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Immunotargeting of *E.coli* TolC outer membrane channel

Cover image: *E.coli* TolC channel (bottom left) in the proximity of a Fab (top right) through the extracellular region. Also the extracellular-view of the close (upper left) and open (bottom right) state of the channel.

Acknowledgments

I would like to show my deepest appreciation to:

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Abstract

TolC is an outer-membrane channel of Gram-negative bacteria, responsible for extrusion of antibiotics and other toxic compounds from the cell and plays a determinant role in bacterial resistance.

Together with two other proteins, an inner membrane efflux pump and a periplasmic fusion protein, TolC forms a complex efflux system. Interestingly the specificity of this complex is defined by the type of efflux pump to which TolC binds to, thus making TolC a common and critical component in a wide range of efflux systems.

This channel, composed by 428 residues, creates a duct in the outer-membrane given its homotrimeric conformation. Structurally, it is composed by a α-helix motif in the periplasmic side and a β-barrel inserted in the outer-membrane. This later harbors an extracellular loop that is a central feature for the purpose of this work.

The main goal of this work was to generate antibodies against *E.coli* TolC channel with the intention of blocking or modulate its extrusion activity. In order to achieve this, three recombinant forms of *E.coli* TolC, two full-length forms and a linear epitope one, were produced and characterized by SDS-PAGE, size exclusion chromatography (SEC), western blot and differential scanning calorimetry (DSC). These results revealed the purity, robustness and stability of the produced proteins, ideal for the immunizations protocols. Produced proteins where then used to immunize experimental model birds, namely quails (*Coturnix japonica*), making using of the unique setup of CNC's Avian Facility.

Antibodies produced were then purified from the egg yolk of hyperimmune birds and biochemical characterized by ELISA and western blot in terms of their target-specificity and reactivity. These antibodies presented good reactivity against both recombinant TolC proteins, specially recognizing the extracellular region of TolC channel.

This work confirmed that the *E.coli* TolC is suitable for immunotargeting using avian antibodies, particularly the extracellular region of the channel. We believe that our results are a good starting point for subsequent characterization of the anti-TolC antibodies through functional efflux assays. Ultimately this will allow the development of therapeutic monoclonal anti-TolC antibodies to be used to fight bacterial resistance.

Table of Contents

Abbreviations

ABC: ATP binding cassette

AMR: Antimicrobial resistance

ATP: Adenosine triphosphate

CNBr: Cyanogen Bromide

CNC: Center for Neuroscience and Cell Biology

DDM: n-Dodecyl-β-D-Maltopyranoside

DNA: Deoxyribonucleic acid

DSC: Differential scanning calorimetry

ELISA: Enzyme-Linked Immunosorbent Assay

EMBL: European Molecular Biology Laboratory

FCA: Freund's Complete Adjuvant

FIA: Incomplete Freund s adjuvant

IBMC/I3S: Instituto de Biologia Molecular e Celular / Instituto de Investigação e Inovação em Saúde

IgG: Immunoglobulin G

IgY: Immunoglobulin Y

IM: intramuscular

IMAC: immobilized ion affinity chromatography

IPTG: Isopropyl β-D-1-thiogalactopyranoside

MATE: Multiple Antibiotic and Toxin Extrusion

MDR: multi drug resistance

MF: major facilitator

MW: Molecular weight

NTP: Nucleoside triphosphate

OM: Outer-membrane

ON: overnight

PAGE: Polyacrylamide gel electrophoresis

PBPs: Penicillin binding proteins

PBS: Phosphate Buffered Saline

PCR: Polymerase chain reaction

PDB: Protein Data Bank

PEG: Polyethylene glycol

PHYRE: Protein Homology/analogy Recognition Engine V 2.0

PNK: T4 Polynucleotide Kinase

RND: resistance-nodulation-division

RT: Room temperature

SEC: size exclusion chromatography

SLIC: Sequence and Ligation Independent Cloning

SMR: small multi drug resistance

TMB: 3,3′,5,5′-Tetramethylbenzidine

TRP: Transient receptor potential

WSPF: water soluble protein fraction

Introduction

The emerging problem of Antimicrobial Resistance (AMR)

Antimicrobial resistance (AMR) is a major challenge to global public health, significantly threating the effective prevention and treatment of an increasing number of infections caused by bacteria, parasites, fungi, and viruses. Every year antimicrobial resistance (AMR) causes over 25 000 deaths in Europe and has a total estimated impact of €1.5 billion due to loss of productivity and increase in healthcare costs (source European Centre for Disease prevention and Control - ECDC) [1][2]. In the US, the impact is currently estimated in 23 000 deaths/year and $55-70 billion (source U.S. Centers for Disease Control and Prevention - CDC). A Welcome Trust review on AMR suggests that the continued rise in antimicrobial resistance by 2050 would lead to 10 million people dying every year, a number that would overcome the ones estimated for major causes of death such as cancer and diabetes [3]. A number of causes responsible for the increase of the AMR phenomenon are currently identified and include 1) the extensive and inappropriate use of antimicrobial drugs in human and veterinary medicine as well as in agro-industry, 2) the effective environmental spread of resistant microbes by food, water systems, trade or travel and importantly 3) the insufficient infection prevention measures together with 4) the lack of new effective antimicrobials or alternative therapies [4].

The evolution of AMR at this alarming rate urges the implementation of different action plans, with one of the main efforts being the development of new antimicrobial drugs.

Antibiotic-resistant Bacteria

Antibiotic-resistant infectious are mediated both by Gram-positive and Gram-negative bacteria. Among the Gram-positive pathogens (Table 1), a global pandemic of resistant *Staphylococcus aureus* and Enterococcus species poses the biggest threat nowadays [4]. Notably in the U.S., Multidrug-resistant *Staphylococcus aureus* (MRSA) kills more people each year than HIV/AIDS, Parkinson's disease, emphysema and homicide combined [5]. Also the spread of resistance in common respiratory Gram-positive pathogens, such as *Streptococcus pneumoniae* is becoming epidemic[6].

Table 1 *- List of major pathogenic Gram-positive bacteria and associated diseases.*

Bacteria	Associated Disease
S.aureus	• Gastroenteritis • Acute bacterial endocarditis • Bacteremia/sepsis
S.pneumoniae	• Pneumonia • Meningitis • Otitis media
C.difficile	• Diarrhea • Pseudomembranous enterocolitis
L.monocytogenes	• Neonatal meningitis • Septicemia

Table 2 *- List of major pathogenic Gram-negative bacteria and associated diseases.*

Bacteria	Associated Disease
E. coli	• Diarrhea • Urinary tract infections • Meningitis in infants • Hospital-acquired pneumonia • Hospital-acquired sepsis
S.typhi	• Typhoid fever
P.aeruginosa	• Pneumonia • Sepsis • Urinary tract infections
K.pneumoniae	• Pneumonia • Sepsis • Urinary tract infections
S.dysenteriae	• Dysentery
N.Meningitidis	• Meningitis • Septocemia
N.gonorrhoeae	• Urethritis • Cervical gonorrhea
C.jejuni	• Secretory or bloody diarrhea
V.cholera	• Cholera
L.pneumophilia	• Pneumonia • Pontiac fever

On the other had a U.S. CDC report from 2013 (Li2015-Ref 6) listing current resistance threats, indicates that multidrug-resistant Gram-negative bacteria constitute a large proportion of the threats. Indeed Gram-negative pathogens (Table 2) are particularly worrisome because resistance is rapidly acquired and occurs to virtually all the antibiotic drug options available [6]. An important fact is that most of the serious Gram-negative

infections actually occur in health care settings and are commonly caused by Enterobacteriaceae (mostly Klebsiella pneumoniae), Pseudomonas aeruginosa and Acinetobacter baumannii[6].

Antibiotics

The modern era of antibiotics started with the discovery of penicillin by Sir Alexander Fleming in 1928 [7][8]. Antibiotics were then first prescribed in 1940, with penicillin being particularly relevant in controlling serious bacterial infections among World War II soldiers [7]. Over the last 50 years a vast number of antibiotics have been developed, each of them targeting bacterial cell mechanisms, responsible for vital functions such as cell-wall or protein synthesis (Table 3). Antibiotics brought major benefits to the global population, not only saving patients' lives but also playing a central role for the achievement of major advances in medicine, such as prevention and treatment of bacterial infections in immunocompromised or chronic patients[4]. Furthermore the impact of antibiotics in developing countries is also reflected on the decrease of morbidity and mortality caused by food-borne and poverty-related infections[6].

Unfortunately the rate of antibiotic development has been accompanied by the rapid evolvement of antibiotic resistance, thus threating our ability to control and eliminate disease-causing bacteria and in particular life-threatening strains.

Table 1 - *Antibiotics classification based on their mechanism of action.*

Cell mechanism affected	Antibiotic classification
Cell wall synthesis	Penicillin, Cephalosporins[9][10]
Protein Synthesis	Anti-30S ribosomal subunit (Aminoglycosides and Tetracyclines) and Anti-50S ribosomal subunit (Macrolides, Chloramphenicol, etc.)[11][12]
DNA Synthesis	Fluoroquinolones (1st, 2nd, 3rd, and 4th generation)[13]
RNA Synthesis	Rifampin[14]
Mycolic Acids Synthesis	Isoniazid[15]
Folic acid Synthesis	Trimethoprim/Sulfonamides[15]

Bacterial mechanism mediating antibiotic resistance

Some bacteria evolve and adapt in order to subvert antimicrobial drugs and thus became resistant. Such bacteria are able to tolerate an antibiotic drug so that the treatment turns ineffective and the infection persist leading to enhancement of clinical manifestation. [16]

Several bacterial resistance mechanisms have been described to date and can be divided essentially in three main groups: 1) mechanisms promoting antibiotic inactivation or modification, 2) mechanisms leading to target modification and 3) mechanisms controlling the efflux of substrates.[17]

Antibiotic inactivation or modification

Enzymes like β-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferases (hydrolases) are known to modulate the antibiotics activity. β-lactamases can hydrolyze compounds such as penicillins, cephalosporins, monobactams and carbapenems, reducing their activity. Another example of a modifying enzyme is erythromycin esterase II, which can hydrolyze a lactone ring of erythromycin A and oleandomycin.[18] [19][20][21]

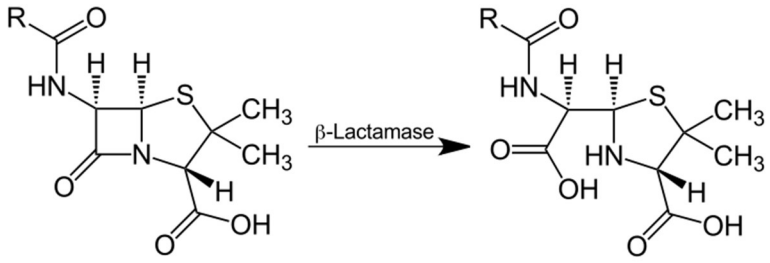

Figure 1 - *Representation of the enzymatic reaction (hydrolysis) performed by beta-lactamase.*

In addition, antibiotics can also be inactivated by transferases that promote binding of adenylyl, phosphoryl, or acetyl groups to the periphery of the antibiotic molecule. This reduces the binding affinity of the modified antibiotic to the 30S and 50S ribosomal subunits, contraposing the ultimate protein synthesis blockage. [22], [23][24].

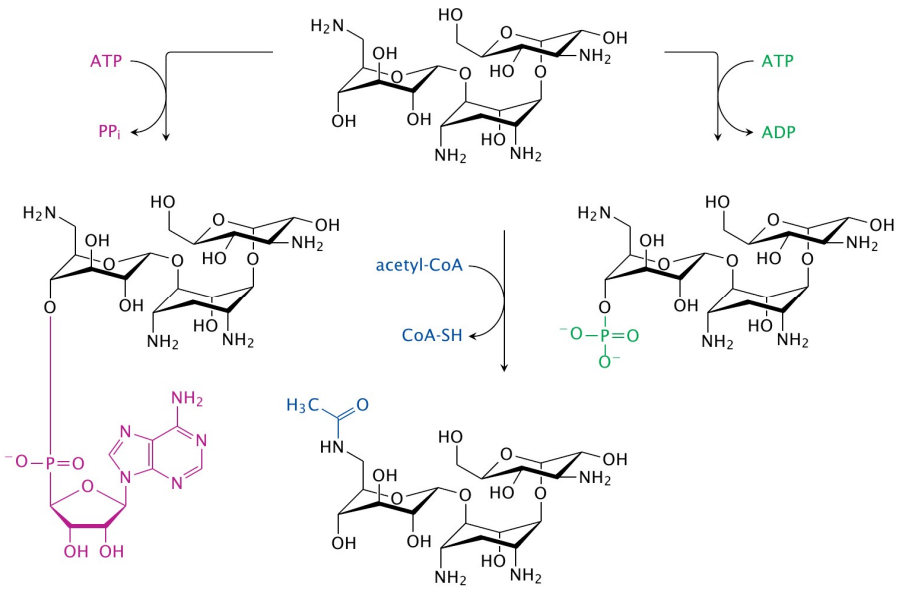

Figure 2 *– Different mechanisms of the inactivation of kanamycin; here represented are the mechanisms of phosphorylation (green), acetylation (blue) and adenylation (pink), both determine the impairment of the antibiotic activity of kanamycin. Adapted from* [25].

Finally, some bacteria are capable of changing the redox state of antibiotics thus inactivating them. This is the case of *S.virginiae* which can change a ketone group from virginiamycin M1 into an alcohol residue preventing it from binding to its target[26].

