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Characterization of a novel *Escherichia coli* IgA-binding antigen impairing neutrophil activation

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ABBREVIATIONS

ADCC: antibody-dependent cellular cytotoxicity

AIDS: acquired immune deficiency syndrome

AP: alternative pathway

BBB: blood-brain barrier

BSA: bovine serum albumin

C4BP: C4b-binding protein

cDNA: complementary deoxyribonucleic acid

CDT: cytolethal distending toxin

CFU: colony forming unit

CNF-1: cytotoxic necrotizing factor 1

CP: classical pathway

CpG: cytosine phosphate guanine

DAEC: diffusely adherent *Escherichia coli*

DFO: deferoxamine mesylate

dIgA: dimeric immunoglobulin A

DNA: deoxyribonucleic acid

DPD: 2,2'-Dipyridyl

EAEC: enteroaggregative *Escherichia coli*

EC: extracellular

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

FH: factor H

FITC: fluorescein isothiocyanate

FMLP: formyl-methionyl-leucyl-phenylalanine

GBS: group B streptococcus

GTP: guanosine triphosphate

HBSS: Hank's balanced salt solution

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV: human immunodeficiency virus

HSA: human serum albumin

IBC: intracellular bacterial community

Ig: immunoglobulin

IgA-BP: immunoglobulin A-binding protein

IL: interleukin

InPEC: intestinal pathogenic *Escherichia coli*

ITAM: immunoreceptor tyrosine-based activation motif

LB: Luria-Bertani

LBSS: luminol-balanced salt solution

LEE: locus of enterocyte effacement

LP: lectin pathway

LPS: lipopolysaccharide

LTB4: leukotriene B4

MAC: membrane attack complex

mRNA: messenger ribonucleic acid

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

pIgR: polymeric immunoglobulin receptor

PLC: phospholipase C

PMN: polymorphonuclear leukocyte

PO: peroxidase

PTK: protein tyrosine kinase

PVP: polyvinylpyrrolidone

QIR: quiescent intracellular reservoir

RNA: ribonucleic acid

ROS: reactive oxygen species

RPMI: Roswell Park Memorial Institute

RT-PCR: reverse transcription-polymerase chain reaction

RU: response unit

Sat: secreted autotransporter toxin

SC: secretory component

SD: standard deviation

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM: standard error of the mean

SIgA: secretory immunoglobulin A

SLR: Sel1-like repeat

SPR: surface plasmon resonance

SSL7: staphylococcal superantigen-like 7

Stx: Shiga toxin

THB: Todd-Hewitt broth

TMD: transmembrane domain

TNF-α: tumor necrosis factor α

tRNA: transfer ribonucleic acid

UPEC: uropathogenic *Escherichia coli*

UTI: urinary tract infection

1.   ABSTRACT

Extraintestinal pathogenic *Escherichia coli* (ExPEC) possess a unique ability to cause disease outside the host intestinal tract and are responsible for a heterogeneous group of disorders, including urinary tract infections, sepsis and neonatal meningitis, that collectively cause considerable morbidity, lost productivity and high healthcare costs. Considering the incidence and also the increasing antibiotic resistance of ExPEC strains, the prevention of extraintestinal *E. coli* 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 against ExPEC 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 ExPEC pathogenesis. In this study, vaccine antigen c5321 has been characterized. Besides being able to induce protection in a sepsis mouse model, it has been shown to be also immunogenic and specifically expressed *in vivo* during *E. coli* infection of the human urinary tract. Antigen expression has also been confirmed under *in vitro* conditions and has been found to be regulated by ion concentration. The determination of the crystal structure of the protein has revealed that c5321 is composed of twelve Sel1-like repeats (SLRs) stacked on top of each other forming an α/α-superhelix, which shows a high structural homology with a protein-protein recognition domain of human O-linked N-acetylglucosamine transferase and with Helicobacter cysteine-rich protein C. SLRs 3 and 4 have been found to participate in octahedral coordination of magnesium, suggesting that ions, in addition to regulating antigen expression, may also have a structural relevance. As

many bacterial SLR proteins, c5321 has demonstrated a capability to interact with the host immune system and, in particular, with human immunoglobulins A (IgA), leading to the inhibition of IgA-mediated neutrophil activation, oxidative burst and chemotaxis. These data suggest that c5321 is a novel *E. coli* IgA-binding protein with specific immunomodulatory properties that could allow the bacterium to avoid clearance by impairing production of reactive oxygen species, as well as neutrophil recruitment to the infection site.

I ceppi patogeni extraintestinali di *Escherichia coli* (ExPEC) sono tipicamente in grado di infettare siti anatomici al di fuori del tratto gastrointestinale dell'ospite, dando luogo a un ampio spettro di malattie, quali le infezioni del tratto urinario, la sepsi e la 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 dai ceppi ExPEC e dell'aumento della resistenza antibiotica, la prevenzione delle infezioni extraintestinali riveste un'importanza sempre maggiore sia in ambito sanitario che in ambito economico. Poiché gli approcci convenzionali si sono dimostrati inadeguati allo sviluppo di un vaccino polivalente, sicuro e altamente immunogenico contro i ceppi ExPEC, è stato scelto di applicare la "reverse vaccinology" per l'identificazione di antigeni vaccinici protettivi e ampiamente conservati. Sebbene alcuni dei candidati selezionati siano stati precedentemente 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 dei ceppi ExPEC. In questo studio, è stato caratterizzato l'antigene vaccinico c5321. Oltre a essere in grado di indurre protezione in un modello animale di sepsi, tale candidato ha dimostrato di essere anche immunogenico e

specificamente espresso *in vivo* durante l'infezione del tratto urinario umano. L'espressione antigenica è stata, inoltre, confermata *in vitro*, rivelando un meccanismo di regolazione dipendente dalla concentrazione di ioni. La risoluzione della struttura cristallografica della proteina ha consentito di dimostrare che l'antigene c5321 è composto da dodici Sel1-like repeats (SLRs) che formano una super-α-elica avente un'elevata omologia strutturale con un dominio di riconoscimento proteico della N-acetilglucosamina transferasi umana e con l'antigene HcpC di *Helicobacter pylori*. Poiché si è osservato che le ripetizioni 3 e 4 sono implicate nella coordinazione ottaedrica di un atomo di magnesio, è possibile ipotizzare che gli ioni, oltre a rivestire un ruolo nella regolazione dell'espressione antigenica, possiedano anche una rilevanza a livello strutturale. Analogamente a molte proteine batteriche costituite da SLRs, l'antigene c5321 è in grado di interagire con il sistema immunitario dell'ospite e, in particolare, con le immunoglobuline umane di classe A (IgA), determinando un blocco dell'attivazione dei neutrofili mediata dalle IgA, nonché del burst ossidativo cellulare e della chemotassi. Il candidato vaccinico c5321 rappresenta, pertanto, un nuovo antigene di *E. coli* in grado di legare le IgA e dotato di specifiche proprietà immunomodulatorie che potrebbero consentire al batterio di eludere i meccanismi di eliminazione dell'ospite, mediante l'inibizione del burst respiratorio e del reclutamento dei neutrofili al sito d'infezione.

2. INTRODUCTION

2.1. Extraintestinal pathogenic *Escherichia coli*

Escherichia coli is able to colonize the gastrointestinal tract of human infants a few hours after birth, usually coexisting with its host in good health and with mutual benefit for decades. These commensal *E. coli* strains rarely cause disease, except when the host is immunocompromised or when the normal gastrointestinal barriers are breached. However, as a versatile microorganism, *E. coli* is able to remodel its genomic repertoire by acquiring and losing virulence attributes that confer an increased ability to adapt to new niches and to cause a broad spectrum of disease. The most successful combinations of factors have persisted and become pathotypes, groups of strains of a single species that cause the same disease using a common set of virulence traits [1].

To date, eight *E. coli* pathotypes have been extensively studied and they can be broadly classified as either intestinal pathogenic *E. coli* (InPEC) or extraintestinal pathogenic *E. coli* (ExPEC). Among the pathogens causing intestinal infections, there are six well-described categories: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). The ExPEC group includes uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) [2].

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 can not 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 [3]. 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 [4-6].

Considering the incidence and also the increasing antimicrobial resistance of ExPEC strains, it is clear that the financial implications and the impact on healthcare systems are concerning and that efforts will be required to reduce the burden of extraintestinal infections caused by *E. coli*.

2.2. How are ExPEC strains different?

The ExPEC strains responsible for most extraintestinal infections are distinct from normal commensal *E. coli* and intestinal pathogenic *E. coli*.

Commensal *E. coli* strains, which constitute the major portion of the normal facultative intestinal flora in most humans and other mammals, typically derive from

phylogenetic groups A or B1 (as defined on the basis of multilocus enzyme electrophoresis) and generally lack the specialized virulence traits that enable pathogenic strains to cause disease within or outside the gastrointestinal tract [3].

In contrast, intestinal pathogenic *E. coli* strains derive from phylogenetic groups A, B1 or 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 2-1) [6, 7].
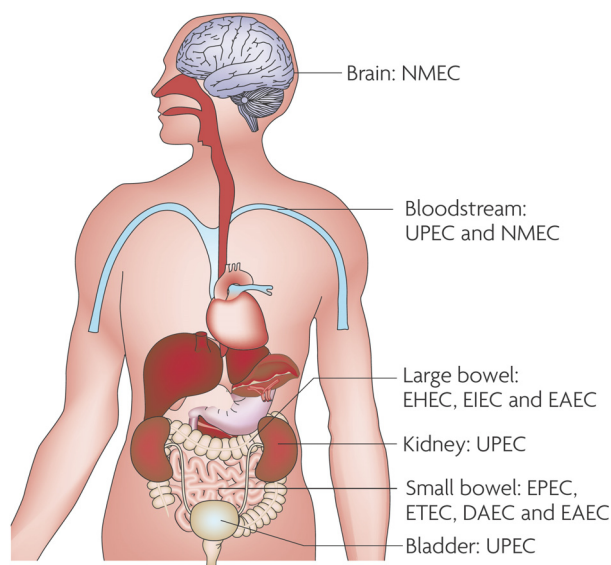
Figure 2-1: Sites of pathogenic *E. coli* colonization (Croxen and Finlay, 2010). Pathogenic *E. coli* colonize various sites in the human body. Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and diffusely adherent *E. coli* (DAEC) colonize the small bowel and cause diarrhea, whereas 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.

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 2-1) [3, 7].

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 [3]. Most of these virulence factors have been acquired by mobile genetic elements.

The loss and gain of mobile genetic elements has a pivotal role in shaping the genomes of pathogenic bacteria [2]. Indeed, it is known that pathogens can evolve and adapt to new niches through the acquisition of virulence attributes by horizontal gene transfer, but also through gene duplication followed by amplification and genome decay, DNA deletions, rearrangements and point mutations [8]. Phages, plasmids, transposons, integrons, insertion sequence elements, islets and large genomic islands, including pathogenicity islands (PAIs), are mobile genetic elements that play a major role in horizontal gene transfer (Figure 2-2) [9]. Genomic islands, for example, are part of the flexible gene pool and their acquisition is crucial in promoting the fitness and survival of a pathogen while it co-evolves with its host [2]. The fact that a great number of genes encoding different functions can be transferred and inserted in block could provide several selective advantages for island-carrying microorganisms: it could help the microorganism to live in the environment (ecological islands) or to persist as saprophyte (saprophytic islands), to colonize the host and provide benefit (symbiosis islands) or just to cause

damage (PAIs) [10, 11]. Of course, the line that separates these conditions can be very subtle, according to the niche and to the right combination of factors.
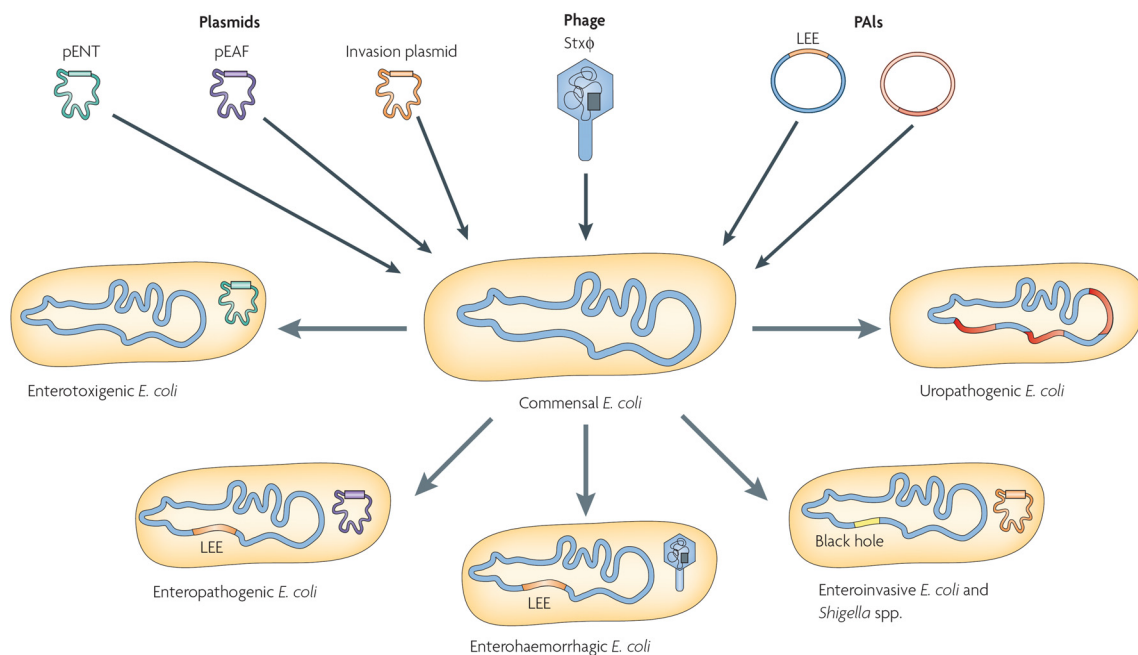
Figure 2-2: Horizontal gene transfer contribution to the evolution of *E. coli* pathotypes (Ahmed et al., 2008). The uptake of mobile genetic elements (phages, virulence plasmids and pathogenicity islands), as well as the loss of chromosomal DNA regions in different *E. coli* lineages, has enabled the 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.

