroduction. Freter's nutrient niche hypothesis theoretically explains the succession of community members of the intestinal microbiota, as well as the ability of enteric pathogens to overcome colonization resistance and thereby invade the microbiota [124]. How invading pathogens compete for nutrients with the established microbial residents is an open question.

A large and growing body of evidence indicates that *E. coli* grows in the intestine on nutrients acquired from mucus. Fluorescence *in situ* hybridization of intestinal thin slices showed that *E. coli* BJ4 [125] and MG1655 [126] are dispersed in the mucus layer. *E. coli* BJ4 grows rapidly in the mouse intestine, with a generation time of 40–80 min [127]. *In vitro*, rapid growth (30-min generation time) occurs in intestinal mucus, but not in luminal contents [128,129] . Among mutants unable to colonize the mouse intestine are those that fail to penetrate mucus, have difficulty surviving in mucus, or have difficulty growing on mucus [130-132].

Virulence factors such as proteases, glycosidases, and mucus secretagogues are produced by these organisms and are believed to be responsible for disruption and depletion of the mucus gel [133]. Enzymes, such as Pic, a serine protease that degrades mucin [134]; StcE, a zinc metalloprotease that cleaves mucin-type O-glycosylated proteins [135]; Hap, a zinc metalloprotease [136] ; TagA, a Hap homolog with metalloprotease activity that is distinct from Hap [137] or mucin-degrading enzymes[138] , degrade mucin oligosaccharides, reduce mucus viscosity and hamper the release of antimicrobial peptides.

The personal repertoire of expression of mucin core proteins and their glycans, mucin allele length, and transient changes in mucin expression and glycosylation in response to infection or stress, as well as variations in environmental conditions may all affect microbial interaction with host mucins and the pathogenic consequences of microbial colonization. Rather than a static barrier, mucins should be considered as a dynamic responsive component of the mucosal barrier that interacts with and responds to other elements of innate and adaptive immunity.

1.8. Vaccines against pathogenic *E. coli*

The prevention of *E. coli* infections is of pressing concern from both the public health and economic perspectives [14]. Indeed, the absence of a broadly protective vaccine against pathogenic *E. coli* strains is a major problem for modern society since some of the diseases caused by these bacteria are associated with high costs to healthcare systems. The overall problem is exacerbated by the increasing antibiotic resistance and the number of recurrent infections [139]. Attempts to develop a broadly protective and safe vaccine against *E. coli* have not been successful so far. The large antigenic and genetic variability of pathogenic *E. coli* species has been a major obstacle to the development of an effective vaccine. Indeed, the difficulty in predicting vaccine coverage and the lack of a correlate of protection, has led to numerous promising pre-clinical data not being confirmed by human studies [140,141].

For many years, *E. coli* pathotypes have been studied in isolation instead of addressing *E. coli* as a single microorganism responsible for human and animal diseases.

Many efforts have already been done to assess protein moieties as putative vaccine candidates against extraintestinal *E. coli* infections. These efforts were logically concentrated on proteins that are surface-exposed and have a potential role in pathogenesis, such as adhesins, iron-regulated outer membrane proteins (OMPs) and toxins [139]. Antibodies directed against adhesins have the promise to enhance the bactericidal activity mediated by complement and professional phagocytes and also to inhibit bacterial binding to host structures, a critical step in the pathogenesis of infection [139]. Systemic immunization with purified P fimbriae [142,143] and synthetic peptides corresponding to the protective epitope of the P fimbrial major subunit PapG [144] conferred protection in a murine pyelonephritis model; immunization with purified P fimbriae or with purified PapDG-complex also conferred protection in a nonhuman primate model [145,146]. Several iron-regulated OMPs have also been assessed to date as potential vaccine candidates, given that many are surface-exposed and iron acquisition is a requisite for pathogenesis [139]. The pore-forming toxin α-hemolysin has been demonstrated to be highly conserved [147]. In a mouse model of pyelonephritis, systemic immunization with purified HlyA was associated with decreased renal damage, but did not affect clearance of *E. coli*. However, its combination with digalactoside-binding pilus was able to prevent both bacterial colonization and renal injury [139,142].

Several whole-cell vaccine approaches have also been tried to prevent extraintestinal *E. coli* infections. Four standardized whole-cell vaccine formulations (Urovac®, OM-89 or Uro-Vaxom®, Urvakol®, Urostim®) have been tested for their efficacy in preventing UTIs [139,148,149]. Although for most of the formulations data are far from convincing and many studies lack of scientific rigor, the potentiality of whole-cell vaccines is high, since they could present multiple antigens, elicit antibodies against conformational and linear epitopes and possess natural adjuvants.

Vaccines are being developed to prevent some of the serious sequel and complications associated with *E. coli*-induced diarrheal illness. Only one vaccine (Dukoral® produced by SBL Sweden) is currently available for the prevention of ETEC diarrhea. This vaccine has been recommended to prevent 'travellers' diarrhea' in people visiting endemic regions from developed countries [150]. Dukoral is primarily designed and licensed to prevent diarrhea due to *Vibrio cholera*, but it contains a recombinant B subunit of the cholera toxin that is antigenically very similar to the LT of ETEC [151]. In an early clinical trial, using a prototype of this vaccine which contained purified cholera B subunit rather than the recombinant form, significant cross protection against ETEC diarrhea was demonstrated [152]. Many alternative vaccine candidates designed specifically to protect people against ETEC are now at various stages of clinical development. These vaccine candidates can be broadly categorized in to two groups: inactivated vaccines containing killed whole cells, purified CF antigens, or inactivated LT; and live attenuated vaccines containing genetically modified, non-pathogenic strains of ETEC, or alternative carrier bacteria expressing the important ETEC antigens [153]. Given the number of antigenically different strains of ETEC, it is likely that a vaccine formulation capable of providing broad protection would need to contain a combination of the most commonly expressed antigens [153].

The spread of infections due to EPEC has been well documented with numerous case studies in hospitals and nurseries [10]; however, no vaccines are currently available to control its spread. Antibodies against EPEC O antigens and outer membrane proteins, such as Bfp and intimin, as well as the secreted proteins EspA, have been found in breast milk [154,155], and protection from mother to infant can be transmitted through colostrum IgA. Purified recombinant versions of EspB and BfpA were capable of eliciting an antibody response in rabbits and showed antigenic potential in humans when reacted with secretory IgA (sIgA) present in the stools of diarrheic pediatric patients [156],indicating that an immune response to these potential vaccine subunits can be produced at an early age [157]. Recently, bacterial ghosts devoid of cytoplasmic contents but expressing all EPEC surface components were constructed and used in vaccination challenge experiments with mice [158]. Vaccinated mice showed 84 to 90% protection when challenged with wild-type EPEC, compared to no protection in control mice. Homologous re-challenge with wild-type EPEC resulted in a reduced severity of disease but had no effect on incidence of diarrhea [159].

Several vaccine strategies have been used against EHEC with variable success in a number of animal models. The strategies have involved the use of recombinant virulence proteins such as Stx, intimin and *E. coli* secreted protein A (EspA) [160]or peptides [161] or fusion proteins of A and B subunits of Stx2 and Stx1 such as Stx2Am-Stx1B [162] or avirulent ghost cells of EHEC O157:H7 [163]. The application of live attenuated bacteria such as *Salmonella* as a carrier for vaccine proteins against mucosal pathogens including EHEC have obvious advantages [164]. As well as protein-based vaccines, DNA vaccines are a

recent development in EHEC prevention, providing encouraging results in a mouse model. Immunization trials carried out with a DNA vaccine expressessing a nontoxic Stx2 mutated form, alone or in combination with another DNA vaccine encoding granulocyte-macrophage colony-stimulating factor, resulted in systemic Stx-specific antibody responses. These antibodies showed toxin neutralization activity *in vitro* and, more importantly, conferred partial protection to Stx2 challenge *in vivo*.

The development of an effective EIEC or *Shigella* vaccine would constitute another preventive and sustainable approach to eliminate the disease burden of bacillary dysentery. Briefly summarized, recent research pursues the design of a multivalent vaccine protecting against the most prevalent serotypes and subserotypes, including *S. dysenteriae 1, S. sonnei*, and all 14 types of *S. flexneri*. Multiple strategies were implemented to engineer both parenteral and mucosal candidate vaccines that have shown various levels of efficacy in clinical trials. The most promising candidates include live attenuated strains of *S. flexneri 2a, S. sonnei*, and *S. dysenteriae 1*, as well as inactivated whole-cell vaccines derived from inactivated *S. sonnei* and *S. flexneri 2a* strains. Subunit-based approaches involve covalent and noncovalent O-polysaccharide protein conjugates targeting *S. flexneri, S. sonnei*, and *S. dysenteriae*, LPS-Ipa-protein complexes protecting against *S. flexneri 2a* and *S. sonnei*, and *S. flexneri 2a*-directed outer membrane vesicles. These candidates, together with major efforts to increase the immunogenicity of mucosal vaccines as well as the selection and design of potent adjuvants and antigen carriers, promise fast progress toward a long-awaited safe and powerful vaccine against *Shigella*

As EAEC proteins are antigenic, it remains possible that a vaccine could be developed, but as of yet, there is none. *In vitro* treatment with lactoferrin inhibits EAEC enteroadhesion and biofilm formation, making it a potential but untested nonantibiotic treatment for the prevention of EAEC [165,166].

In summary, the development of a polyvalent subunit vaccine or a genetically engineered killed whole-cell vaccine will be challenging. However, achieving this goal is important because of the medical and economic burden attributable to ExPEC infections [139].

1.9. The reverse vaccinology approach

Vaccine development followed the same basic principles for more than two centuries. When Edward Jenner inoculated James Phipps with a bovine poxvirus to induce protection against the closely related human pathogen smallpox virus in 1976 and then, almost a century later, Pasteur developed a live attenuated vaccine against rabies, the basic principles for vaccine development were established [167]. These approaches served as guidelines for the development of vaccines throughout the twentieth century, conferring protection against many once lethal infectious diseases. Indeed, all existing vaccines were developed using at least one of the following approaches: killed (inactivated), live attenuated and subunit vaccines, including protein-conjugated capsular polysaccharides, toxoids, cell-free extracts, recombinant proteins and stand-alone capsular polysaccharides. Thanks to those basic principles, several infectious diseases can be prevented by vaccines now. Conventional approaches led to great achievements, such as the eradication of smallpox and the virtual disappearance of diseases like diphtheria, tetanus, poliomyelitis, pertussis, measles, mumps, rubella and invasive *Haemophilus influenzae* B, increasing the life quality and expectancy [168]. Conventional approaches were important to provide the basis of vaccinology, but showed to be time-consuming, leading to years or even decades of research. Inactivation and attenuation were the

first choice for many years, but the difficulty of cultivating some microorganisms *in vitro* and the fact that even attenuation may result in detrimental or unwanted immune responses showed that these approaches are impractical in some instances [169]. Even the purification of specific antigens failed in many cases in providing protective vaccine candidates, since the methods usually employed led to the identification of the most abundant, but also most variable and less suitable, vaccine candidates [170]. Although successful for many pathogens, conventional vaccinology still left many diseases uncontrolled. Considering that even new diseases are sure to emerge through evolution by mutation and gene exchange, interspecies transfer or human exposure to novel environments [171], a faster and more reliable approach must be available to promptly respond to those threats.

The sequencing of the first bacterial genome in 1995 led the vaccine development to enter a new era and to open a new chapter in the vaccine development guidebook. Suddenly, all the proteins encoded by a microorganism were available and for the first time, after more than two centuries, it was possible to identify vaccine candidates without using the conventional vaccinology principles. This new approach, named reverse vaccinology, gives full access to all the proteins that a microorganism can encode and, by computer analysis, allows to identify potential surface-exposed proteins in a reverse manner, starting from the genome rather than the microorganism. The reverse vaccinology approach permits to avoid problems related to non-cultivable microorganisms and also to antigens that are not expressed under *in vitro* conditions, which conferred the most important obstacles for vaccine development. The feasibility of this approach relies on the availability of a high-throughput system for protective immunity screening and also on good correlates of protection. The greatest limitation of the reverse vaccinology approach is represented by the inability to identify non-protein antigens such as polysaccharides, components of many successful vaccines, and glycolipids, a new promising group of vaccine candidates [170]. Nevertheless, reverse vaccinology seemed to be a powerful tool that could help researchers to overcome the obstacles of conventional vaccinology and lead to the discovery and development of novel vaccines against the most concerning emerging diseases.

When many other conventional approaches failed to produce an effective vaccine against meningococcus B, the reverse vaccinology approach appeared as a logical and promising alternative to deliver a vaccine. While the *Neisseria meningitidis* genome sequence was still being assembled, computer analysis allowed the prediction of proteins that could be surface-exposed or homologous to known factors associated with virulence and pathogenesis, leading to the selection of 570 potential vaccine candidates. Successful cloning and expression were obtained in *E. coli* for 350 proteins, which were then purified and used to immunize mice [172,173]. Immune sera were, then, tested in enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS) and Western blot analysis, in order to confirm the surface localization of the antigens, and also in complement-mediated bactericidal assays, to test protein immunogenicity and protective activity, since this kind of assay correlates with protection in humans. Out of 81 proteins found to be strongly positive in at least one of the mentioned assays, 28 showed to be positive in all of them. To confirm the possibility of using these candidates in a vaccine able to protect against heterologous strains, their presence and conservation were tested in a panel of 31 strains of *N. meningitidis* isolated worldwide and over many years. Out of the 28 proteins tested, five showed to be strikingly conserved in the panel, a result not quite expected since they were surface-exposed. In less than two years,

reverse vaccinology achieved what the conventional vaccinology approaches pursued by decades: surface-exposed proteins in *N. meningitidis* B able to induce protection and cross-reactivity among distantly related strains and serotypes and suitable to be used in a universal vaccine against this microorganism, just by extracting the benefits from genome information and applying them in the development of novel vaccines.

The success obtained with the *N. meningitidis* B experience prompted the application of reverse vaccinology to other pathogens, such as *Streptococcus pneumoniae*, *Porphyromonas gingivalis*, *Chlamydia pneumoniae*, *Bacillus anthracis*, *Streptococcus agalactiae*, *Streptococcus pyogenes* and many others, making the reverse vaccinology a routinely classical approach for vaccine development.

However, something unexpected happened when the complete genome of a virulent isolate of *S. agalactiae* (GBS) was sequenced. GBS is one of the leading causes of bacterial sepsis, pneumonia and meningitis in neonates in USA and Europe, and also an emerging cause of infection in the elderly. Conjugate vaccines based on the five major capsular polysaccharides were currently under development, but they were not able to cover all available serotypes. Since conventional approaches failed in providing a universal and efficient vaccine for the most affected groups of patients and since the complete genome sequence of two *S. agalactiae* strains was available in 2002, the classical reverse vaccinology approach sounded logical. But the GBS experience was going to be more challenging. In order to verify the diversity of *S. agalactiae* genome and provide information for a future universal vaccine against this microorganism, comparative genomic hybridization was applied, using the sequenced strain as reference. Approximately 18% of the genes encoded in the sequenced strain was found to be absent from at least one of the other 19 *S. agalactiae* strains tested. The problem is that comparative genomic hybridization is able to provide information only for the genome sequence that is shared among the strains. Therefore, specific genes in the other strains that are absent from the sequenced genome could not be detected. Since it could be a problem for a universal vaccine achievement, this led the classical reverse vaccinology to evolve. Sequencing the genome of only one strain could be not enough anymore to provide the information needed for a universal vaccine development, especially when a high variability is observed. To provide the information requested, six additional genome sequences of *S. agalactiae* were determined. This showed that 1806 genes are shared by all GBS strains, representing the "core genome" that corresponds to approximately 80% of the average number of genes encoded in each strain. The core genome mainly encodes factors for functions that contribute to the major metabolic pathways, the so called housekeeping genes that usually define the identity of a species. The complementary set of genes absent from at least one strain corresponds to the "dispensable genome", probably related to the adaptation of strains to specific environmental conditions by conferring selective advantages. Mathematical extrapolation predicted that, no matter how many strains have been sequenced, each new genome should provide genes that have never been found before. Sequencing additional genomes allowed the estimation of the *S. agalactiae* pan-genome size (the set of genes that will be observed at least once if an infinite number of different strains would be sequenced), corresponding to 2713 genes, of which 907 belong to the dispensable genome, and also allowed the prediction that the pan-genome is going to grow about 33 new genes every time a new strain is sequenced [174,175].

Comparative genome analysis also provided the information necessary to face the quest of providing a universal vaccine against *S. agalactiae*. Computational algorithms predicted 589 surface-

associated proteins, of which 396 belonged to the core genome and 193 were absent from at least one strain. Each protein was tested for protection and four antigens were able to elicit protective immune responses in the animal model, not only by passive immunization and challenge of new-born mice, but also by active maternal immunization and challenge of offspring within the first 48 hours of life [175]. None of these protective antigens could be classified as universal, because three of them were absent from a fraction of the tested strains and the fourth, belonging to the core genome, had a deficient surface accessibility in some strains [176]. The cocktail combining the four best candidates conferred 59-100% protection against a panel of 12 *S. agalactiae* isolates, including the major serotypes, as well as two strains from a less common serotype [175]. Without the determination of additional genome sequences, a universal vaccine against GBS would have been compromised. The comparative genome analysis provided new concepts in delivering universal vaccines by the reverse vaccinology approach, even for microorganisms in which a high variability can be observed, opening the pan-genomic reverse vaccinology era.

Also the sequencing of nonpathogenic bacteria could provide the information necessary for the identification of antigens that could really make the difference in pathogenesis, being responsible for the most strict host-pathogen interactions. In a subtractive comparative genome analysis, called subtractive reverse vaccinology, genes conserved between pathogenic and nonpathogenic strains of a same or even related species could be discarded, leading to the selection of antigens most strictly related to the pathogenesis and, therefore, avoiding any impact on the commensal flora. This approach could also reduce the number of antigens to be tested and, consequently, the time for the delivery of a vaccine. Since the ability of causing disease is frequently related to the integrity of some genes, algorithms must take into account some gene inactivation processes such as frame shifting. Of course, a whole set of factors is usually responsible for pathogenicity, therefore also in this case the sequencing of only one nonpathogenic genome could be not enough for a complete understanding of pathogenicity. This approach could provide the answers that could not be given by the comparison between pathogenic strains [177].

1.10. Identification of protective vaccine candidates against pathogenic *E. coli*

Because reverse vaccinology has shown to be a powerful tool for the identification of protective candidates where conventional approaches to vaccine development had failed, and considering that today multiple genomic sequences of *E. coli* have been completely or partially determined, we decided to apply the subtractive reverse vaccinology approach to identify protective and broadly conserved vaccine antigens against pathogenic *E. coli* [2]. Comparative genome analysis and reverse vaccinology led to the identification of nine antigens capable of inducing protective immune responses against ExPEC strains, several of which are also prevalent in different intestinal *E. coli* pathotypes [178]. We compared the genome of a neonatal meningitis-associated K1 *E. coli*, IHE3034, with those of pathogenic and non-pathogenic *E. coli* strains [2]. We found that the genome of the K1 strain contained 19 genomic islands that were absent from the nonpathogenic strains. These different regions accounted for almost 20% of the total genome, indicating the huge diversity of *E. coli* strains. The most remarkable finding, however, was that these large genomic differences were not only present between the pathogenic and nonpathogenic strains, but also within the pathogenic ExPEC strains, confirming the hypothesis of the individual virulence potential for each ExPEC strain [2,179].

In spite of the huge genomic diversity, by using the subtractive reverse vaccinology approach, we were able to identify nine potential vaccine candidates. Briefly, antigens predicted to be surface-associated or secreted and with no more than three transmembrane domains (TMDs) were selected by bioinformatics analysis of the genomes of the NMEC strain IHE3034 and of the UPEC strains CFT073 [180] and 536 [179]. The presence as well as the level of similarity of these antigens in the nonpathogenic strains MG1655 [181], DH10B and W3110 were used as exclusion criteria. By this approach, we were able to identify 230 potential antigens (black bars in Figure 1-), which were, then, expressed as His-tagged proteins, purified and tested for protection in a sepsis mouse model. In order to improve the solubility or the expression levels of some of the antigens, the genes were expressed as smaller peptides, yielding a total of 270 candidates. Of these, 220 were successfully purified, 69 as soluble and 151 as insoluble proteins [2].

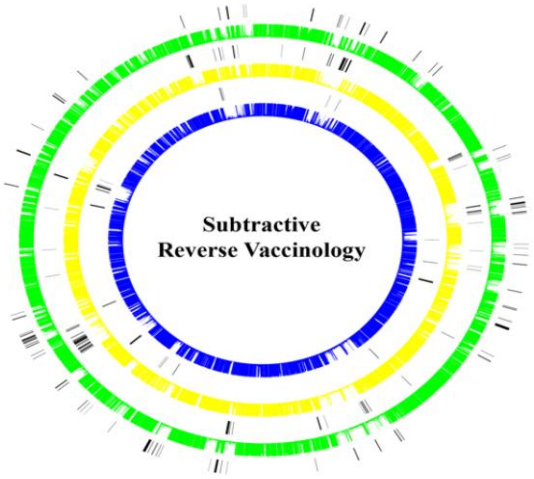

Figure 1-3: The subtractive reverse vaccinology approach (Moriel et al., 2010). The comparison between extraintestinal pathogenic *Escherichia coli* (ExPEC) and nonpathogenic *E. coli* genome sequence is shown (IHE3034, green; CFT073, yellow; 536, blue). Blank regions indicate the low homology shared between ExPEC and nonpathogenic *E. coli* genome sequences. The 230 antigens selected are represented in black,

In vivo protection was evaluated in CD1 mice by subcutaneous injection with three doses of 20 μg for each purified recombinant antigen, in Freud's adjuvant, at days 1, 21 and 35. Two weeks after the last dose, mice were challenged with the homologous strain by intraperitoneal (for IHE3034 and CFT073) or intravenous (for 536) injection. After challenge, animal survival was followed for 4 days and bacteraemia was measured at 20 hours. By using this approach, we identified nine potential vaccine candidates against ExPEC able to confer a protective efficacy (PE) ranging from 13% to 82% in the sepsis mouse model (Table 1-) [2].