Target Modification

Many targets of the antibiotics are enzymes responsible for some crucial processes in bacteria, like for example, synthesis of peptidoglycan. Mutations on the genes that encode proteins responsible for the synthesis of the peptidoglycan, such as penicillin binding proteins (PBPs) lead to a resistance against antibiotics. In this case, due to the fact that β-lactams have a similar structure to the peptidoglycan elements, they bind to PBPs and impair peptidoglycan synthesis. If PBPs get mutated, binding to the β-lactam antibiotics is compromised and peptidoglycan synthesis proceeds normally thus enabling bacteria proliferation.[18]

Mutational events at the level of protein synthesis components targeted by antibiotics can also lead to resistance. Mutations on the 23S rRNA (component of the 50S ribosomal subunit) for example, can confer resistance to certain antibiotics like macrolides, lincosamides, and streptogramin B. [27], [28][29]

Similarly, antibiotics targeting elements belonging to the DNA synthesis machinery, may also become ineffective due to modifications at such element. This is the case of mutations in genes gyrA and parC, genes that encode gyrase and topoisomerase IV, these modify the DNA complex and prevent antibiotic binding.[18]

Efflux systems

Bacterial efflux systems are protein complexes responsible for exporting antibiotics out of the cells in order to keep low intracellular concentrations. This represents one of the main problems of the antibiotic resistance, since the bacteria only achieve resistance after a certain time of exposure to the antibiotic, as they need time to activate other defense mechanisms.[30]

These efflux systems can be divided in 5 families: 1) major facilitator (MF) superfamily, 2) resistance-nodulation-division (RND) family, 3) small MDR (SMR) family, 4) ATP binding cassette (ABC) family and 5) Multiple Antibiotic and Toxin Extrusion (MATE) family. The MF, RND and SMR uses the proton motive force to extrude the drugs while the MATE family uses either proton or sodium ions in exchange. On the other hand, the ABC family utilizes ATP as source of energy.[31] [32]

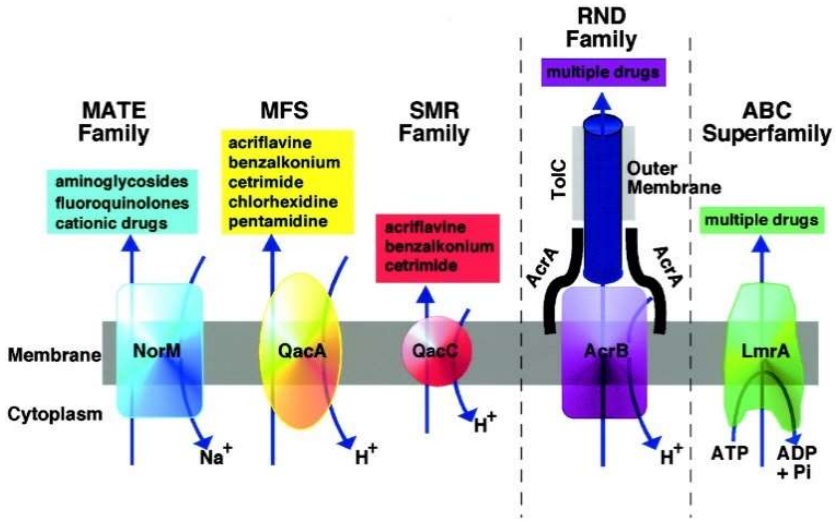

Figure 3 - *Schematic representation of the different Efflux Pump families found in Gram-negative bacteria: Major Facilitator (MF) superfamily in yelow, Resistance-Nodulation-Division (RND) family in purple, Small MDR (SMR) family in red, ATP Binding Cassette (ABC) familyin green and Multiple Antibiotic and Toxin Extrusion (MATE) family in cyan. Adapted from* [33].

The efflux systems in Gram-negative bacteria are generally constituted by three types of proteins [31] [34]:

- The pump – with the function of transporting the antibiotic from the intracellular to the periplasmic zone, this transport is usually driven by the exchange of protons or sodium ions.
- The fusion protein - is the protein responsible to keep the pump and the OM channel together flanking all the complex structure. Located at the periplasmic region, it helps the extrusion of the substrate and it is normally present with two or three units. Peristaltic actions of these proteins facilitate the extrusion of the antibiotic.
- Outer-membrane (OM) channel - this protein is the one that crosses the outer membrane, creating a duct that allows the bacteria to extrude the antibiotic (this is a critical component for antibiotic resistance in Gram-negative bacteria) [35].

These complexes can be specific to antibiotics but most of them are capable to pump-out a wide range un-related drugs, conferring multi drug resistance (MDR) to bacteria [36][37][17].

The presence of the antibiotic promotes a response in the bacteria involving over-expression of efflux pumps. This overexpression allows the bacteria to maintain low intracellular concentrations of antimicrobial drugs while in the meantime the other defense mechanisms are activated. When the sequence of the proteins that bind or internalize the antibiotic is changed, or even the enzymes that alter the structure of the antibiotics is expressed, the antibiotics become unable to bind; after this process the number of efflux pumps return to normal [38] [39][40].

Given their central role in antibiotic resistance, bacteria efflux systems represent a relevant target to combat pathogenic bacteria. Novel drugs enabling blockage or modulation of their activity can be used in antimicrobial strategies, namely in conjunction with antibiotics[30].

TolC outer-membrane channel

Like previously described, the efflux pumps present an OM channel that connects the periplasmic zone to the extracellular domain. In many Gram-negative resistant pathogenic bacteria this connection is made through the TolC protein, however there are also some alternative proteins that can make the connection between the periplasmic side and the extracellular domain like the family of the outer membrane proteins (OprM, OprJ, AprA, etc) [41].

The substrates of this protein are widely unrelated substances such as (tetracycline, chloramphenicol, ethidium bromide, acriflavine, sodium dodecyl sulfate, etc) and they are supplied by ABC, MFS and RND pump families, with the latter being the one most commonly involved[36].

Structural features of TolC channel

The crystal structure of TolC channel was first solved by Koronakis (2000) at 2.1Å resolution. TolC is a 428-residue protein natively assembling as a homotrimer that presents two distinct regions: β-barrel outer membrane region and a helical periplasmic region (Figure 7) [41].

The portion harbored by the outer membrane is approximately 40Å long and is essentially composed of a β-barrel motif, where each monomer contributes with 4 antiparallel β-strands. On the other hand the periplasmic region comprehends a cylinder-shape motif of 140Å in length composed of long α-helical bundles; each monomer contributes with 4 α-helices for the motif. This structure also comprehends a mixed α/β-domain in the middle of the periplasmic region [42][37].

One of the central features of the TolC channel is the small loop region at the extracellular entrance of the duct. This region undergoes conformational changes that is believed are responsible for the opening and the closure of the channel, promoting or blocking substrate efflux [43][44].

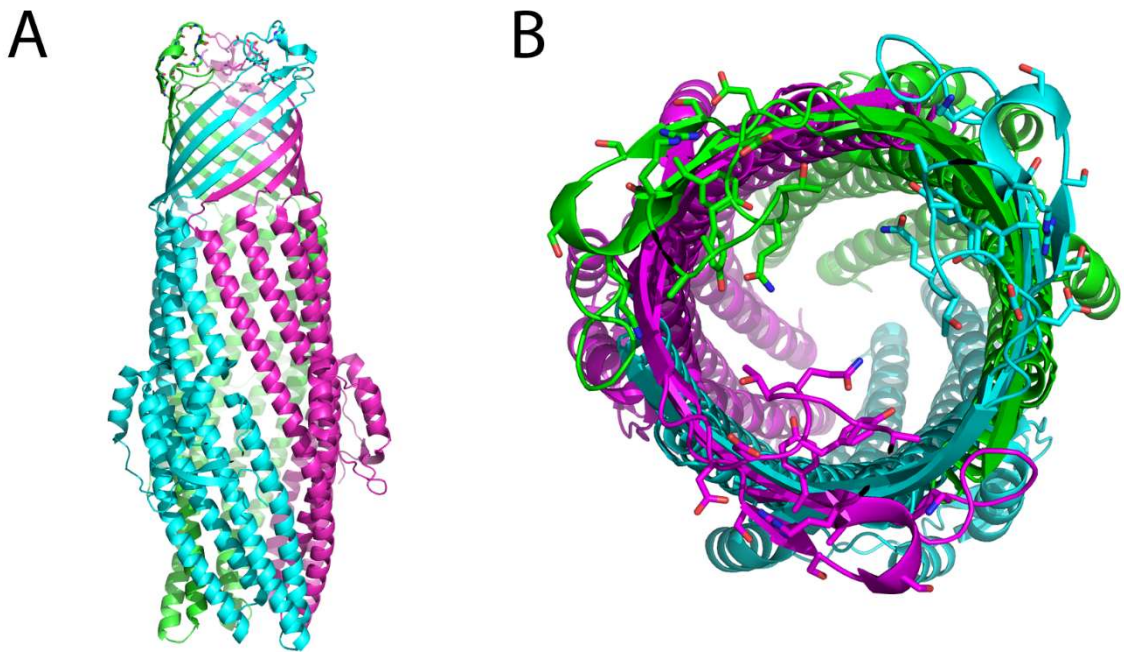

Figure 4 *– Side-view(A) and extracellular-view(B) of E.coli TolC channel (PDB code: 1TQQ). Each TolC monomer is depicted in a different color (green, cyan and purple); the monomers are arranged in an overall trimeric fold characteristic of this duct. On the extracellular-view the surface-exposed loops (termed extracellular loops) present their residue-side chains in the stick-mode representation.*

Interestingly, TolC channel is conserved among many known Gram-negative pathogenic bacteria. However to date, only *E.coli* TolC crystal structure has been solved, thus limiting a thorough comparison with TolCs of other bacteria. Nevertheless, protein sequence alignments and structural homology modeling can bring important insights for such evaluation.

Comparison between TolCs of some pathogenic bacteria (*Escherichia coli, Salmonella enterica, Acinetobacter baumannii, Pseudomonas aeruginosa, Klebsiella pneumoniae, Shigella sonnei, Neisseria meningitides, Yersinia pestis, Campylobacter jejuni* and *Vibrio cholerae*) showed that despite the identity differences between some species (Table 4.), the amino acid sequence similarity (same chemistry) is pretty high resulting in comparable structural homology models as computed by PHYRE algorithm (Protein Homology/analogy Recognition Engine V 2.0)[45]. Figure 8 shows a comparison between *Escherichia coli* TolC crystal structure (green) and the putative *Pseudomonas aeruginosa* TolC structural homology model (red/orange) as predicted by

PHYRE. Notably there is a very good overall structural superposition despite the low sequence identity score (32%).

***Table 2** - Scores and identities of the TolC alignments based on E.coli sequence. These results were obtained using the BLAST alignment tool.*

Bacteria	Max score	Total score	Identity (%)
E.coli	998	998	100
S.enterica	886	886	89
A.baumannii	169	184	30
P.aeruginosa	230	248	32
K.pneumoniae	832	832	83
S.sonnei	994	994	100
N.meningitides	120	139	28
Y.pestis	642	642	75
C.jejuni	58	93	18
V.cholerae	379	379	47

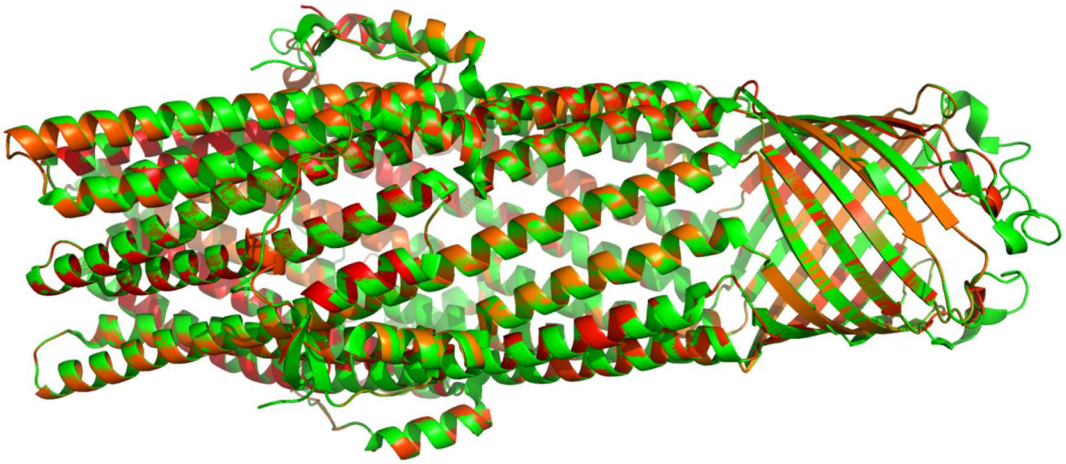

***Figure 5** – Structural superposition of the E.coli TolC crystal structure (green, PDB code: 1TQQ) with the structure-homology model of P.aeruginosa TolC channel (orange, Uniprot ID: P02930) as predicted by PHYRE algorithm (Protein Homology/analogy Recognition Engine V 2.0)* [45].

On the other hand, the alignment of different TolC sequences reveals the presence of highly variable regions. Interestingly this variation happens namely at the level of the extracellular loops (residues 282-289, in E.coli). Figure 9 shows that the loop region varies not only in aminoacid residue composition but also in sequence length. This fact is particularly important because it may predict a specific therapy against each one of the bacterial infections, reducing the unspecific bindings and secondary effects.