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 nonpathogenic members of the same or related species. They are typically associated with tRNA 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 harbored 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 defenses. Despite the similarity in their tertiary structure, each adhesin recognizes

a specific host receptor [12]. 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 [13, 14]. S and F1C fimbriae participate in bacterial adhesion to the lower urinary tract and to the kidney [15]. Type 3 and F9 fimbriae play a role in biofilm formation and can be related to catheter-associated UTIs [16, 17].

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 [18, 19].

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 [20-23]. 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 [24]; secreted autotransporter toxin (Sat), a serine protease autotransporter with vacuolating activity on bladder and kidney cells [25, 26]; Pic and PicU, other type V secreted proteins with serine protease activity [27, 28]; 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 [29-32]; cytolysin A (HlyE, ClyA or SheA), a pore-forming toxin responsible for lysis of mammalian erythrocytes, cytotoxicity towards cultured mammalian cells and also apoptosis in macrophages [33-35]; enterohemolysin (Ehly), not unique for enteropathogenic strains, associated to the induction of apoptosis and damage of intestinal epithelial cells by a detergent-like mechanism [36].

Other proteins are also associated with the virulence of ExPEC strains. For example, the Hek protein causes autoaggregation and can mediate adherence to and invasion of epithelial cells by binding to glycosaminoglycan, in particular heparin [37]. IbeA and IbeT are strictly related to ExPEC strains and are involved in the invasion of brain microvascular endothelial cells [38, 39]. Antigen 43 (Ag43) is associated with a strong aggregation phenotype and with biofilm formation, promoting long-term persistence in the bladder, although its relevance and contribution in the pathogenesis are far from clear [40-43]. UpaG, a trimeric autotransporter, is also able to promote cell aggregation and biofilm formation and its expression results in bacterial adhesion to human bladder epithelial cells [44].

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 [45, 46]. 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 [47], the enterochelin siderophore receptor FepA [48], the hemoglobin and hemin receptor ChuA [49], the ferric yersiniabactin receptor FyuA [50], FitA [51] and IreA [52].

2.3. 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 a 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 signaling pathways that induce invasion and apoptosis (Figure 2-3*A*). 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 [2].
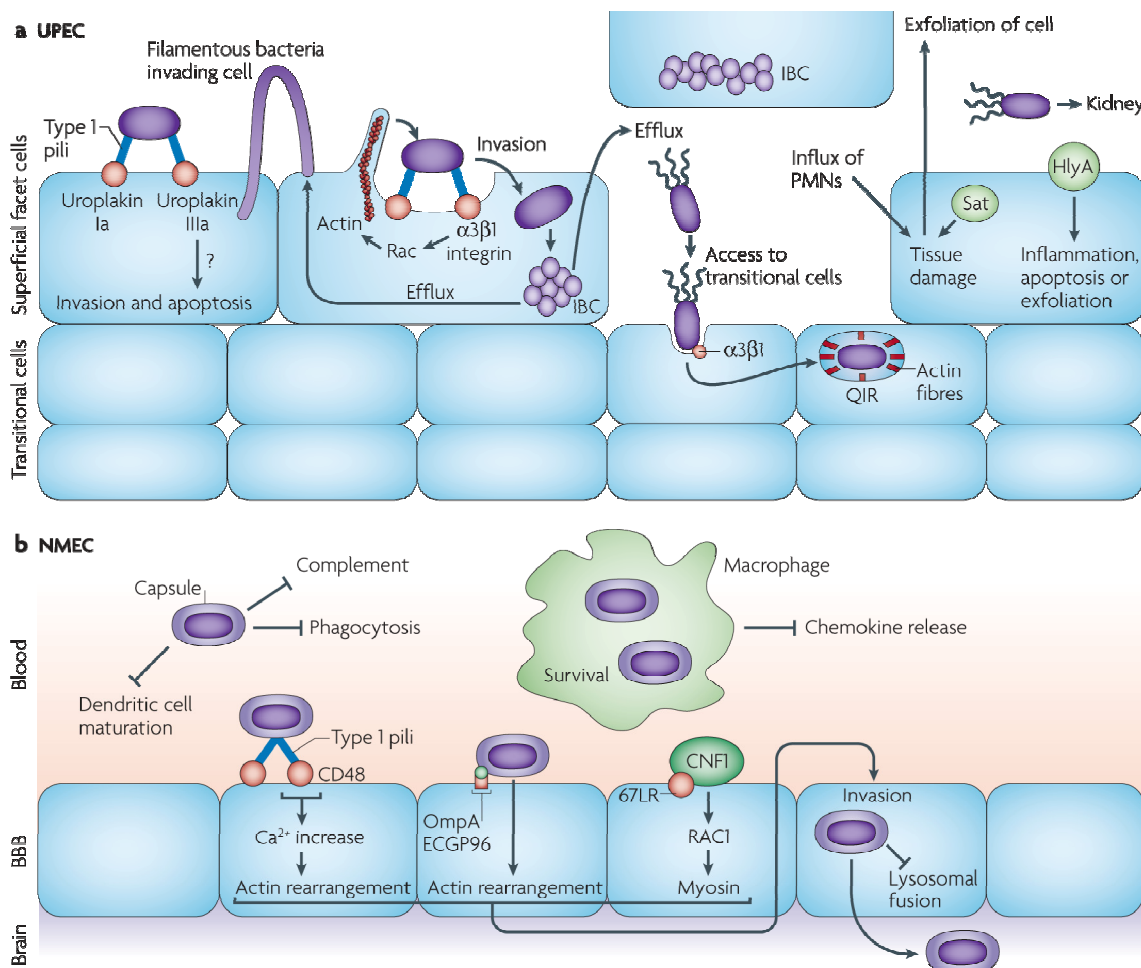
Figure 2-3: Pathogenic mechanisms of ExPEC (Croxen and Finlay, 2010). The different stages of extraintestinal *E. coli* infections are shown. (*A*) UPEC attaches to the uroepithelium through type 1 pili, which bind the receptors uroplakin Ia and IIIa; this binding stimulates unknown signaling pathways that mediate invasion and apoptosis. Binding of type 1 pili to α3β1 integrins also mediates bacteria internalization into superficial facet cells to form intracellular bacterial communities (IBCs). Sublytic concentrations of the pore-forming toxin HlyA can inhibit the activation of Akt proteins and lead to host cell apoptosis and exfoliation. Exfoliation of the uroepithelium exposes the underlying transitional cells to further UPEC invasion, and bacteria can reside in these cells as quiescent intracellular reservoirs (QIRs) that may be involved in recurrent infections. (*B*) NMEC is protected from the host immune response by its K1 capsule and outer-membrane protein A (OmpA). Invasion of macrophages may provide a replicative niche for high bacteremia, allowing the generation of sufficient bacteria to cross the blood-brain barrier (BBB) into the central nervous system. Attachment of NMEC is mediated by type 1 pili binding to CD48 and OmpA binding to ECGP96. Invasion involves CNF-1 binding to 67 kDa laminin receptor (67LR), as well as type1 pili and OmpA binding to their receptors.

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 (Figure 2-3*B*). 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 [2].

2.4. Vaccines against ExPEC

The prevention of ExPEC infections is of pressing concern from both the public health and economic perspectives [7]. Given the large number and diversity of extraintestinal infections and considering also the increasing antibiotic resistance among ExPEC strains, an efficacious vaccine has the potential to have a significant clinical impact on a wide variety of medically important syndromes [53].

ExPEC are typical extracellular pathogens that are inherently resistant to innate host defense factors, such as complement, antimicrobial peptides and phagocytosis in the absence of opsonization. Given the extracellular lifestyle of ExPEC, the development of bactericidal antibodies should lead to protective immunity. However, despite the peaceful coexistence of extraintestinal pathogenic *E. coli* with humans on intestinal mucosal surfaces, the host is unable to develop a protective immune response as a result of natural

colonization. For this reason, a successful immunization strategy needs to be developed in order to confer protection against ExPEC [53].

The development of an efficacious vaccine against extraintestinal *E. coli* presents several challenges. First, although the use of surface polysaccharides has formed the basis for several successful vaccines against extracellular pathogens, this approach is almost impracticable for ExPEC, since more than 80 capsular serotypes exist and more than 150 major variants have been described for the O-antigen moiety of lipopolysaccharide (LPS) in *E. coli* strains. This leads to an antigenic diversity that, combined with the limited immunogenicity and poor immunological memory of non-conjugated polysaccharides, makes the development of a polysaccharide-based vaccine against ExPEC very challenging [53, 54].

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 [53].

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 [53]. Systemic immunization with purified P fimbriae [55, 56] and synthetic peptides corresponding to the protective epitope of the P fimbrial major subunit PapG [57] 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 [58, 59]. Vaccination with type 1 pilus adhesin FimH combined with its chaperone FimC was able to elicit a strong protective immune response, to avoid bacterial binding to human bladder

epithelial cells and to decrease the levels of bacteria recovered from the bladder after challenging. Vaccination of cynomolgus monkeys with the FimCH-complex led to the development of long-lasting IgG responses to FimH, prevented cystitis and inflammation in about 75% of animals challenged with a type 1 piliated ExPEC strain and, at the same time, did not affect the *E. coli* normal flora in the gut [60, 61]. Intranasal immunization with a recombinant truncated FimH adhesin adjuvanted with CpG oligodeoxynucleotides was able to elicit serum IgG titers comparable to that induced by an intramuscular vaccine, but elicited higher vaginal IgA titers [62].

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 [53]. One of the most extensively studied iron-regulated OMPs has been the siderophore receptor IroN; systemic immunization with denatured IroN conferred protection against renal, but not bladder, infection in a mouse UTI model [63].

The pore-forming toxin α-hemolysin has been demonstrated to be highly conserved [64]. 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 [53, 55].

The probability of using these proteins in a stand-alone human vaccine against ExPEC is reduced by their high diversity, site specificity of infection and low distribution. Molecular mimicry of FimH to human lysosomal membrane protein-2 is also responsible for triggering autoimmune responses, an additional obstacle for its use in a vaccine [65].

Several whole-cell vaccine approaches have also been tried to prevent extraintestinal *E. coli* infections. Four standardized whole-cell vaccine formulations have been tested for their efficacy in preventing UTIs. First, Urovac® (SolcoBasel, Basel

Switzerland) consists of ten heat-killed uropathogens; when administered intramuscularly, it induced a significant decrease in recurrent UTIs, but also elicited high levels of adverse reactions that were attributed to endotoxin [53, 66]. Second, OM-89 or Uro-Vaxom® (OM Pharma, Myerlin, Switzerland) is a lyophilized extract from 18 *E. coli* strains and is administered via the oral route; oral therapy with OM-89 was associated with a decrease in recurrent UTIs [67]. Third, Urvakol® (Institute of Microbiology, Czech Republic), which contains inactivated whole cells of *E. coli*, *P. mirabilis*, *P. aeruginosa* and *E. faecalis*, and, lastly, Urostim® (National Center for Infectious and Parasitic Diseases, Bulgaria), which contains lyophilized lysates of *E. coli*, *P. mirabilis*, *K. pneumoniae* and *E. faecalis*, are also both administered orally and have a nonspecific immunostimulatory effect on both innate and acquired immunity [53]. Although for most of the formulations data are far from convincing and many studies lack 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. The idea of using a killed-whole ExPEC strain deficient in capsule and O-antigen resulted in an increased humoral response compared to the wild-type capsulated strain, indicating a higher access to surface-exposed antigens by the antibodies. On the other hand, the development of antibodies against commensal *E. coli* must be carefully considered [53].

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 [53].

2.5. 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 [68]. 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. Table 2-1 shows the vaccines licensed for immunization and distribution in USA, all of them obtained basically by conventional approaches [69]. These 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 [70]. 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 [71]. 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

18

suitable, vaccine candidates [72]. 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 [73], a faster and more reliable approach must be available to promptly respond to those threats.

Table 2-1: Vaccines licensed for immunization and distribution in USA (Moriel et al., 2008).

Vaccine or target	Type
Anthrax	Subunit
Diphtheria	Subunit
Haemophilus influenzae B	Subunit
Hepatitis A	Inactivated
Hepatitis B	Subunit
Human papillomavirus	Subunit
Influenza virus	Inactivated, live
Japanese encephalitis	Inactivated
Measles	Live
Meningococcus	Subunit
Mumps	Live
Pertussis	Subunit
Plague	Inactivated
Pneumococcus	Subunit
Polio	Inactivated
Rabies	Inactivated
Rotavirus	Live
Rubella	Live
Smallpox	Live
Tetanus	Subunit
Tuberculosis	Live
Typhoid	Live, subunit
Varicella	Attenuated
Yellow fever	Live
Herpes zoster	Live

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 [72]. 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 [74, 75]. 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. This profile is completely different from that observed for other microorganisms, such as *Bacillus anthracis*: eight genome sequences were determined for this bacterium, but after the fourth it was verified that the number of new genes added to the pan-genome rapidly converged to zero [76, 77].

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 newborn mice, but also by active maternal immunization and challenge of offspring within the first 48 hours of life [77]. 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 [78]. 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 [77]. 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.

Although the classical reverse vaccinology approach was efficient in providing protective candidates against serogroup B *N. meningitidis*, subsequent analysis showed that NadA, one of the best candidates, is present in only 50% of strains from patients with meningococcal disease and 25% of strains carried by healthy people, indicating that also the pan-genome of this microorganism could have the same features of that of *S. agalactiae* [77]. It is possible that the availability of novel genome sequences of *N. meningitidis* could provide even more candidates that could increase the coverage and efficacy already achieved.

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 frameshifting. 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 [69].

The success of the subtractive reverse vaccinology relies on the clear understanding of the commensalism concept, since the term "commensal" is usually associated with nonpathogenic strains or even probiotics. Commensal bacteria are those that usually colonize an individual without causing disease. Within the commensal flora it is possible to find the "pathogenic commensals" that have the ability to cause disease when the organism is vulnerable. It is important not to confuse those "pathogenic commensals" (like *S. pneumoniae* and *H. influenzae* B) with the "nonpathogenic" ones (like some species of *Lactobacillus*) [79].