Table 1-1. The most protective candidates selected in the mouse model of sepsis (Moriel et al., 2010).

Candidate	Annotation	Protective efficacy, %	*P* value	Solubility	Location	Source
ECOK1_3385	Lipoprotein, putative	82	<0.0001	Soluble	PAI $V_{IHE3034}$	This study
ecp_3827	Hemolysin A	76	<0.0001	Insoluble	PAI I_{536}, PAI II_{536}	(53)
c1275	Hypothetical protein	45	0.0002	Soluble	PAI-CFT073-*serX*	(54)
c5321	Hypothetical protein	33	<0.0001	Soluble	—	This study
ECOK1_3457	TonB-dependent siderophore receptor	25	0.029	Insoluble	—	This study
c0975	Hypothetical protein	24	0.1	Insoluble	Φ-CFT073-b0847	(54)
ECOK1_3374	gspK general secretion pathway protein K	20	0.0009	Soluble	PAI $V_{IHE3034}$	This study
ECOK1_0290	Bacterial Ig-like domain (group 1) protein	15	0.048	Soluble	—	This study
ECOK1_3473	Fimbrial protein	13	0.09	Soluble	—	This study

Candidates ECOK1_3385, ECOK1_3457, ECOK1_3374, ECOK1_0290, and ECOK1_3473 have been amplified from IHE3034; candidates c1275, c5321, and c0975 have been amplified from CFT073; candidate ecp_3827 has been amplified from 536. The infection was performed with the homologous challenge strains. *P* value <0.05, calculated by Fisher's exact test, indicates a significant difference in survival between vaccinated and control groups.

Most of the protective candidates are encoded in large genomic islands and have just a putative or hypothetical function assigned. The feasibility of a universal intervention against all pathogenic *E. coli* strains is highlighted in Fig. 1.5, which shows that all *E. coli* strains sequenced to date could be covered by a vaccine that contains at least four antigens: ECOK1_0290, ECOK1_3385, ECOK1_3457, and c0975.

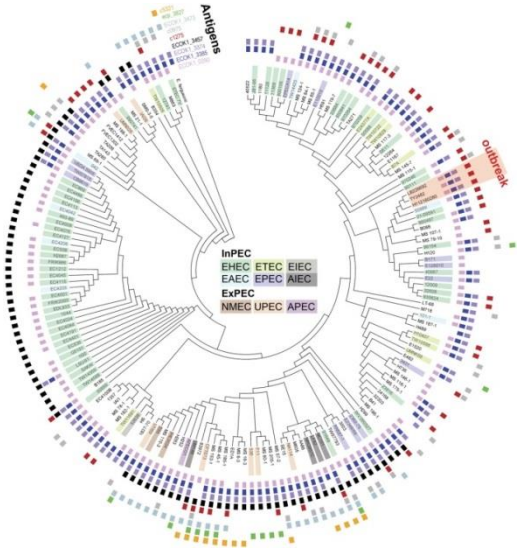

Figure 1-4 The evolutionary relationship and distribution of protective antigens among sequenced *E. coli* strains:(Moriel et al., 2012). The evolutionary history of 170 strains was inferred from MLST data using the neighbor-joining method. The presence of nine protective antigens, identified from reverse vaccinology of ExPEC strains, is shown as colored squares, and they are sorted from the most represented (inner circle) to the least represented (outer circle). Strains were tentatively classified when possible as InPEC (EHEC, ETEC, EIEC, EAEC, AIEC, and EPEC, colored as shown in the legend), ExPEC (NMEC, UPEC, and APEC), and mainly fecal or environmental isolates (not colored).

[183], ECOK1_3457 is involved in iron acquisition [46], and c0975 is annotated as a hypothetical protein. The structural and functional characterization of antigen ECOK1_3385 is the main objective of this thesis.

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Chapter 2

SslE Elicits Functional Antibodies That Impair *In Vitro* Mucinase Activity and *In Vivo* Colonization by Both Intestinal and Extraintestinal *Escherichia coli* Strains

Chapter 2

During the quest for new potential vaccine candidates able to protect against extraintestinal as well as intestinal *E. coli* pathotypes, we recently identified a putative lipoprotein, ECOK1_3385, able to confer protection in a murine model of sepsis and widely conserved in all *E. coli* pathotypes. This protein, also described as SslE (for secreted and surface associated lipoprotein for *E. coli*) and formerly known as YghJ, appears to be required for biofilm formation and for virulence of enteropathogenic *E. coli* (EPEC), although more recent evidences indicate that SslE has no effect on adherence and biofilm formation in atypical EPEC strains.

SslE has been recently associated to the M60-like extracellular zinc-metalloprotease sub-family which is implicated in complex glycan recognition. By applying a number of *in vitro* bioassays and comparing wild type and knockout mutant strains, we have now demonstrated that SslE specifically contributes to degradation of mucin substrates. Mucinolytic activity, fully restored by complementing *E. coli* with a plasmid carrying the wild-type *sslE* gene, was affected in the mutant strain complemented with a plasmid carrying a triple mutation in the putative metallopeptidase domain (M60-like) of *sslE*. Since we have previously demonstrated that SslE is immunogenic in a mouse model of sepsis, we tested whether antibodies against this antigen may also impair mucinase activity *in vitro*. We found that antibodies raised against SslE are able to inhibit translocation of different *E. coli* strains through a mucin-based matrix, suggesting that they may possess functional properties also *in vivo*. By using well-established animal models, we proved that immunization with recombinant SslE significantly reduced gut, kidney and spleen colonization by both intestinal and extraintestinal strains and impaired *E. coli* systemic spread.

In vitro anti-mucinase activity exerted by SslE polyclonal antibodies, corroborated by a reduced colonization of caecum in mice immunized with recombinant SslE, support the hypothesis that the impairment of mucin cleavage may account for the mechanisms of protection from *E. coli* infections in both gut and urinary tract. In addition, by a global amino acid sequence alignment of 424 sequences, we found that SslE sequence variability was present and distributed along the entire protein sequence and that two main branches represent SslE clades (variant I and II).

In this study, we demonstrated that antibodies raised against variant I possess cross-functional properties versus other SslE sub-variants. Taken together, these data strongly support the importance of SslE in *E. coli* colonization of mucosal surfaces. Moreover, the widespread distribution and conservation of SslE, together with the capacity to elicit functional antibodies, assessed both *in vitro* and *in vivo*, strongly support the potential coverage of such an antigen against both intestinal and extraintestinal pathogenic *E. coli* strains.

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SslE Elicits Functional Antibodies That Impair *In Vitro* Mucinase Activity and *In Vivo* Colonization by Both Intestinal and Extraintestinal *Escherichia coli* Strains

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Abstract

SslE, the Secreted and surface-associated lipoprotein from *Escherichia coli*, has recently been associated to the M60-like extracellular zinc-metalloprotease sub-family which is implicated in glycan recognition and processing. SslE can be divided into two main variants and we recently proposed it as a potential vaccine candidate. By applying a number of *in vitro* bioassays and comparing wild type, knockout mutant and complemented strains, we have now demonstrated that SslE specifically contributes to degradation of mucin substrates, typically present in the intestine and bladder. Mutation of the zinc metallopeptidase motif of SslE dramatically impaired *E. coli* mucinase activity, confirming the specificity of the phenotype observed. Moreover, antibodies raised against variant I SslE, cloned from strain IHE3034 ($SslE_{IHE3034}$), are able to inhibit translocation of *E. coli* strains expressing different variants through a mucin-based matrix, suggesting that SslE induces cross-reactive functional antibodies that affect the metallopeptidase activity. To test this hypothesis, we used well-established animal models and demonstrated that immunization with $SslE_{IHE3034}$ significantly reduced gut, kidney and spleen colonization by strains producing variant II SslE and belonging to different pathotypes. Taken together, these data strongly support the importance of SslE in *E. coli* colonization of mucosal surfaces and reinforce the use of this antigen as a component of a broadly protective vaccine against pathogenic *E. coli* species.

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Introduction

Pathogenic *E. coli* can be broadly classified as either extraintestinal pathogenic *E. coli* (ExPEC), the main cause of urinary tract infection (UTI), newborn meningitis and sepsis, or as intestinal pathogenic *E. coli* (InPEC) causing diarrhoeagenic infections. Among the intestinal pathogens there are at least six well-described groups: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) [1]. The plasticity of the *E. coli* genomes, due to the ability to gain or lose virulence attributes by horizontal gene transfer, allows these organisms to colonize different sites. Indeed, *E. coli* possesses an array of virulence factors which include various adhesins, capsule, iron-transporters, toxins and proteases (reviewed in [1]). However, recent studies have suggested that the pathogenesis of *E. coli* is considerably more complex than previously appreciated involving additional virulence factors [2,3]. The absence of a broadly protective vaccine against pathogenic *E. coli* is a major problem for modern society since diseases caused by these bacteria are associated with significant human suffering and high healthcare costs. The overall problem is exacerbated by the rising rates of multi-drug resistant strains and by the emergence of new sequence types and hypervirulent strains [4–9]. We have recently proposed ECOK1_3385 as a promising vaccine candidate able to confer protection in a murine model of sepsis [10,11]. This protein, described as SslE (for secreted and surface-associated lipoprotein from *E. coli*) and formerly known as YghJ [12,13], appears to be required for biofilm formation and for virulence of EPEC strains [14], although more recent evidence indicates that SslE has no effect on adherence and biofilm formation in atypical EPEC strains [15]. Thus, the function of

Author Summary

Escherichia coli are the predominant facultative anaerobe of the human colonic flora. Although intestinal and extraintestinal pathogenic *E. coli* are phylogenetically and epidemiologically distinct, we recently proposed a number of protective antigens conserved in most *E. coli* pathotypes. In this study, we have elucidated the function of the most promising of these antigens, SslE, which is characterized by the presence of a M60-like domain representative of a new extracellular zinc-metalloprotease sub-family. In particular, *in vitro* analysis of the ability of an *sslE* knockout mutant strain to transverse an agar-based mucin matrix revealed that SslE is essential to *E. coli* mucinase activity. Evidence showing that SslE induces functional antibodies, preventing both *in vitro* mucin degradation but also *in vivo* gut, kidney and spleen colonization, further support the hypothesis that SslE may facilitate *E. coli* colonization by favoring the penetration of the sterile inner mucus layer leading to interaction with host cells. Finally, the ability of SslE to also induce protective immunity against sepsis, linked to its presence among different pathotypes, supports the use of such an antigen as a broadly protective *E. coli* vaccine candidate.

SslE remains to be fully elucidated. However, it is known that SslE is secreted through a type II secretion system (T2SS), an exporting apparatus typically used by Gram-negative bacteria to secrete virulence determinants [16]. Two T2SSs exist in *E. coli*, designated as alpha (T2SSα) and beta (T2SSβ) [17]. The T2SSβ operon is composed of three genes (*yghJ*, *pppA*, and *yghG*) upstream of *gspC*β. The first gene, *yghJ*, encodes for the SslE protein. A functional T2SSβ secreting a cognate SslE protein was recently studied in the non-pathogenic *E. coli* W strain [18]. Recently, it was reported that SslE belongs to a new sub-family of extracellular zinc-metallopeptidases, characterized by a M60-like zinc-metalloprotease domain HEXXHX(8,24)E [19], that is distantly related to known viral enhancin zinc-metallopeptidases. The baculovirus enhancin protein Vef is able to digest intestinal mucins, facilitating the attachment and entry of the virus into epithelial cells [20].

Using biochemical and functional assays, we demonstrated that SslE is involved in *E. coli* degradation of mucin substrates. In addition, antibodies raised against SslE variant I from ExPEC strain IHE3034 were able to inhibit translocation of different *E. coli* pathotypes through a mucin-based matrix, suggesting a possible mechanism for *in vivo* protection. This hypothesis was corroborated by the fact that in mouse models of intestinal and urinary tract colonization, SslE variant I induced protective immunity also against *E. coli* strains expressing variant II. The widespread distribution and conservation of SslE, together with the ability to elicit functional antibodies, assessed both *in vitro* and *in vivo*, strongly support the potential of the SslE antigen to provide coverage against both intestinal and extraintestinal pathogenic *E. coli* strains.

Results

SslE localizes on *E. coli* surface at distinct foci

It has been recently reported that although SslE is secreted by a T2SS, it is also found on the bacterial cell surface [10,14]. Confocal analysis of Z-stack images of an ExPEC strain IHE3034 stained for SslE and deconvoluted using Volocity Software, revealed that the antigen is translocated on the bacterial surface at specific foci (Fig. 1A). Of interest, we observed that only a small proportion of bacteria (3% of total number) expressed the antigen on the surface (Fig. 1A). We determined that this phenotype is attributable to the polysialic acid capsule (K1 antigen) that it responsible for masking SslE on the bacterial surface (Fig. S1). The *sslE* deletion mutant strain (IHE3034Δ*sslE*) did not show any surface labeling (Fig. 1B), confirming the specificity of the signal. Complementation of the mutant strain with a pET24b+ plasmid carrying the *sslE* gene (including the promoter region) restored antigen surface localization (Fig. 1C). To exclude the possibility that the SslE signal at the bacterial surface could be partially attributed to the re-association of the secreted form of the protein to the membrane, we co-cultured the IHE3034 wild-type (WT) strain with the IHE3034Δ*sslE* strain engineered to express the GFP fluorescent protein. Staining of bacteria using SslE antibodies conjugated to FITC, revealed that the antigen was exclusively detected on the surface of the WT strain and not on the fluorescent bacteria, indicating that no SslE re-association occurred (data not shown).

SslE is involved in *in vitro* mucin degradation by IHE3034 strain

As recently reported by Nakjang and collaborators [19], HEXXHX(8,24)E is the full putative metalloprotease core motif of SslE (residues: 1304–1322; SslE accession number: YP_006102500),

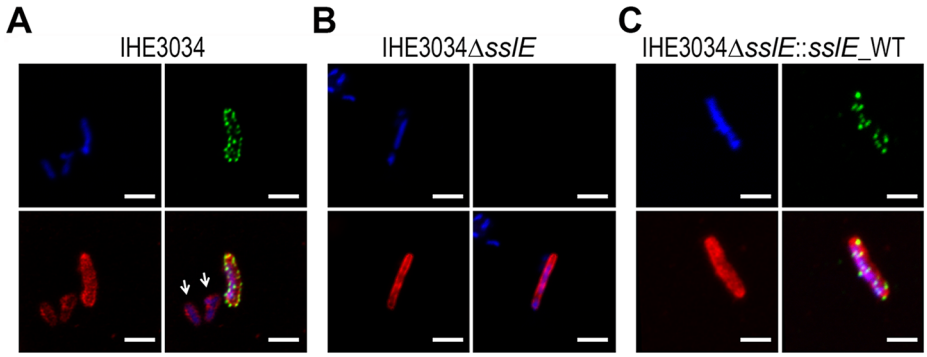

Figure 1. SslE surface localization on the ExPEC strain IHE3034. Confocal images of (A) IHE3034 wild-type, (B) IHE3034Δ*sslE* knockout mutant and (C) IHE3034Δ*sslE*::*sslE*_WT complemented strain. SslE was detected using specific anti- SslE antibodies raised in rabbits and visualized using a fluorescent secondary antibody (green). Antibodies to whole-IHE3034 bacteria and a fluorescent secondary antibody (red) and DAPI (blue) were used to visualize bacteria and chromosomal DNA, respectively. White arrows indicate bacteria negative for SslE staining. Bars, 1 μm.
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exclusively present in a recently characterized zinc metallopeptidase sub-family possessing mucinase activity [19]. The pattern "HEXXHX(8,24)E" consists of a conserved glutamate residue localized 8 to 24 amino acids from the "HEXXH" motif. To investigate the putative mucinolytic activity of SslE, we have applied a number of *in vitro* assays previously reported to specifically detect mucinase activity in bacteria [21–24]. The first approach is based on the use of bacteria grown on agar plates containing 0.5% bovine submaxillary mucin followed by amido black-staining [25,26]. Plates containing the IHE3034 WT strain incubated for 24 h revealed clear zones of mucin lysis (Fig. 2A). However, no cleared areas were detected when the IHE3034Δ*sslE* knockout (KO) strain was added to the plates, indicating the specific contribution of SslE to the mucinase activity. Incubation of mucin-based plates with the complemented strain IHE3034Δ*sslE*::*sslE*_WT carrying the WT *sslE* gene fully restored the wild-type phenotype as assessed by the lack of amido black staining. To investigate the role of the M60-like core motif in mucin lysis, we transformed the IHE3034Δ*sslE* strain with the pET24b+ plasmid carrying a triple mutation in the putative metallopeptidase motif of SslE (**YV**VG**Y** vs. HEVGH). In particular, we introduced hydrophobic elements in the HEXXH motif (Y and V), which by reducing the charge of the enzymatic task are likely to impair the mucinase activity. Testing of this mutant by the amido black assay revealed a phenotype comparable to the *sslE* KO strain (Fig. 2A). These data were further confirmed by the *In Vivo* Imaging System (IVIS-Perkin Elmer) technology which allowed the visualization of bacterial migration through the agar-mucin matrix at different time points, using IHE3034 strains engineered for constitutive expression of a luciferase operon (p*lux*) [27] (Fig. 2B). Briefly, a mid-log bacterial culture of the bioluminescent strains was loaded in a well created at the center of a mucin-agar plate and bacterial distribution was detected after 24 h of incubation. IHE3034(p*lux*) and IHE3034(p*lux*)Δ*sslE*::*sslE*_WT strains, but not IHE3034(p*lux*)Δ*sslE* and IHE3034(p*lux*)Δ*sslE*::*sslE*_mut, were able to spread beyond the site of the initial inoculum (Fig. 2B), confirming that SslE-dependent mucin degradation enables diffusion of *E. coli* through the agar.

Antibodies against SslE prevent the ability of IHE3034 to cross a mucin-based matrix in a dose-dependent manner

To test the hypothesis that anti-SslE antibodies may also inhibit mucinase activity *in vitro*, we developed an *in vitro* system to quantify the ability of strain IHE3034 WT to transverse a mucin-based gel matrix. An agar-based matrix gel containing 10% bovine submaxillary gland mucins was reconstituted in a 1 mL syringe and bacterial aliquots (10^8 CFU) were layered on top of the gel and statically incubated for 3 h at 37°C in a vertical position to allow bacterial translocation. At the end of the incubation period, gel fractions were eluted from the bottom of the syringe, diluted and plated for CFU determination. After confirming the impaired phenotype of the *sslE* KO strain in traversing the mucin matrix compared to the isogenic WT strain (~2.5 Log reduction) (Fig. 3A), we tested the ability of polyclonal antibodies generated by subcutaneous immunization of rabbit with the full length recombinant SslE from ExPEC strain IHE3034 (anti-$SslE_{IHE3034}$) to reduce bacterial translocation through the mucin agar-gel syringe. Anti-SslE IgGs and IgAs in the rabbit serum were measured by ELISA (Fig. S2A and B). A significant dose-dependent inhibition of bacterial translocation was observed when the mucin-gel matrix was polymerized together with anti-SslE antibodies (dose range 1:50 to 1:1350) (Fig. 3B). At a dilution of 1:50 the inhibitory effect of SslE antibodies was evident in all fractions collected, while higher

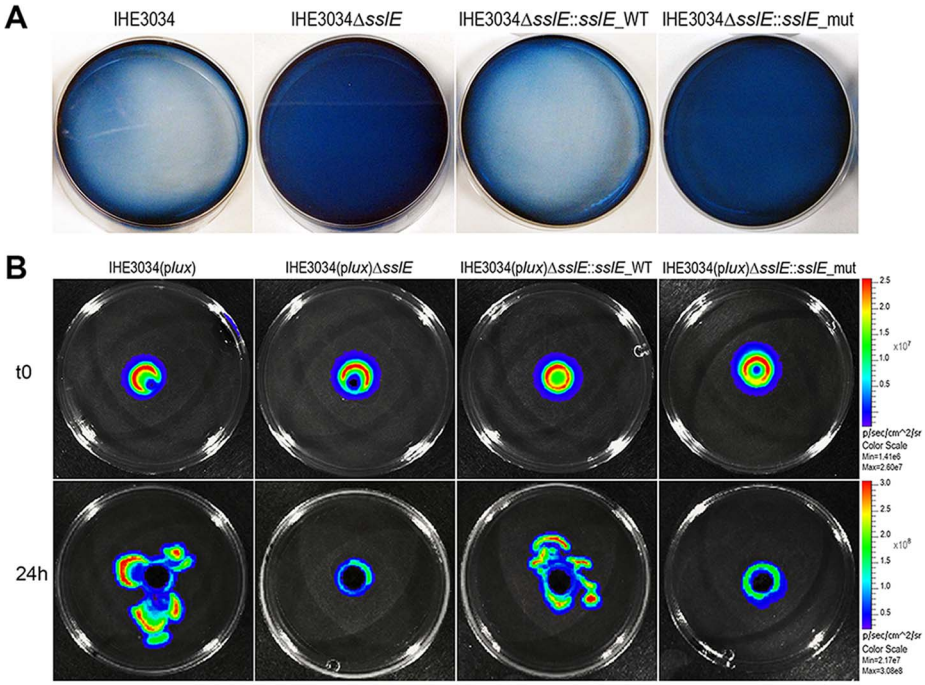

Figure 2. SslE mucinolytic activity. (A) Mucin lysis (clear plates) was assessed by amido black staining. IHE3034 wild-type, IHE3034Δ*sslE* knockout mutant, IHE3034Δ*sslE*::*sslE*_WT (complemented with the *sslE* wild-type gene), and IHE3034Δ*sslE*::*sslE*_mut (complemented with the *sslE* gene mutated in the putative metallopeptidase motif), were grown on plates containing 0.5% bovine submaxillary mucin (SIGMA) and stained with 0.1% (wt/vol) amido black in 3.5 M acetic acid for 30 min and destained with 1.2 M acetic acid. (B) The four strains were engineered for constitutive luciferase expression (p*lux* operon) and mucinolytic activity was detected by the *In Vivo* Imaging System (IVIS) technology. Bacterial migration in the mucin-agar plates is shown, from the point of inoculum (time zero; t0) to growth at 24 hours (24 h).
doi:10.1371/journal.ppat.1004124.g002