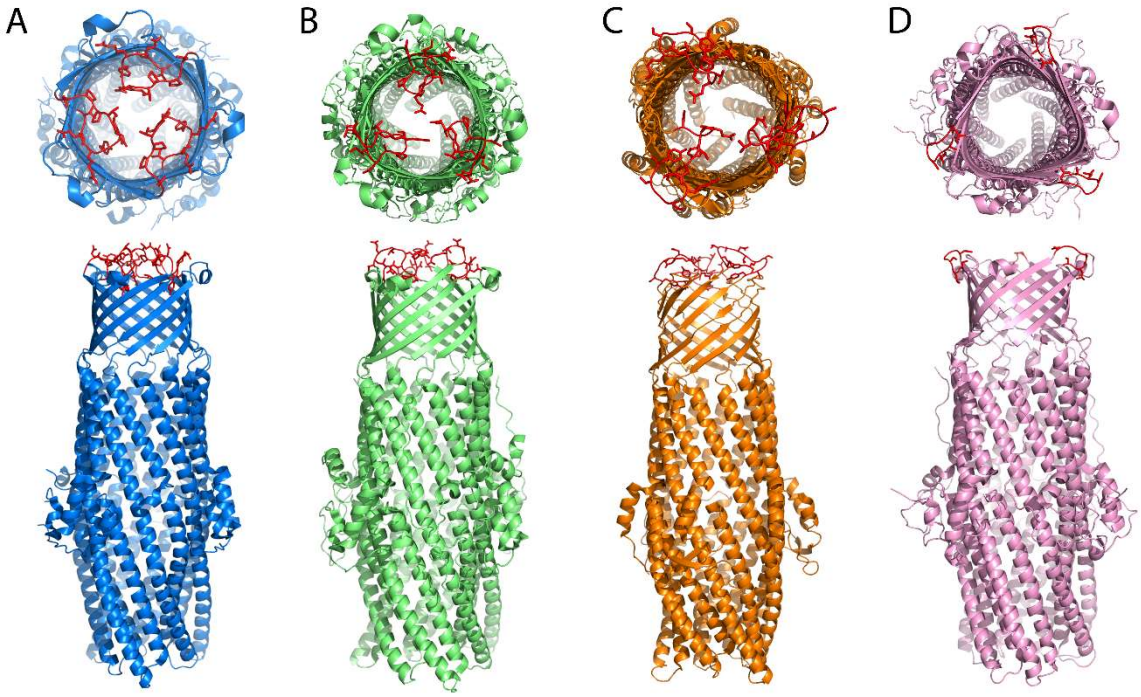

Figure 6 *– Extracellular-view (upper pannel) and side-view (bottom pannel) of the crystal structures of different TolC-like proteins, showing the overall structural similarity with E.coli TolC channel. A) Vibrio cholerae VceC (blue, PDB code: 1YC9), B) Campylobacter jejuni CmeC (green, PDB code: 4MT4), C) Salmonella enterica ST50 (yellow, PDB code: 5BUN) and D) Neisseria gonorrhoeae MtrE (pink, PDB code: 4MT0).*

Phyre2 analysis of *E.coli* TolC also revealed some TolC-like proteins with its structure already been resolved (Figure 6). Similarly to the *E.coli* TolC, these channels also occur as an homotrimer with a β-barrel motif located in the outer membrane and a α-helical domain in the periplasmic region. Just like the TolC, they are responsible for the extrusion of small molecules, like ions, but also bigger molecules as proteins, lipids and others.

Taken together, the intrinsic features of TolC and its central role in the antibiotic extrusion mechanism, make the channel an attractive target for modulation of the detoxification ability of resistant bacteria. Controlling the extrusion of drugs across TolC, will ultimately open new possibilities for modulation of antimicrobial bacterial resistance.

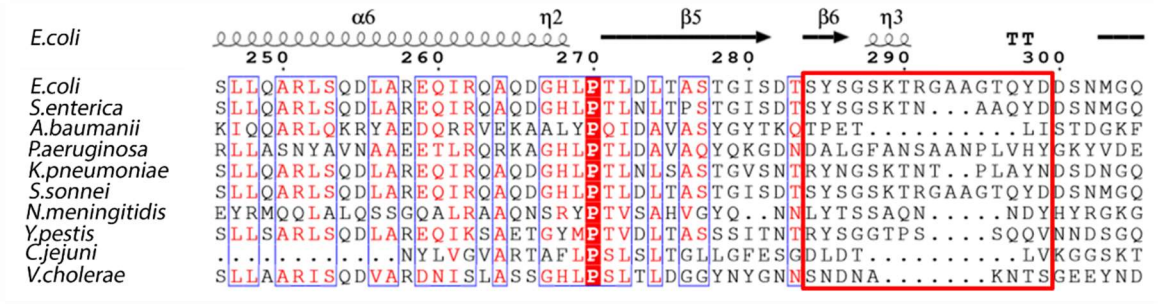

***Figure** 7 - Protein sequence alignment of TolC channels of different Gram-negative bacteria, shows a highly variable residue composition of this extracellular loop region (residues 284-299, highlighted with a red box). The absence of sequence conservation in this region supports the hypothesis of strain-specific targeting. Escherichia spp. (Uniprot ID: P02930), Salmonella enterica (Uniprot ID: V5KL83), Acinetobacter baumanii (Uniprot ID: B0VDE7), Pseudomonas aeruginosa (Uniprot ID: G3XCK3), Klebsiella pneumoniae (Uniprot ID: B5XU48), Shigella sonnei (Uniprot ID: Q3YXK5), Neisseria meningitidis (Uniprot ID: A0A125WEC3), Yersinia pestis (Uniprot ID: P58411), Campylobacter jejuni (Uniprot ID: Q0PBE5) and Vibrio cholera (Uniprot ID: Q9K2Y1).The secondary structural elements of E.coli TolC crystal structure (PDB code: 1TQQ) are presented on the top of the alignment.*

Avian antibodies

Mammalian antibodies obtained essentially from rabbits, mice and goats, have been used for decades for medical and research applications. The use of bird models (e.g. chicken, goose and quails) for antibody production has also been exploited since the 90s, but is still largely neglected mainly due to genetic divergence between birds and humans when compared with other mammals.[46] However the production of antibodies in birds, namely chickens, holds a strong potential due to several advantages:[47]

- Chicken antibodies can be collected from egg-yolk thus in a much less invasive fashion then mammalian blood collections.
- The egg yolk contains only one type of antibodies – the IgY isotype, counterpart of mammalian IgG - making it simple to collect and purify.
- The phylogenetic divergence from humans can be used as an advantage, since chickens allow the production of antibodies against highly conserved mammalian protein and notably less antigen is required for its production (Table 5);
- Productivity is around 18 times greater than rabbits when compared to the amount of protein obtained (mg) per animal weight;

- Processing of eggs is already carried out at industrial scale; thus chickens represent a cost-effective source of specific antibodies, whose production is largely scalable.

Table 3 *- Comparison between the production of antibodies in mammals and chickens.*[48]

	Mammalian IgG	Chicken IgY
Sampling	Invasive	Non-Invasive
Amount of Antibody	~ 150mg / 20 ml production bleed (every 3 weeks)	~100 mg IgY per egg (5-7 eggs per week)
Amount of Antibody / Month	~ 200 mg	~1500 mg
Amount of Specific Antibody	~ 5%	5-10%
Protein A/G Binding	Yes	No
Reactivity with Mammalian IgG	Yes	No
Cross Reactivity with Mammalian Rheumatoid Factor	Yes	No
Activation of Mammalian Complement System	Yes	No

Despite of all the advantages egg-yolk derived antibodies (IgY) are not exactly the same as mammalian IgG. Both have two lights and two heavy chains where the light chains of both IgY and IgG are constituted by one constant and one variable domain, while the heavy chain of IgG have one variable domain and three constant domains and the heavy

chain of IgY as a variable domain, but one more constant domain making it four constant domains[47].

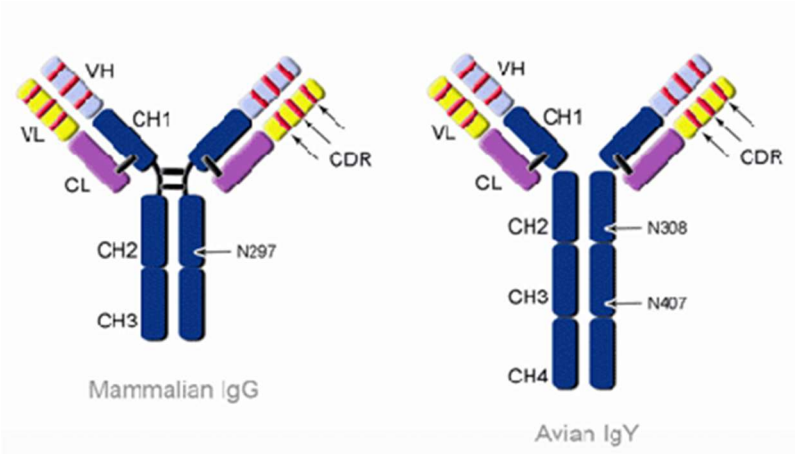

Figure 8 - *Differences between mammals IgG and hens IgY. VL=Variable domain of Light chain; VH=Variable domain of heavy chain; CL=Conserved domain of light chain; CH=Conserved domain*

Therapeutic and prophylactic applications of IgY antibodies

Avian IgY antibodies have been extensively use in a number of therapeutic and prophylactic applications to treat and/or control bacterial, viral and fungal infections [49]. IgY antibodies against *P.aeruginosa* (PA) have been administrated prophylactically through mouth rinse in cystic fibrosis patients resulting in significant reduction or impairment of PA colonization [50], [51]. In another study, a similar oral treatment with IgY antibodies raised against *C.albicans,* successfully prevented candidiasis in immunocompromized children undergoing leukemia treatment [52]. Moreover relevant examples of IgY therapies are found using animal models; for example intranasal administrations of IgY antibodies against H5N1 virus prevented the mortality of 100% of the mice against lethal challenge with H5N1 [53]. Other examples of therapeutic and prophylactic applications of avian IgY antibodies in microbial infections are described in Table 6. Given the high number success cases reported and validated using IgY for immunotherapies, the approach is reemerging in the pharmaceutical industry that is revisiting and refining the use of avian antibodies to produce alternative antimicrobial (biological) drugs[47].

***Table 6** – Therapeutic applications avian IgY antibodies in bacterial, viral and fungal infections.*

Type of infection		IgY Therapeutic/prophylactic effect
Bacterial	*P.Aeruginosa*	Reduce PA adhesion
	E.coli (ETEC)	Treatment of gastrointestinal infections
Viral	Influenza virus	Prevented death in mice
	Human rotavirus (HRV)	Prevented diarrhea in mice
Fungal	*C.albicans*	Reduce colonization in vivo

Production of antigen-specific IgY

Antigen-specific IgY can be obtain through hens' immunization as thoroughly described by many works. The procedure involves intramuscular injections of the antigen combined with oil-based adjuvants such as Freund's complete and incomplete adjuvant (FCA and FIA). A standard immunization protocol includes 4 injections of the antigen with 2 weeks of interval between each one. Egg-yolk samples are collect during a period that extends from one week before (Day: -7) the first immunization (Day: 0), up to 40 to 80 days after immunization start. The antigen-specific response is monitored by analyzing the egg-yolks collected along the immunization procedure, avoiding invasive animal bleeds.[47]

This protocol is very important since it enables the production a hyperimmune bird. These birds are critical for the generation of novel immunological repertoires, since the animals are challenged with a unique set of external antigens of known origin. By collecting the lymphoid organs where the antibody producing B-cells maturate (spleen, femur bone marrow and Bursa of Fabricius) it is possible to recover the genetic material that encodes the antibody repertoire and in this way produce monoclonal antibodies targeting the initial set of antigens.[47]

Antibodies against membrane channels

Many antibodies (monoclonal and polyclonal) produced in mammals have been utilized for the modulation of channels activity[54], namely antibodies against voltage-gated potassium channels that reduce cell current[55], proliferation, migration[56] and tumor growth[57], but also against voltage-gated sodium channels[58][59] and TRP channels[60][61] as shown in Table 6.

Also an anti-TolC antibody produced in rabbit was used to suppress the proliferation of chloramphenicol resistant *E.coli* cells. In this case, treatment with the anti-TolC serum were able to block the proliferation of the cells in the presence of the antibiotic obtaining a inhibiting rate similar to the deletion of this channel. [43]

Table 6 *– Antibodies used to modulate the activity of mammalian ion channels.*

Target	**Antibody**	**Effect**
Voltage-gated potassium channel	Anti-K_v 1.2	Reduce whole cell current
	Anti-K_v 3.1	Reduce proliferation and migration
	Anti-K_v 10.1	Reduce tumor growth and increase cell death
Voltage-gated sodium channels	Anti-Na_v	Shift the voltage-dependence of activation and inactivation
	Anti-Na_v 1.5	Reduce whole cell current
TRP channels	Anti-TRPM3	Reduce whole cell current
	Anti-TRPC1	Reduce store-operated calcium entry

Materials and Methods

Expression and purification of full-length E.coli TolC (residues 1-450)

A plasmid containing a gene encoding *E.coli* TolC residues 1-450 (Uniprot ID: P02930) obtained from gene synthesis (GeneArt Gene Synthesis, Thermo Fisher) was already available in the laboratory. Briefly, the pET23-a plasmid was used and TolC gene was inserted using NdeI and Xho1 restriction enzymes. The resulting construct encodes for full-length *E.coli* TolC (residues 1-450) harboring a C-terminal the 6xHis-tag. Expression of recombinant *E.coli* TolC (1-450) was performed in *E.coli* BL21 star after transformation of competent cells with the plasmid construct described above. Cells were grown until O.D.600= 0.6 and induced with 0.5mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). After 3h of expression cells were harvested by centrifugation at 4000g for 15min. Cell pellets were ressuspended in Buffer A (50mM Tris-HCl pH 8.0 with 150mM NaCl) and submitted to three cycles of cell disruption on a high pressure homogenizer (Avestin Emulsiflex C3).