The structure and composition of the flora are the result of a selection at both microbial and host levels, leading to a mutual cooperation and stability of this complex system, allowing the flora to form a natural barrier that has numerous protective (pathogen displacement, nutrient competition, receptor competition, etc.), structural (barrier fortification, IgA induction, immune system development, etc.) and metabolic effects (metabolization of dietary carcinogens, synthesis of vitamins, ion absorption, etc.) [80]. Hence, the delivery of vaccines against microorganisms that belong to the commensal flora is not an easy task, even for the "pathogenic commensals". It has been shown that the polysaccharide heptavalent vaccine against *S. pneumoniae* can lead to the replacement with non-vaccine serotypes, but still able to cause infection, and that the polysaccharide-conjugated vaccine against *H. influenzae* B led to the increase of serotype F incidence [80]. The consequences of a vaccine against commensal flora components are usually unexpected, since reducing the level of competition could promote the emerging of other "pathogenic commensals" [81]. Considering that virulence, fitness and colonization factors could be overlapping and dependent on the niche and environmental

conditions, understanding the "nonpathogenic commensals" could prevent the development of vaccines with an impact on the commensal flora.

2.6. Identification of protective vaccine candidates against ExPEC

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 ExPEC [82]. Because most of the ExPEC genomes available up to now are from UPEC strains, we decided to sequence a neonatal meningitis-associated K1 *E. coli*, IHE3034 [83]. The comparison of this genome with those of pathogenic and nonpathogenic *E. coli* strains facilitated the identification of unique genomic features and of broadly protective vaccine candidates [82].

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 [82, 84].

In spite of the huge genomic diversity, by using a subtractive reverse vaccinology approach, we were able to identify nine potential vaccine candidates against ExPEC. Briefly, antigens predicted to be surface-associated or secreted and with no more than three transmembrane domains (TMDs) were selected by bioinformatic analysis of the

genomes of the NMEC strain IHE3034 and of the UPEC strains CFT073 [85] and 536 [84]. The presence as well as the level of similarity of these antigens in the nonpathogenic strains MG1655 [86], DH10B and W3110 were used as exclusion criteria. By this approach, we were able to identify 230 potential antigens (black bars in Figure 2-4), 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 [82].
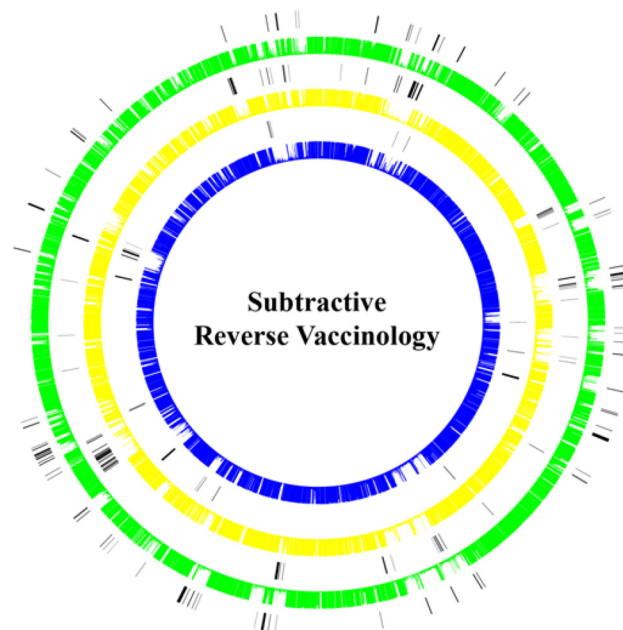

Figure 2-4: The subtractive reverse vaccinology approach (Moriel et al., 2010). The comparison between extraintestinal pathogenic *Escherichia coli* (ExPEC) and nonpathogenic *E. coli* genome sequence is shown (IHE3034, green; CFT073, yellow; 536, blue). Blank regions indicate the low homology shared between ExPEC and nonpathogenic *E. coli* genome sequences. The 230 antigens selected are represented in black, indicating their position in the genome from which they have been selected.

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 bacteremia 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 2-2) [82].

Table 2-2: 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. Among them, two seem to be associated with a type 2 secretion system, including the most protective candidate ECOK1_3385, which is a putative lipoprotein, and ECOK1_3374, which corresponds to subunit K of the secretion apparatus. Other protective candidates have been previously described, for example hemolysin A (HlyA, ecp_3827), a pore-forming toxin secreted by a type 1 secretion system [87], and FitA (ECOK1_3457), which participates in iron uptake and is up-regulated in iron-restricted media [51]. Other candidates may be involved in bacterial adhesion, for example ECOK1_0290, which shares 94% similarity with the ETEC intimin-like protein EaeH [88], and ECOK1_3473, which is a putative fimbrial subunit potentially related to a recently described ExPEC adhesin EA/I involved in the colonization of chicken's lung [89]. However, the remaining candidates, c1275, c5321and c0975, are just annotated as hypothetical proteins and their putative functions still need to

be characterized [82]. The structural and functional characterization of antigen c5321 is the main objective of this thesis.

2.7. Sel1-like repeat proteins: the versatile helix

Sel1-like repeat (SLR) proteins are solenoid proteins with a typical modular architecture consisting of α/α-repeats of 36 to 44 amino acids that are stacked on top of each other forming an extended superhelical macromolecule with a continuous hydrophobic core. The SLR consensus sequence, although characterized by a variable length, highlights conserved alanine and glycine residues at positions 3, 8, 14, 24, 32, 39, 40 and 43; the glycine residues at positions 14, 24 and 43 facilitate sharp turns in loop regions and small residues at positions 3, 8, 32, 39 and 40 allow tight packing of α-helices [90].

In the SMART database release 5.0, SLRs have been identified in 858 protein sequences of bacteria and eukaryotes with an average frequency of 4.8 repeats per protein, but unfortunately only few of them have functional annotations [91]. The origins and domain compositions of SLR proteins with functional annotations are summarized in Figure 2-5. The comparison of SLR proteins is complicated by the extremely low sequence identity, which makes the identification of functional homologues difficult [90].

Although SLR proteins possess a broad spectrum of cellular functions, they frequently participate in protein-protein interactions, assembly of macromolecular complexes and signal transduction pathways. Indeed, SLRs have been identified in several eukaryotic proteins involved in cell cycle regulation, transcriptional control, mitochondrial and peroxisomal protein transport, neurogenesis, protein folding and degradation, as well as in many bacterial proteins involved in pathogen adaptation to its specific host, immunomodulatory functions, bacterium-host interactions and resistance to

bactericidal defense mechanisms and anti-microbial agents. Therefore, the SLR domain may represent an ancient protein-protein interaction module that has been recruited by different proteins and adapted for specific functions [90, 92, 93].
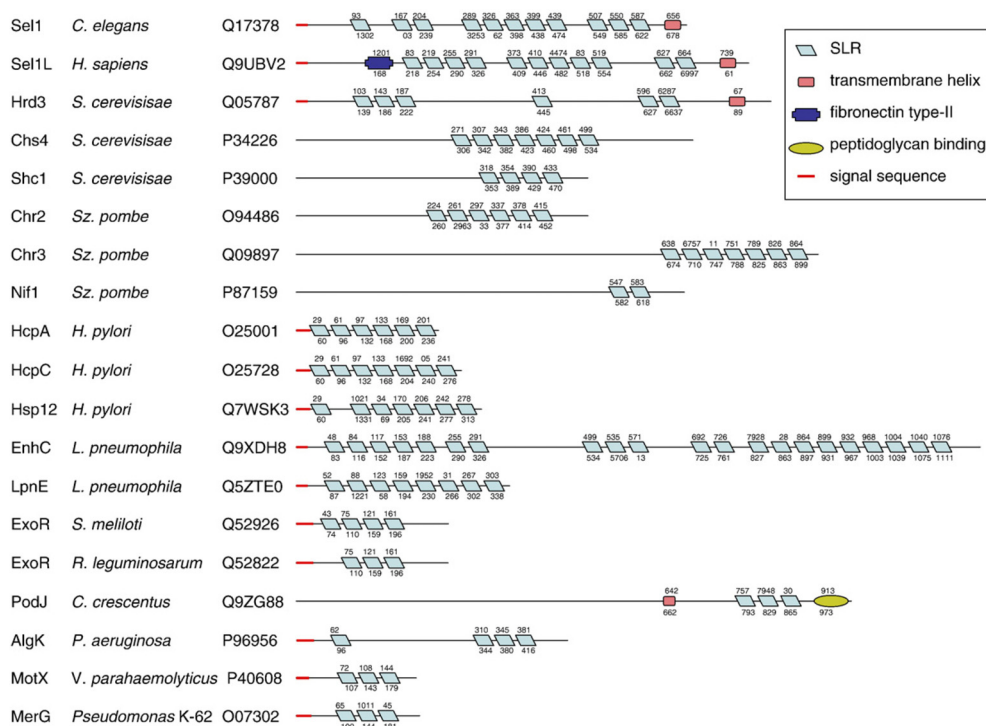

Figure 2-5: List of SLR proteins with functional annotations (Mittl and Schneider-Brachert, 2007). Protein names, origins and Uniprot identifiers are given in columns 1 to 3. In the last column the domain composition is depicted. Numbers refer to the first and last residues of the structural units.

2.8. IgA, IgA Fc receptor and bacterial IgA-binding proteins

IgA represents the most prominent antibody class at mucosal surfaces and the second prevalent antibody class in human serum. While present in the circulation as a monomer, at mucosal sites IgA is produced by local plasma cells in the lamina propria as dimeric IgA (dIgA), containing a joining J chain responsible for disulphide bridge formation between IgA monomers. By binding to the polymeric immunoglobulin receptor (pIgR) expressed on the basolateral membrane of mucosal epithelial cells, dIgA is endocytosed and actively transported through the cell. At the apical membrane, the

external domains of pIgR are cleaved off and the complex is released into secretions. The remainder of pIgR that binds covalently to dIgA is called the secretory component (SC) and the SC-dIgA complex is referred to as secretory IgA (SIgA) [94, 95].

In humans, two subclasses of IgA, termed IgA1 and IgA2, exist and they mainly differ by the presence or absence of a 13 amino acids hinge region. This region, exclusively present in IgA1, has many O-linked glycosilation sites and is a target for at least two families of bacterial proteases. IgA2 is not susceptible to such proteolysis and bears two additional N-linked carbohydrate chains [94]. IgA in serum is composed of 90% IgA1 and 10% IgA2, whereas for SIgA the subclass proportions vary with mucosal site, ranging from 80 to 90% IgA1 in nasal and male genital secretions, through 60% IgA1 in saliva, to 60% IgA2 in colonic and female genital secretions [96].

Secretory and serum IgA are molecules with different biochemical and immunochemical properties produced by cells with different organ distribution [97]. By inhibiting adherence of pathogenic microorganisms to the mucosal wall, SIgA is the primary mediator of immunity in mucosal areas. SIgA is a hydrophilic, negatively charged molecule due to the predominance of hydrophilic amino acids in its Fc region and to the abundant glycosilation of both IgA and SC. SIgA can, therefore, surround microorganisms with a hydrophilic shell that is repelled by the mucin glycocalix at mucosal surfaces. SIgA is also able to agglutinate microbes and interfere with bacterial motility by interacting with flagella. In addition, SIgA interacts with bacterial products such as enzymes and toxins, neutralizing their action (Figure 2-6) [94]. Binding of SIgA to antigens, however, does not trigger inflammatory responses, which is beneficial because immune responses against food antigens and commensal bacteria at mucosal sites could induce chronic inflammation [98]. Whereas the role of SIgA is established in mucosal immunology, the function of serum IgA antibodies is mostly unknown. Serum

IgA is considered a "discrete housekeeper", because IgA immune complexes can be removed by the phagocytic system with little or no resulting inflammation. Moreover, in the absence of antigen, monomeric serum IgA displays anti-inflammatory activity and is capable of inhibiting functions such as IgG-induced phagocytosis, chemotaxis, bactericidal activity, oxidative burst and cytokine release. [99].
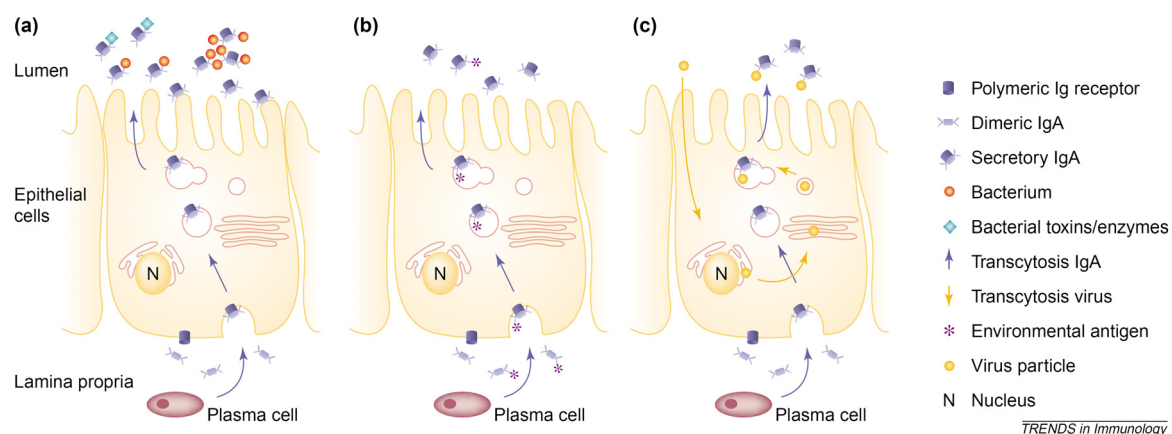

Figure 2-6: Proposed functions of IgA in the mucosal area (van Egmond et al., 2001). Plasma cells in the lamina propria produce dimeric IgA (dIgA) that binds to the polymeric Ig receptor (pIgR), which is expressed on the basolateral membranes of epithelial cells. This complex is transported through epithelial cells to the lumen, where the pIgR is cleaved, releasing secretory IgA (SIgA). (*a*) SIgA shields the mucosa from bacterial penetration, agglutinates bacteria, interferes with their motility and neutralizes bacterial products. (*b*) Antigens in the lamina propria are bound by IgA and can be transported through epithelial cells via the same route as free dIgA, ridding the mucosa of excess antigens. (*c*) During transcytosis IgA can intercept virus antigens and interfere with viral synthesis and/or assembly, thus neutralizing viruses intracellularly.

Although IgA has by far been considered an anti-inflammatory antibody, recent data support its capacity to trigger a plethora of inflammatory functions by interacting with the myeloid IgA Fc receptor FcαRI (CD89), which is constitutively expressed on cells of the myeloid lineage, including neutrophils, monocytes, macrophages, eosinophils, interstitial dendritic cells and Kuppfer cells. A number of cytokines and other agents can modulate FcαRI expression, for example receptor upregulation on neutrophils occurs via a calcium-dependent signaling pathway in response to formyl-methionyl-leucyl-phenylalanine (FMLP), IL-8 and tumor necrosis factor α (TNF-α) [94, 99].