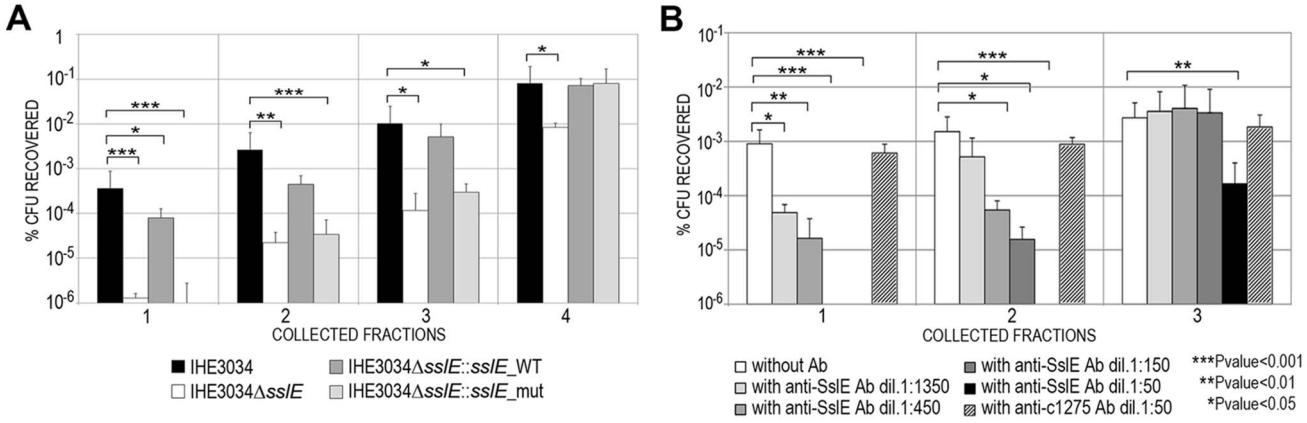

Figure 3. Anti-SslE antibodies impair translocation of *E. coli* through a mucin-gel matrix. (A) IHE3034 wild-type, the IHE3034Δ*sslE* knockout mutant, IHE3034Δ*sslE*::*sslE*_WT (complemented with the *sslE* gene wild-type) and IHE3034Δ*sslE*::*sslE*_mut (complemented with *sslE* gene mutated in the putative metallopeptidase motif), were loaded on the top of a mucin-gel matrix column polymerized in a 1 ml syringe. After 3 hours at 37°C, eluted fractions were collected and plated for CFU counting. The results were reported as percentage of CFU recovered in four different fractions, sequentially eluted from the bottom of the column, with respect to the initial inoculum. (B) Dose-dependent inhibition of IHE3034 translocation through a mucin-gel matrix by anti-SslE antibodies. Serial dilutions (range 1:50–1:1350) of antibodies were used for inhibition. Antibodies against the unrelated ExPEC c1275 were used as a negative control at the dilution 1:50. Translocation was reported as the percentage of CFU recovered with respect to the initial inoculum for three sequentially eluted fractions. P values were determined using a two-tailed unpaired Student's significance test.
doi:10.1371/journal.ppat.1004124.g003

dilutions principally affected bacterial translocation in the first two fractions. A higher dilution of 1:4050 did not show an inhibitory effect in any of the collected fractions (data not shown). The specificity of the inhibition was confirmed by the absence of an effect when using an antiserum against the unrelated ExPEC antigen c1275 [10], at the lowest dilution (Fig. 3B). On the other hand, since antibodies against a fragment of SslE, C-$SslE_{IHE3034}$, lacking the M60-like motif (Fig. S3A), were still capable of impairing IHE3034 translocation through the mucin layer (Fig. S3B), it is not possible to establish whether polyclonal antibodies have a direct or an indirect effect on SslE activity.

Antibodies against variant I SslE from IHE3034 inhibit *E. coli* mucin translocation in strains expressing variant II SslE

As previously reported, SslE can be divided into two main variants [10]. Three hundred and eighteen *E. coli sslE* sequences were added to the 96 previously analyzed by Moriel *et al.* [10] (Table S1) and global amino acid sequence alignment revealed that sequence variability was present and distributed along the entire protein sequence. Overall, amino acid sequence identity ranged from 86–100%, with the HEXXHX(8,24)E core motif fully conserved in all sequences analysed. A total of 155 *E. coli* unique protein sequences were identified and used to construct a phylogenetic tree (Fig. 4 and Table S2). The two main branches denoted the presence of two SslE clades (encoding for two variants: I and II). To understand whether antibodies raised against variant I can cross-inhibit the mucinolytic activity of other SslE sub-variants, we selected a number of strains producing SslE variant II and belonging to different pathotypes. We tested the ability of an antiserum against SslE from strain IHE3034 ($SslE_{IHE3034}$, belonging to variant I) to prevent the translocation of intestinal and extraintestinal strains expressing SslE belonging to variant II. In particular, we selected an EPEC strain (IC50), a SEPEC (septicemic-associated *E. coli* belonging to ExPEC) strain (IN1S), an ETEC strain (GL53) and the EAHEC strain (LB226692) recently identified to be responsible for the 2011 German *E. coli* outbreak. $SslE_{IHE3034}$ antiserum inhibited the ability of all *E. coli* pathotypes tested (expressing SslE variant II) to traverse the mucin-based matrix (Fig. 5).

SslE promoter is functional in a mouse model of intestinal colonization

The evidence that antibodies against $SslE_{IHE3034}$ are functional and recognize different variants allows us to postulate that a vaccine containing this antigen may have the potential to protect against most pathogenic *E. coli* species. In order to test the protective efficacy of $SslE_{IHE3034}$ (variant I), we set up a mouse model of intestinal colonization using the ETEC GL53 strain. Mice were intragastrically infected with the bioluminescent GL53-P*em7-luxCDABE* strain [27] and consistent bioluminescent signals were detected in the abdominal region until to 72 hours post-infection by the *In Vivo* Imaging System (IVIS) (Fig. 6A). As observed for other intestinal *E. coli* pathotypes [28,29], bacterial infection mainly occurs in the caecum tract (Fig. 6B). This is consistent with data obtained by both CFU counts from infected intestinal ileum and caecum tracts (Fig. 6C) and confocal imaging of tissues (Fig. 6D and E). After setting up the GL53 intestinal colonization, we evaluated the functionality of the *sslE* promoter *in vivo*. 2D bioluminescent signal in the abdominal region could be observed when the luciferase expression was driven by the *sslE* promoter (Fig. 7A), compared to the positive control GL53-P*em7-luxCDABE*. As expected, GL53 transformed with the luciferase promoterless plasmid gave no signal (Fig. 7A) [27]. In addition, 3D analysis confirmed that the signal was predominantly associated with the intestine (Fig. 7B). *SslE* transcription in GL53 colonizing bacteria was further evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR), confirming that the *sslE* promoter is active *in vivo* (Fig. 7C).

Variant I SslE induces cross-protective immunity against variant II SslE expressing strains in intestinal colonization, UTI and sepsis mouse models

Cross-protective efficacy was evaluated by immunizing 30 mice intranasally with the recombinant variant I SslE and challenging

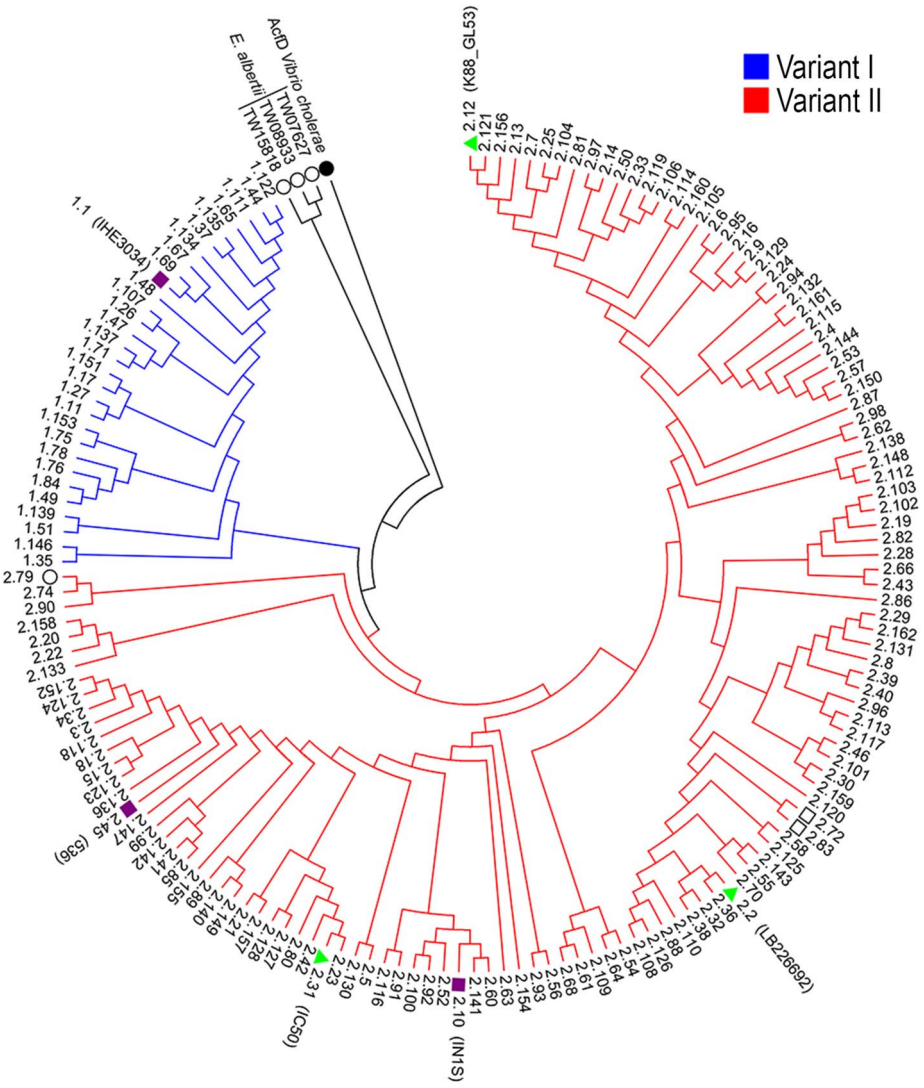

Figure 4. Phylogenetic tree of SslE from a panel of *E. coli* isolates. The phylogenetic tree of 155 unique *E. coli* SslE protein sequences was inferred using the neighbor-joining method. Two main SslE variants are highlighted with different colors: blue for variant I and red for variant II. Purple bullets refer to ExPEC and green triangles to InPEC isolates used in experiments. Black circles indicate SslE amino acid homologues of *Vibrio cholerae* and *Escherichia albertii* used as outgroup sequences. The tree also includes two sequences from *Escherichia fergusonii* (black square). The strains are designed with patterns made by a first number relative to the SslE main variant and a second number corresponding to the sub-variant (see table S1 and table S2 for immediate identification). Phylogenetic analysis were conducted by MEGA4 software [52].
doi:10.1371/journal.ppat.1004124.g004

them with the ETEC strain GL53 (expressing variant II SslE). Following immunizations with 30 μg of recombinant $SslE_{IHE3034}$ at days 1, 21 and 35, mice were infected by oral gavage with 5×10^7 CFU of GL53 at day 49. Intestinal caecum tracts were collected at day 51, serial dilutions of the homogenized tissues were plated and the CFU numbers were enumerated. A statistically significant reduction (2.5 Log) in the mean value of GL53 bacterial counts in the caecum was observed in mice immunized with the $SslE_{IHE3034}$ antigen versus those treated with saline (Fig. 8A). Anti-SslE responses in protected mice consisted of antibodies belonging to both IgG and IgA isotypes (Fig. S2C and D).

To further support the observation that $SslE_{IHE3034}$ (variant I) induces heterologous protection, we considered two alternative models: a murine model of ascending UTI and a murine sepsis model. In the UTI model, 30 mice were intranasally inoculated with either cholera toxin (CT) alone (as an adjuvant) or an $SslE_{IHE3034}$-CT mixture. Following three immunizations (days 0, 7, 14), animals were transurethrally challenged on day 21 with the UPEC strain 536 (expressing SslE variant II) and protection was assessed at 48 h post infection by determining the CFUs in the urine, bladder, kidneys and spleen. $SslE_{IHE3034}$ immunization led to a significant reduction in median CFU/g ($P = 0.0394$) in the kidneys and a more evident protection in the spleen with a 2.0 Log reduction in median CFU/g ($P = 0.0006$) (Fig. 8B). In the sepsis model, systemic *E. coli* infection was performed as recently reported [10]. Active immunization with $SslE_{IHE3034}$ followed by challenge with the SEPEC strain IN1S (expressing SslE variant II) provided significant protection from mortality (60% survival, $P < 0.0001$) (Fig. 8C).

Discussion

E. coli is a well-adapted human pathogen which uses the gut as a preferential niche and, as for other intestinal microorganisms, it

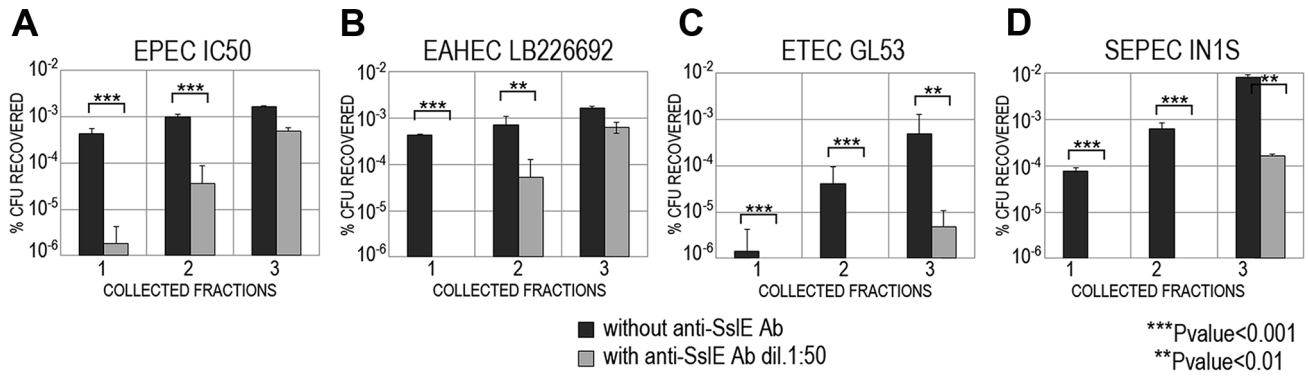

Figure 5. Cross-inhibition of *E. coli* translocation through a mucin-gel matrix by anti-SslE$_{IHE3034}$ (belonging to variant I) antibodies. EPEC IC50 (A), EAHEC LB226692 (B), ETEC GL53 (C), SEPEC IN1S (D) strains carrying variant II were loaded on top of the gel-mucin matrix column and bacterial translocation with or without anti-SslE$_{IHE3034}$ antibodies was assessed in three sequentially collected fractions. For each strain, translocation was reported as the percentage of CFU recovered with respect to the initial inoculum.
doi:10.1371/journal.ppat.1004124.g005

persists in this region due to its ability to exploit a number of metabolic substrates and to stay in the outer mucus layer where commensal bacteria normally reside. Recent studies [30–33], including those reported by our group [34,35], have postulated that this microorganism has adapted to the human body by developing a sophisticated network of virulence and colonization factors. Among these adhesins, iron-uptake systems and IgA binding proteins may allow *E. coli* to out-compete the many species occupying an overcrowded environment such as the intestine. In this scenario, our finding that SslE contributes to *E. coli* mucinase activity suggests the involvement of this antigen in landscaping the *E. coli* territory allowing the establishment of a long lasting colonization. Indeed, shaping of the intestinal microbial community by the mucosa does not only depend on goblet cells secreting antimicrobial proteins, but also on a number of metabolic substrates vital to mucus-degrading bacteria [36–38]. In our study, the diminished capacity of the *sslE* mutant strain to translocate through a mucin-rich matrix *in vitro* suggests that SslE activity may facilitate bacterial penetration of the mucosal surface, including the inner mucus layer, to reach the underlying host epithelium. Although these data do not exclude that the catabolism of such glycoproteins may also contribute to an increased fitness of *E. coli* in the outer mucus layer, the pathogenic strains that are armed with immune evasion virulence factors may use SslE as a spearhead to penetrate the sterile inner mucus layer so as to intimately adhere to the epithelial cells of the host.

The core motif, HEXXH, present in SslE is conserved in all families of the Clan of peptidase named MA (M for metallo) although it might also be present by chance in proteins with no peptidase activity [39,40]. Using the full putative metalloprotease domain of the ExPEC variant of SslE (residues: 1082–1382) to search the Pfam-A protein families database, we confirmed that the entire top 100 hits (E-value<8e-35) were M60-like domains (Pfam ID: PF13402). This domain is exclusively present in a recently characterized zinc metallopeptidase sub-family that possesses mucinase activity [19]. The multiple sequence alignment of the best hits showed the extended motif of the M60-like domain (Supporting information Fig. S4). These hits were mainly bacterial proteins from Gamma proteobacteria, and they have comparable sequence lengths to ExPEC SslE (~1460–1520 a.a.). Interestingly, the majority of these proteins were predicted to be outer membrane lipoproteins that are N-terminally anchored to the outer membrane, which implies that these mucinases are dedicated to digestion of extracellular host glycoproteins. However, although our data support the hypothesis for the contribution of SslE to *E. coli* colonization by a mechanism likely to involve mucin degradation, we were not able to obtain direct evidence for such an enzymatic activity. Indeed, we observed that recombinant SslE binds to Zinc, but is unable to cleave a number of putative metalloprotease-target molecules including gelatin, casein, fibrinogen, and different collagens (data not shown). However, since bacterial metalloprotease activities are known to depend on different parameters (such as pH, temperature, salt concentration, etc.) [41,42], further screenings for appropriate *in vitro* conditions will be required.

The large antigenic and genetic variability of pathogenic *E. coli* species has been a major obstacle to the development of a broadly protective vaccine. Indeed, the difficulty in predicting vaccine coverage and the lack of a correlate of protection, has led to numerous promising pre-clinical data not being confirmed by human studies [43–47]. By comparing the genome of an ExPEC strain causing neonatal meningitis to those of other ExPEC and nonpathogenic strains, we have recently proposed a number of well conserved protective antigens. Among them the most promising candidate was SslE, which due to its conservation in both intestinal and extraintestinal strains was proposed as a universal vaccine candidate. The anti-mucinase activity exerted by anti-SslE polyclonal antibodies *in vitro*, corroborated by a reduced colonization of caecum in mice immunized with recombinant SslE, further support the hypothesis that the impairment of mucin cleavage may account for the mechanisms of protection from *E. coli* infections in both the mucosal tissues of the gut and the urinary tract [48,49]. In addition, antibodies generated against SslE variant I showed cross-functional properties versus strains expressing variant II. Since polyclonal antibodies raised against full-length SslE are able to cross-inhibit antigen functional activity, we hypothesized that they may target conserved domains of SslE potentially involved in the metalloprotease activity. However, only a few strains were tested and further studies using a larger panel of clinically relevant strains would be needed to confirm such an assumption.

In conclusion, the contribution of SslE to *E. coli* mucinolytic activity *in vitro*, and SslE mediated protection against intestinal and urinary tract colonization *in vivo*, indicate the importance of SslE as a novel colonization factor and a valid target for intervention strategies against disease caused by this important human pathogen.