Extraction of the TolC from cell membranes was performed by incubating whole cell lysates with detergents, overnight at RT with gentle agitation. Two different commercial detergents were used in independent assays 1) n-Dodecyl-β-D-Maltopyranoside (DDM; 40mM extraction concentration) and 2) Triton X-100 (5% v/v extraction concentration). Cleared supernatant containing solubilized TolC was obtained after ON extraction by centrifugation at 35000g for 45min. This was then applied to Chelating Sepharose Fast Flow column (GE Healthcare), previously prepared with immobilized cobalt ions according to the manufacturer recommendations and equilibrated in buffer A supplemented with 1mM DDM (or 0.5% triton) and 10mM of Imidazole. All steps were performed using a column flow rate of 1mL/min. After loading the cleared lysate the column was washed with buffer A supplemented with 1mM DDM (or 0.5% triton) and 20mM of Imidazole and final protein elution was performed with the same buffer supplemented with 150mM of Imidazole. The fraction containing TolC was concentrated and applied to a size exclusion chromatography (Superdex 200 10/300). This column was previously equilibrated with buffer A supplemented with 1mM DDM (or 0.5% triton). Fractions of 1mL were collected and analyzed by SDS-PAGE. Those corresponding to TolC (with a clear band at ~45kDa) were pooled together and concentrated to 1mg/mL.

Mass spectrometry (MS)

Mass spectrometry analysis for identification of *E.coli* TolC 1-450, was performed at the Mass Spectrometry Unit of the Center for Neuroscience and Cell Biology.

Cloning E.coli TolC extracellular epitope (residues 282-298) in fusion with a carrier protein

E.coli TolC extracellular epitope (residues 282-298) was cloned using a Sequence and Ligation Independent Cloning (SLIC) method. Briefly, two oligos each coding for half the TolC residues 282-298 epitope sequence, namely:

Sense oligo,
5'-TGCGGCACCACGGGTTTTTGAACCGCTATATGAGCCATTTTCGTCTGAAAGGCTTTGTGCG-3'
anxd antisense oligo,
5'-GGTACACAGTATGATGATCACCACCACCACCACCACTGACGCCATTAACCTGATGTTCTGGGG–3' were primarily phosphorylated using T4 Polynucleotide Kinase (PNK) for 60min at 37°C. The reaction was then stopped by inactivation of PNK at 60°C for 20min. The phosphorylated oligos were further used to linearize a pCoofy plasmid (EMBL) harboring the carrier protein with the following reaction: 10µL 5x Phusion buffer; 0.5µL Template (35ng); 1.5µL Forward Primer (10µM); 1.5µL Reverse Primer (10µM); 0.5µL Phusion; 1µL dNTPmix; ddH_2O to a final volume of 50µL. Linearization reaction was carried under the following conditions:

Table 8 *– PCR conditions used for the linearization reaction.*

Segment	Cycles	Temperature	Time
1	1	98°C	1 minute
2	25	98°C	30 seconds
		53°C	30 seconds
		72°C	9 minutes
3	1	72°C	10 minutes

Linearization reaction was then treated with DpnI for 1h at 37°C, followed by a ligation reaction at 16°C overnight using T4 Ligase. The final ligation product was used to transform DH5α cells. The following day, colonies from plate were ressuspended in ddH_2O and submitted to the following colony PCR: 2µL 10x Dream Taq buffer; 0.5µL Template (ressuspended colonies); 1µL Forward Primer (10µM); 1.5 Reverse Primer

(10µM); 0.25µL Dream Taq; 0.4µL dNTPmix; ddH_2O to a final volume of 20µL. The reaction took place under the following conditions:

Table 9 *– PCR conditions used on colony PCR.*

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	30	95°C	30 seconds
		52°C	30 seconds
		72°C	1.5 minutes
3	1	72°C	5 minutes

Products were then analyzed in agarose gel and positive colonies were selected and grown over night in 5mL LB. The next day, plasmid mini prep was made using the NZYMiniprep kit. New TolC-epitope clones were confirmed by sequencing (GATC sequencing services).

Purification of Carrier-E.coli TolC extracellular epitope (residues 282-298) fusion

The fusion construct Carrier-TolC (282-298) was expressed in *E.coli* BL21 star (DE3). Expression was induced at O.D.600= 0.6 with 0.5mM IPTG for 3h. Cells were then harvested by centrifugation at 4000g for 15min. Cellular pellet was ressuspended in Buffer B (50mM Tris-HCl pH 7.5 150mM KCl) and submitted to three cycles of cell disruption on a high pressure homogenizer (Avestin Emulsiflex C3), followed by a centrifugation at 35000g. Soluble fraction was then applied to Chelating Sepharose Fast Flow column (GE Healthcare), previously prepared with immobilized cobalt ions according to the manufacturer recommendations and equilibrated in buffer B supplemented with 10mM of Imidazole. All steps were performed using a column flow rate of 1mL/min. After loading the soluble fraction the column was washed with buffer B supplemented with 20mM of Imidazole and final protein elution was performed with the same buffer supplemented with 150mM of Imidazole. Collected Carrier-TolC (282-298) was then concentrated for further analysis. Due to the instability of the Carrier-TolC epitope fusion, it wasn't possible to run on a size exclusion chromatography.

Carrier-TolC epitope fusion was also purified using a nucleotide-immobilized affinity column. After expression as described above, the soluble fraction was incubated with the affinity column beads ON at 4°C under gently agitation. On the next day, beads

were washed with Buffer B and protein was eluted with the same buffer supplemented with 5mM of competing nucleotide. Protein elution peak was collected and concentrated.

Immunization of experimental quails

Three female quails (*Coturnix japonica*) with 8-12 week of age were selected for the immunization protocols. Quails were given *ad libitum* water and food with a light cycle of 16L/8D until stabilization of the egg-laying cycle. Bird's body weight was monitored every immunization/boost day. Eggs were collected daily and registered at an egg-laying Table.

Birds were immunized with intramuscular (IM) injections in the pectoral muscles with the following periodicity: immunization at D0 (day 0) and three additional boosts every 15 days (D15, D30 and D45). Injectable suspensions were prepared by mixing purified recombinant immunogens and Freund Adjuvant (Sigma) in 1:1 (v/v) ratio followed by extrusion through an emulsifying syringe until stable emulsions were obtained; Complete Freund Adjuvant (CFA, Sigma) or Incomplete Freund Adjuvant (IFA, Sigma) were used to prepare emulsions respectively for immunization and boosts. An average amount of 100µg of purified recombinant immunogens were administered per immunization/boost.

Purification of Anti-TolC Antibodies

Standard Purification

For standard purification of anti-TolC IgY antibodies from quail hyperimmune eggs, 12,5mL of egg yolk were diluted in 87,5mL of ddH_2O. After a brief stir, pH of the solution was adjusted to 4.8-5.0 and submitted to a ON freeze-thaw cycle. Water soluble protein fractions (WSPF) were then collected by centrifugation at 4000g for 30min in a falcon centrifuge. Antibodies present in the sample were then precipitated with 8-12% PEG 6k and harvest by a centrifugation at 4000g. IgY pellet was ressuspended in 5mL of PBS and applied to a preparative size exclusion chromatography (Superdex 200 26/600). Eluted fractions of 5mL were collected, analyzed by SDS-PAGE and those containing the antibody were pooled and concentrated for further analysis.

Immunogen-affinity Purification

Immunogen-affinity purification of anti-TolC IgY antibodies followed the same procedure of standard purification until WSPF sample. Then WSPF was loaded into a CNBr column (previously coupled with the TolC 282-298) at a flow rate of 1mL/min flow. Elution was performed with a 0.1M glycine pH2.7 and peak was collected in fractions of 1mL with immediate pH adjustment to 7.4 using an excess of 1M Tris-HCl pH 7.4. Fractions were then analyzed by SDS-PAGE and those containing the antibody were pooled together, concentrated and applied to a size exclusion chromatography column Superdex 200 10/300. Fractions of 500µL were collected and analyzed by SDS-PAGE and those containing the IgY were pooled for further analysis.

Differential Scanning Calorimetry (DSC) analysis

The thermal stability of the produced immunogens was tested by Differential Scanning Calorimetry (DSC). All DSC measurements were carried out with a VP-Cap DSC calorimeter equipped with an auto sampler using deep 96-well plates. Two concentrations of each purified immunogen were analyzed in duplicate: for TolC 1-450, c1= 0.13mg/ml (0.96µM, oligomer) and c2= 0.06mg/ml (0.44µM, oligomer) were used and for TolC 282-298 and c1= 0.7mg/ml (4.78µM, oligomer), c2= 0.3mg/ml (2.05µM). Purification buffers used for sample dilutions and for reference in the DSC runs were respectively, 50mM Tris-HCl pH 8.0, 150mM NaCl, 1mM DDM for TolC 1-450 and 50mM Tris-HCl pH 7.5, 150mM KCl and 5mMATP for TolC 282-298. Samples were scanned from 25°C to 100°C at a rate of 1.5°C/min. DSC thermograms were normalized considering the molar concentrations of the native oligomeric state of each sample, TolC 1-450 oligomer MW= 135kDa, TolC 282-298 oligomer MW= 146.4 kDa.

Enzyme-Linked Immunosorbent Assays (ELISA)

ELISA assays were performed using the purified TolC antigen for ELISA multi-well plate coating. This was done by incubating 0.5µg of antigen per well in 0.05M carbonate buffer pH 9.6, for 2h at 37°C. Wells were then washed 3x with PBS-T (0.05% Tween 20) and blocked ON at 4°C with PBS-T (0.05% Tween 20) with 3% BSA. The next day, antibody-containing samples were incubated with appropriate dilutions in PBS-T (0.05% Tween 20) with 1% BSA for 1h at 37°C and subsequently washed three times with PBS-T (0.05% Tween 20). Conjugated secondary antibody was incubated in PBS-T

(0.05% Tween 20) 1%BSA for 30min at 37°C followed by three wash steps with PBS-T (0.05% Tween 20). Development of the plates was done using 3,3′,5,5′-Tetramethylbenzidine (TMB) reagent at RT for 30min and reaction was stopped with 0.5M H_2SO_4. Titers of anti-TolC specific antibody were evaluated by reading the absorbance at 450nm.

Western-Blot assays

Western-Blot assays were performed using samples of purified TolC immunogens; 1 µg were loaded per lane on a 1mm 12% polyacrylamide gel and samples were run at 120V for 5min (stacking gel) followed by 180V for approximately 30min (running gel). Proteins were then transferred to a nitrocellulose membranes using Trans-Blot Turbo (Bio-Rad) with 20min at 25V. Membrane were then blocked with TBS-T (0.1%Twenn 20) 5% milk for 1h at RT or ON at 4°C. Incubation with the primary antibody, using dilutions described in Table 10, was performed in TBS-T (0.1%Twenn 20) 5% milk for 1h at RT or ON at 4°C. This step was followed by three consecutive washes with TBS-T (0.1%Tween 20) 0.5% milk for 5min and membrane was then incubated with the conjugated secondary antibody (Rabbit anti-chicken IgY, Sigma) on TBS-T (0.1%Tween 20) 0.5% milk for 1h at RT. After this, membrane was washed again three consecutive times with TBS-T (0.1%Twenn 20) for 5min and development was performed using ECF or ECL Western Blotting Reagent (GE Healthcare), depending on the conjugated secondary antibody used.

Table 10 *– Antibody dilutions used in western blots assays.*

Antibody	**Dilution**
Anti-His	1:10000
Anti-TolC 1-450 standard purified	1:10000
Anti-TolC 1-450 immunogen-affinity purified	1:2000
Anti-TolC 282-298 standard purified	1:1000

Collection and storage of lymphoid organs from hyperimmune quails

At the end of immunization protocols (~3 months, ~Day83-90) birds were euthanized with an intraperitoneal injection of an overdose of sodium pentobarbital (Eutasil 20%, Ceva, Sante Animale). Lymphoid organs, namely spleen and femur bone

marrow were collected and immediately immersed in RNALater Stabilization reagent (LifeSciences) and stored at -20ºC for subsequent total RNA extraction procedures.

Ethics Statement: All experimental procedures performed with birds were reviewed and approved by the local ethics committee and competent authority for animal protection (CNC ORBEA), to comply with all the requirements of Directive 2010/63/EU on the protection of animals used for scientific purposes.

Bacterial strains

***Table 11** – Antibody dilutions used in western blots assays.*

Strain	Description
BL21 Star™(DE3)	F- ompT hsdSB (rB-mB-) gal dcm rne131 (DE3)
OverExpress C41(DE3)	F – ompT hsdSB (rB- mB-) gal dcm (DE3)
OverExpress C43(DE3)	F – ompT hsdSB (rB- mB-) gal dcm (DE3)

Expression bacterial strains used in this work are described above in Table 11. Strains C41(DE3) and C43(DE3) are derived from BL21(DE3) with uncharacterized mutations. These strains were phenotypically selected where the mutations acquired prevented cell death associated with expression of many recombinant toxic proteins.

Goals and aims

TolC channel plays an important role in maintain the low antimicrobial intracellular concentrations inside the MDR bacterial cells. Alongside with the other proteins from the efflux complex, the TolC channel is responsible for the extrusion of antibiotics and other toxic compounds. Therefore, impairing this activity will most likely prevent the survival of MDR bacteria to antimicrobial treatment.