FcαRI consists of two extracellular (EC) Ig-like domains, a transmembrane (TM) region, which is crucial for association to the signaling FcR γ-chain, and a short cytoplasmic tail devoid of recognized signaling motifs. All forms of IgA are ligands for FcαRI, but the binding capacities differ. Although monomeric and dimeric IgA are able to bind FcαRI with moderate affinity (K_a approximately 10^6 M^{-1}) in the boundaries of their domains Cα2 and Cα3, IgA immune complexes bind avidly. Furthermore, because of the partial overlap of the IgA binding site for either FcαRI or pIgR, interaction of SIgA with FcαRI is partially hampered due to SC steric hindrance. However, SIgA binding is increased when complement receptor 3 (Mac-1, CD11b/CD18) functions as co-receptor. Crystallographic studies demonstrated that one IgA molecule can simultaneously bind two FcαRI molecules and that the IgA binding site on FcαRI is located in EC1 [95].

Binding of IgA immune complexes induces pro-inflammatory responses which require association of FcαRI with the FcR γ-chain subunit, as the cytoplasmic tail of FcαRI does not bear any known signaling motif. FcR γ-chain contains an immunoreceptor tyrosine (Tyr)-based activation motif (ITAM), which is essential for initiation of activatory signals. Cross-linking of FcαRI by IgA immune complexes is able to induce relocation of FcαRI into lipid rafts, which are rich in signaling molecules, such as the Src protein Tyr kinase (PTK) p56^{lyn}, which are proposed to function as signaling platforms on the cell membrane. Upon FcαRI triggering, the Tyr in the FcR γ-chain ITAMs are phosphorylated by p56^{lyn}, followed by p72^{syc} association with phosphorylated ITAM and modulation of a multimolecular adapter complex containing Cbl, Shc, SHIP, Grb2, SOS, SLP-72 and CrkL. Additionally, both phosphoinositide kinases, such as phosphoinositide 3-kinase (PI 3-kinase) and phospholipase Cγ2, and serine/threonine kinases, like protein kinase C (PKC) α, PKCε and protein kinase B α, are recruited. The interconnected signaling pathways triggered by FcαRI cross-linking couple upstream FcR γ-chain ITAM

phosphorylation to different cellular processes, such as gene expression by activation of several transcription factors (including NF-κB, AP-1 and Sp1), phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), respiratory burst, calcium release, degranulation, internalization of IgA complexes followed by antigen presentation, and release of cytokines, chemotactic factors and inflammatory lipid mediators [95, 98, 99].

The fact that numerous pathogens have evolved means to specifically evade or subvert the IgA immune response is an indicator of the importance of IgA in protection against infection. These evasion mechanisms include proteins that bind specifically to IgA inhibiting its action, as well as specific proteases that inactivate IgA through cleavage [96]. Important examples of IgA-binding proteins (IgA-BPs) include proteins Arp4 and Sir22 of group A *Streptococcus*, which are members of the M protein family [100, 101], and β-protein, an unrelated protein expressed by group B *Streptococcus* [102, 103]. These streptococcal proteins interact with the IgA Cα2-Cα3 domain interface, which is essentially the same site recognized by FcαRI. Therefore, the IgA-BPs can inhibit IgA binding to FcαRI, as well as the triggering of FcαRI-mediated respiratory burst [104], allowing the bacterium to evade elimination mechanisms that would normally be elicited by IgA through the interaction with FcαRI [97]. Another example of IgA-BP is represented by staphylococcal superantigen-like 7 (SSL7), a secreted toxin of *Staphylococcus aureus* which is able to bind the Cα2-Cα3 interface of monomeric IgA1 and IgA2 and also SIgA, competitively inhibiting FcαRI binding [105]. Since defense against *S. aureus* depends on preventing adherence to mucosal surfaces by SIgA and other opsonins, the ability to perturb IgA-mediated clearance presumably provides the bacterium with increased opportunities for colonization [97]. It is remarkable that several host receptors and bacterial molecules interact with the same region at the interface of the Cα2 and Cα3 domains in the IgA Fc. Presumably this region in intrinsically suited to

protein-protein interactions and has been conserved as a binding site for key host receptors. Various pathogens have, then, targeted this region with their own proteins that often block interaction with host receptors, thereby subverting the protective antibody response [96].

3. MATERIALS AND METHODS

3.1. 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 adult participants.

3.2. Bacterial strains and culture conditions

E. coli CFT073 is a prototypic UPEC strain isolated from the blood and urine of a patient with acute pyelonephritis [106]. For *in vivo* studies in the sepsis mouse model, the strain was routinely cultured in Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 37°C with agitation and aeration. For real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) studies, the strain was cultured in different growth media. M9 minimal medium (3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 0.2 mM $CaCl_2$, 1 mM $MgSO_4$, 0.2% glucose) supplemented with 0.3% casamino acids and 0.2% tiamine-HCl (M9CA) was used as reference condition. Bacteria from an overnight M9CA culture were inoculated into fresh medium at an optical density at 600 nm (OD_{600}) of 0.1 and were grown up to 7 hours at 37°C with agitation and aeration; approximately 10^9 bacteria were collected every hour for RNA isolation. For iron-limiting conditions, CFT073 was cultured in M9CA medium supplemented with 200 μM 2,2'-Dipyridyl (Sigma-Aldrich) or 100 μM deferoxamine mesylate (Desferal; Sigma-Aldrich), whereas, for calcium and magnesium limitation, the growth medium was supplemented with 100 μM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich); approximately 10^9 bacteria were collected after 3 hours (exponential phase) and 6 hours

(stationary phase) for RNA isolation. For growth in whole human blood, approximately 10^9 bacteria from an overnight M9CA culture were pelleted by centrifugation at 3500 rpm for 5 minutes and were resuspended in 3 ml of heparinized freshly isolated human blood; whole blood infected with bacteria was incubated for 1 hour (up to exponential phase) and 2 hours (up to stationary phase) at 37°C with gentle shaking to avoid sedimentation. For growth in human urine, midstream urine collected from 6 male and female healthy donors was pooled and sterilized through a 0.22 μm pore filter; bacteria from an overnight urine culture were used to inoculate human urine at $OD_{600} = 0.05$ and were grown statically without aeration at 37°C for 3 hours (exponential phase) and 6 hours (stationary phase) for RNA isolation.

3.3. Open reading frame prediction and gene identification

Open reading frames (ORFs) were predicted as previously described [74, 107]. All ORFs were searched with FASTA3 against all ORFs from the complete genomes and matches with a fasta P value of 10^{-15} were considered significant [82].

3.4. Antigen selection

Vaccine candidates were selected based on different criteria: low identity between ExPEC (IHE3034, 536 and CFT073) and nonpathogenic (DH10B, W3110 and MG1655) ORFs, low number of TMDs, and subcellular localization. Proteins sharing an identity and overlap with nonpathogenic strains above 90% and 80%, respectively, were discarded, as well as proteins with a cytoplasmic prediction and a high number of TMDs (>3). Protein subcellular localization was predicted by bioinformatic tools [108, 109] and also proteomic approaches [82, 110].

3.5. Cloning and expression of antigen c5321

c5321 gene was amplified by PCR from the CFT073 genomic DNA template, cloned in pET-21b vector (Novagen) and transformed in DH5α-T1 chemically competent cells (Invitrogen) for propagation. BL21(DE3) chemically competent cells (Invitrogen) were used for protein expression. Candidate c5321 was cloned and expressed as His-tagged fusion protein without the predicted signal sequence. Protein purification was performed from the bacterial soluble fraction using nickel-affinity chromatography as already described [74, 82].

3.6. Mice immunization and lethal challenge

Active protection of c5321 was evaluated in a sepsis mouse model. CD1 out-bred mice (8-10 females per group, at least 3 groups per antigen, 5 weeks old, purchased from Charles River Italia) were immunized by subcutaneous injections at days 1, 21 and 35 with 20 µg of recombinant protein in 150 µl of saline solution using complete and incomplete Freund's adjuvant (Sigma-Aldrich). Positive control mice were immunized with 10^8 heat-inactivated (65°C for 30 minutes) bacteria in Freund's adjuvant, whereas negative control mice were immunized with Freund's adjuvant in saline solution. Immunized animals were challenged at day 49 with a lethal dose of homologous strain CFT073 causing 80% to 100% killing in control mice. Heparinized blood samples were collected from survived mice at 20 hours after challenge to determine bacteremia levels and the mortality was monitored for 4 days after challenge [82].

Vaccine protection was expressed as percent PE calculated as [1 − (% dead in vaccine group / % dead in negative control group) x 100]. Statistical analysis was carried out by two-tailed Fisher's exact test comparing the number of survived animals in

vaccinated groups with the number of survived animals in negative control groups. *P* values <0.05 are considered as significant [82].

3.7. ELISA with patient sera

Recombinant c5321 was diluted in phosphate buffered saline (PBS) to a concentration of 5 μg/ml and 100 μl per well were dispensed into microtiter plates (96-well Nunc MaxiSorp; Nunc). After an overnight incubation at 4°C, wells were washed with PBS containing 0.05% Tween-20 and blocked with 200 μl of a 3% (wt/vol) polyvinylpyrrolidone (PVP) solution for 1 hour at 37°C. Diluted patient sera were incubated for 2 hours at 37°C and, subsequently, wells were washed and incubated with alkaline phosphatase-conjugated goat anti-human IgG (Sigma-Aldrich) diluted in PBS containing 1% bovine serum albumin (BSA). Plates were incubated for 30 minutes at 37°C and, then, washed. IgG binding was revealed by adding a chromogen-substrate solution containing p-nitrophenylphosphate (Sigma-Aldrich). After 30 minutes, the reaction was stopped with 4 N NaOH and plates were read at 405 nm.

3.8. RNA purification and real-time quantitative RT-PCR

Prior to RNA isolation, two volumes of RNAprotect Bacteria Reagent (Qiagen) were added to one volume of bacterial culture to immediately stabilize the *in vivo* gene expression profile in bacteria. After RNA stabilization, cells from blood cultures were harvested by centrifugation and incubated with 5 volumes of Erythrocyte Lysis (EL) buffer (Qiagen) for 15 minutes on ice. Cells were, then, centrifuged at 4500 rpm for 6 minutes at 4°C and resuspended with 2 volumes of EL buffer to complete erythrocyte lysis. For total RNA extraction, cells were disrupted by treatment with 15 mg/ml

lysozyme (Sigma-Aldrich) and 2 mg/ml proteinase K (Qiagen) for 10 minutes at room temperature. RNA isolation was completed with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and integrity were assessed by measurement of the A260/A280 ratios and electrophoretic analysis.

Prior to RT-PCR, contaminating DNA was removed from RNA samples by treatment with RQ1 RNase-Free DNase (Promega). RT-PCR was carried out on 500 ng total RNA using ImProm-II™ reverse transcriptase (Promega) and random primers (Promega) for first-strand cDNA synthesis.

Real-time quantitative RT-PCR was performed using FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics) in a LightCycler® 480 II Real-Time PCR System (Roche Diagnostics). All samples were run in duplicate on 96-well optical PCR plates (Roche Diagnostics). The specific primers used to amplify cDNA fragments corresponding to c5321 were: 5'-AAAGATGAGCAACAGGCCGCAATC-3' (forward) and 5'-ATCACGTTCTACGCCCAAGCCATA-3' (reverse). After an initial denaturation at 95°C for 10 minutes, in the subsequent 40 cycles denaturation was performed at 95°C for 15 seconds, followed by primer annealing at 60°C for 30 seconds and a final extension at 72°C for 30 seconds. The specificity of the amplified fragment was demonstrated by the melting curve, where a single peak was observed for each sample amplified with c5321 primers. For absolute quantification of transcript levels, serial dilutions of an external homologous standard (plasmid DNA carrying the cloned target sequence) were used to generate a standard curve. Data analysis was carried out using LightCycler® 480 1.5 software (Roche Diagnostics). The Second Derivative Maximum Method was applied for quantification.

3.9. Crystallization and structure determination

Structure was solved by SAD. Data were integrated and scaled in iMosflm. Experimental phasing was performed with Phenix (AutoSol) and followed by automated model building in Phenix (AutoBuild) and CCP4 (Buccaneer), and by refinement in Phenix (phenix.refine) and CCP4 (Refmac5). Data collection and crystallographic refinement statistics are presented in Table 3-1.

Table 3-1: Crystallographic statistics.

Parameter	Value
Space group	$P2_1$
Unit cell dimensions (Å)	
a; b; c (Å)	48.99; 58.52; 88.38
α; β: γ (°)	90.0; 103.9; 90.0
Wavelength (Å)	0.9791
Resolution range (Å)	42.90-1.68
Unique reflections	54866
R_{merge} (%)	11.7
Data completeness (%)	99.5
Multiplicity	7.2
I/σ	12.9
R (%)	20.2
R_{free} (%)	23.0
R.m.s deviations	
bond lengths (Å)	0.006
bond angles (°)	0.928
No. total atoms	3806
No. protein atoms	3675
No. water atoms	131
Ions (Mg^{2+})	1
Ramachandran plot	
Most favored regions (%)	95.6
Additional allowed regions (%)	4.4
Generously allowed regions (%)	0
Disallowed regions (%)	0

3.10. Complement-dependent binding assay

S. aureus strain KV27 was grown to an OD_{660} of 0.5 in Todd-Hewitt broth (THB) and washed in $HEPES^{++}$ buffer (20 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, 2.5 mM $MgCl_2$) with 0.1% BSA. Bacteria were, then, incubated with normal human serum (NHS) and 20 μg/ml recombinant c5321 for 30 minutes at 37°C with shaking at 600 rpm. Heat-inactivated (56°C for 30 minutes) NHS was used as control. Bacteria were washed with PBS containing 0.1% BSA. Recombinant c5321 was detected using mouse anti-His-tag monoclonal antibodies (Novagen) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Dako). Fluorescence of 10,000 bacteria was measured by flow cytometry (FACSCalibur, Becton Dickinson). The same experiment was also performed using complement-depleted sera (ΔC2, ΔC3, ΔC4, ΔC5, ΔC6, ΔC7, ΔC8, ΔC9) purchased from Calbiochem. For sera repletion, C3 was purified from human plasma as already described [111], while the purified components C2, C4, C5, C6, C7, C8 and C9 were purchased from Calbiochem.