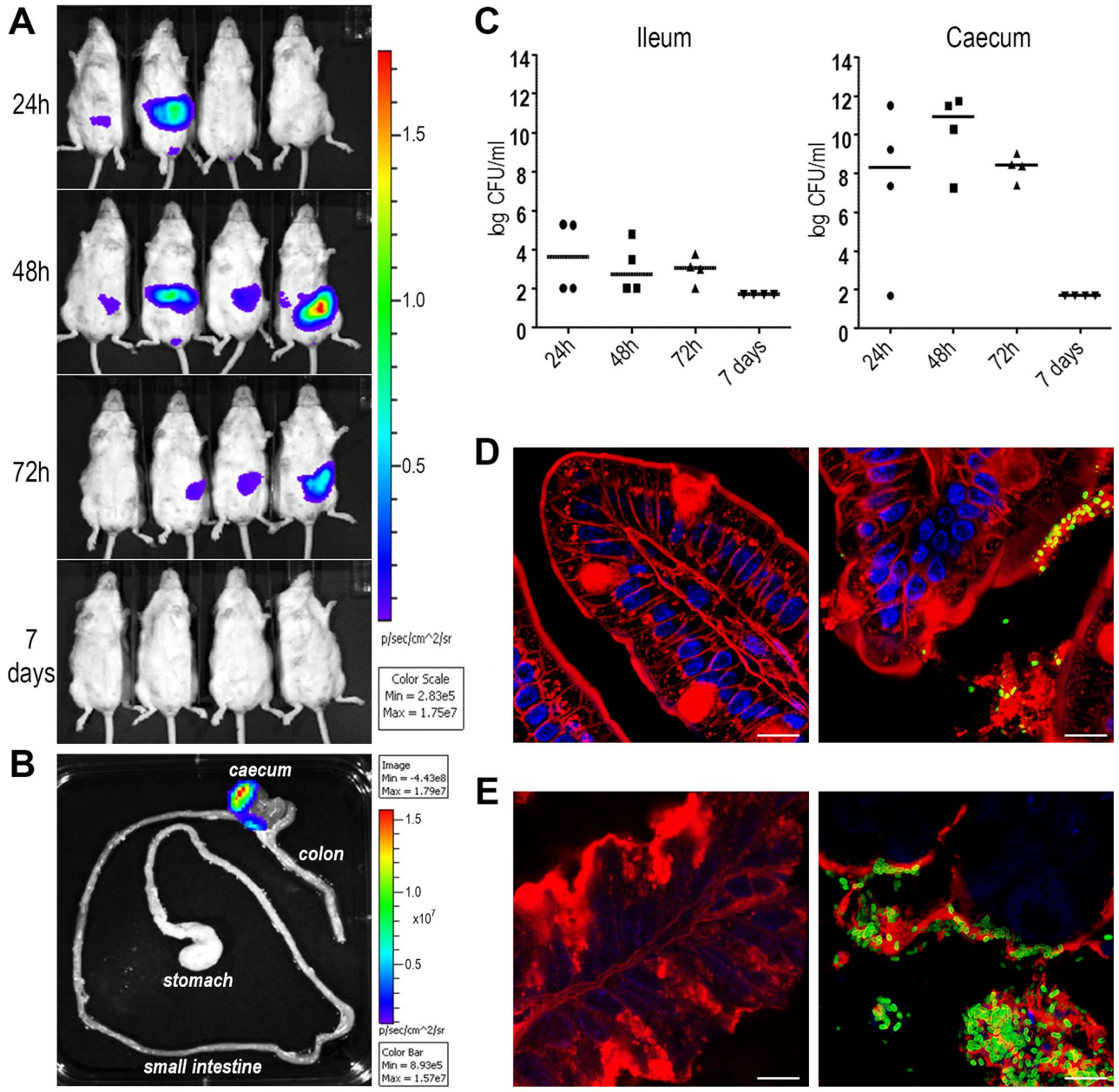

Figure 6. ETEC strain GL53 is able to colonize the mouse intestine. (A) A group of 4 mice was infected with bioluminescent GL53 strain and monitored up to 7 days by IVIS System. The data displayed illustrate the results of a representative experiment. (B) Distribution of the bioluminescent GL53 strain in the intestinal tract of infected mice shows bacterial accumulation in the caecum. (C) Quantitative analysis of intestinal colonization by GL53. Briefly, ileum and caecum at 24 h, 48 h, 72 h and 7 days post infection were homogenized and plated for CFU counts. Symbols represent single mice and the median is shown as bars for each time point. (D) Confocal staining of uninfected (left panel) and GL53 infected (right panel) ileum and (E) images of uninfected (left panel) and infected caecum (right panel). Tissues were visualized with the red fluorescent Wheat Germ Agglutinin (Alexa Fluor 568-WGA, Life Technologies) and nuclei with the blue fluorescent DAPI. Bacteria were detected using polyclonal antibodies against GL53 and visualized by green Alexa Fluor 488-conjugated secondary antibody (Life Technologies). Bars: 10 µm.
doi:10.1371/journal.ppat.1004124.g006

Materials and Methods

Ethics statement

Animal studies regarding intestinal colonization and sepsis models were carried out in compliance with current Italian legislation on the care and use of animals in experimentation (Legislative Decree 116/92) and with the Novartis Animal Welfare Policy and Standards. Protocols were approved by the Italian Ministry of Health (authorization 236/2010-B) and by the local Novartis Vaccines and Diagnostics Animal Welfare Body (authorization AEC 201010). Animal studies for urinary tract infection experiments were conducted according to protocol #08999 approved by the University Committee on the Care and Use of Animals at the University of Michigan Medical School. The approved procedures are in compliance with University guidelines, State and Federal regulations, and the standards of the "Guide for the Care and Use of Laboratory Animals".

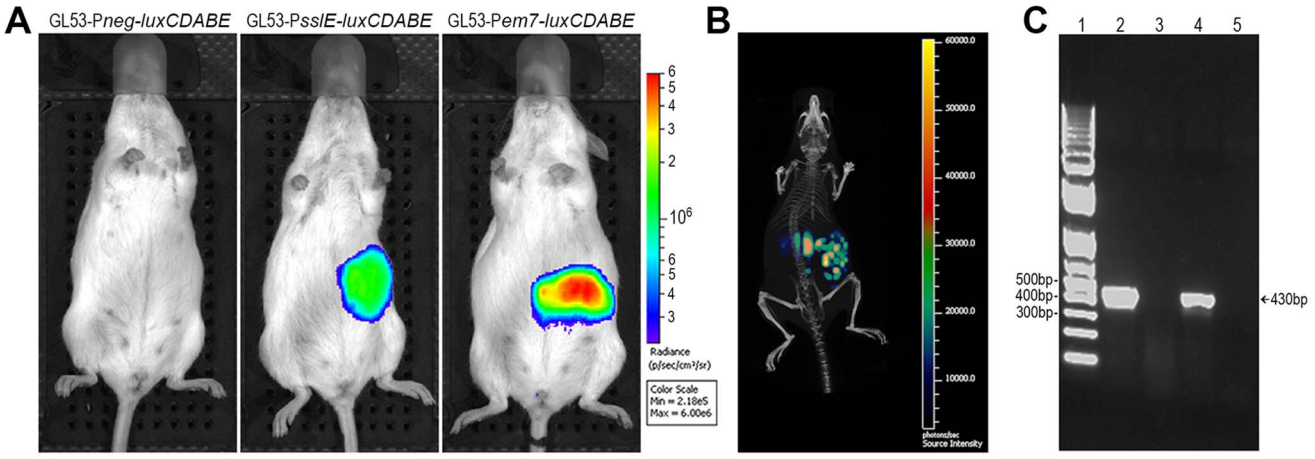

Figure 7. The *sslE* promoter is functional in an intestinal model of colonization. (A) 2D *in vivo* imaging at 24 hours of mice intragastrically infected with GL53-P*neg-luxCDABE* (promoterless control vector), with the bioluminescent derivative GL53-P*sslE-luxCDABE* and with the GL53-P*em7-luxCDABE* (positive control). (B) 3D image reconstruction showing *sslE*-promoter driven luciferase expression in *E. coli* localized in the intestinal tract. (C) RT-PCR of RNA purified from: *in vitro* lab-grown GL53 bacteria (lane 2, positive control); caecum tract of uninfected mice (lane 3, negative control); GL53 bacteria recovered from infected mice (lane 4); GL53 bacteria recovered from infected mice without the RT step (lane 5). 1 Kb Plus DNA Ladder (Life Technologies) is shown in lane 1.
doi:10.1371/journal.ppat.1004124.g007

Bacterial strains and culture conditions

Genomic DNA was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma) according to the manufacturer's instructions. ExPEC strain IHE3034 (serotype O18:K1:H7) was isolated in Finland in 1976 from a case of human neonatal meningitis [50]. Strains were cultured in Luria-Bertani (LB) broth at 37°C with agitation and aeration. *E. coli* DH5α-T1R (Invitrogen) was used for cloning purposes and *E. coli* BL21(DE3) (Invitrogen) was used for expression of His-tagged fusion proteins. The clones carrying a specific antibiotic resistance cassette were

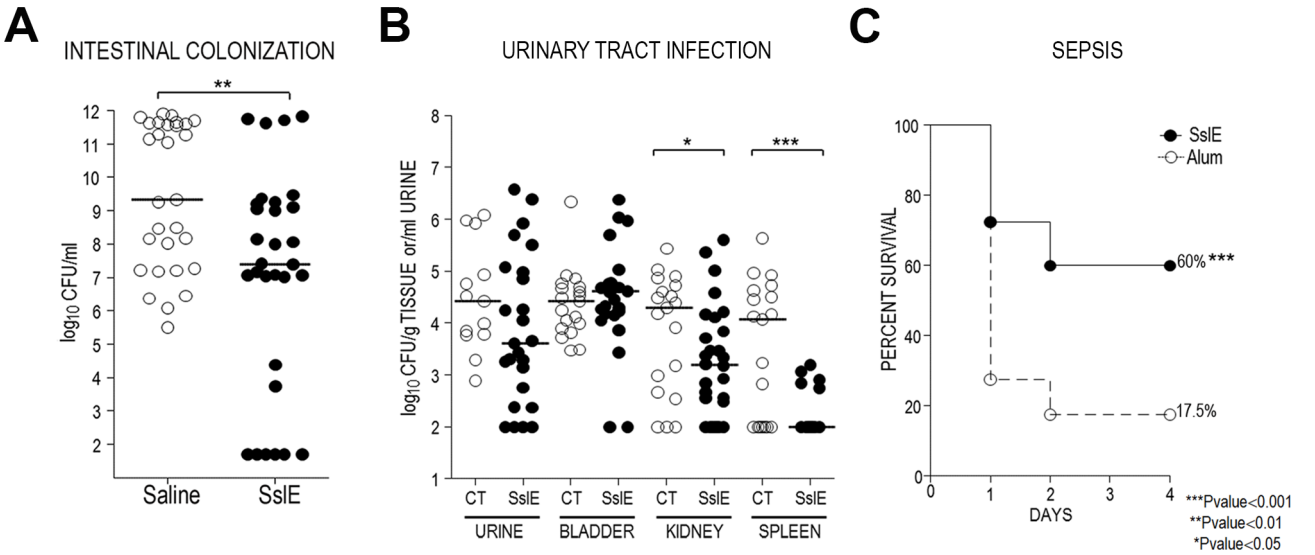

Figure 8. $SslE_{IHE3034}$ induces cross-protection in intestinal colonization, UTI and sepsis models. (A) Thirty CD1 mice were intranasally immunized with 30 μg of $SslE_{IHE3034}$ at days 1, 21 and 35. Saline was used in the negative control groups. Challenge was done by oral gavage with 5×10^7 CFU of strain GL53 at day 49. Serial dilutions of the homogenized intestinal caecum tract were plated and the CFU number was enumerated. Statistical significance of protection was obtained using the Mann Whitney test. (B) $SslE_{IHE3034}$ prevents the spread of the UPEC strain 536 into the kidneys and spleen in an ascending model of urinary tract infection. Thirty mice were immunized intranasally with 10 μg cholera toxin (CT) alone or with 100 μg of $SslE_{IHE3034}$ at a 10:1 ratio of antigen:CT (day 1). After two boosts of 25 μg antigen (10:1 ratio of antigen to CT) or CT alone (day 7 and 14), mice were transurethrally challenged with 10^8 CFU of strain 536 at day 21. After 48 h, bladder, kidneys and spleen were harvested and homogenized. Bacteria in urine and in the tissue homogenates were enumerated by plating serial dilutions. Symbols represent CFU/g tissue or CFU/ml urine of individual mice, and bars indicate median values. P values were determined using the nonparametric Mann-Whitney significance test. (C) $SslE_{IHE3034}$ protects against the SEPEC strain IN1S in a sepsis mouse model. CD1 out-bred mice were immunized by subcutaneous injections at day 1, 21, and 35 with 20 μg of recombinant $SslE_{IHE3034}$ formulated with alum or alum alone. Immunized animals were challenged at day 49 with a sublethal dose of heterologous strain IN1S and survival was monitored for up to 4 days. The results are indicated as percentage of survival out of a total number of 40 mice. P values were determined using the nonparametric Mann-Whitney significance test.
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grown in the presence of kanamycin (50 μg/ml) or ampicillin (100 μg/ml).

Construction of *sslE* deletion mutant and complemented strains

The isogenic *sslE* knockout mutant strain was constructed by replacement of the entire gene by an antibiotic resistance cassette. The upstream and the downstream regions of the *sslE* gene were amplified by PCR with the primers 1–2 and 3–4 (Table S3), using IHE3034 chromosomal DNA as template, and cloned into the pBluescriptKS (Stratagene). The kanamycin resistance cassette was inserted between the two flanking regions in the plasmid. The resulting plasmid was used to electroporate the target strain. Single transformants were confirmed by PCR and Western blotting. Complemented strains were obtained by transformation of the *sslE* mutant with *sslE*_WT and *sslE*_mut recombinant plasmids, carrying the *sslE* wild-type gene or the gene mutated in the putative metallopeptidase motif. For amplification of the *sslE* gene, *E. coli* IHE3034 genomic DNA was used with the primers 5 and 6 (Table S3). The triple mutation (mut) (H1274Y+E1275V+H1278Y) was obtained by two overlapping PCRs performed with primers 7, 8 and 9 (Table S3). Finally, the p*sslE*_WT and p*sslE*_mut constructs were generated carrying the *sslE* predicted promoter region upstream of the *sslE* gene. The two clones harboring these plasmids were produced by a PIPE method [51] that is based on the transformation of HK100 *E. coli* cells with a mix of a vector/insert PCR. The vector PCR was performed using the *sslE*_WT and *sslE*_mut templates with primers 10 and 11 (Table S3), while the insert PCR was obtained with *E. coli* IHE3034 genomic DNA template and primers 12 and 13 (Table S3).

Confocal staining of pathogenic *E. coli* bacterial cells

E. coli strains were grown to exponential phase in LB medium and fixed in PFA 1% for 20 min on a poly-L-lysine-coated slide (Thermo scientific). After a blocking step in PBS+1% BSA, slides were incubated with anti-SslE rabbit serum and then with a donkey anti-rabbit IgG Rhodamine RedX-conjugated antibody (Jackson Immuno-Research Laboratories). IHE3034 bacteria were localized using mouse polyclonal antibodies raised against whole cell IHE3034, and the green fluorescent Alexa Fluor488 goat anti-mouse IgG. The samples were mounted using the Pro-Long Gold antifade reagent containing the blue-fluorescent nuclear counterstain DAPI (Invitrogen). Images were acquired using a 100× oil objective (1.4 n.a.) mounted on a Zeiss LSM710 confocal microscope. In the pictures the signal from SslE was pseudocoloured in green, while the signals from bacteria are shown in red. Z-stacks of images were deconvoluted using Volocity Software (Improvision).

In vitro mucinase activity assays

1. **Amido black assay.** Pathogenic *E. coli* from mid-log culture phase were incubated on LB-agar plates containing 0.5% bovine submaxillary mucin (SIGMA), stained with 0.1% (wt/vol) amido black in 3.5 M acetic acid for 30 min and destained with 1.2 M acetic acid.
2. **Detection of mucinase activity by IVIS.** IHE3034 wild-type, IHE3034Δ*sslE* knockout mutant, IHE3034Δ*sslE*::*sslE*_WT and IHE3034Δ*sslE*::*sslE*_mut were transformed by electroporation with the pGEN-*luxCDABE* plasmid (Amp^R) expressing luciferase (p*lux* strains). Plasmid stability was assessed in all strains by CFU counting on LB and LB-Amp_{100} plates. A soft gel mucin-based matrix was allowed to polymerize in round 5-ml plates. 5×10^7 CFU of mid-log *E. coli* p*lux* strains were loaded into wells cut in the middle of the plates and incubated statically at 37°C. Starting inocula were plated to determine the loaded CFU. Pictures were acquired by IVIS 100 time 0 and at 24 h.
3. **Mucin gel degradation assay.** 10^8 CFU of pathogenic *E. coli* strains from mid-log culture phase were layered on top of 1 ml soft gel-matrix polymerized together with 10% submaxillary gland mucin (SIGMA) in 1 ml syringes and incubated statically for 3 h at 37°C in a vertical position (starting inocula were determined as CFU at time 0). 100 μl fractions were sequentially collected from the bottom of the syringe, diluted and plated. The counts were calculated as % of recovered bacteria in each fraction, sequentially eluted from the column, with respect to the starting inoculum. Data presented are the mean of three independent experiments performed in duplicate. Antibody inhibition of mucin degradation was performed by adding different sera dilutions to the soft gel mucin-based matrix before the polymerization.

PCR amplification and sequence variability analysis of *sslE* gene

Amplification and sequencing of the *sslE* gene was performed as previously described [10]. Assembly, alignment and comparison of the SslE deduced amino acid sequence was performed with GENEIOUS V6 software (Biomatters. Available from http://www.geneious.com/). In addition to the 96 *sslE* sequences used by Moriel *et al.* [10], 318 *E. coli sslE* sequences were included. The final dataset comprised 414 isolates which comprised EXPEC, InPEC and faecal isolates. Further, sequences relative to unknown *E. coli* pathotypes were extracted from the NCBI database (Table S1). 155 unique SslE protein sequences were selected using GENEIOUS V6 software. The phylogenetic tree was inferred from the alignments by the neighbor-joining distance-based method implemented on MEGA4 [52].

Cloning, expression and purification of SslE recombinant protein

The *sslE* gene was amplified by PCR from the IHE3034 genomic DNA template, cloned into the pET-21b vector (Novagen) and transformed into DH5α-T1R chemically competent cells for propagation. BL21(DE3) chemically competent cells were used for His-tagged protein expression. The protein was purified by nickel chelating affinity chromatography using a HisTrap HP column (GE Healthcare) followed by anionic exchange chromatography. The purified protein was finally dialyzed in phosphate-buffered saline (PBS) and stored at −20°C.

In vivo monitoring of sslE promoter activity

The P*sslE-luxCDABE* plasmid was obtained by replacing the constitutive P*em7* promoter of the pGEN-*luxCDABE* with the *sslE* putative promoter region. To obtain the predicted *sslE* promoter region, a 484-bp fragment was amplified from IHE3034 genomic DNA by PCR using the primers 14 and 15 (Table S3). Chemically competent DH5α cells (Life Technologies) were used for transformation and ampicillin (Amp_{100}) was used as a marker of selection. The resulting P*sslE-luxCDABE* plasmid was confirmed by sequence analysis and used to transform the ETEC strain GL53 by electroporation, resulting in the GL53-P*sslE-luxCDABE* strain. Ten-week old CD1 female mice (Charles River) were infected intragastrically with 5×10^5 CFU of either the bioluminescent ETEC GL53-P*sslE-luxCDABE* strain or GL53-P*neg-luxCDABE*

(promoterless control vector). Imaging of mice anesthetized with isofluorane (4% initially, 1.5% during image acquisition) was performed with an IVIS Spectrum CT Imaging System (Perkin Elmer). Detection of 2D bioluminescent signals was carried out without filters (open), binning 8 and times of acquisition from 1 s to 1 min. 3D images were acquired with six filters (500, 520, 560, 580, 600 and 620 nm), using the same binning and acquisition times and reconstructed by the Living Image software (version 4.3.1).

Reverse transcriptase-PCR

The GL53 infected caecum was homogenized using gentleMACS Dissociator (MiltenyiBiotec) in 10 ml PBS. After filtration and centrifugation, the pellet was incubated for 5 minutes at room temperature in 3 ml of RNA protect Bacteria Reagent (Qiagen). After cell lysis, total RNA was purified using the RNeasy Mini kit (Qiagen) and an additional DNase treatment was done using the TURBO DNA-free kit (Applied Biosystem), according to the manufacturer's protocols. Purity of RNA was assessed by electrophoresis on agarose gels. Reverse transcription and amplification of an *sslE* fragment with the primers 16 and 17 (Table S3) from RNA were performed using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase kit (Invitrogen).

Mouse model of intestinal colonization

Five-week old CD1 mice were intranasally immunized with 30 µg of SslE antigen at days 1, 21 and 35. Saline was used as a negative control. Fourteen days after the last immunization mice were streptomycin-treated (for 2 days) to eradicate the resident flora and then they were infected by oral gavage with 5×10^7 CFU/400 µl of strain GL53/Amp^r. Forty-eight hours after challenge, mice were euthanized and the intestinal caecum tract was recovered and homogenized. Serial dilutions of the suspension were plated on LB/Amp$_{100}$ plates and the CFU were enumerated. Statistical significance of protection was determined using the Mann Whitney test.

Urinary tract infection model

Female CBA/J mice, 6 to 8 weeks old, were transurethrally inoculated as previously described [53]. Purified antigen was mixed with cholera toxin (CT) (Sigma) at a ratio of 10:1. The vaccine was administered intranasally in a total volume of 20 µl/animal (10 µl/nostril). Animals received a primary dose on day 0 of 100 µg antigen (containing 10 µg CT) or 10 µg CT alone. Two boosts of 25 µg antigen (mixed with 2.5 µg CT) or 2.5 µg CT alone were given on days 7 and 14, and mice were challenged on day 21. *E. coli* 536 suspensions in phosphate-buffered saline (PBS) (50 µl/mouse) were delivered transurethrally using a sterile 0.28-mm-inner-diameter polyethylene catheter connected to an infusion pump (Harvard Apparatus), with a total inoculum of 10^8 CFU/mouse. For determination of CFU, organs were aseptically removed from euthanized animals at 48 h post inoculation and homogenized in PBS with a GLH homogenizer (Omni International). Bacteria in tissue homogenates were enumerated by being plated on LB agar containing 0.5 g/liter NaCl using an Autoplate 4000 spiral plater (Spiral Biotech), and CFU were determined using a QCount automated plate counter (Spiral Biotech). Blood was collected as necessary from anesthetized mice by an infraorbital bleed using 1.1- to 1.2-mm Micro-Hematocrit capillary tubes (Fisher), and serum was separated using Microtainer serum separator tubes (Becton Dickinson). The animals were ≤15 weeks old at the conclusion of all experiments.