Hereupon the main goal of this work was to produce antibodies in an avian host system, capable of recognizing the *E.coli* TolC channel namely the extracellular loop. For this purpose, several objectives were proposed:

1. Production and characterization of TolC immunogens suitable for antibody generation
    a. Recombinant expression of full-length and epitope forms
    b. Purification by characterized technologies
    c. Characterization by size exclusion chromatography (SEC), SDS-PAGE and differential scanning calorimetry (DSC)
2. Immunization of birds model for production of anti-TolC specific antibodies
3. Isolation of egg yolk antibodies and biochemical characterization of their target specificity and reactivity
    a. Isolation of egg yolk IgY antibodies
    b. Enrichment in target-specific IgY
    c. Characterization of anti-TolC by ELISA and western blot techniques.

Results

Expression and purification of E.coli TolC immunogens

E.coli TolC 23-450 truncated immunogen

In order to obtain antibodies against the TolC channel (UniProt ID:P02930), a truncated TolC form was created for the immunization protocol. This truncation was performed in the N-terminus of the protein deleting residues 1 to 22 which removed the signal peptide that redirects the protein to the outer membrane. Previous lab experiments revealed that expression of this TolC form was more appropriate in *E.coli* BL21 star (DE3) however the protein revealed to be insoluble and by this we decided to purify it from the acrylamide gel.

Truncated TolC (23-450) was expressed in *E.coli* BL21 star (DE3) carrying a pET23a with the truncated TolC gene. Expression last for 3h and was followed by a centrifugation at 4000g for cell harvesting. In the time course expression analysis it is possible to observe an increasing amount of overexpressed TolC (45kDa band) over the time as shown in Figure 9. Since the protein is not being expressed in a soluble form, purification of this form of TolC was performed from the polyacrylamide gel. A fraction of the cellular pellet was ressuspended and run in a 15% acrylamide gel. The gel was negatively stained with a 0.2M $CuCl_2$ as a standard method described for protein band extraction for immunization purposes, so the bands corresponding to the TolC (~45kDa) could be cut out from the gel and processed. With this method, protein band remain unstained allowing band excision followed by mechanical homogenization and temperature treatment to recover protein content from the acrylamide. This mixture was finally filtered to remove the acrylamide from the sample and a SDS-PAGE analysis revealed a good purity grade (Figure 3) ideal for immunizations.

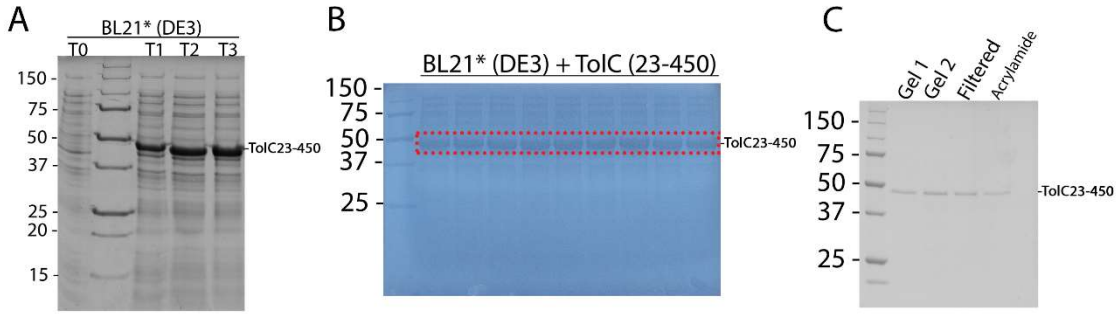

Figure 9 – Expression and purification of TolC 23-450. A) *SDS-PAGE analysis of the time course expression in BL21 star (DE3) of recombinant E.coli TolC 23-450. T0, time of expression induction; T1, T2 and T3 time point of 1h, 2h and 3h of expression repectively.* ***B)*** *Negative stained acrylamide gel for protein band extraction. Lanes containing equal samples of TolC 23-450 expression culture.* ***C)*** *SDS-PAGE analysis of the samples along the purification process of TolC 23-450. Gel 1 and Gel 2, bands extracted from two different acrylamide gels; Filtered, purified sample from acrylamide gel; Acrylamide, sample of the remaining protein fraction in the acrylamide after the purification process. TolC 23-450 with a molecular weight around 45kDa.*

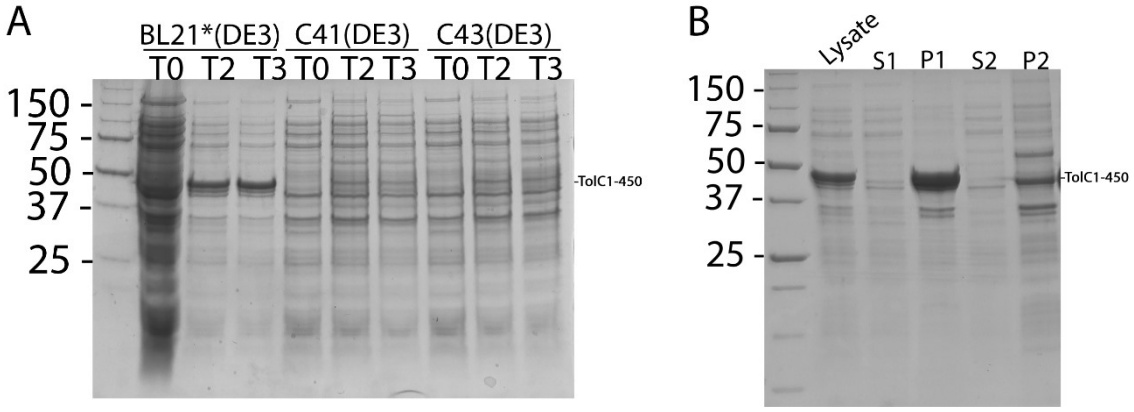

Figure 10 – Expression and membrane enrichment of TolC 1-450. A) *SDS-PAGE analysis of the time course expression in BL21 star (DE3), C41(DE3) and C43(DE3) of recombinant E.coli TolC 1-450. T0, time of expression induction; T2 and T3 time point of 2h and 3h of expression repectively.* ***B)*** *SDS-PAGE analysis of the sample from membrane enrichment. Lysate, sample of the cell expression culture lysate; S1 and P1 samples of the supernatant and pellet of the centrifugation at 8000g respectively; S2 and P2 samples of the supernatant and pellet of the centrifugation at 100000g respectively. TolC 1-450 with a molecular weight around 45kDa.*

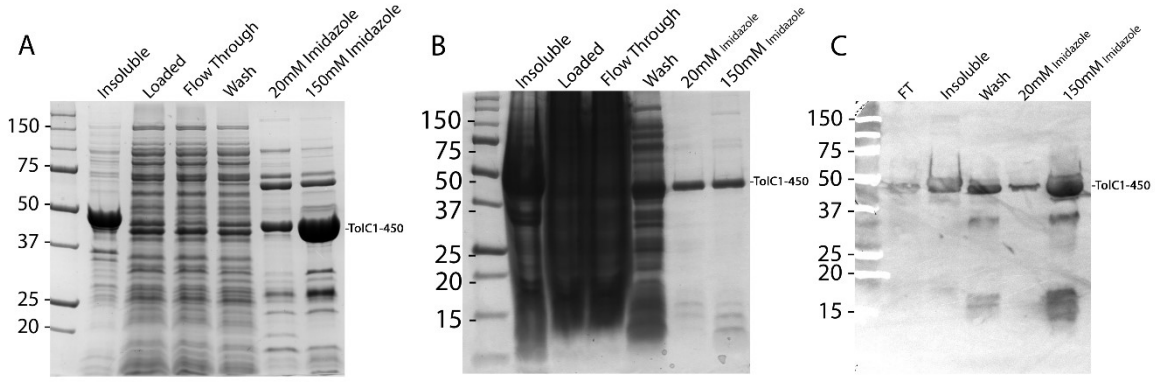

Figure 11 – Purification of TolC 1-450. A) *SDS-PAGE analysis of the samples along the affinity*

purification of the TolC 1-450 extracted with Triton X-100. ***B)*** *SDS-PAGE analysis of the samples along the affinity purification of the TolC 1-450 extracted with DDM.* ***C)*** *Western blot analysis validating the purification of the TolC 1-450 extracted with DDM with an anti-His antibody in a dilution of 1:10000. Insoluble, insoluble detergent fraction; Loaded, soluble detergent sample loaded into the IMAC; Flow Through and FT, soluble detergent sample after being loaded into the IMAC; Wash, sample of the wash step of the IMAC; 20mM Imidazole and 150mM Imidazole, samples corresponding to the elution with 20mM and 50mM Imidazole respectively. TolC 1-450 with a molecular weight around 45kDa.*

E.coli TolC 1-450 full-length immunogen

In order to raise antibodies against the TolC channel, a full-length form of the TolC was purified. This one includes the signal peptide that redirects the protein to the membrane allowing their correct folding and stabilization.

A vector containing the sequence for the TolC 1-450 was already constructed in the laboratory however the expression protocols for this protein were not optimized yet. Thus, regarding the fact that the overexpression of a membrane protein could compromise the integrity of the membrane we selected three strains for expression tests: BL21 star, C41 and C43 *E.coli* cells. *E.coli* C41 and C43 strains were selected due to their robustness and efficiency for the expression of toxic and membrane proteins.

Time course expression analysis revealed that *E.coli* BL21 star (DE3) presented the best expression results for the TolC 1-450 (with a MW of ~45kDa) and due to this results, this was the selected strain to proceed for subsequent steps (Figure 10).

Regarding the expression results, we moved on to the purification of TolC 1-450 with 2 different approaches: 1) A membrane fraction enrichment and 2) Direct extraction from cell lysates.

In our initial approach we tried to enrich the membrane fraction containing overexpressed TolC thus improving the final membrane protein yield for subsequent extraction purposes. For this we performed a membrane preparation by 2 steps centrifugation approach: a first spin at 8000g to remove major cell debris followed by a second spin at 100000g to harvest the membrane fraction (Figure 5).

Unexpectedly, as shown in Figure 10, a significant amount of the overexpressed protein remained in the insoluble fraction of the first centrifugation. This result indicated that the majority of TolC is being expressed as an insoluble protein and by this not being redirected to the membrane. Since membrane enrichment did not significantly increased

the TolC fraction, extraction of the protein from the membrane preparations was not performed.

The second purification approach was based on direct extraction of the membrane protein from bacterial lysates in an attempt to decrease the loss of protein during sample processing. For this purpose we used two different commercial detergents: n-Dodecyl β-D-maltoside (DDM) and Triton X-100. Detergent extractions were followed by immobilized ion affinity chromatography (IMAC) for protein recovery.

Figure 11A) shows the purification IMAC following Triton X-100 extraction. Because we are working with a membrane protein, the corresponding band is not perfectly distinguished in the soluble fraction ("Loaded") due to the low amounts of proteins in the membrane fraction. However IMAC affinity column allowed an efficient enrichment of the protein as shown in the elution fraction ("150mM Imidazole") revealing a good amount of membrane protein in opposition to the previous method.

Despite the successfully extraction and enrichment in TolC, the purity grade was not very good, with the elution sample containing a lot of contaminants. This process yielded 0.75-1.0mg of purified TolC per 1L culture

Following the same IMAC procedure, using DDM as solubilizing agent, also revealed good amounts TolC in the solubilized membrane fraction. Interestingly, this resulted in a clear main band in the eluted fraction, thus reflecting an overall higher protein purity grade when compared to the Triton X-100 elution fraction (Figure 11). Similarly to the extraction with Triton X-100, the utilization of DDM did not lowered the outcome of the process, yielding also 0.75-1.0mg of purified TolC per 1L culture.

The purified recombinant version of *E.coli* TolC 1-450 immunogen was analyzed by mass spectrometry that confirmed the identity of protein.

E.coli TolC 282-298 Extracellular Epitope

To produce antibodies that would bind the extracellular domain of the TolC channel, a fusion protein composed by the extracellular residues of the TolC channel and a carrier capable of presenting those residues was produced.

Table 6 *- Fusion constructs generated for the recombinant production of TolC 282-298 the control APRc 168-178.*

Fusion Constructs	Linker	Epitope sequence
Carrier-TolC 282-298	-G-	**SYSGSKTRGAAGTQYDD**HHHHHH
Carrier-APRc 165-178	-GS-	**YTRTYLTANGENKA**HHHHHH

For this purpose we used a proprietary protein carrier developed at Instituto de Biologia Molecular e Celular (IBMC/I3S, Porto). This technology allows for multicopy presentation of recombinant immunogens thus promoting higher avidity and immunogenicity.

Cloning procedure used as template an available plasmid encoding the protein carrier. We use the sequence ligation independent cloning (SLIC) procedure, as described in the methods section, to introduce the sequence coding for TolC extracellular Epitope giving rise to the final protein fusion construct described in Table 6.

Products were then analyzed in agarose gel and positive colonies were selected and grown over night in 5mL LB. The next day, plasmid mini prep was made using the NZYMiniprep kit. New TolC-epitope clones were confirmed by sequencing (GATC sequencing services).