3.11. Complement ELISAs

Complement ELISAs were performed as previously described [112, 113] with minor modifications. ELISA plates (MaxiSorp; Nunc) were coated overnight with 1 μg/ml IgM (Calbiochem) for the classical pathway (CP), 10 μg/ml mannan (*Saccharomyces cerevisiae*; Sigma) for the lectin pathway (LP), or 20 μg/ml LPS (*Salmonella enteriditis*; Sigma) for the alternative pathway (AP) in 0.1 M sodium carbonate buffer (pH 9.6). For the CP and LP, plates were blocked with 1% BSA in PBS with 0.05% Tween-20 for 1 hour at 37°C, whereas for the AP plates were blocked with 4% BSA in PBS with 0.05% Tween-20. For the CP and LP, samples were diluted in $GHEPES^{++}$ buffer (20 mM

HEPES, 140 mM NaCl, 0.1% gelatin, 5 mM $CaCl_2$, 2.5 mM $MgCl_2$), while for the AP samples were diluted in GHEPES/MgEGTA buffer (20 mM HEPES, 140 mM NaCl, 0.1% gelatin, 5 mM $MgCl_2$, 10 mM EGTA). NHS was pre-incubated with 20 µg/ml recombinant c5321 for 15 minutes at 37°C and subsequently added to the plates for 1 hour at 37°C. Deposited C3b and C5b-9 were detected using anti-C3c WM1 (American Type Culture Collection) and anti-C5b-9 (Santa Cruz Biotechnology) antibodies, respectively, followed by peroxidase (PO)-conjugated goat anti-mouse IgG (BioRad).

3.12. Hemolysis assay

The CP hemolytic assay was performed as previously described [114] with minor modifications. NHS was pre-incubated with 20 µg/ml recombinant c5321 for 15 minutes at 37°C. Subsequently, opsonized (with anti-sheep IgM) sheep erythrocytes (Alsever) were incubated with c5321-treated serum in HEPES^{++} buffer (20 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, 2.5 mM $MgCl_2$). After 1 hour at 37°C, samples were centrifuged and the absorbance of the supernatants at 405 nm was measured. The AP hemolytic assay was performed as described above, using rabbit erythrocytes (Alsever) with HEPES/MgEGTA buffer (20 mM HEPES, 140 mM NaCl, 5 mM $MgCl_2$, 10 mM EGTA).

3.13. ELISA with human IgA

To study the binding of c5321 to IgA, human serum IgA (Calbiochem) or secretory IgA (Sigma) were diluted in PBS to a concentration of 10 µg/ml and 100 µl per well were dispensed into microtiter plates (96-well Nunc MaxiSorp; Nunc). After an overnight incubation at 4°C, wells were washed with PBS containing 0.05% Tween-20 and blocked with 250 µl of a 2.7% (wt/vol) PVP solution for 2 hours at 37°C. After

washing, wells were incubated with serially diluted recombinant c5321 for 2 hours at 37°C. Binding of c5321 was detected using mouse anti-c5321 monoclonal antibodies and PO-conjugated rabbit anti-mouse IgG (Dako), followed by the addition of a chromogen-substrate solution containing o-phenylenediamine dihydrochloride (Sigma-Aldrich). After 20 minutes, the reaction was stopped with 12.5% H_2SO_4 and the difference between absorbance at 492 nm and absorbance at 630 nm was measured.

3.14. Surface plasmon resonance

All surface plasmon resonance (SPR) experiments were performed using a Biacore T100 (GE Healthcare) equilibrated at 25°C. Human IgA antibodies previously exchanged in PBS or recombinant c5321 in PBS were first covalently immobilized by amine coupling on a carboxymethylated dextran sensor chip (CM-5, GE Healthcare). IgA or c5321 concentrated 10 μg/ml in 10 mM sodium acetate buffer, pH 5.0, were injected at 5 μl/min until approximately 2000 or 1000 response units (RU) were captured, respectively. The blank (reference) surface was constructed similarly, except that antibodies or protein were omitted. Different concentrations of c5321 or IgA in running buffer (PBS containing 0.05% Tween-20, pH 7.4) were injected over immobilized IgA or c5321, respectively. Following each injection, sensor chip surfaces were regenerated with a 30-second injection of 10 mM glycine, pH 2.2. Resulting sensorgrams were analyzed by the BIAevaluation Software provided by the manufacturer.

3.15. PMN isolation

Human PMNs were isolated from heparinized venous blood of healthy volunteers by Ficoll-Paque[TM] PLUS (GE Healthcare) density gradient centrifugation. Cell viability was determined by trypan blue exclusion.

3.16. Respiratory burst measurement

PMN respiratory burst activity was measured by chemiluminescence. Briefly, neutrophils were diluted in Hank's balanced salt solution (HBSS) with 0.1% human serum albumin (HSA) to a final concentration of 10^5 neutrophils/ml. IgA were pre-incubated with recombinant c5321 for 15 minutes at 37°C and subsequently mixed with neutrophils. Following addition of Luminol-balanced salt solution (LBSS), the oxidative burst was measured every 30 seconds for 60 minutes at 37°C by chemiluminescence using a luminometer (Berthold Technologies, Wildbad, Germany).

3.17. Chemotaxis assay

To measure neutrophil chemotaxis (directed migration), bottom chambers of Transwell supports were filled with supernatants deriving from PMNs incubated (20 minutes at 37°C) with IgA or c5321-treated IgA. Neutrophils were added to the upper chambers. After 1 hour at 37°C, cells that had migrated towards the lower compartments were quantified by flow cytometry.

3.18. Neutrophil activation, lysis and immunoblots

Neutrophil activation by FcαRI cross-linking was performed by incubating cells (5 x 10^6) in RPMI 1640 (Invitrogen) with IgA or c5321-treated IgA for 1 minute at 37°C.

Cells were recovered by centrifugation at 16,000x*g* for 30 seconds at 4°C, washed twice in PBS and lysed in 1% (v/v) Triton X-100 in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, in the presence of a protease inhibitor cocktail (0.2 mg/ml sodium orthovanadate, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 10 mM phenyl methyl sulfonyl fluoride). Equal amounts of proteins from each sample (measured using the Bio-Rad DC Protein Assay Kit) were resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.45 μm nitrocellulose filters (Whatman GmbH). Prestained molecular mass markers (Invitrogen) were included in each gel. Immunoblots were carried out using anti-phosphotyrosine (clone 4G10) antibody (Millipore) and PO-conjugated rabbit anti-mouse antibody (Dako) according to the manufacturer's instruction and a chemiluminescence detection kit (Pierce). All blots were reprobed with loading control antibody after stripping (anti-actin, clone C4; Millipore).

4. RESULTS

4.1. c5321 is a protective vaccine candidate against ExPEC

To identify potential vaccine candidates against ExPEC, we applied the subtractive reverse vaccinology approach and we selected, by bioinformatic analysis of the IHE3034, 536 and CFT073 genomes, those antigens which were predicted to be surface-associated or secreted and to have no more than three TMDs. To prevent an impact on the commensal flora, proteins sharing an identity and overlap with nonpathogenic strains above 90% and 80%, respectively, were discarded. By this approach, we selected 230 antigens, which were, then, expressed as His-tagged proteins, purified and tested for protection in a sepsis mouse model. At the end of this screening, we identified nine potential vaccine candidates, able to elicit a significant level of protection compared to the control group. Among them, c5321 induced an average PE of 33% after challenge with the homologous UPEC strain CFT073. We also observed a good correlation between protection and bacteremia levels at 20 hours (Figure 4-1).
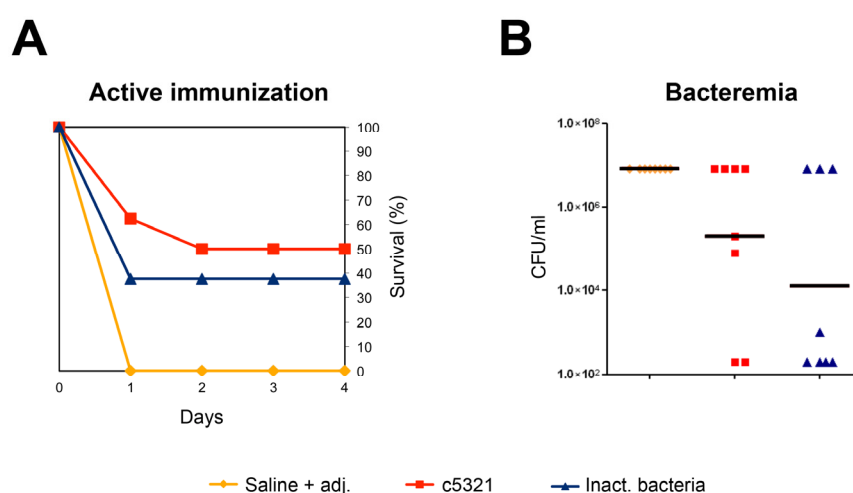
Figure 4-1: Protection induced by antigen c5321 in the sepsis mouse model. (*A*) Mice (10 per group) were actively immunized with recombinant c5321, heat-inactivated CFT073 and saline solution with Freund's adjuvant. Following challenge with the strain CFT073, survival of mice was monitored for 4 days. (*B*) Bacterial counts in the blood were determined at 20 hours by counting the colony forming units (CFUs) in the blood detected by 10-fold serial dilutions and plating method. Bacteremia mean values in each group were determined assuming that mice dead before 20 hours had the maximum level of CFU causing mouse death. A representative experiment is shown.

4.2. c5321 is recognized by human sera from patients affected by UTIs

To define the relevance of c5321 during *E. coli* infections, we collected a panel of 22 human sera from patients suffering from *E. coli* UTIs and bleeded at hospital entrance. In order to evaluate the *in vivo* immunogenicity of c5321 and to confirm its expression during key events in pathogenesis, serum IgG titers against the antigen were measured by ELISA. As shown in Figure 4-2*A*, IgG titers against c5321 were significantly higher compared to those found in a panel of healthy human donors, suggesting that the antigen is immunogenic and specifically expressed *in vivo* during *E. coli* infections.

To better define the kinetic of c5321 expression during bacterial growth phases, the levels of c5321 transcript in the UPEC strain CFT073 were evaluated by real-time quantitative RT-PCR (Figure 4-2*B*). A time course analysis of bacteria grown in M9CA medium revealed that the levels of c5321 mRNA remain constant up to 3 hours of growth (exponential phase) and decline during stationary phase (from 4 to 7 hours). These data not only confirm c5321 expression *in vitro*, but also postulate a growth phase-dependent mechanism of regulation.
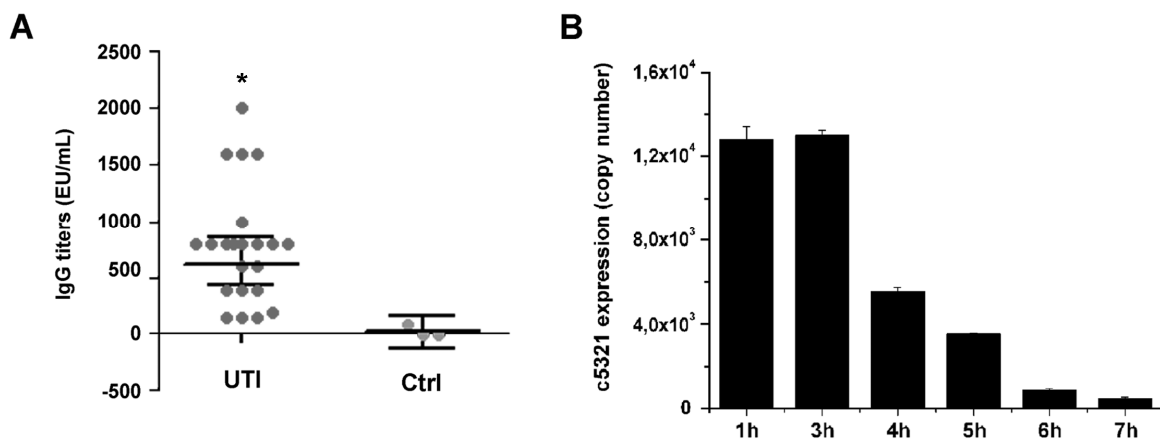

Figure 4-2: Human sera from UTI patients recognize antigen c5321. (*A*) Immunoreactivity of human sera from UTI patients towards recombinant c5321. ELISA was performed by coating the recombinant antigen on microtiter plates and overlaying wells with specific human sera. IgG binding was revealed by alkaline phosphatase-conjugated goat anti-human IgG. Sera from healthy human volunteers were used as IgG control levels. The data represent the mean ± SD of 22 human sera. Titers are reported as serum dilutions. *Comparison of c5321 IgG titers between healthy human volunteers and UTI patients: p≤0.05 (paired Student's t-test). (*B*) *In vitro* kinetic of c5321 expression. c5321 transcript levels in the UPEC strain CFT073 grown in minimal medium were evaluated by real-time quantitative RT-PCR at different time points ranging from 1 to 7 hours. The graph represents a typical experiment out of three performed with similar results.