Sepsis mouse model

CD1 outbred mice were immunized by subcutaneous injections at day 1, 21, and 35 with 20 µg of recombinant SslE$_{IHE3034}$ formulated with alum or alum alone. Immunized animals were challenged at day 49 with a sublethal dose of a heterologous strain and survival was monitored for up to 4 days. The results are indicated as the percentage of survival from a total number of 40 mice. P values were determined using the nonparametric Mann-Whitney significance test.

Statistical analysis

Mean values, standard deviation values, and the P values associated to two-tailed unpaired Student's t test were calculated using the Microsoft Excel application. A P value<0.05 was considered statistically significant.

Supporting Information

Figure S1 Polysialic acid capsule interferes with SslE detection on *E. coli* K1 IHE3034. (A) Flow cytometry detection of K1 capsule on wild-type strain IHE3034 (left panel) and acapsulated IHE3034Δ*kps* strains (right panel) by anti-capsule monoclonal antibody SEAM12 (blue lanes). Serum from animals immunized with PBS was negative control (red). (B) SslE surface detection on wild-type IHE3034 (left panel) and its derivative lacking the capsule IHE3034Δ*kps* (right panel) by anti-SslE immune sera (blue lines) compared to the PBS negative control (red). (C) Titration of binding by an anti-SslE rabbit serum on both IHE3034Δ*kps* acapsulated (red) and IHE3034Δ*kps*Δ*sslE* (blue) strains. (D) Confocal microscopy images of SslE surface localization on IHE3034Δ*kps* and (E) IHE3034Δ*kps*Δ*sslE*. Bacteria were visualized with both DAPI (DNA marker, blue) and FM4–64 Dye (membrane marker, red). SslE was detected using the anti-SslE rabbit serum and a fluorescent secondary antibody (green). Merged images are also displayed. Bars: 2 µm.
(TIF)

Figure S2 IgG and IgA antibody response following SslE immunization. Immunoglobulin levels were quantified by ELISA. Briefly, 100 ng/well of purified SslE was incubated with serial dilution of sera for 2 h at 37°C. Following detection with Alkaline Phosphatase (AP) conjugated secondary antibody, OD_{405} values were plotted in the titration curves. (A) IgG and (B) IgA response derived from serum of immunized rabbit (circles) compared to negative control (square). (C) IgG and (D) IgA response in pool of sera derived from immunized mice (circles) compared to the negative control (square). Each point represents the means ± standard deviations.
(TIF)

Figure S3 Polyclonal antibodies against the truncated C-SslE impair *E. coli* translocation through a mucin matrix. (A) Schematic representation of the C-SslE truncated form lacking the Zn-metalloprotease domain compared to the full-length SslE protein. (B) Inhibition of wild-type IHE3034 translocation through a mucin-gel matrix by anti-C-SslE antibodies compared to negative controls.
(TIF)

Figure S4 Comparison of the SslE core motif with other M60-like members. The figure reports a multiple sequence alignment of the SslE core motif of the zinc metallopeptidase M60-like domain versus the best hits that were found when searching the Pfam-A database. The extended core motif is shown by a dotted square and the conserved residues of the core motif are indicated with an

asterisk. The species names are followed by the Uniprot accession codes in brackets.
(TIF)

Table S1 List of strains used for global SslE amino acid sequence alignment.
(PDF)

Table S2 List of SslE unique sequences.
(PDF)

Table S3 List of primers used in the study.
(PDF)

Methods S1 Detailed description of the experimental procedures relative to the data reported in Fig. 6, Fig. S1 and Fig. S2.
(DOCX)

Text S1 Polysialic acid capsule interferes with SslE detection on *E.coli* K1 IHE3034. By comparing the SslE-specific signal between IHE3034 WT and the IHE3034Δkps deletion mutant by FACS and confocal imaging analysis, we demonstrated that the K1 capsule clearly interferes with the anti-SslE antibody accessibility and recognition of the protein on the bacterial surface.
(DOCX)

Text S2 IgG and IgA antibody response following SslE immunization. We observed that subcutaneous immunization of rabbit with recombinant SslE generated a high response in terms of IgG, while IgA values were low.
(DOCX)

Text S3 Polyclonal antibodies against the truncated C-SslE impair *E. coli* translocation through a mucin matrix. We postulated that antibodies against the N-terminal portion of the protein lacking the M60-like zinc-metalloprotease motif are still able to impair *E. coli* translocation through the mucin matrix, suggesting an indirect inhibitory activity.
(DOCX)

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Author Contributions

Conceived and designed the experiments: BN MV MS LS MRF HLTM MP. Performed the experiments: BN MV RR AP AS CJA PD LB SB. Analyzed the data: BN MV MS LS MRF GB MM YA SB. Contributed reagents/materials/analysis tools: MDV VR IB GT MF. Wrote the paper: MS LS BN MP KLS HLTM MRF PD.

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Chapter 3

Pathogenic *E. coli* Exploits SslE Mucinase Activity to Translocate Through the Mucosal Barrier and Get Access to Host Cells

Chapter 3

E. coli is a well-adapted human commensal which uses the gut as a preferential niche. As for other intestinal microorganisms, the capacity of *E. coli* to persist in this district depends on the ability to exploit a number of metabolic substrates and to stay in the outer mucus layer where commensal bacteria normally reside. In this scenario, the recent characterization of SslE, a novel *E. coli* mucinase, has opened new outlooks on the way this mucosal pathogen adapts to the inhospitable environment of human intestine. In chapter 2 we have demonstrated that SslE is involved in the degradation of mucin substrates, here we show that the interaction of SslE with mucus plays a relevant role in *E. coli* colonization of the gut and in the pathogenic events associated. This was achieved through the development of an *in vitro* model of mucus-secreting cells resembling the intestinal mucosa with the presence of both gel forming and secreted mucins. We proved that SslE expression facilitates *E. coli* penetration of mucus and the consequent access to target molecules on host cells, where proinflammatory responses are induced. We observed that bacteria with an impaired expression of SslE get trapped in the sticky mucosal matrix witch restrict their ability to spread, leading them to a reduced infectiveness. Of importance, our evidence that SslE expression is augmented when bacteria are in contact with the differentiated mucus producing cells not only postulates the specificity of SslE for mucosal surfaces, but also its active role during colonization process. Moreover, we found that IHE3034 wild-type strain exhibited a growth advantage over the IHE3034Δ*sslE* in minimal medium containing mucin, suggesting that SslE may provide some metabolic benefits to the bacterium. Furthermore, we found that SslE-mediated penetration of the mucosal barrier allows *E. coli* interaction with epithelial cells and induces Il-8 secretion promoting a pro-inflammatory response at the mucosal level. These results support the hypothesis that *SslE* contributes to *E.* coli exploitation of mucins as an environmental cue to modulate both metabolism and virulence.

Pathogenic *E. coli* Exploits SslE Mucinase Activity to Translocate Through the Mucosal Barrier and Get Access to Host Cells

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ABSTRACT

SslE is a zinc-metalloprotease involved in the degradation of mucin substrates and recently proposed as a potential vaccine candidate against pathogenic *E. coli*. In this paper, by exploiting a human *in vitro* model of mucus-secreting cells, we demonstrated that bacteria expressing SslE have a metabolic benefit which results in an increased growth rate postulating the importance of this antigen in enhancing *E. coli* fitness. We also observed that SslE expression facilitates *E. coli* penetration of the mucus favouring bacteria adhesion to host cells. Moreover, we found that SslE-mediated opening of the mucosae contributed to the activation of pro-inflammatory events. Indeed, intestinal cells infected with SslE-secreting bacteria showed an increased production of IL-8 contributing to neutrophil recruitment. The results presented in this paper conclusively designate SslE as an important colonization factor favouring *E. coli* access to both metabolic substrates and target cells.

Chapter 3

INTRODUCTION

E. coli is mainly regarded as a commensal microorganism retaining probiotic property [1]. However, some bacteria possess virulence factors that allow them to cause intestinal and extra-intestinal diseases [2]. Pathogenic *E. coli* species principally reside in the gut, but could also migrate to distal organs such as bladder and kidney, where they can cause urinary tract infections and sepsis. *E. coli* pathogenesis is characterized by IL-8 secretion and a strong infiltration of polymorphonuclear leukocytes [3-6]. In order to colonize or invade intestinal epithelium, *E. coli* must penetrate the mucus barrier and then either attach to the apical surface of epithelial cells or release toxins that disrupt epithelial integrity [7]. The mucus layer, largely composed of mucins, contains various digestive enzymes and antimicrobial peptides as well as immunoglobulins. The inner layer is densely packed, firmly attached to the epithelium, and devoid of bacteria. In contrast, the outer layer is movable and has an expanded volume that favours bacterial colonization [8,9]. Notably, bacterial pathogens have evolved mechanisms to circumvent this mucus hurdle and directly access the epithelial surface [10,11].

The recent description of SslE as a novel *E. coli* mucinase [12,13], has opened new outlooks on the mechanisms used by this important mucosal pathogen to adapt to the intestine. SslE (ECOK1_3385) is a promising vaccine candidate identified by using a subtractive reverse vaccinology approach [14].The antigen is characterized by the presence of a M60-like domain representative of a new extracellular zinc-metalloprotease sub-family which is implicated in glycan recognition and processing. SslE is a 160 kDa mucin-binding protein able to degrade intestinal mucins including Muc2, Muc3 and bovine submaxillary mucin [12,13]. However, the contribution of this protein to *E. coli* adaptation to the host still remains controversial. Indeed, SslE also appears to be required for biofilm formation in an EPEC strain [15], although this was not confirmed in an atypical EPEC strain [16]. Thus, the function of SslE remains to be fully elucidated.

In the present study, we show that SslE expression not only increases bacterial growth in the presence of mucosal substrates but it also facilitates *E. coli* penetration of the mucus. The evidence that SslE expressing bacteria have an enhanced access to the apical epithelial surface was corroborated by an increased pro-inflammatory response. These results further support the pivotal role of SslE during *E. coli* colonization of the intestinal mucosa.

MATERIALS AND METHODS

Antibodies, reagents and recombinant proteins

Antibody against muc-5AC and muc-3 were from Sigma-Aldrich (Milan, Italy), Anti-muc2 and muc3 antibodies were from Abcam, anti-muc1 was from Thermo Fisher Scientic, Alexa Fluor 568 anti-mouse secondary antibody and ProLong Gold Antifade Reagent with DAPI were obtained from Invitrogen.

Cells were maintained Dulbecco's Modified Eagle Medium (DMEM) or in Roswell Park Memorial Institute medium (RPMI), supplemented with 10% heat-inactivated fetal bovine serum, non-essential amino acids and 2 mM L-glutamax (Invitrogen Ltd, Paisley, UK). Blood neutophils were isolated by stratifying whole blood on Ficoll-Paque Plus (GE Healthcare).

For cDNA preparation we used Directzol RNA kit (Zymo Research) and TURBO DNase (Life Technologies), the real time analyses were performed in PCR plates using FastStart Universal SYBR Green Master (Roche Diagnostics).

Ethics statement

The institutional review board of the Department of Health Service at Novartis Vaccines and Diagnostics (Siena, Italy) approved the study and the use of human samples from the volunteers. Written, informed consent was obtained from the healthy donors (available from authorized blood banks).

Bacterial strains and culture conditions

ExPEC IHE3034 (serotype O18 K1:H7), was isolated in Finland in 1976 from a case of human neonatal meningitis [17]. Strains were cultured in Luria-Bertani broth at 37°C with agitation and aeration. *SslE* deletion mutant and complemented strains have been previously described [12]. Bacterial growth was performed by sub-culturing overnight broth cultures into the appropriate medium and reading the optical density at 600 nm (OD_{600}) at various time points. Growth in minimal medium was performed in M9 medium with 1% glucose; 0.05% glucose was employed during experiments in which mucin was added. Mucus was pooled from confluent HT29-MTX at 13,000g for 30 min at 4°C [11]. The clones carrying a specific antibiotic resistance cassette were grown in the presence of kanamycin (50 µg/ml) or erythromycin (50 µg/ml).

Cell culture

HT29-MTX human intestinal epithelial cells [18] derived from a colonic carcinoma were progressively adapted to a galactose-containing media [19], for experiments cells were grown as 2-dimensional (2D) monolayers on collagen coated Transwell inserts (0.4-µm pore size; BD Biosciences) and, unless stated otherwise, allowed to differentiate for 21 days.

Polymorphonuclear neutrophils were purified from buffy coats by density gradient centrifugation (400 × g for 30 min at room temperature) on Ficoll-Paque Plus, followed by centrifugation (250 × g for 10 min at 4°C) on a 3% (wt/vol) dextran solution. After osmotic lysis of erythrocytes, cells were resuspended in RPMI 1640 supplemented with 10% FBS and incubated at 37°C in a humidified atmosphere with 5% CO2

Measurement of trans-epithelial electrical resistance (TEER)

The integrity of polarized HT29-MTX monolayers was checked by measurement of Trans Epithelial Electrical Resistance (TEER) using an EVOMAX meter and STX-2 probe (World Precision Instruments). TEER was measured at different time points over a 21 day culture period and expressed as Ω/cm^2.

RNA isolation and RT-PCR

Total RNA was isolated both from HT29-MTX cells and from bacteria using Directzol RNA kit and was treated with DNase. The RNA was ethanol precipitated and dissolved in 30 µl RNase-free water. Real-time quantitative PCR was performed in a LightCycler 480 II real-time PCR system (Roche Diagnostics). All samples were run in triplicate on 96-well optical PCR plates. The specific primers used to amplify cDNA fragments are listed in Table 1. After an initial denaturation at 95°C for 10 min, denaturation in the subsequent 40 cycles was performed at 95°C for 15 s, followed by primer annealing at 60°C for 30 s and a final extension at 72°C for 30 s. For relative quantification of gene expression, the starting mRNA copy number of the unknown samples was determined using the comparative threshold cycle ($\Delta\Delta C_T$) method, as previously described [20], and levels of the different transcripts were normalized to 16S rRNA or β-actin, used as housekeeping genes.

Bacterial growth in HT29-MTX intestinal epithelial cell mucus and infection assay

Three set of transwells of polarized HT29-MTX cells were infected with *E. coli* at a multiplicity of infection (MOI) of 100. After 2 hrs of incubation wells were washed and a total association assay was performed on one set of wells. The remaining two sets of wells were further incubated at air-liquid interface for 24 or 48 h, and then CFU counting was performed. The same experiment was run on non-differentiated HT29-MTX cells (non-polarized and no mucus), as control. For the infection assays polarized HT29-MTX cells were grown on transwell filters as described above. Infections were performed in triplicate in DMEM without serum at a MOI of 100 bacteria per cell at 37°C, 10% CO2, for 4 hrs. Then wells were washed and 10 mM N-acetylcysteine (PBS 0.2 mM calcium chloride, 0.5 mM magnesium chloride and 15 mM glucose) was added for 1 h with agitation at 70 rpm, to remove the mucus layer after the *E. coli* infection period [21]. To evaluate the bacteria trapped into mucus, serial dilutions of the NAC medium were plated. For the quantitative determination of the cell-associated *E. coli*, infected cells were lysed with 1% saponin for 10 min, and serial dilutions of the cell lysates were made.

Enzyme-linked immunosorbent assay (ELISA)

Post *E. coli* infection, HT29-MTX cell supernatant was collected and analyzed for IL-8 using a Human IL-8 ELISA Kit (R&D Systems, QuantakineR) according to the manufacturer's directions. The intra-assay coefficient of variation (CV) and the inter-assay CV were <5%.

Chemotaxis assay

To measure neutrophil chemotaxis, bottom chambers of transwell supports were filled with supernatants deriving from HT29-MTX cells infected for 4 hours with IHE3034 or IHE3034Δ*sslE* strains. Neutrophils (2.5×10^5) were added to the upper chambers. After 1h at 37°C, cells that had migrated toward the lower

compartments were quantified by flow cytometry. Cells were analysed with a LSRII flow cytometer (Beckton-Dickinson) by using Floujo software.

Statistical Analysis

Mean values, standard deviation values and non-parametric Mann-Whitney U test were calculated using the GraphPad Prism 6 application. A level of $P<0.05$ was considered statistically significant.

RESULTS

SslE expression is modulated by contact with differentiated mucus producing cells

To dissect the contribution of SslE to *E. coli* infection of mucosal surfaces, we used an *in vitro* model based on polarized and fully differentiated HT29-MTX human colonic epithelial cells [18]. The degree of epithelial polarity was monitored by measuring trans-epithelial electrical resistance (TEER) (Fig. 1A), while the expression of both secreted and cell-surface mucins was assessed by qRT-PCR and confocal microscopy analysis (Fig. 3-1B and S3-1). To investigate whether the interaction with intestinal epithelial cells modulates SslE expression, we examined the transcription profile of bacteria adhering to differentiated HT29-MTX cells. To this end polarized cells were infected for 30 min and then SslE expression in bacteria adhering to mucus-producing cells was compared with that of bacteria growing in medium alone. As reported in Fig. 3-2A, a statistically significant increase in *sslE* transcript was observed during the interaction of *E. coli* with epithelial cells. Western blot analysis of supernatants from bacteria incubated in medium alone or HT29-MTX cells confirmed an increased production of SslE in the presence of differentiated cells (Fig. S3-2). To further understand whether bacteria-cell contact or factors released in the medium were responsible for gene activation, the level of SslE transcription of cell-adhering bacteria was compared with that of planktonic bacteria collected from the supernatant. Under such conditions, we clearly demonstrated that SslE expression is significantly increased only in cell-adhering bacteria (Fig. 3-2A) suggesting that host cells surface components are required to trigger the activation of the gene. Ultimately, the contribution of cell glycocalyx to the protein up-regulation was evaluated by comparing the level of SslE expression upon the interaction with differentiated mucus-producing cells *versus* non-differentiated ones. Interestingly, transcription of the *sslE* gene was not affected by the contact with the non-differentiated cells (Fig. 3-2B). Collectively, these data postulate that host cell differentiation status is crucial for the modulation of SslE expression.

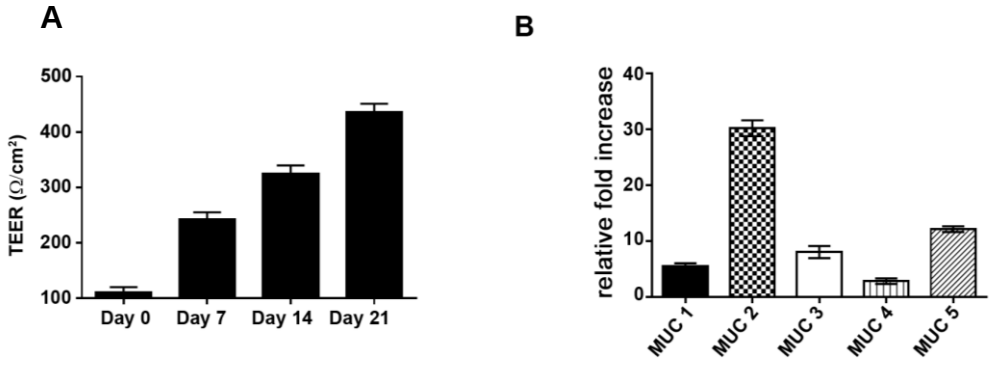

Fig. 3-1. Kinetics of transepithelial electrical resistance in HT29-MTX cells over a 21 day period of differentiation. (A) TEER values were measured at different time points throughout a 21 day period of differentiation. (B) RT-PCR analysis of MUC mRNAs. After 21 days of differentiation, HT29-MTX mRNA was isolated, and cDNA was used to compare the level of MUC gene expression. Data are represented as relative fold increase of MUC mRNA in differentiated mucus-producing cells *versus* non-differentiated

(control) cells. Control cells were assigned a value of 1.0. Levels of the different transcripts were normalized to β-actin, used as a house keeping gene. Error bars represent the SD.

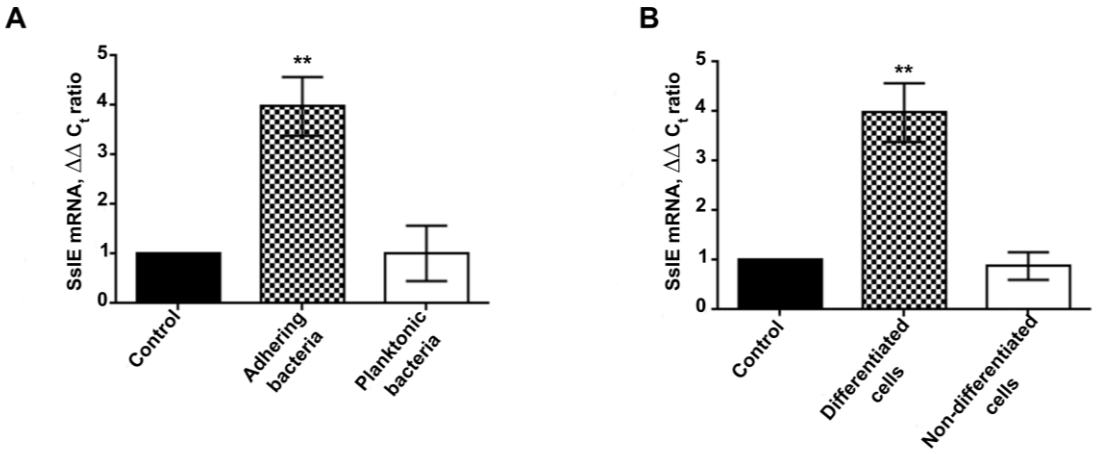

Fig. 3-2. Modulation of SslE gene expression upon interaction with intestinal epithelial cells. (A) SslE transcription level in bacteria adhering to differentiated cells and in planktonic organisms. (B) SslE transcription level upon the interaction with differentiated or not differentiated HT29-MTX cells. Starting mRNA copy number of the unknown samples was determined using the comparative $\Delta\Delta_{CT}$ method, and levels of the different transcripts were normalized to 16S rRNA, used as a housekeeping gene. Error bars represent the SD. **, $P \leq 0.01$. n= 3.