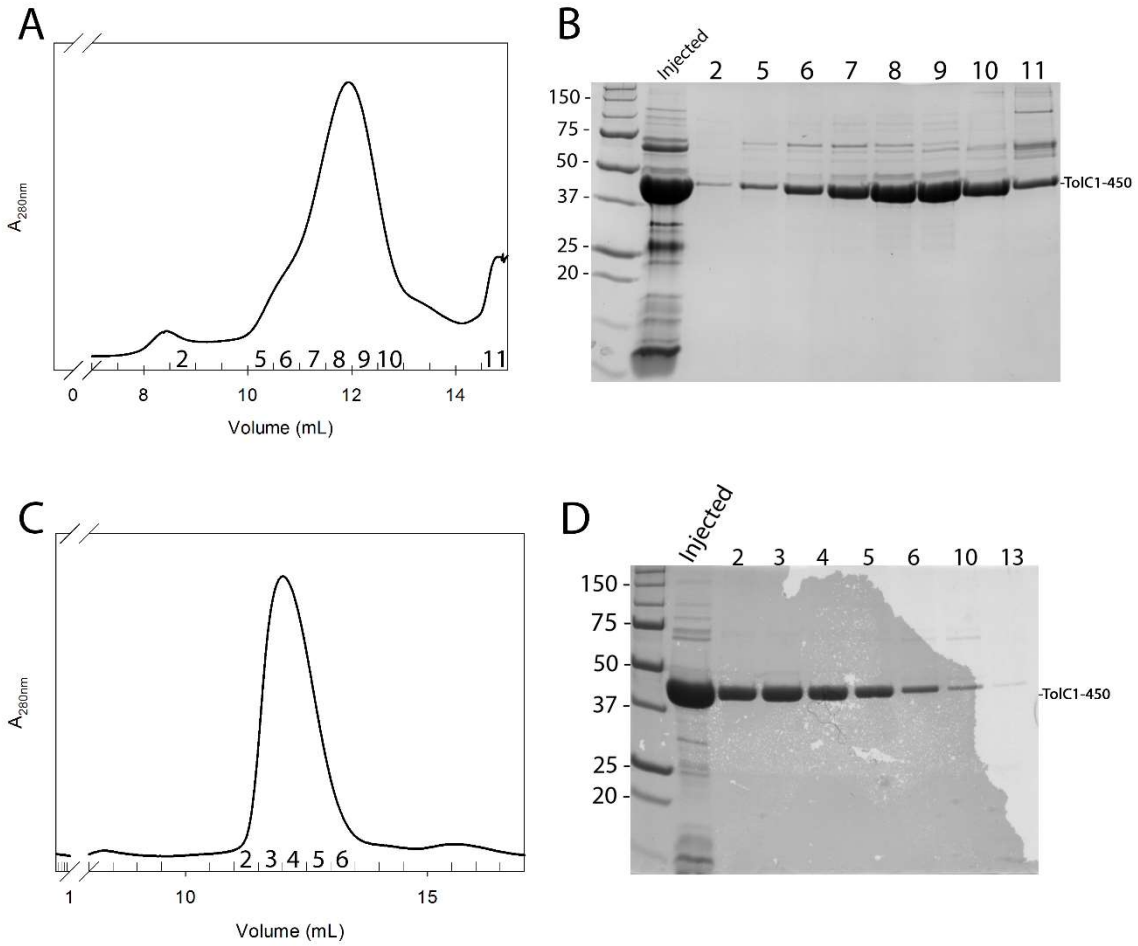

Figure 12 – Size exclusion chromatography (SEC) of recombinant E.coli TolC 1-450. A) *Elution profile of recombinant purified TolC 1-450 extracted with Triton X-100.* ***B)*** *SDS-PAGE analysis of the SEC eluted fractions. Injected, sample that was injected into the SEC; 2, 5,6,7,8,9,10 and 11, samples of the fractions collected in the SEC.* ***C)*** *Elution profile of recombinant purified TolC 1-450 extracted with DDM.* ***D)*** *SDS-PAGE analysis of the SEC eluted fractions. Injected, sample that was injected into the SEC; 2,3,4,5,6,10 and 13, samples of the fractions collected in the SEC. Elution volume of 12mL corresponds to the TolC homotrimeric conformation (~150kDa). TolC 1-450 with a molecular weight around 45kDa.*

Regarding the expression of TolC Epitope (282-298), it was performed in BL21 star (DE3) cells carrying the above described vector containing the TolC Epitope gene. As presented in fig. 12 it revealed good expression levels for TolC as indicated by 16kDa band. For purification of TolC Epitope we used two distinct protein affinity technologies: the first one used an affinity resin with immobilized nucleotides and the second technology with immobilized ions (IMAC). The first explores the high affinity of the carrier to the immobilized nucleotide while the second makes use of the C-terminal HisTag of the TolC fusion protein.

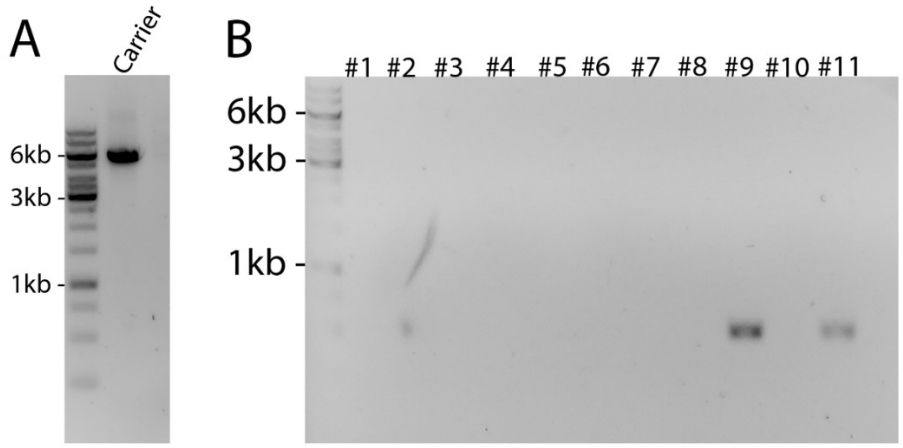

Figure 13 – Construction of carrier-TolC 282-298 plasmid. A) *Agarose gel analysis of the plasmid linearization reaction. A single band at 6kb indicating that the plasmid was successfully linearized. Carrier, sample obtained after the linearization reaction.* ***B)*** *Agarose gel with colony PCR products. After the ligation reaction, E.coli DH5α cells were transformed with the resulting product and a colony PCR was performed using a generic forward primer (T7 – that initiates before the carrier) and a specific reverse primer (the same reverse primer used to linearize the plasmid)A single band at ~500bp indicating that the TolC 282-298 was inserted in the plasmid. #1 to #11, samples of colonies from ON growth after PCR reaction.*

Briefly for the purification of TolC Epitope through the immobilized nucleotide resin, cell lysate supernatant was left incubating with the resin overnight at 4°C under gently agitation. Wash and elution of the affinity column was performed in the following day using a competing nucleotide and analyzed by SDS-PAGE (Figure 14).

This procedure yielded 3-4mg per 1L of culture with a purity grade of 90-95% (fig. 13). Noteworthy, we observed some instability and precipitation of the protein in the eluted fraction, thus we did not proceed to gel filtration analysis, to prevent column blocking issues.

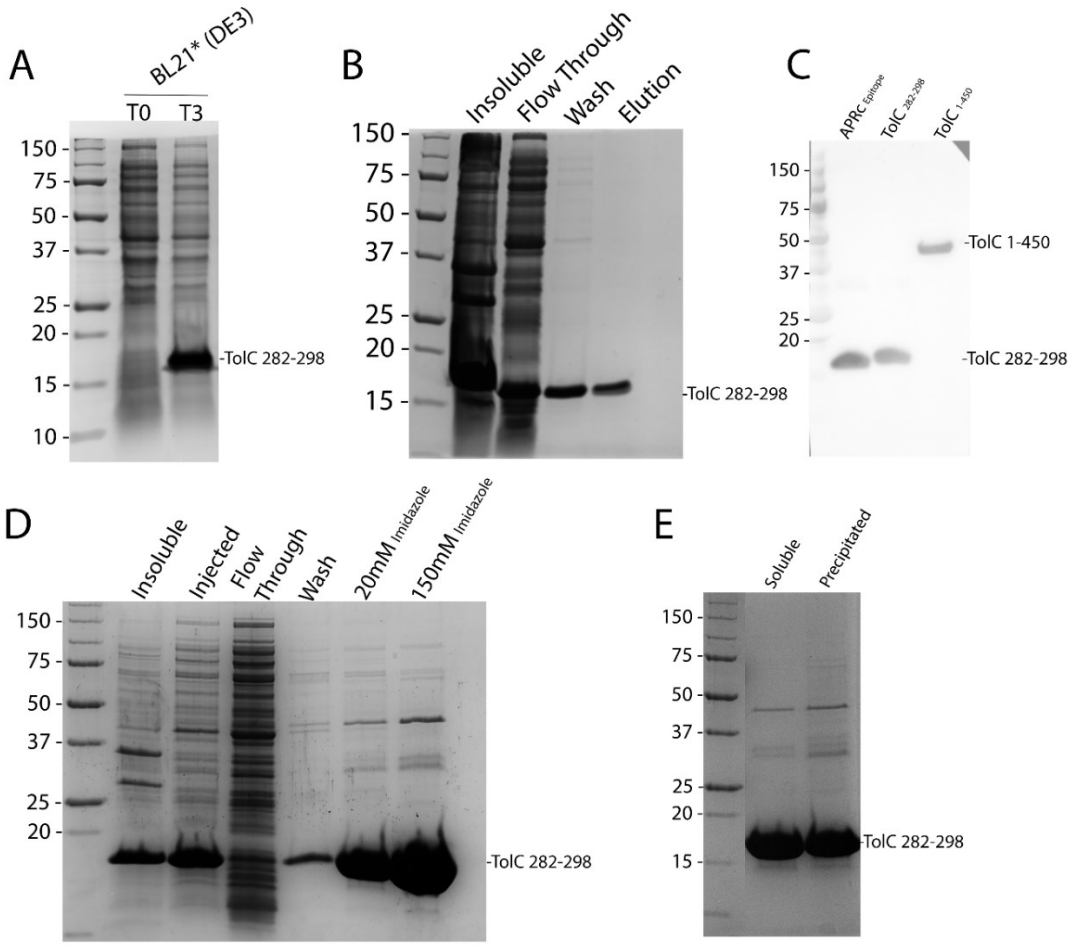

Figure 14 – Expression and Purification TolC 282-298. ***A)*** *SDS-PAGE analysis of the time course expression in BL21 star (DE3), of recombinant carrier-TolC 282-298. T0, time of expression induction; T3 time point of 3h of expression.* ***B)*** *SDS-PAGE analysis of the samples along the purification of the TolC 282-298. Insoluble, water insoluble fraction; Flow Through, water soluble sample after being loaded into the affinity column; Wash, sample of the column wash step; Elution, TolC 282-298 purified sample.* ***C)*** *Western blot analysis validating the integrity of all produced proteins using anti-His antibody in a dilution 1:10000. APRc Epitope, sample of the purified APRc Epitope; TolC 282-298, sample of the purified TolC 282-298; TolC 1-450, sample of the purified TolC 1-450.* ***D)*** *SDS-PAGE analysis of the samples along the IMAC purification of the TolC 282-298. Insoluble, water insoluble fraction; Injected, soluble sample loaded into the IMAC; Flow Through, soluble sample after being loaded into the IMAC; Wash, sample of the wash step of the IMAC; 20mM Imidazole and 150mM Imidazole, samples corresponding to the elution with 20mM and 50mM Imidazole respectively.* ***E)*** *SDS-PAGE analysis of purified TolC 282-298 with IMAC after dialysis. Soluble, sample of the fraction that remained soluble after dialysis; Precipitated, sample of the precipitated fraction after dialysis solubilized with guanidine. TolC 1-450 with a molecular weight around 45kDa; TolC 282-298 and APRc Epitope with a molecular weight of ~18kDa.*

Since the carrier-TolC fusion had also a 6 His Tag in the C-terminus (TABLE 6), purification was also possible through this affinity tag. We used a standard IMAC procedure which resulted in a final yield of 60mg per 1L of culture, though it also revealed a very unstable behavior as shown by protein precipitation. Nevertheless we tried to stabilize the purified fraction through overnight dialysis by reducing the high osmolality

of the solution due to the presence of 150mM imidazole. The following day, the dialyzed sample was centrifuged, and pellet and supernatant analyzed by SDS-PAGE (Figure 14). Consistent with this analysis, protein concentration measurements revealed that half the protein purified through the IMAC column dropped of solution. Therefore, we did not proceed to gel filtration analysis.

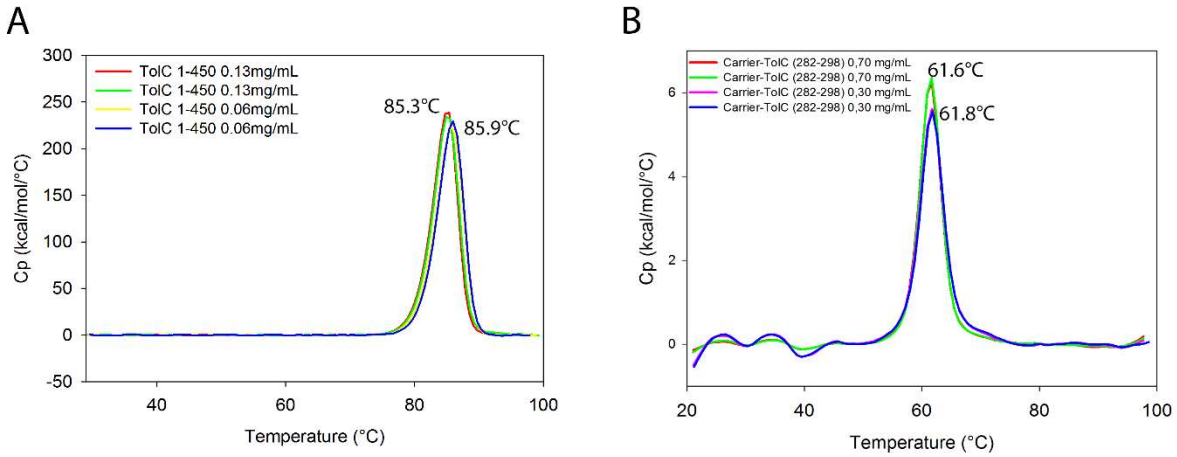

Figure 15 - Differential scanning calorimetry (DSC) analysis of the recombinant produced TolC 1-450 and TolC 282-298. A) *DSC analysis of E.coli TolC 1-450 analyzed in duplicate revealing a consistent Tm of 85.3-85.9°C.* ***B)*** *DSC analysis of carrier-TolC 282-298 analyzed in duplicate revealing a consistent Tm of 61.6-61.8°C. Both profiles reveal the stability and robustness of the purified proteins.*

Generation of hyperimmune birds

Purified TolC immunogens (TolC 23-450, TolC 1-450 and TolC 282-298) were used to immunize experimental laying quails (*Coturnix japonica*).