4.3. Ions play a role in the regulation of c5321 expression

Human niches colonized by *E. coli* vary in ion content and this may affect the adaptation of the bacterium to its host. Indeed, ions are fundamental to bacterial physiology, as they can modulate both metabolism and virulence. To evaluate c5321 expression in physiologically relevant conditions, such as those mimicking the environment encountered *in vivo* by ExPEC during infection, the transcript levels in whole human blood and urine were measured by real-time quantitative RT-PCR. As shown in Figure 4-3, the levels of c5321 mRNA significantly decreased compared to those measured in M9CA medium, suggesting that in iron-limiting environments, such as blood and urine, the antigen expression may be down-regulated. To evaluate the potential contribution of iron to the regulation of c5321 expression, we analyzed the antigen mRNA levels in the strain CFT073 grown up to exponential phase in the presence of 2,2'-Dipyridyl and Desferal, both strong iron chelators. To assess the role of other ions, the transcript levels were measured also during bacterial growth in the presence of EDTA, a well known calcium and magnesium chelator. We observed that both 2,2'-Dipyridyl and Desferal significantly reduced ($p \leq 0.05$) the levels of c5321 mRNA compared to the reference medium alone. The presence of EDTA also significantly affected c5321 expression, although at a lower extent (Figure 4-3). No differences in transcript levels were observed in any condition tested when bacteria were grown up to stationary phase (data not shown). These data suggest that ions may play a role in *E. coli* physiology by regulating c5321 expression.
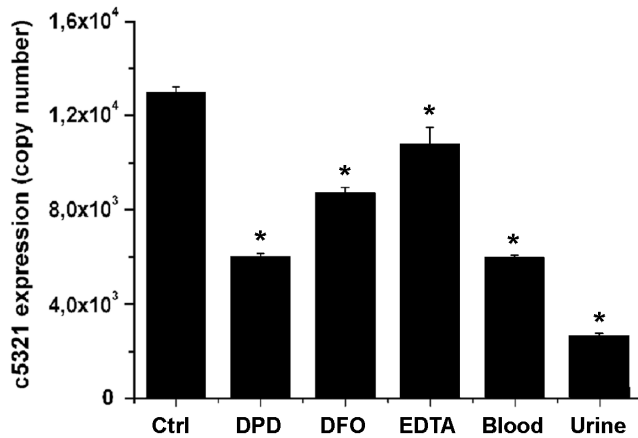
4.4. c5321 belongs to the family of SLR proteins

Since no functional information was available in the literature on c5321, which was just annotated as hypothetical protein, we tried to get some clues from the protein structure. By using the PHYRE bioinformatic tool, we predicted that c5321 three-dimensional structure was composed of SLRs stacked on top of each other forming an α/α-superhelix. This prediction was, then, confirmed by solving the antigen crystal structure, which revealed that c5321 is composed of twelve SLRs (residues 1 to 428), followed by a C-terminal helix (residues 428 to 456) and a tail (residues 456 to 464) (Figure 4-4). The antigen also showed a high structural homology with a protein-protein recognition domain of human O-linked N-acetylglucosamine transferase and with Helicobacter cysteine-rich protein C (HcpC) (Figure 4-5).


```
REPEAT_1    -NVNLEQLKQKAESGEAKAQLELGYRYFQ-GNETTKD-
REPEAT_7    -TQSRVLFSQSAEQGNSIAQFRLGYILEQ-GLAGAKEP
REPEAT_4    -AESVKWFRLAAEQGRDSGQQSMGDAYFE-GDGVTRDY
REPEAT_6    -AISAQWYRKSATSGDELGQLHLADMYYF-GIGVTQDY
REPEAT_3    -AQAVIWYKKAALKCLPQAQQNLGVMYIIE-CNGVKVDK
REPEAT_10   -KKAVEWFRKAAAKGEKAAQFNLGNALLQ-GKGVKKDE
REPEAT_2    LTQAMDWFRRAAEQGYTPAEYVLGLRYMN-GEGVPQDY
REPEAT_11   -QQAAIWMRKAAEQGLSAAQVQLGEIYYY-GLGVERDY
REPEAT_5    -VMAREWYSKAAEQGNVWSCNQLGYMYSR-CLGVERND
REPEAT_8    -LKALEWYRKSAEQGNSDGQYYLAHLYDK-GAEGVAKN
REPEAT_9    REQAISWYTKSAEQGDATAQANLGAIYFRLGSE--EEH
REPEAT_12   -VQAWAWFDTASTNDMN----LFQTENRNITEKKLTA-
```

Figure 4-4: SLRs of c5321. The protein is composed of 12 SLRs (residues 1 to 428). The repeats generally follow the SLR consensus sequence, except repeat 12, which adopts the fold but deviates from the consensus sequence. Some of the matching positions with HcpC are colored in cyan. A pronounced presence of tryptophan residues and large hydrophobic residues in general (in green) is observed. Some of the other locations of hydrophobic residues within the consensus sequence are represented in orange. Positively and negatively charged residues are indicated in blue and red, respectively.
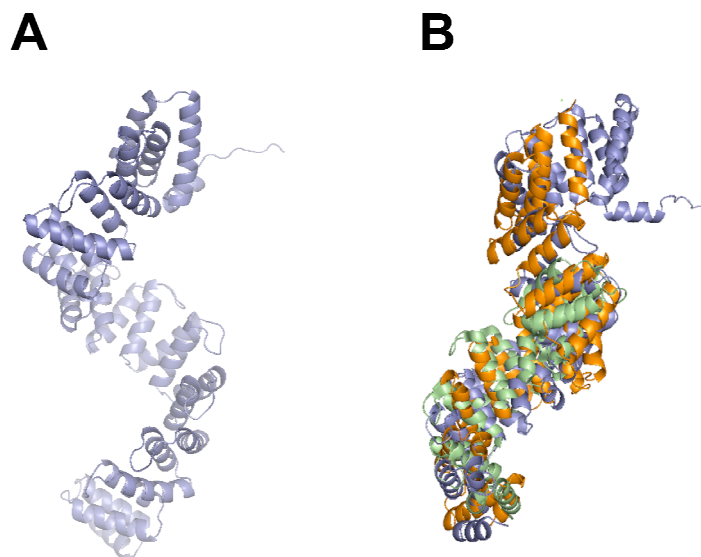
Figure 4-5: Crystal structure of c5321 and structural homologies. (*A*) Crystal structure of c5321. The protein is composed of 12 SLRs (residues 1 to 428), followed by a C-terminal helix (residues 428 to 456) and a tail (residues 456 to 464). Structure was solved by SAD. Data were integrated and scaled in iMosflm. Experimental phasing was performed with Phenix (AutoSol) and followed by automated model building in Phenix (AutoBuild) and CCP4 (Buccaneer), and by refinement in Phenix (phenix.refine) and CCP4 (Refmac5). (*B*) Superposition of c5321 (residues 2 to 464, shown in blue) on 1W3B (protein-protein recognition domain of human O-linked N-acetylglucosamine transferase, shown in orange) and 1OUV (HcpC, shown in green). High structural homology is observed. Packing angles for helices are different among the structures, as well as the length of the loops connecting the repeats.

An interaction between the C-terminal tail of one molecule with the concave region of SLRs 8, 9 and 10 of a second molecule was observed in the crystal structure of c5321. This could potentially be reflective of an oligomerization mode or a binding mode to partner proteins. The HcpC crystal structure also revealed a prominent contact between the C-terminal coil and the concave surface of the symmetry-related superhelix, which could be important either for HcpC recognition of proteins with similar C-terminal tails, or for the auto-recognition and formation of oligomers. Indeed, the aggregation of HcpC was shown to occur and to be strongly dependent on pH and ionic strength [115].

Furthermore, Mg^{2+} ions were present at relatively high concentrations (200 mM) in the crystallization conditions and c5321 SLRs 3 and 4 were found to participate in octahedral coordination of the ion. These data suggest that ions, in addition to playing a physiological role by regulating antigen expression, may also have a structural relevance.

The determination of c5321 structure, however, did not provide any evident clue to infer the protein function. Indeed, although SLR proteins share similar α-helical conformations, they are characterized by an extremely low sequence identity, which complicates the identification of functional homologues, and also by the propensity to participate in a broad spectrum of biological processes. Even the structural homology with HcpC could not suggest any functional role for c5321, as the *Helicobacter* antigen belongs to a family of proteins that lacks a detailed functional characterization [115]. However, considering that several bacterial SLR proteins are reported to be involved in host-pathogen interactions, immunomodulatory functions and immune evasion strategies [90], we decided to investigate potential interactions of c5321 with host immune system components.

4.5. c5321 is able to bind human complement components

The complement system represents a crucial component of the innate immune defense against infections. Therefore, the ability of avoiding or preventing killing by complement is an important determinant of microbial pathogenicity. In order to inhibit classical and lectin pathways of complement activation many microbes have evolved the ability to bind to host C4b-binding protein (C4BP), whereas protection from alternative pathway is mainly provided by factor H (FH) capturing. Another bacterial complement evasion strategy is to capture and inactivate C3, the central molecule of all complement pathways [116]. For example, M proteins of *S. pyogenes* are able to bind C4BP and FH, contributing to the capacity of the bacterium to evade phagocytosis [117, 118]. Another FH-binding protein was identified in *N. meningitidis*, the meningococcal vaccine antigen GNA1870 [119]. Instead, the extracellular fibrinogen-binding protein (Efb) from *S.

aureus can bind the C3d domain of the C3 molecule, thereby inhibiting C3b deposition on the surface of *S. aureus* [120].

By ELISA, we showed that c5321 is not able to interact with C4BP and FH (data not shown). However, we demonstrated that the antigen can bind to bacterial surfaces in a complement-dependent manner. Indeed, when *S. aureus*, which served just as surface that allows complement deposition, was incubated with recombinant c5321 and NHS, the antigen could be detected after bacterial washing, whereas no protein binding occurred in the presence of heat-inactivated NHS, suggesting that c5321 is able to interact with active complement components deposited on the surface of bacteria (Figure 4-6).
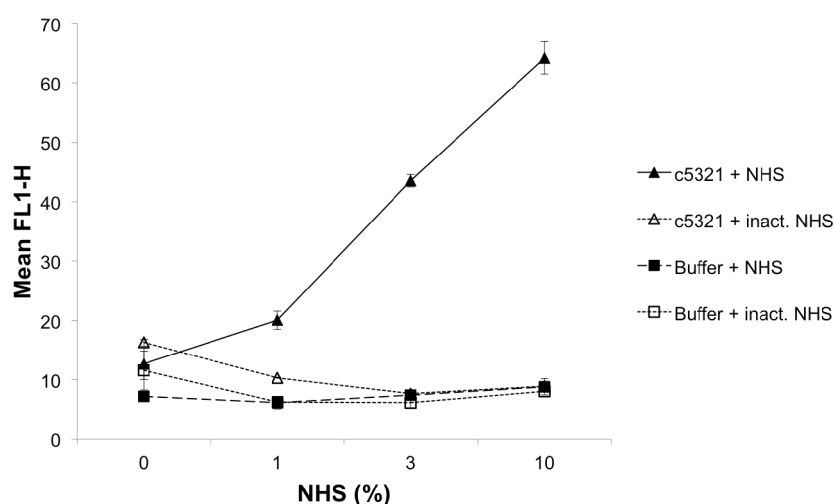

Figure 4-6: Complement-dependent binding assay. *S. aureus* strain KV27 was incubated with NHS and 20 μg/ml recombinant c5321 for 30 minutes at 37°C. Heat-inactivated (56°C for 30 minutes) NHS, as well as buffer alone, were used as control. After washing, recombinant c5321 was detected using mouse anti-His-tag monoclonal antibodies and FITC-conjugated goat anti-mouse IgG. Fluorescence of 10,000 bacteria was measured by flow cytometry. The graph shows that c5321 is able to bind to *S. aureus* only when active complement components are deposited on the bacterial surface. Results represent mean values ± SEM of two independent experiments.

In order to identify the specific components interacting with c5321, we performed the same assay using complement-depleted sera instead of NHS. We found that c5321 binding to *S. aureus* surface decreased with ΔC3, ΔC5, ΔC6 and ΔC7 sera, and it was restored when the respective complement components were added to the depleted sera,

suggesting that c5321 is able to interact with C3, C5, C6 and C7 (Figure 4-7). On the

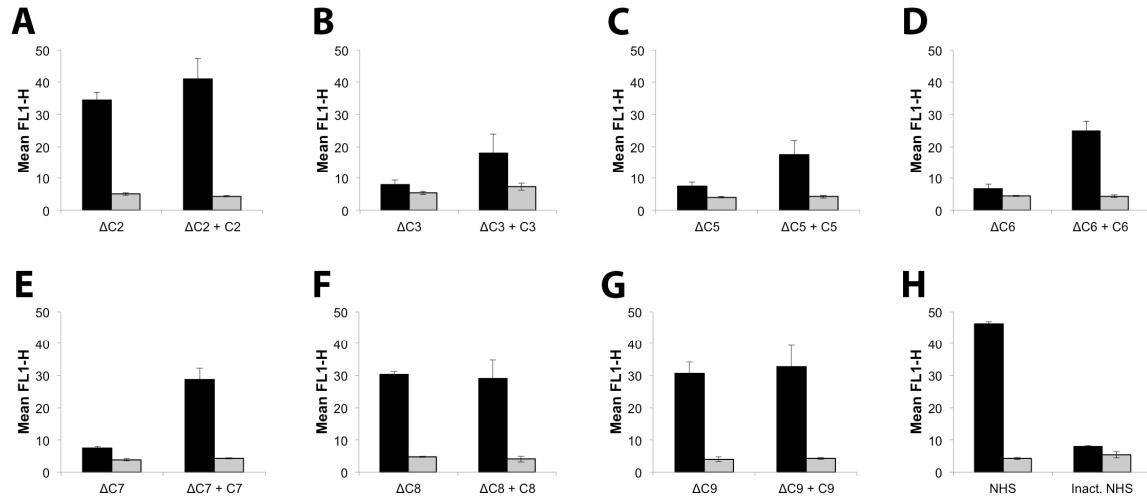contrary, antigen binding to bacterial surface was not dependent on C2, C8 or C9.

Figure 4-7: Complement-dependent binding assay with depleted sera. *S. aureus* strain KV27 was incubated with 10% depleted serum and 20 µg/ml recombinant c5321 for 30 minutes at 37°C. NHS and heat-inactivated NHS were used as controls (panel *H*). After washing, recombinant c5321 was detected using mouse anti-His-tag monoclonal antibodies and FITC-conjugated goat anti-mouse IgG. Fluorescence of 10,000 bacteria was measured by flow cytometry. Binding of c5321 (black bars) was compared to that of buffer alone (grey bars). The graphs show that c5321 binding to *S. aureus* decreased with ΔC3, ΔC5, ΔC6 and ΔC7 sera, and it was restored when the respective complement components were added to the depleted sera (panels *B, C, D* and *E*, respectively), suggesting that c5321 is able to interact with C3, C5, C6 and C7. On the contrary, antigen binding to bacterial surface was not dependent on C2, C8 or C9 (panels *A, F* or *G*, respectively). Results represent mean values ± SEM of two independent experiments.