SslE mucinase activity facilitates E. coli colonization of the apical cell surface by improving bacterial growth rate

To assess whether SslE expression could increase the overall fitness of *E. coli*, we performed growth curves of IHE3034 wild-type and IHE3034*ΔsslE* knock-out mutant strains in M9 minimal medium containing mucus harvested from HT29-MTX cells. As shown in Fig. 3-3A, the presence of mucins boosted the growth ability of the wild type strain which reached a plateau at OD_{600}= 0.9 (stationary phase), whereas growth rate of the *sslE* mutant strain was unaffected. Similar results were obtained using the IHE3034Δ*sslE::sslE*_WT complemented strain carrying a WT *sslE* gene and HE3034Δ*sslE::sslE*_mut carrying a triple mutation in the metallopeptidase motif (**YV**VG**Y** vs. HEVGH) [12] (Fig. 3-3B). To demonstrate that SslE-mediated mucin degradation by increasing bacterial growth rate facilitates colonization of apical surfaces, we compared IHE3034 wild-type and IHE3034*ΔsslE* mutant strains for the ability to reside on mucus producing cells. As shown in Fig. 3-4, infection with WT and *ΔsslE* strains for 2 hours resulted in almost an equal bacteria binding to both non-differentiated and differentiated HT29-MTX cells (Fig. 3-4A and 4B, respectively). However, prolonged incubations at air liquid interface for up to 24 and 48 hours, revealed statistically significant differences in the number of WT bacteria growing on mucus producing cells compared to the *sslE* mutant (Fig. 3-4B). Such differences were abrogated when WT and *ΔsslE* infections were performed using non-differentiated cells (Fig. 3-4A).These data suggest that SslE expressing bacteria may increase their fitness by using mucosal apical glycoproteins (including mucins).

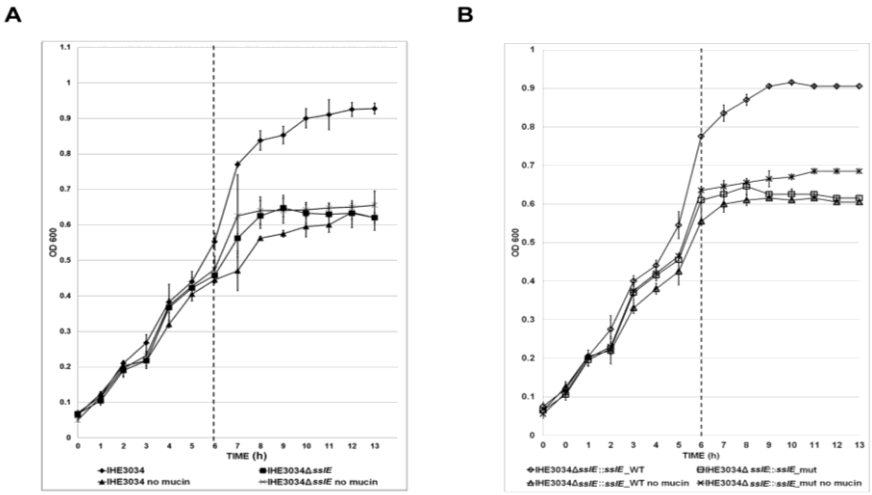

Fig.3-3. Growth curves of strain IHE3034 in the presence of mucin. Growth curves of (A) IHE3034 and IHE3034Δ*sslE* strains (B) IHE3034Δ*sslE::sslE*_WT and IHE3034Δ*sslE::sslE*_mut, are shown in M9 minimal medium with and without the addition of mucin harvested from HT29-MTX cells after 6 h of incubation (Dash line). Measurements were performed in triplicate.

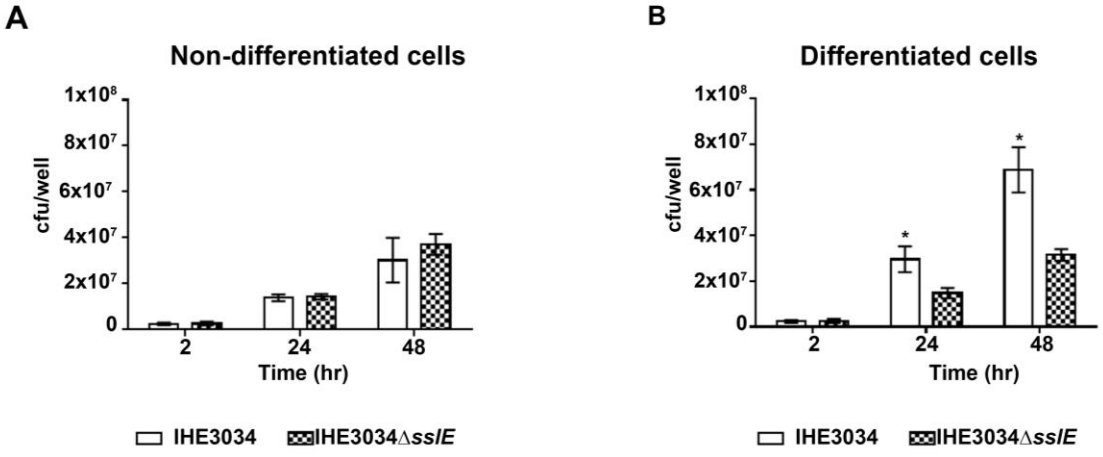

Fig. 3-4. *E. coli* growth rate in association with HT29-MTX cells. Using the transwell system, three sets of non-differentiated cells (A) and three sets of differentiated-mucus–producing- cells (B) were infected with WT and *sslE* KO bacteria for 2 hrs. Medium was removed to eliminate non-adhering bacteria and two sets of wells were used to do a total association assay, while the other wells were further incubated for 24 and 48 hrs at air liquid interface. At the end of the incubation period a total association assay was performed. The data presented are means ± standard deviations for 3 replicate experiments (n= 9).*P≤0.05. Error bars, SD.

SslE contributes to E. coli translocation of the mucosal barrier in vitro

In order to further reinforced our previous finding on the mucolytic activity of the protein, observed using gel matrix [12], and to evaluate its role in host colonization process, we tested SslE activity in our *in vitro*

gut model. Polarized monolayers of mucus producing cell were infected for 4 hours with the wild-type IHE3034, its isogenic derivative IHE3034Δ*sslE*, IHE3034Δ*sslE::sslE*_WT and HE3034Δ*sslE::sslE*_mut. Addition of N-acetyl cysteine (NAC) at the end of the incubation period allowed us to remove the apical mucus layer and to distinguish between the bacteria trapped in it and the bacteria adhering to underlying cells (Fig. 3-5A). As shown in Fig. 3-5B, the *sslE* deficient strain was less efficient in reaching the cell surface compared to the isogenic WT. Indeed, a high number of IHE3034Δ*sslE* and IHE3034Δ*sslE::sslE*_mut bacteria were recovered from the mucus fraction, while both WT and the complemented strains were mainly associated to the cells underlying the mucus layer. These data confirm that SslE facilitates *E. coli* penetration of mucus and allows bacteria to get access to the host cells surface. Of interest, co-infection experiments using IHE3034 WT and IHE3034Δ*sslE* strains revealed that the expression of SslE by the WT strain complements the ability of the *sslE* deficient strain to get access to the apical cell surface of the HT29-MTX polarized epithelium (Fig. 3-5C).

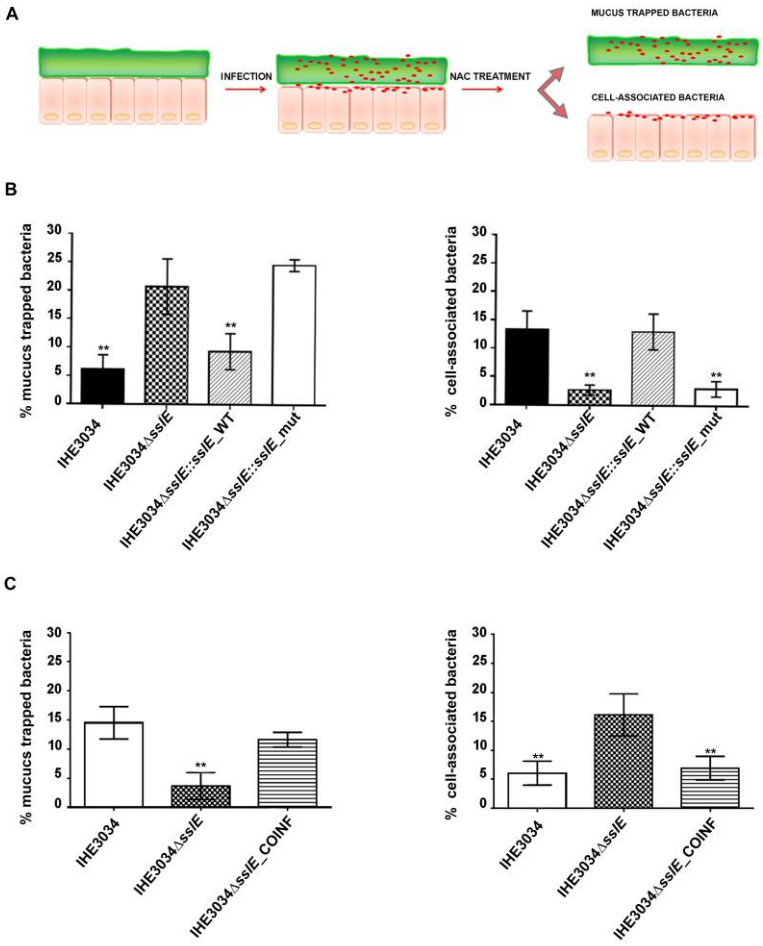

Fig. 3-5. SslE contributes to the capacity of IHE3034 strain to reach the surface of mucus-producing epithelial cells. (A) Schematic representation of the experimental procedure. (B), Percentage of mucus trapped (left panel) and cell associated (right panel) **I**HE3034 (WT), IHE3034Δ*sslE* (KO), IHE3034Δ*sslE::sslE*_WT (COMPL) and IHE3034Δ*sslE::sslE*_mut (MUT) bacteria after 4hrs of infection.

n =4, **P≤0.01; Error bars, SD; percentages were calculated on recovered CFU respect to the starting inoculum. (C) Co-infection experiments: percentage of mucus trapped (left panel) and cell associated (right panel) **I**HE3034 (WT), IHE3034Δ*sslE* (KO) or IHE3034Δ*sslE* plus IHE3034 (KO_COINF) bacteria. Strains were plated on both non-selective and selective plates to differentiate WT and Δ*sslE* for CFU counts. *n* =4, **P≤0.01; Error bars, SD;

SslE-mediated opening of the mucosae contributes to the activation of pro-inflammatory events

As a result of the interaction with pathogens, the enterocytes act as immunocompetent cells and secrete various signalling molecules, such as cytokines and chemokines. Previous work from Svanborg's group [6] has clearly shown that *E. coli* species targeting epithelial cells induce soluble mediators leading to the recruitment of inflammatory cells that participate directly in the clearance of bacteria. To further prove that SslE expression favours *E. coli* access to epithelial cells, we investigated whether this phenotype correlates with an increased pro-inflammatory response. To this end, supernatants derived from HT29-MTX cells infected with the wild-type IHE3034 or its isogenic Δ*sslE* mutant strains were collected. We observed that both bacterial strains were able to stimulate IL-8 release compared to negative control (supernatant of uninfected cell). Of note, WT strain induced a statistically significant higher level of IL-8 compared to the Δ*sslE* strain (Fig. 3-6A); further supporting our hypothesis that SslE is indirectly involved in promoting inflammation. Since IL-8 is a well-known chemo-attractant able to stimulate neutrophil recruitment [6], we compared supernatants derived from cells infected with WT and Δ*sslE* bacteria for their ability to promote neutrophil migration. As expected, flow cytometric analysis revealed that WT-derived supernatant induced a higher PMN migration compared to KO (Fig. 3-6B).

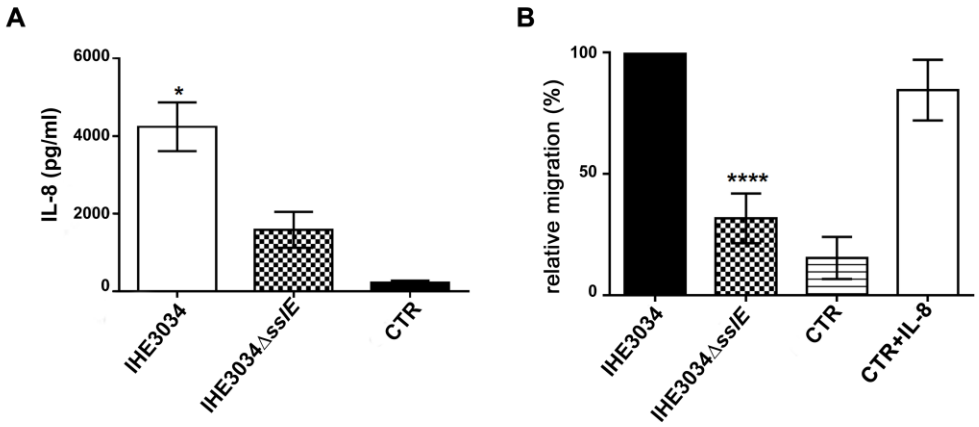

Fig. 3-6. SslE induces IL-8 secretion and stimulates neutrophil chemotaxis. (A) IL-8 levels in supernatants from the apical compartments of polarized HT29-MTX cells infected with IHE3034 WT and IHE3034Δ*sslE* KO bacteria. Data show mean chemokine concentrations in culture supernatants representative of three independent experiments *P≤0.05. Error bars, SD. (B) To measure neutrophil chemotaxis, bottom chambers of transwell supports were filled with supernatants deriving from HT29-MTX infected cells. Neutrophils were added to the upper chambers. After 1 h at 37°C, cells that had migrated toward the lower compartments were quantified by flow cytometry. DMEM has been used as a negative

control and recombinant IL-8 as a positive control. The graph represents a typical experiment out of three performed with similar results. **** $P \leq 0.0001$. Error bars, SD

DISCUSSION

Multiple mucus layers overlying gut epithelium act as microbial sensing and intrinsic defence systems that counteract against infective intruders. They protect from hurdle colonization, invasion, and systemic dissemination of both symbiotic and pathogenic microorganisms [22]. An important aspect in the understanding of microbial pathogenesis is the recognition of the different strategies evolved by microbes to circumvent the mucosal barrier and cause disease [23]. A number of virulence factors such as proteases, glycosidases, and mucin secretagogues are produced by these organisms and are believed to be responsible for disruption and depletion of the mucus gel [24,25]. Serine protease such as Pic [7], zinc metalloproteases (StcE, Hap) [26,27], as well as mucin-degrading enzymes [28], metabolize mucin oligosaccharides, reduce mucus viscosity and release antimicrobial peptides. The recent identification of SslE, a novel *E. coli* mucinase [12,13], has opened new outlooks on the way this mucosal pathogen adapts to the inhospitable environment of human intestine. To elucidate the contribution of SslE to *E. coli* pathogenesis, we developed an *in vitro* model based on colonic HT29-MTX cells grown on transwell inserts. This system resembles the intestinal mucosa with the presence of both gel forming and secreted mucins. In particular, we demonstrated that SslE facilitates penetration of the mucus layer and the consequent access to the underlying epithelial cells. These results further reinforce the hypothesis recently formulated by our group (confirmed also by Fleckenstein and colleagues [12,13]) for a role of SslE in the colonization of the intestine. Notably, the use of the N-acetyl cysteine allowed us to precisely distinguish between bacteria trapped into the mucus network and bacteria bound to underlying cells. Our results clearly establish a cause-effect relationship between SslE expression and mucus barrier penetration. In particular, we show that bacteria with an impaired expression of SslE get trapped in the sticky mucosal matrix witch restrict their ability to spread, leading them to a reduced infectiveness. Of importance, our evidence that SslE expression is augmented when bacteria are in contact with the differentiated mucus-producing cells not only postulates the specificity of SslE for mucosal surfaces, but also its active role during colonization process.

The ability of indigenous microflora to multiply at a rate that allows counteracting the turnover and the erosion of the mucus layer is crucial for their capacity to persist in an overcrowded niche such as the human gut. In this context, mucins represent preferential substrates for several human colonizers including avirulent *Escherichia coli* [29], *Salmonella typhimurium* [30], *Clostridium perfringens* [31], *Bacteroides* species [32] and virulent *Shigella flexneri* [33]. The fact that SslE expression, by mediating mucins catabolism, not only favours *E. coli* diffusion but also enhances its growth rate, suggest a broad impact of this protein on the overall bacterial fitness. Of importance, this was demonstrated in the presence of mucins extracted from cells as well as when bacteria were in direct contact with the mucus film lying on top of differentiated HT29-MTX cells. However, whether the advantage of SslE results from the release of glycans from intestinal mucus and/or from degradation of the mucin protein backbone remain to be elucidated.

In view of our findings, we propose a model for SslE involvement in *E. coli* translocation of the mucus layer and the consequent triggering of a pro-inflammatory immune response. Briefly, *E. coli* microorganisms, that reside in the outer mucus layer, by their intrinsic mucinase activity may access to the inner mucus barrier

and migrate towards cellular targets (Fig. 3-7). The direct contact of bacteria with the intestinal epithelium leads to the secretion of chemokines and stimulates neutrophil transmigration.

The fact that SslE appears to be also secreted by commensal *E. coli* species raises an important issue on the role of this antigen in the adaption to the host. Indeed, commensals are rarely found in the inner mucus barrier of the gut suggesting that *in vivo* SslE activity alone may not be sufficient to allow mucus penetration, but may require the synergy with other proteases expressed by pathogenic strains. We are far from understanding this aspect and more studies are needed to unravel the different strategies used by this pathogen to get access to host cell targets.

In conclusion, by providing new clues on the role of SslE in the interplay between *E. coli* and host cells, we will not only increase the current understanding on *E. coli* pathogenesis, but also better define the mechanism at the base of the protective response induced by this promising vaccine candidate.

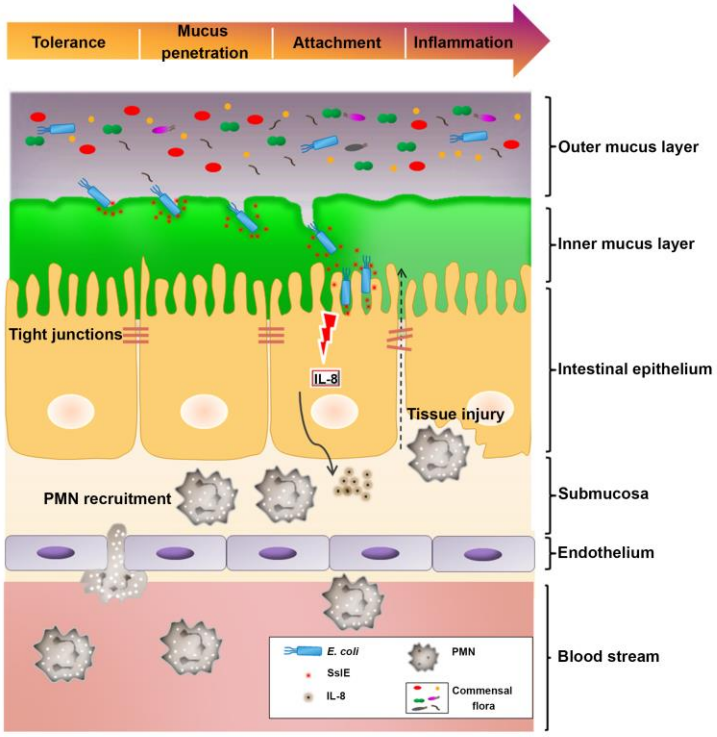

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Fig. 3-7. Schematic representation of the contribution of SslE to *E. coli* pathogenesis. Gut mucus forms two layers, an inner firm mucus layer devoid of bacteria, and an outer layer that is not sterile and is a major habitat for commensal bacteria. *E. coli* can penetrate this barrier through the SslE-mediated enzymatic degradation of the mucus, targeting epithelial cells. This interaction will eventually lead to IL-8 release and neutrophils recruitment.

SUPPORTING INFORMATION LEGENDS

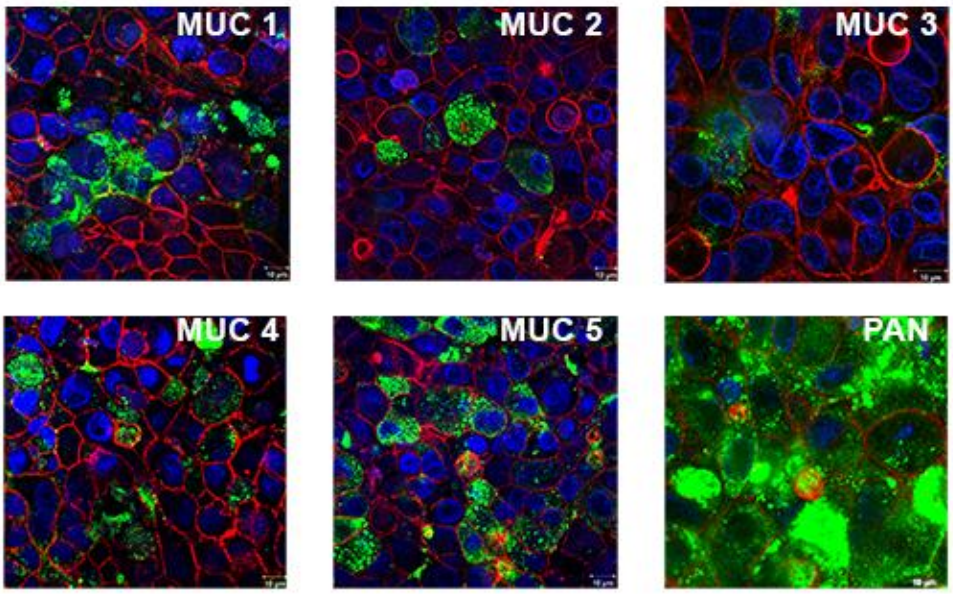

Fig. S3-1. Characterization of the extracellular mucus layer of HT29-MTX cells. HT29-MTX cells grown on transwell filters for 21 days were stained with specific antibodies for MUC1, MUC2, MUC3, MUC4, MUC5AC and PAN anti-gastric mucin. The mucins are stained in green and the actin skeleton in red. DAPI (blue) staining was used to visualize cell nuclei.