For each protocol a group of 3 birds was allocated; 100µg of immunogen were used per bird per injection with one immunization followed by 3 boosts every 14 days.

Bird welfare along the immunization protocol was essentially monitored by two parameters: bird body weight and egg laying capacity (Figure 16) with the last being a well described parameter reflecting the fitness of birds.

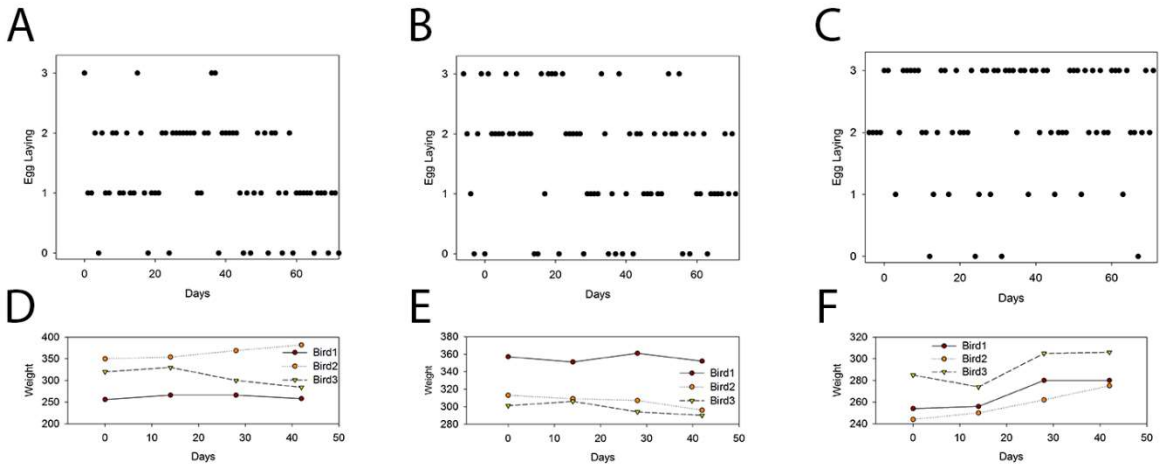

Figure 16 – Birds welfare along the immunization process. *Laying gg capacity of birds immunized with **A)** TolC 23-450, **B)** TolC 1-450 and **C)** TolC 282-298. Dots represent the number of eggs laid each day along the process. Birds weight monitored along the process for the immunization protocols with **D)** TolC 23-450, **E)** TolC 1-450 and **F)** TolC 282-298. Measurement made in the immunization days (D0, D14, D28 and D42).*

As shown in Figure 16, the egg laying capacity of the birds used in the protocols was quite different. Since all of them used 3 birds, we expected an average of 3 eggs per day. Indeed we obtained this average for the TolC 282-298 protocol but a lower average of 2 eggs per day for the TolC 23-450 and TolC 1-450 protocols.

Bird weight remained relatively stable during the protocols, indicating that the immunizations did not significantly altered quails welfare.

To test the antigen-specific response developed by the hyperimmune birds, samples of laid eggs were prepared and analyzed by ELISA. For this, 96-well plates were coated with 0.3µg of TolC 1-450 per well and serial dilutions of the water soluble protein fraction (WSPF) of each sample were analyzed (Figure 17).

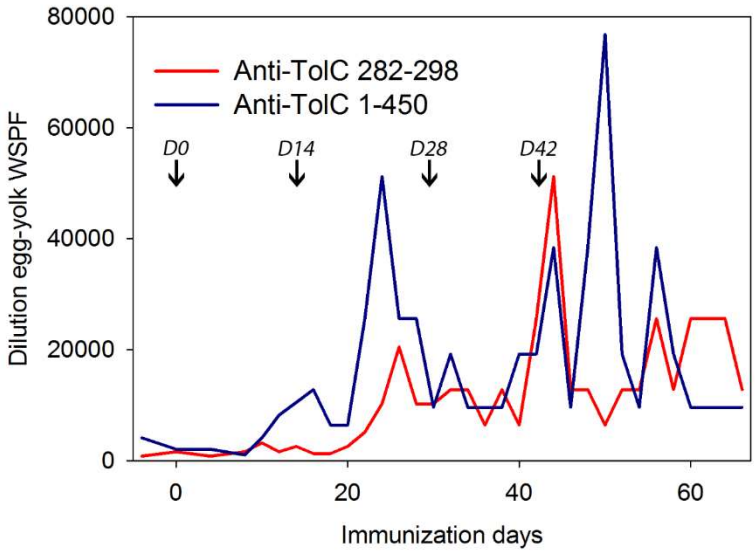

Figure 17 - Monitoring Anti-TolC titer in hyperimmune eggs. *Titers obtained for independent immunization procedures using either TolC 1-450 (blue) or TolC 282-298 (red) are shown. High antibody titers are obtained between day-40 and day-60 of the procedures. Black arrows indicate different rounds of immunization/boosts.*

The titer plots obtained for each immunization procedure presented the 2 peak profile expected for standard antigen-specific response (Figure 17): a first peak between day 20 and 30 (between the 2nd and the 3rd immunization); and a second peak of higher titer between the day 40 and 60. A plateau of high titer is also maintained at the end of each procedure, revealing a persistent TolC-specific hyperimmune response.

Taken into account this titer profile, 2 pools of egg yolk were made per immunization protocol: for TolC1-450 protocol pool 1 – Days22-38 and pool 2 – Days40-58; and for TolC Epitope protocol pool 1 – Day24-40 and pool 2 – Day42-64. These egg yolk pools were stored at 4°C until further antibody purification and characterization steps.

Purification of anti-TolC antibodies

Standard purification method

Standard purification of antibodies from egg yolk, followed a protocol already implemented in the laboratory.

Typical purification of egg yolk IgY antibodies is made in two steps, a water dilution step and a PEG precipitation step. The first is intended to precipitate the lipoproteins abundant in the egg yolk while the second intends to selective precipitate the IgY content. As expected, most of the contaminants present in the egg yolk were removed by this procedure as shown in the SDS-PAGE analysis (Figure 18). Indeed, as clear in Figure 18, the PEG precipitation sample is enriched in IgY (Heavy chain 68kDa band and Light chain 25kDa band) although not presenting a desired purity grade.

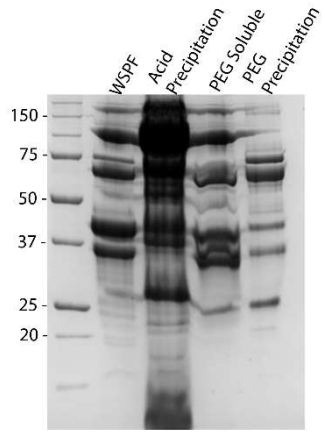

Figure 18 - Purification steps of standard antibody purification. *WSPF, water soluble protein fraction obtained from egg yolk; Acid precipitation, insoluble fraction of egg yolk; PEG Soluble and PEG precipitation, Soluble and insoluble fraction obtained from PEG precipitation respectively.*

Therefore, in order to improve the IgY sample purity for downstream characterization we performed an additional purification step using a size exclusion chromatography (SEC) column Thus, PEG precipitation sample was ressuspended and injected into a preparative Superdex 200 26/600 column. As shown in Figure 19, SEC elution profile and corresponding SDS-PAGE analysis demonstrated an effective isolation of the IgY from other protein contaminants. IgY elutes at 165mL while other lower molecular weight contaminants eluting in separate peaks.

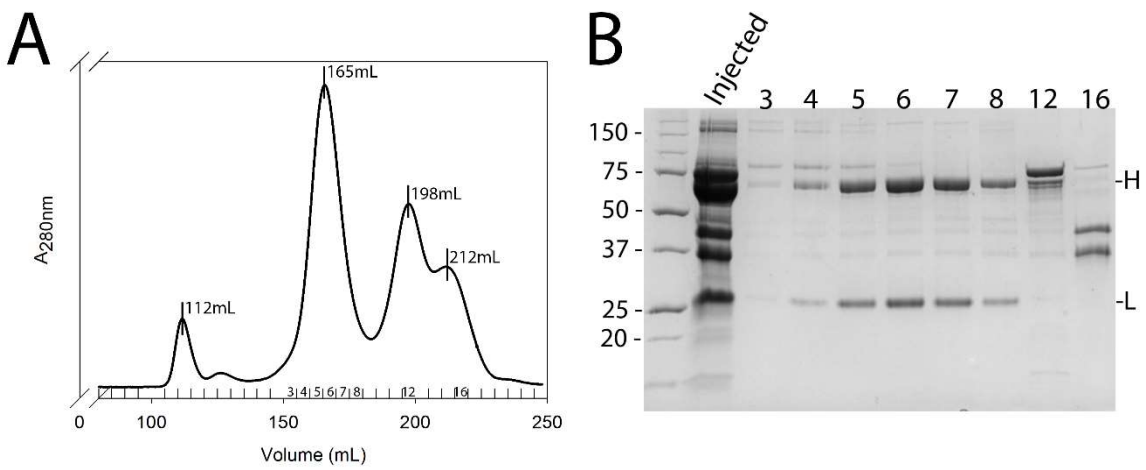

Figure 19 –Standard purification of IgY anti-TolC (1-450) antibody. A) *Size exclusion chromatography (SEC) profile of anti-TolC (1-450). The antibody elutes at around 165ml in a preparative SEC column.* ***B)*** *SDS-PAGE analysis of the SEC fractions; Injected, sample of the fraction that was injected into the SEC; 3,4,5,6,7,8,12 and 16 samples of the fractions obtained in the SEC. H, indicates antibody heavy chain (68KDa) and L, indicates antibody light chain (25kDa).*

Purification process described above was used for both anti-TolC 1-450 and anti-TolC Epitope with the yield of each procedure reported in Table 7.

Table 7 *- Yield of the anti-TolC 1-450 and anti-TolC 282-298 antibody purification methods.*

Antibody	Purification method	Yield (mg)
Anti-TolC 1-450	Standard	3.10
Anti-TolC 1-450	Immunogen-affinity	0.075
Anti-TolC 282-298	Standard	6.45

Immunogen-affinity purification method

We also used an immunogen-affinity purification method to enrich the anti-TolC fraction able to specifically recognize the extracellular TolC Epitope. For this purpose we immobilized the carrier-TolC Epitope fusion protein on a cyanogen bromide (CNBr) column and recovered the anti-epitope specific fraction from a WSPF of TolC 1-450 protocol. Eluted fraction of CNBr column presented a single peak highly enriched in IgY as shown in SDS-PAGE analysis (Figure 20). Nevertheless, due to the presence of small amount of contaminants (Figure 20C)) we proceed with a purification refinement using SEC.