4.6.   No biological relevance has been found for complement binding

To analyze whether c5321 binding to complement components could result in the

blocking of the complement cascade, we tested c5321 activity in a well-described

complement ELISA, where the different complement pathways and activation steps can

be assessed separately [112, 113]. In particular, the activation of the CP, LP and AP can

be measured by incubating human serum in microtiter plates coated with IgM, mannan

and LPS, respectively. We specifically evaluated the effect of c5321 on the CP, LP and

AP, at the level of both C3b and C5b-9 deposition. We found that the antigen is not able

to block any of the complement pathways at the level of C3b deposition. We observed

only a slight decrease in C5b-9 formation via the CP, although C5b-9 deposition was not affected by c5321 in the other pathways (Figure 4-8).

To further assess the effect of c5321 on complement activation in human serum, we tested the antigen in CP- and AP-mediated hemolytic assays, in which membrane attack complex (MAC)-dependent killing of sheep and rabbit erythrocytes, respectively, is used as readout for complement activity. However, no hemolysis inhibition was observed in the presence of c5321 (Figure 4-9).

We also demonstrated that c5321 does not block C3b and C5b-9 deposition on bacteria and does not inhibit complement-mediated opsonophagocytosis (data not shown).

Considering all these data, no evidence was found to support a biological significance of the interaction between c5321 and complement components.
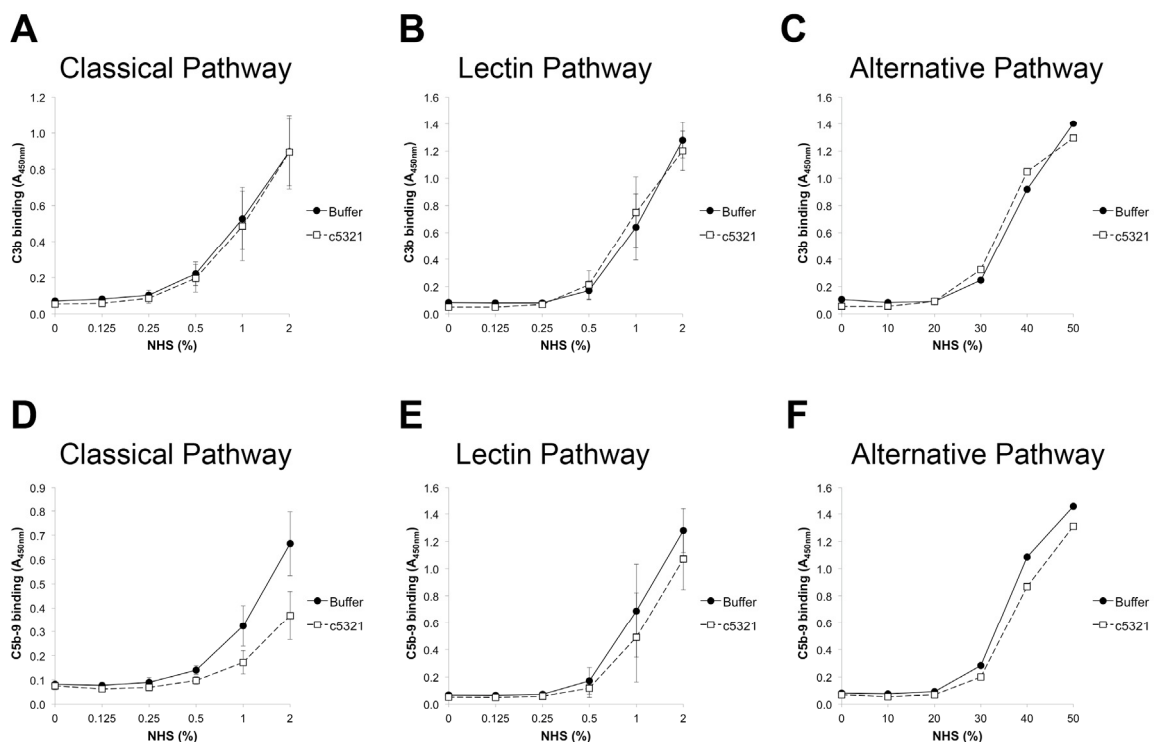

Figure 4-8: Complement ELISAs. Human serum was incubated with recombinant c5321, or buffer alone, and complement activation via the CP, LP and AP was determined by ELISA. c5321 is not able to prevent C3b deposition via the CP (panel *A*), LP (panel *B*), or AP (panel *C*). C5b-9 formation was not inhibited in the LP (panel *E*) and AP (panel *F*), whereas it showed a slight reduction in the CP (panel *D*). For the CP and LP, the results represent mean values ± SEM of three independent experiments. For the AP only a single experiment is shown.
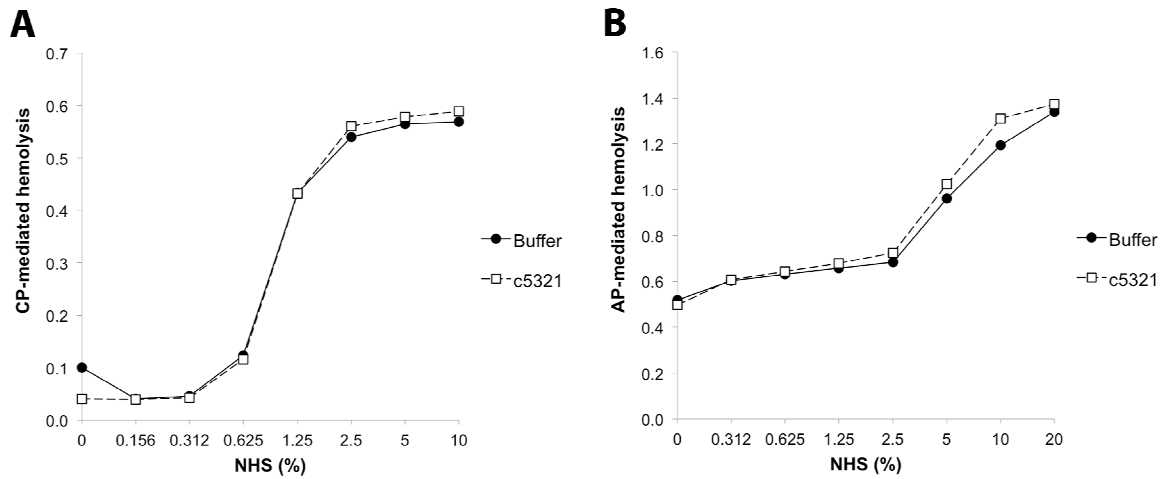
Figure 4-9: Hemolysis assays. NHS was pre-incubated with c5321 for 15 minutes at 37°C. Treated serum was mixed with opsonized sheep erythrocytes or rabbit erythrocytes and incubated for 1 hour at 37°C. Erythrocytes were pelleted and the absorbance of the supernatants at 405 nm was measured. The graphs show that c5321 is not able to inhibit CP-mediated (panel *A*) and AP-mediated (panel *B*) hemolysis.

4.7. c5321 binds specifically to human IgA

Since we could not find a functional role for complement binding, we decided to screen other soluble components of the human immune system, such as immunoglobulins, for their ability to interact with c5321. By ELISA we observed a dose-dependent binding of the antigen to both human serum and secretory IgA, and we estimated that the dissociation constant (K_d) is approximately 10^{-7} M for both interactions (Figure 4-10).
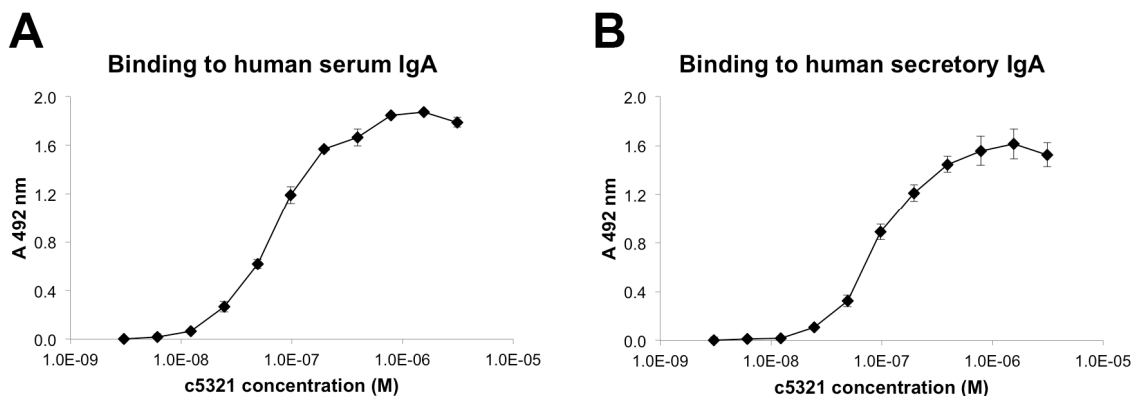

Figure 4-10: ELISA showing c5321 binding to human IgA. Wells of ELISA plates were coated with IgA and incubated with a dilution series of c5321. Binding of c5321 was detected using mouse anti-c5321 monoclonal antibodies and PO-conjugated rabbit anti-mouse IgG. ELISA data show that c5321 is able to bind to both human serum IgA (*A*) and human secretory IgA (*B*) with an estimated dissociation constant of approximately 10^{-7} M. The graphs represent a typical experiment out of three performed with similar results.

ELISA results were confirmed also by Biacore analysis. Indeed, to verify and compare c5321 binding to human serum and secretory IgA, both protein and antibodies were immobilized on a CM-5 sensor chip by amine coupling and interactions were monitored by SPR. Although it was not possible to detect any binding between immobilized c5321 and IgA in solution injected over the surface, a dose-dependent interaction was observed when both IgA forms were bound to the sensor chip and c5321 was injected at increasing concentrations, ranging from 1 nM to 1 μM (Figure 4-11).
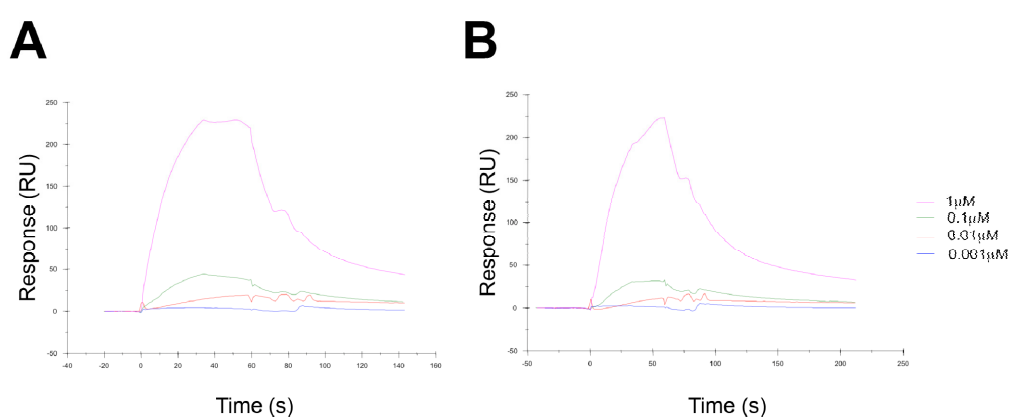
Figure 4-11: SPR experiments showing c5321 binding to human IgA. Human serum IgA (*A*) and secretory IgA (*B*) were immobilized on a CM-5 sensor chip by amine coupling and c5321 was injected over the surfaces at increasing concentrations. The graphs show that c5321 is able to bind both IgA forms in a dose-dependent manner.

The injection of increasing concentrations of c5321 over the immobilized protein also showed a dose-dependent response, confirming the ability of the protein to interact with itself, as previously demonstrated by the crystal structure (Figure 4-12).
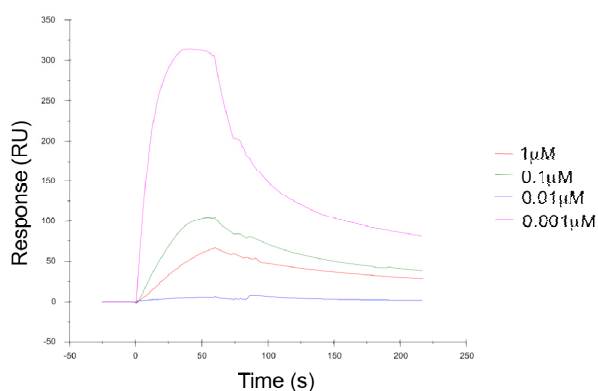
Figure 4-12: SPR experiment showing the interaction of c5321 with itself. Increasing concentrations of c5321, ranging from 1 nM to 1 μM, were injected over the immobilized protein. The graph shows that c5321 is able to bind to itself in a dose-dependent manner.

4.8. c5321 is able to inhibit IgA effector functions

Serum and secretory IgA interact with different host receptors, including FcαRI, also known as CD89, which is a key mediator of IgA effector functions. IgA molecules clustered on the surface of a pathogenic target can trigger various elimination processes through engagement of FcαRI present on neutrophils, monocytes, eosinophils, some macrophages and dendritic cells. These mechanisms include phagocytosis, ADCC, release of cytokines, chemotactic factors and inflammatory lipid mediators, degranulation, and production of reactive oxygen species (ROS) [96].

To study the significance of c5321 interaction with IgA, we investigated respiratory burst activity of human neutrophils by chemiluminescence. Both serum and secretory IgA triggered ROS production, although serum IgA-induced oxidative burst was significantly higher. We found that c5321 clearly inhibited the neutrophil respiratory burst mediated by both IgA forms. In particular, 20 μg/ml of c5321 were sufficient to induce a 2-fold decrease and a 6- to 10- fold decrease in the oxygen radical production triggered, respectively, by serum and secretory IgA (Figure 4-13).