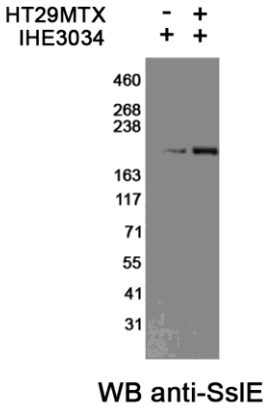

Fig. S3-2. SslE production is enhanced upon contact with epithelial cells. Immunoblot analysis of SslE expression in S/N of bacteria incubated with medium alone (control) or differentiated HT29-MTX cells. A representative experiment out of 3 performed is shown. Molecular weight markers are indicated on the left column.

TABLE

Table1. Primers pair used in this study

Gene	Primers Pair	Reference
SslE	F-CTCATCTTCCTTGCCCTCTTC	This study
	R- TCATGGAGTCGAGTTGCAGA	This study
16S	F- ACGTGCTACAATGGCGCATA	Ref-[34]
	R- TCATGGAGTCGAGTTGCAGA	Ref-[34]
Muc1	F-TCAGCTTCTACTCTGGTGCACAA	Ref-[35]
	R-ATTGAGAATGGAGTGCTCTTGCT	Ref-[35]
Muc2	F-CTGCACCAAGACCGTCCTCATG	Ref-[36]
	R-GCAAGGACTGAACAAAGACTCAGAC	Ref-[36]
Muc3	F-AGTCCACGTTGACCACTGC	Ref-[37]
	R-TGTTCACATCCTGGCTGGCG	Ref-[37]
Muc4	F-CGCGGTGGTGGAGGCGTTCTT	Ref-[36]
	R-GAAGAATCCTGACAGCCTTCA	Ref-[36]
Muc5	F-TGATCATCCAGCAGCAGGGCT	Ref-[36]
	R-CCGAGCTCAGAGGACATATGGG	Ref-[36]
ACTIN	F-GCTATCCCTGTACGCCTCTG	Ref-[38]
	R-CTCCTTCTGCATCCTGTCGG	Ref-[38]

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Concluding remarks

Concluding remarks

Pathogenic *E. coli* strains represent a major health threat associated with relevant financial implications, as they are able to cause a wide variety of medically important syndromes, including diarrheal illness, UTIs, sepsis, neonatal meningitis, intra-abdominal infections, nosocomial pneumonia, osteomyelitis, cellulitis and wound infections. Considering the increasing antimicrobial resistance, the number of recurrent infections and the high incidence in susceptible subpopulations, the prevention of *E. coli*-induced diseases could have a significant clinical and economic impact [1]. Although many efforts have already been done to develop a successful immunization strategy, a highly immunogenic, broad protective and safe vaccine against *E. coli* is still not available.

Because reverse vaccinology has demonstrated to be a powerful tool where conventional approaches to vaccine development had failed, we determined the sequence of a neonatal meningitis-associated K1 strain and we compared it to the available genomes of pathogenic and nonpathogenic *E. coli* strains, in order to identify protective and broadly conserved vaccine antigens against *E. coli* infections. This comparative genome analysis, named "subtractive reverse vaccinology", led to the identification of nine potential vaccine candidates, most of which have just a putative or hypothetical function assigned, indicating that still much work has to be done to better understand the mechanisms of *E. coli* pathogenesis [2]. In this work, the functional characterization of protective antigen ECOK1_3385 has been discussed.

Co-evolution between human gut and microorganism have led to a symbiotic relationship in which bacteria make essential contributions to human nutrient metabolism and in return occupy a nutrient-rich environment . In this context, the intestinal mucosa plays an active role [3,4] with goblet cells secreting both mucins and antimicrobial proteins helping to eliminate bacteria that penetrate the mucus layer [4,5]. In fact, the intestinal mucus barrier consists of two layers: a thick outer mucus layer that is typically hosting commensal microbes and a thinner inner mucus layer that is rich with innate immune factors and therefore highly sterile and difficult to penetrate by microbes [6]. *E. coli* is a well-adapted human commensal which uses the gut as a preferential niche. As for other intestinal microorganisms, the capacity of *E. coli* to persist in this district depends on the ability to exploit a number of metabolic substrates and to stay in the outer mucus layer where commensal bacteria normally reside.

Recent studies, have postulated that *E. coli* has adapted to the human body by developing a sophisticated network of virulence and colonization factors working in a concert manner. Adhesins, iron-uptake systems, IgA binding proteins and, here reported, mucinases may allow *E. coli* to out-compete the many species occupying an overcrowded environment such as the intestine [7,8]. The intestinal mucosa not only is critical for intestinal homeostasis but also serves as an infectious foothold for the microbiota and invading pathogens. Therefore, the gut epithelium uses multiple defense mechanisms against microbes, including the luminal microbiota, a mucus layer, epithelial integrity, epithelial cell turnover and innate or acquired immune responses. Despite the presence of these defensive systems, pathogenic bacteria can invade spaces that are usually devoid of microbes [9,10]. In this scenario, SslE mucinolytic activity may facilitate bacterial penetration of mucosal surfaces including the inner mucus layer to reach the underlining host epithelium. Our finding suggests the involvement of such an antigen in landscaping *E. coli* territory allowing the establishment of a long lasting occupancy. Indeed, mucus layer not only prevents direct contact of intestinal cells with microbes but also constitutes a metabolic substrate for mucus-degrading bacteria

[13]. We demonstrated that SslE expression not only increases bacterial growth in the presence of mucosal substrates but it also facilitates *E. coli* penetration of the mucus. The evidence that SslE expressing bacteria have an enhanced access to the apical epithelial surface was corroborated by an increased pro-inflammatory response. These results further support the pivotal role of SslE during *E. coli* colonization of the intestinal mucosa.

The anti-mucinase activity exerted by anti-SslE polyclonal antibodies *in vitro*, corroborated by a reduced colonization of caecum in mice immunized with recombinant SslE, further support the hypothesis that the impairment of mucin cleavage may account for the mechanisms of protection from *E. coli* infections in both gut and urinary tract. This study by combining *in vitro* evidence for the contribution of SslE to *E. coli* mucinolytic activity and *in vivo* protection data suggesting the importance of SslE in intestinal and urinary tract colonization, support the emerging interest for this antigen as a novel colonization factor and a valid target for intervention strategies against disease caused by this important human pathogen.

SslE belongs to a large and diverse family of eukaryotic and prokaryotic proteins containing putative metalloprotease domains [14]. Interestingly, Enhancin, the prototype molecule of the family of proteases most closely related to SslE, was isolated from an insect virus and targets insect intestinal mucins [15,16] . It bears a canonical HEXXH metalloprotease motif within a domain (M60-like pfam13402) that is strongly associated with pathogens and commensal organisms that colonize mucosal surfaces [17]. The significant homology between SslE and *V. cholera* AcfD [18] is worthy of comment. AcfD has anti-vibriocidal activity [19], and is required for efficient intestinal colonization of *Vibrio cholerae*. Given the presence of an Enhancin-like protease domain, it has been suggested that AcfD is a mucinase [17]. To our knowledge this has not been tested experimentally. AcfD along with two other lipoproteins encoded by *V. cholerae*, TcpC, and ToxR-activated gene A (TagA), are regulated by the ToxR virulence regulon [20]. Interestingly, TagA [21], encoded on the same *V. cholerae* pathogenicity island (VPI) that encodes AcfD, also is a secreted mucinase. Notably, the hemagglutinin protease of *V. cholerae* is also a mucinase that is secreted via the T2SS [22,23], responsible for secretion of CT. Therefore, *V. cholerae* appears to be equipped with multiple enzymes with the ability to degrade intestinal mucins, and similar to *E. coli* it uses the T2SS to export both an enterotoxin and one or more mucinases that facilitate the toxin delivery. The identification of SslE homologues in a variety of enteric pathogens with known mucinases might suggest that multiple mucin-degrading enzymes could contribute cooperatively to virulence. A variety of different diarrheagenic *E. coli* produce mucinases [24-26], and the recent O104:H4 outbreak strain, also encodes Pic, an established mucinase shared by enteroaggregative *E. coli* and *Shigella flexneri* [27,28].

SslE is part of a dynamic complex between *E. coli* and the mucosa. Interestingly, SslE appears to be also secreted by commensal *E. coli* species, raising an important issue on the role of this antigen in the adaption to the host. Indeed, commensals are rarely found in the inner mucus barrier of the gut suggesting that *in vivo* SslE activity alone may not be sufficient to allow mucus penetration, but may require the synergy with other proteases expressed by pathogenic strains. *Escherichia coli* is a paradigm for a versatile bacterial species which comprises harmless commensal as well as different pathogenic variants with the ability to either cause intestinal or extraintestinal diseases in humans and many animal hosts. We are far from understanding this aspect and more studies are needed to clarify the different strategies used by this pathogen to breach the physical barrier imposed by mucins get access to host cell targets.

Concluding remarks

As our understanding of *E. coli* pathogenesis evolves, new approaches to vaccine development emerge. Likewise, current information regarding the nature of *E. coli* commensalism in the intestine is inadequate, illustrated by the very limited number of "commensal" genomes presently available. Given the apparent paucity of pathotype-specific vaccine targets, it will be important to determine whether highly conserved antigens such as SslE could be effectively targeted as vaccine candidates.

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Appendix

The *Neisseria meningitidis* ADP-ribosyltransferase NarE enters human epithelial cells and disrupts epithelial monolayer integrity

Appendix

NarE is a 16 kDa ADP-ribosyltransferase identified for the first time in type B *Neisseria meningitidis* (MenB) associated to a subset of hypervirulent strains with 100% sequence identity. Since many pathogenic bacteria utilize ADP-ribosylating toxins to modify and alter essential functions of eukaryotic cells, this antigen immediately appeared of interest for MenB. NarE structure has been recently solved and structural features confirmed the presence of a catalytic site homologous to that of *E. coli* heat-labile enterotoxin (LT) and cholera toxin (CT). These data were supported by *in vitro* assays demonstrating that this protein retains the capacity to hydrolyze NAD and to transfer ADP-ribose moiety to arginine residues in target protein acceptors. NarE is also able to perform auto-ADP-ribosylation. Despite the absence of predicted leader peptide, the protein was shown to be efficiently exported in the periplasmic space of Meningococcus, but could not be detected in the supernatant fraction of bacterial culture.

In this study we tried to elucidate the contribution of NarE to MenB pathogenesis by focusing our attention on the effect of NarE on human epithelial cells. We used Chang cells that represent an established model to study *N. meningitidis* interaction with host cells. Initially, we demonstrated that NarE binds to cells in a saturable fashion, suggesting the existence of a specific receptor. Furthermore, *in vitro* ADP-ribosylation studies pointed out that actin may act as a possible target for NarE activity. It is known from the literature that proteins with ADP-ribosylating activity on actin may have the capacity to alter cell morphology. Indeed, we observed that NarE is able to reduce cellular monolayer–electrode impedance, suggesting a possible role of the protein in altering cell morphology. Furthermore, NarE was able to cause apoptosis in epithelial cells by activating caspase-3 and inducing nuclear fragmentation. As a control, the NarE-R7K mutant, which is devoid of the enzymatic activity, did not induce any significant change of cellular structures, indicating a direct link between ADP-ribosyltransferase activity and actin remodelling. Finally, we demonstrated that NarE, and not NarE-R7K, is able to induce Chang cells apoptosis by a caspase-3 activation.

The data reported in this study suggest that NarE may contribute to *N. meningitidis* pathogenesis by affecting cellular integrity of host tissues. Considering that damage of monolayer integrity is an important step in *Neisseria* pathogenesis allowing bacteria to cross the epithelium and reach the bloodstream., our data strongly suggest a potential role of NarE in *Neisseria* colonization and spreading

The Neisseria meningitidis ADP-ribosyltransferase NarE enters human epithelial cells and disrupts epithelial monolayer integrity

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ABSTRACT

Many pathogenic bacteria utilize ADP-ribosylating toxins to modify and impair essential functions of eukaryotic cells. It has been previously reported that *Neisseria meningitidis* possesses an ADP-ribosyltransferase enzyme, NarE, retaining the capacity to hydrolyse NAD and to transfer ADP-ribose moiety to arginine residues in target acceptor proteins. Here we show that upon internalization into human epithelial cells, NarE gains access to the cytoplasm and, through its ADP-ribosylating activity, targets host cell proteins. Notably, we observed that these events trigger the disruption of the epithelial monolayer integrity and the activation of the apoptotic pathway. Overall, our findings provide, for the first time, evidence for a biological activity of NarE on host cells, suggesting its possible involvement in Neisseria pathogenesis.

INTRODUCTION

Neisseria meningitidis is a Gram-negative, aerobic, non-motile, non-sporulating, usually encapsulated and piliated bacillus. It is restricted to humans and generally colonizes the nasopharynx of 8–20% of healthy individuals, however in a small proportion of infected patients, the bacterium crosses the mucosal barrier and reaches the bloodstream, giving rise to meningitis or fulminant septicaemia [1]. Masignani et al. have identified, through a profile-based computational approach, an ADP-ribosyltransferase protein, NarE, which shares structural homologies with *E. coli* heat-labile enterotoxin (LT) and cholera toxin (CT)[2]. NarE possesses both ADP-ribosylating and NAD-glycohydrolase activities, confirmed by the evidence that, in the presence of an ADP-ribose acceptor, NarE acts as a transferase whereas in the absence of the acceptor it acts as a NAD glycohydrolase [3]. Furthermore NarE undergoes auto-ADP-ribosylation [4].

Mono ADP-ribosylation is a post-translational modification of proteins, shared by eukaryotes and prokaryotes, which modulates protein function [5]. Mono-ADP-ribosyltransferases (ADPRTs) catalyze the transfer of a single ADP-ribose group of β-nicotinamide adenine dinucleotide (NAD^+) to protein/peptide target acceptors with the release of nicotinamide (Nam) at the same time [6]. In pathogenic bacteria, proteins known to belong to this class of enzymes are generally classified as toxins since they alter or impair essential functions of host eukaryotic cells [7,8]. On the basis of the ADPRTs targets, at least three groups of ADP-ribosylating toxins can be identified. One group causes ADP-ribosylation of G proteins. Members of this group are cholera toxin (CT) [9], *E. coli* heat-labile enterotoxin (LT) [10] and pertussis toxin (PT) [11], which, through modification of regulatory G proteins, impair signal transduction. The second group includes diphtheria toxin (DT) [12] and *Pseudomonas* exotoxin A (ExoA) [13] that target elongation factor 2 (EF-2), thus inhibiting protein synthesis. A large third group of bacterial toxins modulates actin cytoskeleton directly, by covalent modification of actin, as C2 toxin of *C. botulinum* [14], Iota toxin of *Clostridium* [15], VIP2 toxin of *Bacillus cereus* [16], and SpvB of *Salmonella* [17], or indirectly, by covalent modification of Rho GTPases, as C3 exoenzymes of *Clostridium*, *Bacillus cereus* and *S.aureus* [18,19] exoenzyme S (ExoS) di *P.aeruginosa* [20].

Each group of toxins provides the bacterial pathogen with a selective advantage in modulating cell host response and resistance to infection, therefore they have been extensively characterized.

The *narE* gene is present only in a subset of hypervirulent clusters, ET-5 and Lineage 3 complexes, suggesting its involvement in *Neisseria meningitidis* pathogenesis [3]. However, no evidence of NarE toxic activity has been provided so far and its function remains to be fully elucidated. In the present report, we show that NarE specifically targets human epithelial cells. We demonstrated that NarE is internalized into human epithelial cells and gains access to the cytoplasm. Furthermore, through its ADP-ribosylating activity, NarE targets host cell proteins, alters epithelial monolayer integrity and initiates the apoptotic pathway responsible for cell death. Collectively, our data provide for the first time evidence of the biological role of this enzyme and suggest its potential contribution during colonization of upper respiratory tract and spreading of infection.

Materials and methods

Cells, antibodies, reagents and recombinant proteins

Chang human epithelial cell line (HeLa contaminant) was purchased from the American Type Culture Collection (ATCC, CCL-20.2). Chang cells were maintained in minimum essential medium Eagle (MEM, Invitrogen Ltd, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Ltd, Paisley, UK), 15mM L-glutamine and antibiotics. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere. In order to produce NarE polyclonal antiserum, CD1 mice were immunized with 10 µg of purified protein formulated with AlOH, as an adjuvant. The recombinant protein was given intraperitoneally (day1), a second (day 21) and a third (day 35) booster doses were administered. Blood samples were taken on day 49. Antibody against cleaved caspase-3, anti-GAPDH and anti-Laminin were from Cell Signaling Technology (Beverly, MA). Antibody anti-ADAM10 was from Abcam, anti-cytokeratin was from Invitrogen and anti-actin was from Biosource. Mouse antibodies against MHCI were from Biolegend, anti-Lamp1 from Abcam, Rabbit antibodies anti-EEA1 were from Novus Biologicals. Alexa 488- or Alexa 568-conjugated secondary anti-rabbit or anti-mouse goat antibodies were from Molecular Probes. Recombinant NarE was conjugated with fixable Alexa-Fluor-546 according to manufacterer's instructions (kit A10237, Life Technologies).

CHX and NAD were purchased from Calbiochem (Merck Biosciences GmbH, Schwalboch, Germany). NarE and NarER7K recombinant proteins were cloned, expressed and purified as described [3]. In both preparations the endotoxin content, determined using a LAL assay (QCL-1000 kit from Lonza), was below 0.5 EU/mg protein.

FACS analysis

To measure NarE binding to Chang cells, $2x10^5$ cells were incubated with different concentrations of NarE or NarER7K (ranging from 0,01 to 100 µg/ml) in serum-free DMEM in 96-well culture plates for 1h on ice. Cells were then washed and incubated on ice with anti-NarE serum for 30 min. After washing, cells were stained with FITC conjugated secondary antibodies. Cells were analysed with a Canto II flow cytometer (Beckton-Dickinson) by using Floujo software. The geometric mean fluorescence intensity (MFI) for each population was calculated.

In vitro ADP-ribosylation assay

For cell-free ADP-ribosylation assay, 50 µg of whole-cell lysate were used. ADP-ribosylation reaction was performed for 30 min at 37°C with 1 µg NarE or NarE-R7K in a buffer containing 20 mM Tris-HCl (pH 8), 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 10 µM biotinylated NAD^+ (Trevigen) and Complete protease inhibitor (Roche, Basel, Switzerland) (according to the manufacturer's manual). The reaction was stopped by adding 4x NuPAGE LDS Sample Buffer (Invitrogen) and 10x NuPAGE Reducing Agent (Invitrogen) and boiling the samples for 5 min at 100°C. The samples were subjected to SDS-PAGE and subsequently the biotin-ADP-ribosylated proteins were transferred to a nitrocellulose membrane and visualized with HRP-conjugated streptavidin.

Immunofluorescence microscopy and pulse-chase experiments

Chang cells ($5x10^4$/well) were plated on 8-wells Lab-Tek II Chamber Slide System (Thermo Scientific) and incubated overnight at 37°C 5% CO2. For NarE binding and to visualize morphological effects, cells were incubated at for different times with NarE/NarER7K. After washing with PBS, cells were fixed in paraformaldehyde 2% for 20 min at RT (room temperature) and then, after three washes with PBS, permeabilized with TritonX 0.1% plus saponin 1% for 20 min at RT. Cells were washed three times with PBST (PBS 1x, TritonX 0,1%) and incubated for 30 min at RT with Duolink II Blocking Solution 1X (Olink Bioscience). After washing with PBST twice, cells were incubated for 15 min at RT with mouse anti-NarE serum. Cells were washed again with PBST twice and incubated for 10 min at RT with secondary antibody Alexa Fluor 568 anti-mouse. Subsequently, cells were washed with PBS twice and, after drying at RT, one drop of ProLong Gold Antifade Reagent with DAPI (Invitrogen) was added. For pulse-chase experiments, in order to detach not internalized NarE bound to the plasma membrane of Chang cells, they were first incubated with NarE for 1 h, then they were washed with ice-cold phosphate-buffered saline (PBS) containing 500 mM NaCl and 0.5 % acetic acid and three times with complete medium; before fixation the cells were incubated for 1 h. For the analysis of the subcellular localization of NarE, the slides were analyzed by Confocal microscopy, performed on a LSM700 (Carl Zeiss, Inc.) and LSM 510 (Carl Zeiss, Inc.) confocal microscopes using a Plan-Neofluar 63X objective. Detectors were set to detect an optimal signal below the saturation limits. Images were processed with Zen 2009 image software (Carl Zeiss, Inc.).

xCELLigence system: time- and dose-dependent cell response profiles of Chang cells

The effect of NarE on epithelial barrier function was measured with an electrical cell-substrate impedance sensing system (Applied BioPhysics). In total, 1×10^5 Chang cells were seeded in 8W10E Cultureware and incubated at 37°C in a carbon dioxide incubator. Resistance of the monolayer was recorded until a stable resistance of approximately 600–1000 Ω was documented prior to the addition of purified protein.