Since cross-linking of FcαRI by IgA leads also to the release of leukotriene B4 (LTB4) [95, 121], which is a potent neutrophil chemoattractant, we tested the ability of c5321 to inhibit PMN chemotaxis. We demonstrated that serum and secretory IgA were equally capable of inducing neutrophil migration and that c5321 caused approximately 65% and 27% inhibition of serum IgA- and secretory IgA-induced neutrophil chemotaxis, respectively, suggesting that, in addition to blocking ROS production, c5321 could also interfere with neutrophil recruitment to the infection site (Figure 4-14).
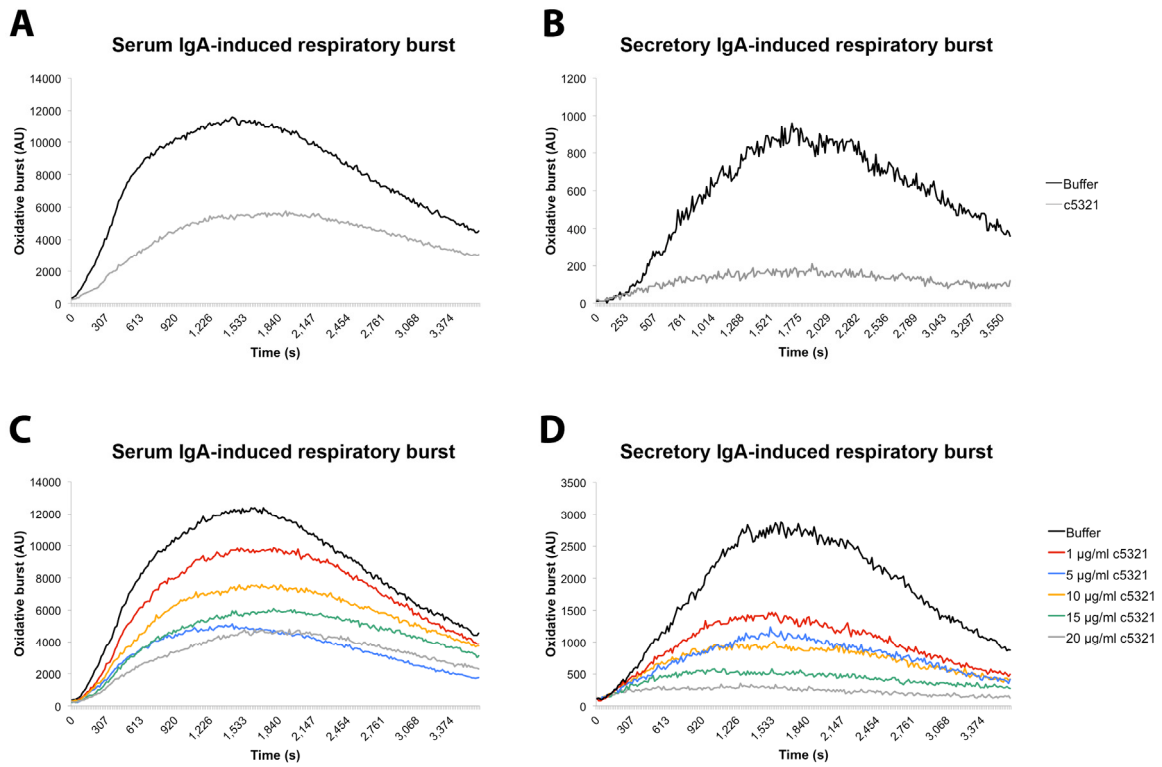
Figure 4-13: c5321 is able to inhibit IgA-induced neutrophil respiratory burst. ROS production by PMNs was measured by chemiluminescence every 30 seconds for 60 minutes at 37°C. The graphs show that c5321 (at a concentration of 20 μg/ml) inhibits both serum IgA-induced and secretory IgA-induced oxidative burst (*A* and *B*, respectively). The inhibitory effect of c5321 is dose-dependent (*C* and *D*). The graphs represent a typical experiment out of three performed with similar results.
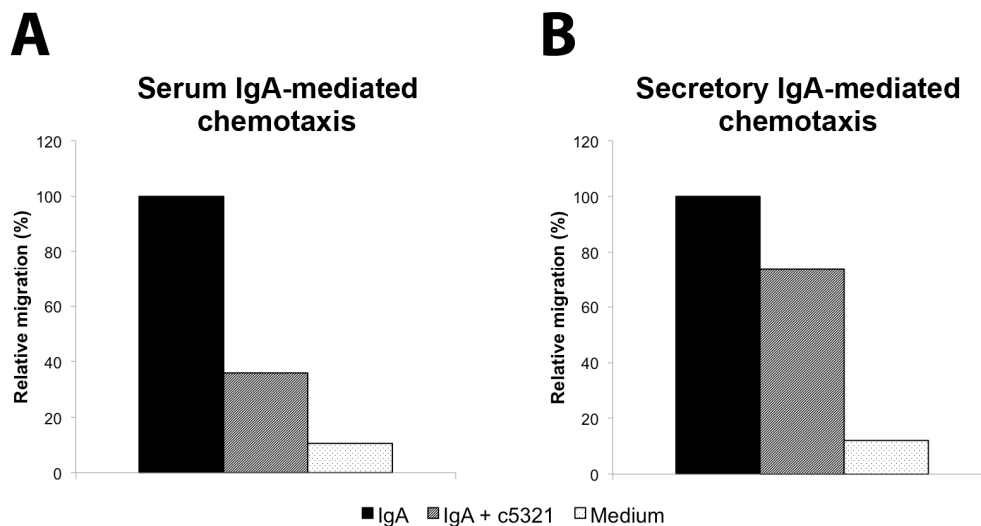

Figure 4-14: c5321 is able to inhibit IgA-induced neutrophil chemotaxis. To measure neutrophil chemotaxis, bottom chambers of Transwell supports were filled with supernatants deriving from PMNs incubated with IgA or c5321-treated IgA. Neutrophils were added to the upper chambers. After 1 hour at 37°C, cells that had migrated towards the lower compartments were quantified by flow cytometry. The graphs show that c5321 is able to cause a 65% inhibition of serum IgA-induced migration (*A*) and a 27% inhibition of secretory IgA-induced migration (*B*). The graphs represent a typical experiment out of two performed with similar results.

Although most of bacterial IgA-BPs can block IgA-mediated effector functions by competitive inhibition of FcαRI, we observed that, in the presence of c5321, IgA molecules were still able to bind to neutrophils (data not shown). Therefore, also considering that c5321 can not bind PMNs (data not shown), we hypothesized that the interaction of c5321 with IgA, albeit not establishing a competitive binding mechanism for FcαRI inhibition, could interfere with a productive FcαRI signaling. Indeed, effector functions of IgA are controlled by FcαRI signal-transduction pathways, which rely on the recruitment of multiple classes of signaling molecules, such as tyrosine kinases, phosphoinositide kinases and serine-threonine kinases [122].

To investigate whether c5321 could interfere with IgA-mediated FcαRI signaling, we analyzed the general tyrosine phosphoprotein pattern in neutrophils stimulated with IgA and we observed that serum and secretory IgA were equally capable of activating PMNs. However, pre-incubation of secretory IgA, but not serum IgA, with c5321 resulted in dose-dependent modifications in the tyrosine phosphorylation level of specific cytosolic proteins, including PLCγ (Figure 4-15). This suggests that c5321 binding to secretory IgA could actually hamper a productive FcαRI signaling, leading to inhibition of neutrophil activation. However, this mechanism can not explain the ability of c5321 to also block the effector functions of serum IgA.
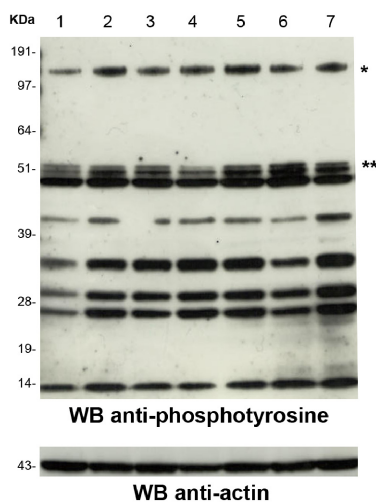

Figure 4-15: c5321 is able to interfere with FcαRI signaling. Neutrophil activation was performed by incubating cells with IgA or c5321-treated IgA for 1 minute at 37°C. Cells were lysed in 1% Triton X-100 in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, in the presence of a protease inhibitor cocktail. Equal amounts of proteins from each sample were resolved by 10% SDS-PAGE and transferred to 0.45 μm nitrocellulose filters. Immunoblots were carried out using anti-phosphotyrosine antibody and PO-conjugated rabbit anti-mouse antibody. Blots were reprobed with loading control antibody after stripping (anti-actin). The blot shows the general tyrosine phosphoprotein pattern in PMNs stimulated with: medium (lane 1); SIgA (lane 2); SIgA pre-incubated with 10 μg/ml c5321 (lane 3); SIgA pre-incubated with 30 μg/ml c5321 (lane 4); serum IgA (lane 5); serum IgA pre-incubated with 10 μg/ml c5321 (lane 6); serum IgA pre-incubated with 30 μg/ml c5321 (lane 7). The interaction of c5321 with SIgA resulted in dose-dependent modifications in the tyrosine phosphorylation level of specific cytosolic proteins, including PLCγ (*) and others not yet identified (**). PLCγ identity was confirmed by reprobing blot with anti-PLCγ1 antibody after stripping (data not shown). The blot represents a typical experiment out of three performed with similar results.

5. DISCUSSION

The absence of a safe and broadly protective vaccine against ExPEC is a major problem for modern society because some of the diseases caused by these bacteria, such as UTIs, are associated with high healthcare costs and others, such as sepsis, are associated with high infant mortality [6]. In addition, extraintestinal *E. coli* infections are significantly increasing in susceptible subpopulations, such as patients with diabetes, multiple sclerosis, HIV/AIDS, catheters and spinal cord injuries, pregnant women, children and the elderly [5]. Considering also the increasing antibiotic resistance and the number of recurrent infections, the prevention of the diseases caused by ExPEC is of pressing concern from both the public health and economic perspectives [7].

Since attempts to develop an efficacious ExPEC vaccine by conventional approaches had failed, we took advantage of the growing genomic information available on *E. coli* pathotypes to identify broadly protective antigens against extraintestinal *E. coli* infections. Indeed, we determined the sequence of a neonatal meningitis-associated K1 strain and we compared it to the available genomes of ExPEC and nonpathogenic strains. By using a subtractive reverse vaccinology approach, we identified nine potential vaccine candidates able to induce protective antibodies against a homologous strain in a mouse model of sepsis [82].

One of the most interesting antigens identified was c5321, which was able to induce a significant protection by active immunization of mice and challenge with the uropathogenic strain CFT073. The *in vivo* immunogenicity of the antigen was confirmed also during *E. coli* infection of the human urinary tract, as hospitalized patients suffering from UTIs were found to possess IgG titers against c5321 significantly higher compared to those of healthy individuals.

Besides being specifically expressed *in vivo* during *E. coli* infections, c5321 was also shown to be expressed *in vitro* with a growth phase-dependent mechanism, as transcript levels were higher in exponential phase and declined during stationary phase. In addition, we observed that in iron-limiting conditions, as well as in the presence of calcium and magnesium chelators, c5321 mRNA levels significantly decreased, suggesting that antigen expression could be affected by ion concentration. Considering that human niches colonized by *E. coli* vary in ion content, which can modulate both bacterial metabolism and virulence, we hypothesized that ions may play a role in *E. coli* physiology by regulating c5321 expression.

Since no functional annotations could be assigned to c5321 based on gene homology, we tried to get some clues by solving the crystal structure of the protein and we found that it is composed of twelve SLRs stacked on top of each other forming an α/α-superhelix, which showed a high structural homology with a protein-protein recognition domain of human O-linked N-acetylglucosamine transferase and with Helicobacter cysteine-rich protein C. The C-terminal tail of one molecule of c5321 was found to interact with the concave region of SLRs 8, 9 and 10 of a second molecule, suggesting a possible oligomerization mode of the protein. Interestingly, SLRs 3 and 4 were shown to participate in octahedral coordination of magnesium, suggesting that ions, in addition to playing a physiological role, may also have a structural relevance.

Considering that bacterial proteins belonging to the SLR family are frequently involved in immune evasion strategies and immunomodulatory functions, we investigated potential interactions of c5321 with different components of the human immune system. Since the ability of avoiding or preventing killing by complement is an important determinant of microbial pathogenicity, we tested c5321 capability to bind complement components and we observed a positive interaction with C3, C5, C6 and C7. However,

considering that this binding did not result in the blocking of the complement cascade, it was not possible to find any evidence supporting a biological relevance for this kind of interaction.

On the contrary, we demonstrated that c5321 was also able to specifically bind human IgA and that this interaction resulted in the inhibition of the IgA effector functions mediated by the neutrophil receptor FcαRI. Indeed, by ELISA and SPR, we observed a dose-dependent binding of c5321 to both human serum and secretory IgA, with an estimated K_d of approximately 10^{-7} M. Most strikingly, this interaction was found to be able to hamper the neutrophil respiratory burst activity and chemotaxis, which are both inflammatory processes triggered upon cross-linking of FcαRI by IgA. Although c5321, unlike most of bacterial IgA-BPs, did not seem to block IgA-mediated effector functions by competitive inhibition of the neutrophil receptor, we showed that c5321 binding to secretory IgA, but not serum IgA, was able to interfere with the FcαRI signal-transduction pathway, leading to modifications in the tyrosine phosphorylation level of neutrophil cytosolic proteins.

These results suggest that c5321 is a novel *E. coli* IgA-BP with specific immunomodulatory properties that allow the bacterium to avoid clearance by inhibiting the neutrophil oxidative burst, as well as the recruitment of immune cells to the infection site. Therefore, the data reported in this thesis not only propose a novel protective vaccine candidate against ExPEC, but also open new perspectives on the defense mechanisms developed by pathogenic *E. coli* in order to survive in the host.

6. CONCLUSIONS

Extraintestinal 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 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 ExPEC-induced diseases could have a significant clinical and economical impact. Although many efforts have already been done to develop a successful immunization strategy, a highly immunogenic, broad protective and safe vaccine against ExPEC 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 ExPEC and nonpathogenic strains, in order to identify protective and broadly conserved vaccine antigens against extraintestinal *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 ExPEC pathogenesis.

In this study, the functional characterization of protective antigen c5321 has been discussed. The vaccine candidate has been shown to be immunogenic not only in the sepsis mouse model, but also during human UTIs, suggesting its relevance in *E. coli* pathogenesis. Antigen c5321, which can be classified as a SLR protein, has revealed a specific capability to interact with human IgA, leading to the inhibition of the IgA effector

functions mediated by the neutrophil receptor FcαRI, such as PMN respiratory burst activity and chemotaxis. Although c5321, unlike most of bacterial IgA-BPs, does not compete with the antibody for receptor binding, it has been demonstrated that, upon interaction with IgA, this *E. coli* antigen is able to impair neutrophil activation by blocking the FcαRI signal-transduction pathway.

Further studies will be needed to elucidate the protein's mechanism of action; however, these data suggest that c5321 is a novel IgA-BP contributing to ExPEC evasion of the host immune system.

7.   ACNOWLEDGMENTS

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