Cell treatments, lysis, and immunoblots

For immunoblot analysis, cells ($2x10^6$ cells/well) were plated in a 6-well dish, grown overnight at 37°C to reach a subconfluent condition, and incubated with NarE/NarER7K at 37°C in a humidified atmosphere with 5% CO2 for different time. Cells were recovered by centrifugation at 16,000Xg for 30 sec at 4°C, washed 2X in PBS and lysed in 1% (v/v) Triton X-100 in 20 mM Tris-HCl pH 8, 150 mM NaCl (in the presence of0.2 mg/ml Na orthovanadate, 1 mg/ml pepstatin, leupeptin, and aprotinin, and 10 mM phenyl methyl sulfonyl fluoride). Alternatively, for subcellular protein fractionation, cells were collected, washed in ice-cold PBS and processed by Thermo scientific subcellular protein fractionation Kit. Protein content of isolated fractions, corresponding to cytoplasmic, membrane, nuclear and pellet fraction, was determined by BCA assay. Equal amounts of proteins from were resolved by NuPage Novex 4-12% Bis-Tris Gels (Invitrogen) and transferred to 0.45-mm nitrocellulose filters nitrocellulose membrane by means of iBlot Gel Transfer Device (Invitrogen). Prestained molecular mass markers (Invitrogen) were included in each gel. Immunoblots were carried out using primary antibodies and peroxidase-labeled secondary antibodies

according to the manufacturers'instructions and a chemiluminescence detection kit (Pierce). Blots were scanned using a laser densitometer (DuoscanT2500 Agfa, Milan, Italy) and quantified using the ImageQuant5.0 software (Molecular Dynamics, Sunnyvale, CA). Data were normalized to loading controls.

Statistical analysis

Mean values, standard deviation values and Student's t test (unpaired) were calculated using the Microsoft Excel application. A level of $P<0.05$ was considered statistically significant.

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RESULTS

NarE binding to human epithelial cells leads to protein internalization

The biological function of NarE was assessed on Chang human epithelial cells, a well-established *in vitro* model to study *N. meningitidis* interaction with the host [21,22]. Purified recombinant protein was added at increasing concentrations to epithelial cells and the binding was measured by flow cytometric analysis. As shown in Fig. 1A, NarE, is able to bind Chang cells in a concentration dependent manner, with a rapid increase in binding at lower concentrations, a kinetic probably reflecting NarE interaction with a high affinity receptor. Similar results were obtained when the enzymatically inactive variant of the toxin, NarER7K, which is devoid of the ADP-ribosylation activity, was used [23] (data not shown). The evidence that NarE is able to bind to host epithelial cells was further demonstrated by confocal analysis (S1). Of interest, we observed that NarE not only is able to bind to target cells but it can also be internalized. Indeed, confocal microscopy analysis of cells extensively washed with high-salt acidic buffer (which allows the removal of surface-associated proteins) revealed that NarE is found intracellularly within numerous perinuclear structures (Fig.1B).

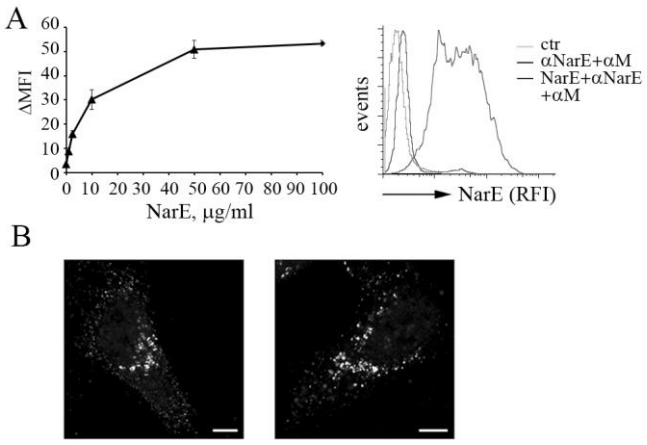

Fig. 1. NarE binding to human epithelial cells leads to protein internalization. (A) Flow cytometric analysis of NarE binding to Chang cells. The data are expressed as the difference between the MFI of cells incubated with NarE, anti-NarE antibodies (α-NarE), anti–mouse Ig FITC-labelled secondary antibodies (α-M), and the MFI of cells incubated with primary and secondary antibodies in the absence of NarE (□MFI). A representative concentration response curve (n > 3) and FACS profile (NarE 1μg/ml) are shown. RFI, relative fluorescence intensity. (B) Chang cells were plated on coverslips and incubated with 10 μg/ml of recombinant NarE for 1 h. Next, the cells were washed with a high-salt and acid buffer to rinse the non-internalized NarE. Finally, the cells were fixed and stained with an anti-NarE antibodies (Fig.1B, *left panel*) or further incubated for 1h at 37°C before being processed (Fig.1B, *right panel*). All the images were acquired using with the same imaging conditions. Three independent experiments were performed. Scale bar is 10 μm.

NarE is internalized through an endocytic traffic route

To further characterize the intracellular trafficking of NarE, Chang cells were incubated with recombinant fluorescently labelled NarE (Alexa 546-NarE) for 1h at 37°C. Cells were then processed by immunofluorescence analysis to assess the co-localization of NarE with established markers of intracellular

compartments. Confocal analysis showed that NarE is distributed in punctate structures at the peripheral and juxtanuclear region of the cell (Fig.2a). Notably, these structures were found to co-localize with the major histocompatibility complex Class I (MHCI) protein (Fig. 2b), a marker of recycling endosomes, and EEA1 (Fig. 2c), a marker of early endosomes (Eyster et al. 2009). No co-localization was observed with LAMP-1, a lysosomal-associated membrane protein (Fig. 2d). These data postulate that NarE is actively internalized into epithelial cells through an endocytic traffic route.

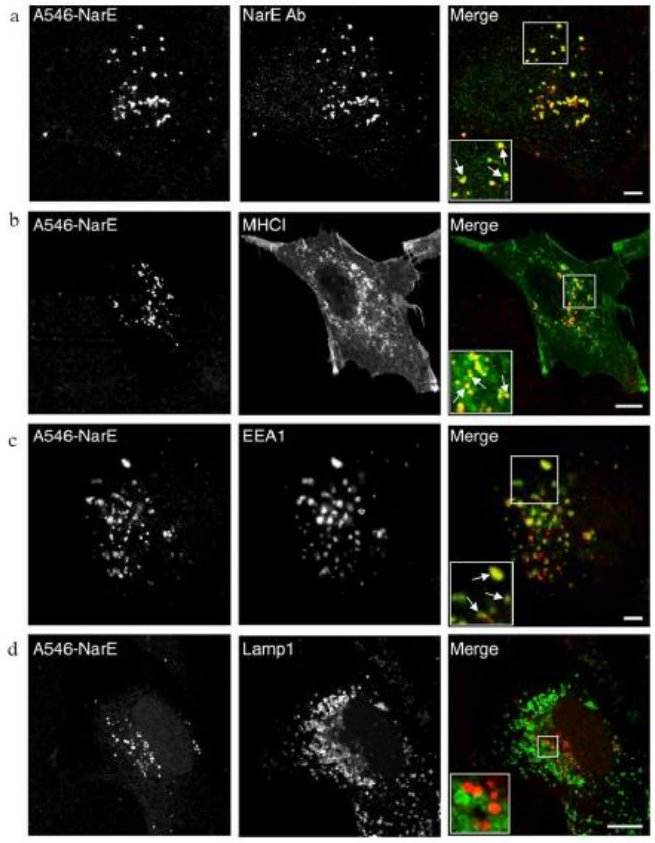

Fig. 2. Immunofluorescence analysis of NarE subcellular localization. Chang cells were plated on coverslips and incubated for 1 h with (10 µg/ml) Alexa 546-NarE. Then, the cells were fixed and labelled with antibodies against NarE (a), MHCI (b), EEA1 (c) and Lamp1 (d). Areas of colocalization of Alexa 546-NarE with the signals detected using antibodies against NarE, MHCI and EEA1 are magnified in the inserts and highlighted by white arrows. Scale bar is 10 µm.

NarE gains access to the cytoplasm and targets host cell proteins

We then hypothesized that NarE internalization and consequent association to the endocytic route, may allow this ADP-ribosyl transferase to gain access to the cytosol. To this end we isolated cytoplasmic, membrane, nuclear soluble and cytoskeletal fractions from Chang cells incubated with NarE for different time points. As shown in Figure 3A, Western blotting analysis revealed that already at 1h, NarE

accumulated in the cytosolic fraction and remained in this compartment until 6h. To identify putative cellular substrate/s ADP-ribosylated by NarE, whole-cell lysates were incubated with NarE or NarE-R7K in presence of biotinylated NAD^+ (or with ^{32}P-NAD^+, data not shown). Interestingly, as shown in Figure 3B, incubation of cell extracts with NarE, but not with the genetically inactivated mutant NarER7K, resulted in the increase of ADP-ribosylated proteins, indicating that NarE specifically targets Chang cell proteins.

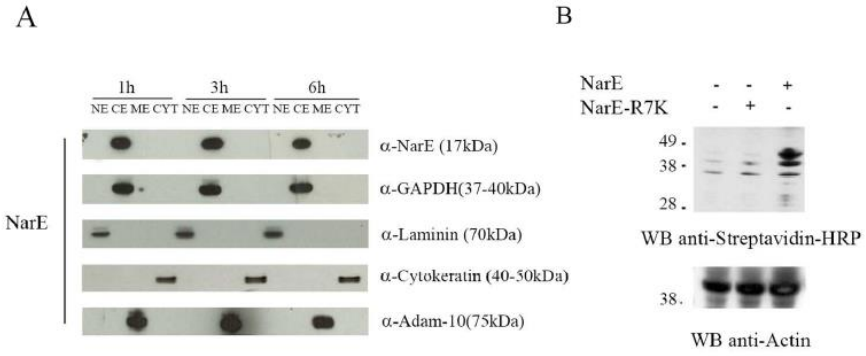

Fig.3. NarE gains access to the cytoplasm and targets host cell proteins. (A) Chang cells, grown to confluence, were exposed to NarE (1µg/ml) or saline (control) for 1 h washed and further incubated for1, 3 and 6h. At the end of the incubation period cytoplasmic (CE), membrane (ME), nuclear soluble (NE) and cytoskeletal (PE) fractions were isolated and processed for western blot analysis. A mouse polyclonal anti-NarE antibody was used. Polyclonal antibodies anti-GAPDH, anti-ADAM10, anti-Laminin and anti-Cytokeratin were used to check the purity of each fraction. HRP-conjugated secondary antibodies were used before developing in chemiluminescence. The molecular weights of the single proteins are in brackets. (B) Immunoblot analysis of the ADP-ribosylation state of NarE substrates in whole-cell extracts from Chang cells treated with NarE (1µg/ml) for 30 min at 37°C. Biotin-ADP-ribose labelled proteins were identified using an anti-streptavidin HRP antibody. The results of a representative experiment are shown. Anti-actin was use as loading control. The position of the molecular mass markers are indicated on the left.

NarE impairs human epithelial monolayer integrity and induces cell rounding

Since NarE ADP-ribosylates Chang substrates, we then examined whether it could alter essential functions of eukaryotic cells. To this end electrical cell-substrate impedance sensing (xCELLigence) was used to measure trans-epithelial electrical resistance. Impedance reflects the status of the cell monolayer, including confluency, viability, and junction functionality [24,25]. Chang cells were grown to confluence on xCELLigence inserts and the experiments were started when transepithelial resistance (TEER) was found constant between two measures taken 24 hours apart. Cells were then incubated with NarE or NarE-R7K. Vehicle was used as negative control. Changes in monolayer integrity were monitored every 15 min. As shown in Fig.4A Chang monolayer treated with 1µg/ml NarE demonstrated a rapid and progressive loss of resistance. Similar results were obtained when *Clostridium difficile* toxin, TcdA, a well-known cytotoxin affecting actin cytoskeleton [7,26], was used. Neither vehicle nor NarER-7K altered monolayer resistance.

In the attempt to correlate changes in monolayer integrity with cytopathic events, NarER7K and NarE treated cells were visualized by light microscopy. As shown in Fig. 4B NarE, as well as TcdA, our positive control, induced morphological changes as the cells rounded up within 4h (Fig.4B). On the contrary, host cells exposed to the mutant form of the protein did not show any changes in their morphology, indicating

that induction of the cell-rounding phenotype of Chang cells required a functional ADP-ribosylating domain.

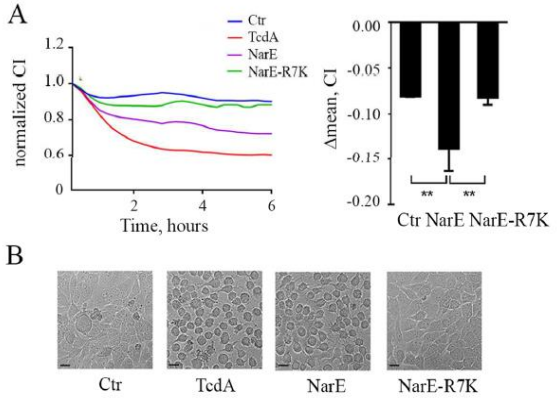

Fig. 4. NarE impairs human epithelial monolayer integrity and induces cell rounding. (A) Top left, Chang monolayer was treated either with NarE or NarER7K (1µg/ml) and barrier resistance was continuously measured using an electric cell-substrate impedance sensing (xCELLigence) system. TcdA (100ng/ml) was used as positive control, while saline was used as negative control. CI, Cell Index (arbitrary unit for electric impedance measurement). Top right, Variation of Chang cells CI after 6 h of treatment with NarE or NarE-R7K (1µg/ml). The results are expressed as the difference between normalized CI before agents addition (CI = 1) and CI value after 6 h of incubation averaged from three independent experiments, each performed on duplicate samples. Error bars represent the SD. **, $P \leq 0.01$. (B) Effect of NarE treatment on the morphology of Chang cells. Chang cells were incubated at 37°C with NarE , NarER7K (1µg/ml) , TcdA as positive control (100ng/ml) or left untreated as control. After 4 hours, pictures were taken to assess the change in morphology. Bar, 20 µm.

NarE induces apoptosis in Chang cells

We next investigated whether NarE-induced cell damages correlate with the activation of cell death pathways. Among a wide range of factors controlling apoptotic cell death, caspase-3 activation plays a key role and its activation requires proteolytic processing of its inactive zymogene into activated p17/19 and p12 fragments [27]. Therefore in order to assess NarE effect on the apoptotic pathway, Chang cells were incubated with NarE, NarE-R7K or cycloheximide, CHX, (as positive control) for various times and then caspase-3 cleavage, which is an indication of its activation state, was evaluated. Activation of caspase-3 was observed after incubation with NarE for 2-6 h (Fig. 5A). As expected, NarE-R7K, which is devoid of the ADP-ribosylation activity, did not induce caspase-3 activation.

The development of apoptosis was further assessed using Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL). To this end Chang cells were treated with NarE for up to 24h. The appearance of fluorescence in the nuclei of NarE-treated cells showed evidence of apoptosis (Fig5B, *right panel*), whereas no DNA fragmentation was observed in cells treated with NarER7K or in control cells (vehicle only). Following incubation with NarE, the amount of apoptotic cells significantly increased as shown by elevation of fragmented DNA (calculated as specific apoptosis as percentage of total cells) in Fig5B (*left panel*).

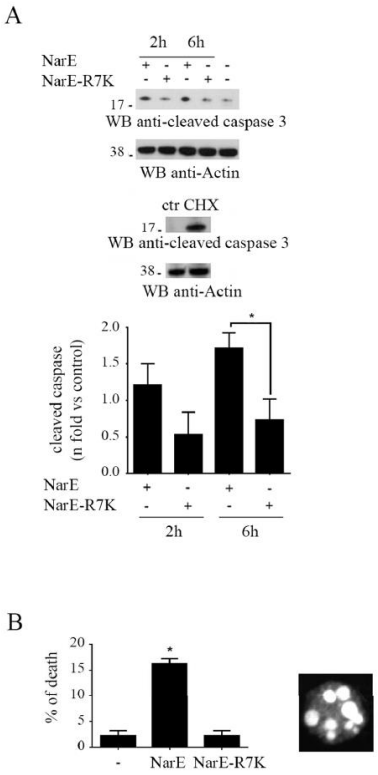

Fig. 5. NarE induces apoptosis in Chang cells. (A) Top panel, Immunoblot analysis, using a specific antibody to detect caspases-3 cleavage, in postnuclear supernatans from Chang cells incubated for the indicated times in the presence or absence of either 1 μg/ml NarE or NarE-R7K. Bottom panel, Quantification by laser densitometry of the relative levels of caspase-3 cleavage (fold activation vs untreated controls) in Chang cells in the presence of 1 μg/ml NarE or NarER7K (n=3).*P≤0.05. Error bars, SD. (B) Fluorescence microscopy analysis of apoptotic cells treated with NarE. The data are expressed as percentages of TUNEL-positive cells (n = 2; *P≤0.05; percentages were calculated on 100 cells/sample)

DISCUSSION

In this paper, we focus on the biological functions of NarE when getting in contact to epithelial cells. In particular, we reported that NarE binds to Chang cells in a dose dependent and saturable manner, and that these events trigger the internalization of this ADP-ribosyl transferase that once in the cytoplasm, exerts its enzymatic activity on host cell proteins. This results in the alteration of cell morphology and epithelial monolayer integrity leading to the activation of caspase-3, an apoptosis executer (cytotoxic effect). Indeed, by using the catalytically inactivated mutant NarE-R7K, we have shown that ADP-ribosylating activity of NarE is responsible for both the cytopathic and the cytotoxic effect. Notably, similar events are reported for various bacterial toxins targeting mammalian cells (e.g. C. botulinum C2, C. perfringens iota toxin and S. enterica SpvB), which by inducing loss of adhesion and cell rounding lead to the activation of the apoptotic pathaway [7,28,29].

ADP-ribosyltransferase activities have been observed in many prokaryotic and eukaryotic species and in viruses. The best characterized mono-ADP-ribosylation reactions are those catalyzed by bacterial toxins, such as diphtheria, cholera, pertussis and clostridial toxins, which act by modifying crucial host cell proteins such as the α-subunit of heterotrimeric GTP-binding (G) proteins, the small GTPase Rho, monomeric actin and elongation factor 2 (EF-2), resulting in permanent activation or inactivation of the cell functions modulated by these protein substrates. Consequently, these toxins have been characterized extensively for their activity and represent some of the best understood microbial mediators of disease and have been used as components of vaccines able to prevent associated diseases [30,31].

By in silico studies Masignani and colleagues identified an ADP-ribosyltransferase in MC58 strain of N. meningitidis serogroup B. They demonstrated that this protein is able to hydrolase NAD and to transfer the ADP-ribose group to small guanidine compounds like agmatine and arginine and so they called it NarE, N. meningitidis ADP-ribosylating enzyme. Interestingly, they found that, despite the absence of a predicted leader peptide, NarE efficiently accumulates in the periplasm of Meningococcus [3]. Further, they showed that the narE gene is present only in a subset of hypervirulent strains of bacteria.

An open question is how NarE, being a periplasmic protein, could achieve contact with host cells. NarE lacks a leader peptide as well as a gene coding for the translocation/receptor-binding subunit B but since it is a really small proteins it maybe can reach the periplasmic space of Meningococcus by translocating across the cytoplasmic membrane through natural membrane pores [3]. However, in order to exert a toxic activity, NarE has to contact host cells. For this reason, once in the periplasm, a second step is required to allow the release of the protein. A possible hypothesis would be that NarE follows a pathway similar to that described for LT of E. coli [32] and it could be released in association with vesicles. Internalization of vesicles could then allow intoxication of the host cells. Alternatively, the export through the outer membrane could occur following cell contact or could just happen upon lysis of the bacterium [3].

Even if the mechanism by which NarE reaches host cells is still unknown, our data clearly highlight that the protein is able to bind to epithelial cells and alter their integrity suggesting a possible role of NarE in first stages of N. meningitidis pathogenesis, indeed disruption of epithelial barrier is a key step for a successful colonization of upper respiratory tract and spreading of infection. Further investigations are needed to

elucidate the pathway followed by this newly identified ADP-ribosylating enzyme to get to epithelial cells and its potential role in the virulence and pathogenesis of meningococcal species.

SUPPORTING INFORMATION LEGENDS

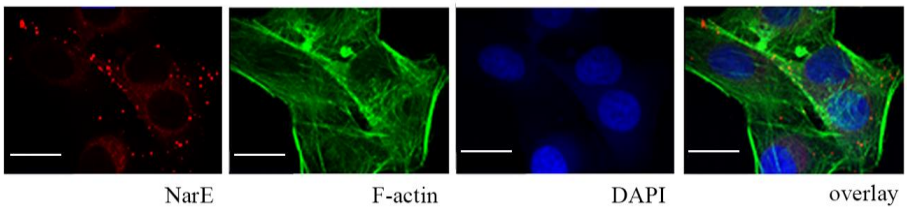

Fig.1S. NarE binds to Chang cells. Immunofluorescence analysis of NarE binding to Chang cells. Cells were either untreated or incubated with 1ug/ml NarE at 37°C for 1 hour and then fixed and stained. Representative confocal images are shown. Cells were co-stained with anti-NarE (red), phalloidin (green) and DAPI (blue). Bar, 20 µm

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