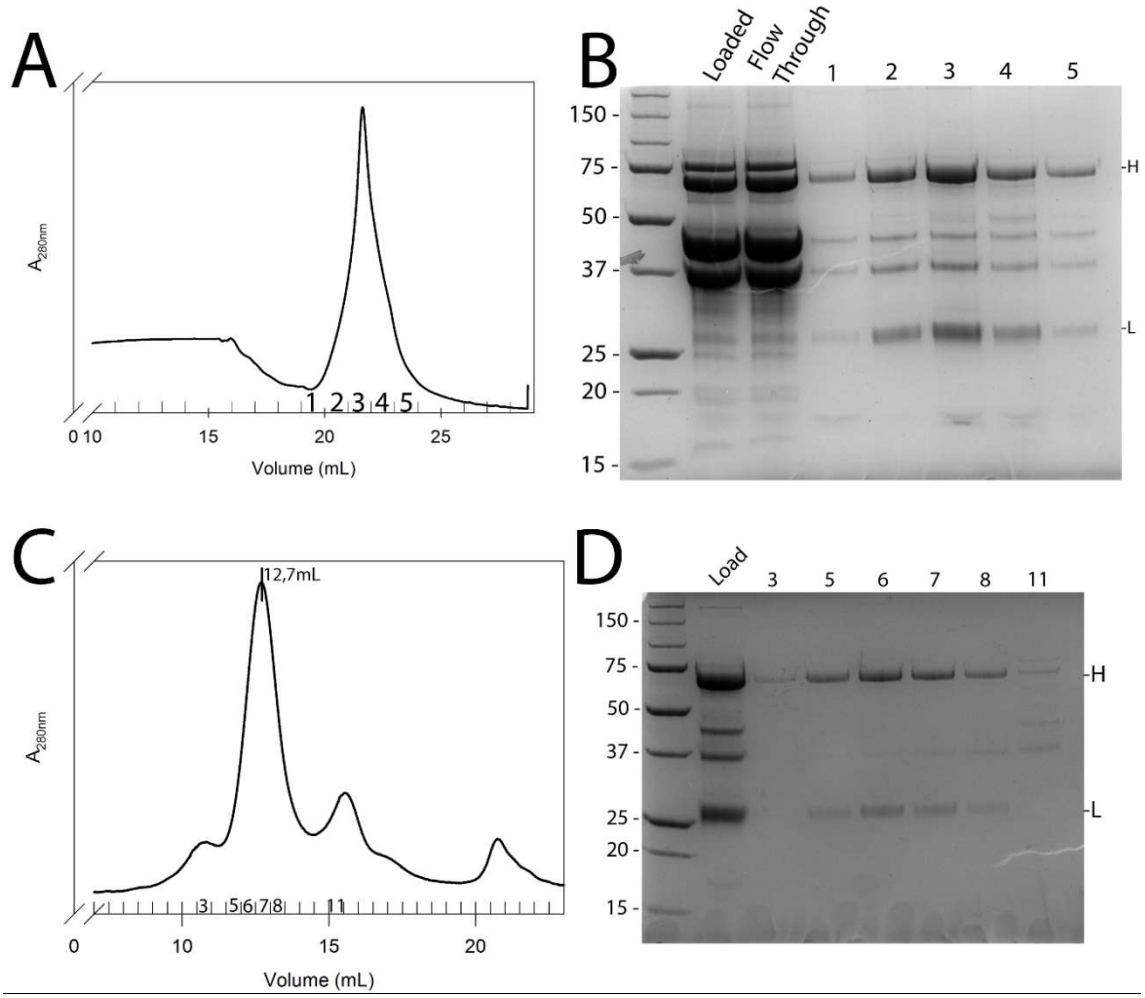

Figure 20 – Immunogen-affinity purification of IgY anti-TolC (1-450) antibody. A) *Anti-TolC antibody purified using a CNBr affinity column, prepared with immobilized recombinant E.coli TolC epitope 282-298.* ***B)*** *SDS-PAGE analysis of the fraction obtained in the purification step. Loaded, sample of the WSPF loaded into the CNBr affinity column; Flow Through, sample of the WSPF after being loaded into the CNBr affinity column; 1,2,3,4 and 5, sample of the fractions collected in the affinity purification.* ***C)*** *Size exclusion chromatography profile of the immunogen-purified anti-TolC antibody.* ***D)*** *SDS-PAGE analysis of the collected SEC fractions. Load, sample of the fraction that was injected in the SEC; 3, 5,6,7,8 and 11, samples of the eluted fractions obtained in SEC. H, indicates antibody heavy chain (68KDa) and L, indicates antibody light chain (25kDa).*

As expected, the elution volume of the antibody was at 12,5mL and nearly all the contaminants were removed from the sample, achieving a purity grade over 95%. Fractions containing the IgY were pooled together and concentrated for further characterization (Table 7).

Characterization of Anti-TolC antibodies

Anti-TolC antibodies produced and purified as described in the previous sections were further characterized in terms of target-specificity and reactivity by western blot and ELISA assays.

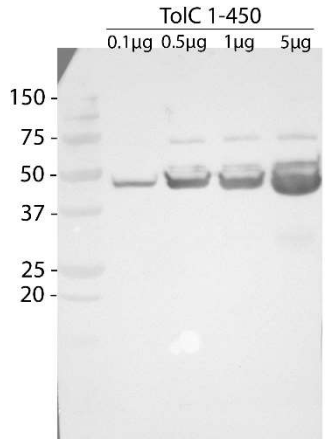

Figure 21 - Western blot validation of produced anti-TolC 1-450 *through standard purification in a dilution of 1:10000. Lanes containing crescent amounts of recombinant purified TolC 1-450.*

ELISA titer evaluation from the previously described produced antibodies (Table 7) revealed the highest measurable dilution for the antibodies was ~1:160000 for the, ~1:64000 anti-TolC 1-450 immunogen-affinity purified and ~1:96000 for the anti-TolC 282-298 standard purified (Figure 22).

Therefore western blots analysis of anti-TolC 1-450 standard purified revealed very high sensitivity being able to detect 0.1µg of the recombinant purified TolC 1-450 (Figure 22).

Western-Blot analysis also validated specificity of the produced antibodies against both recombinant TolC forms, using as negative control a rickettsial protease epitope (APRc) showing no reactivity (Figure 22). Note that the signal obtained for the APRc control with the Anti-TolC epitope 282-298 results from the fact that both epitopes were fused to the same carrier protein for expression purposes which could not be detected by both antibodies produced against TolC 1-450.

***Table 7** - Yield of the anti-TolC 1-450 and anti-TolC 282-298 antibody purification methods.*

Antibody	Starting antibody concentration (mg/mL)	ELISA dilution	Effective concentration to detect 0,5µg TolC (ng/mL)	Relative reactivity
Anti-TolC 1-450 standard purified	3,88	1:160000	24,25	1
Anti-TolC 1-450 immunogen-affinity purified	0,50	1:64000	7,81	3,22
Anti-TolC 282-298 standard purified	4,30	1:96000	44,79	0,54

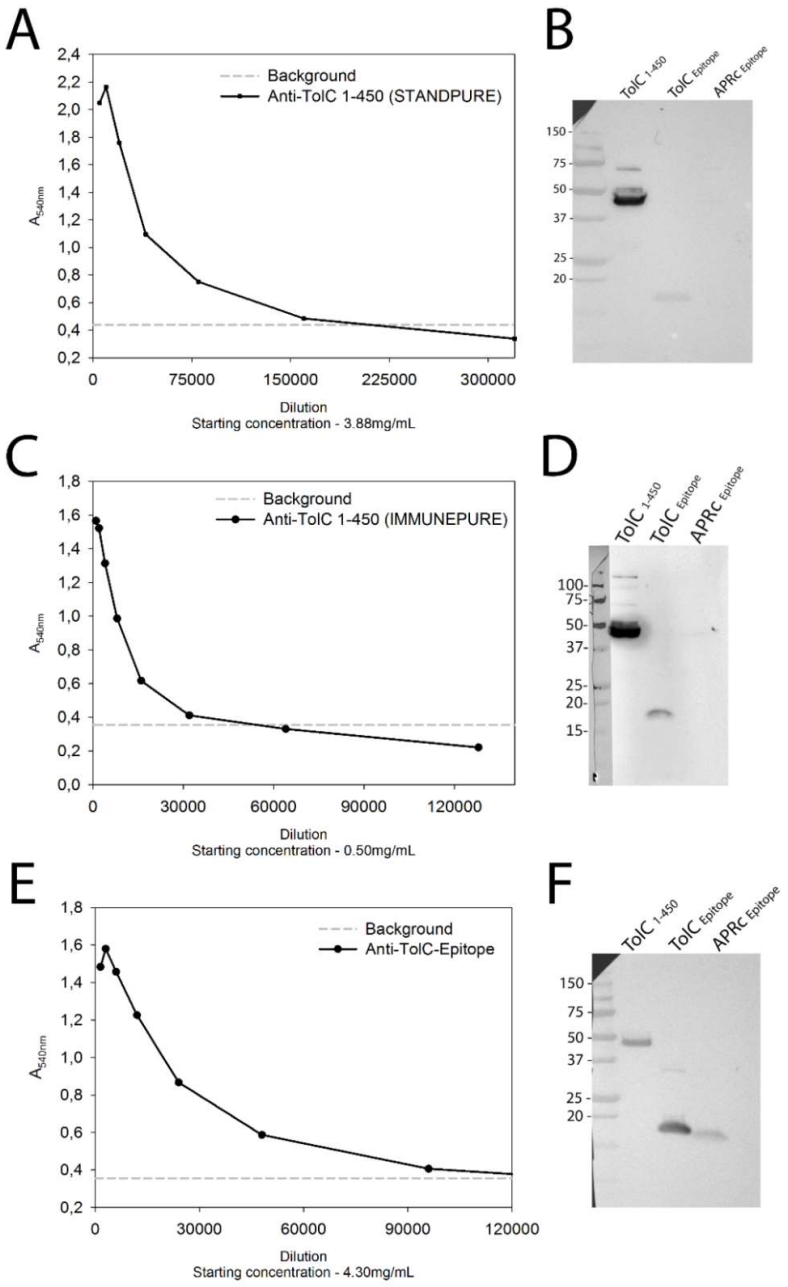

*Figure 22 – **Target-specificity and reactivity of purified anti-TolC antibodies.** **A)** ELISA titer evaluation for Anti-TolC 1-450 purified by standard method. **B)** Western-Blot analysis validating specificity of the Anti-TolC 1-450 standard purified against both recombinant TolC forms, along with the control APRc Epitope. WB was performed using a 1:10000 dilution of the antibody. **C)** ELISA titer evaluation for Anti-TolC 1-450 purified by immunogen-affinity method. **D)** Western-Blot analysis validating specificity of the Anti-TolC 1-450 immunogen-affinity purified against both recombinant TolC forms, along with the control APRc Epitope. WB was performed using a 1:2000 dilution of the antibody. **E)** ELISA titer evaluation for Anti-TolC 282-298 purified by standard method. **F)** Western-Blot analysis validating specificity of the Anti-TolC 282-298 standard purified against both recombinant TolC forms, along with the control APRc Epitope. WB was performed using a 1:1000 dilution of the antibody.*

Discussion

The present work intended to raise antibodies that would bind to the TolC channel and block or modulate its extrusion activity. This well described mechanism is responsible for keeping low concentrations of antimicrobial drugs inside resistant bacteria. Thus it was necessary to rationalize a structure-based approach capable of impairing the extrusion activity across the channel. For this we used the available crystal structures of *E.coli* TolC channel, namely the ones deposited in the Protein Data Bank with the PDB codes 1TQQ and 2VDD. This structures allowed us to identify surface-exposed regions susceptible of antibody targeting. Indeed as clear in Figure 23, TolC presents an extracellular region composed by a 3-fold arrangement of a 17 residues loop. Therefore, we decided to design two different immunogens to mimic this region: 1) one based on a linear epitope and 2) a second one based on the native homotrimeric structure of the channel. Both approaches will allow not only the generation of antibodies that recognize the extracellular region (1) but also its complex structural arrangement (2).

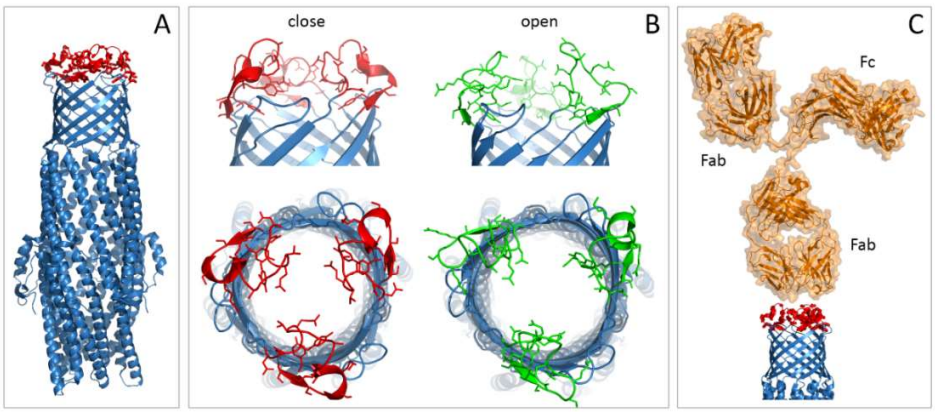

Figure 23 *– E.coli TolC structures A) Resolved E.coli TolC closed structure with extracellular loop colored red. B) Lateral and extracellular view comparing the open and closed state of the E.coli TolC structure. PDB codes 1TQQ and 2VDD repectively. C) Modeling the overall binding of a mammalian IgG immunoglobulin (PDB code: 1IGT) to E.coli TolC channel.*

For the native *E.coli* TolC we worked with two recombinant forms for production purposes: a full-length form (residues 1-450) and a truncated form (residues 23-450) lacking the N-terminal signal peptide that directs the protein for membrane insertion. The last one, had been previously tested in the laboratory, and showed good levels of protein overexpression though in insoluble form (inclusion bodies). For this reason we decided to follow a well described procedure for immunization purposes, involving protein extraction from polyacrylamide gels. Indeed this allows the production of antibodies

against linear epitopes of the protein immunogen but, given the fact that this gets denatured during SDS-PAGE, structural antibodies are most likely lost. Our results indicate that gel extraction was not very efficient, with a good fraction of protein remaining in the acrylamide gel after processing, thus compromising the available amount for downstream immunizations. This was reflected on the corresponding ELISA assays, with very low anti-TolC signal being obtained for this immunogen as shown in annex 1.

Expression and purification of the other *E.coli* TolC full-length form (residues 1-450) available in the laboratory, was implemented in the context of the present work. In contrast with the previous one, overexpressing this form allowed us to obtain a detergent soluble fraction of *E.coli* TolC conformation with much less formation of inclusion bodies. Protein extraction from the membranes was performed with two different detergents in independent experiments: DDM (n-Dodecyl β-D-maltoside) and Triton X-100. Both detergents allowed similar extraction yields but we observed significant differences at the level of purity. Size exclusion purified samples from DDM procedure were further characterized by DSC. A single peak melting profile with a transition temperature at 85°C revealed a high robustness and stability of the full-length TolC immunogen. The amount of TolC immunogen obtained were sufficient for the purpose of this work. Nevertheless results obtained in parallel using an homologous TolC from cyanobacteria showed improvements of protein production yield by lowering the overexpression temperature to 20°C and increasing the $O.D_{600}$ of induction to 1.0. These optimization are likely to work for also *E.coli* TolC and should be considered for further protein preparations.

For the design of the linear epitope immunogen, we looked for optimal way to present the 17 residues loop present in the extracellular domain of the *E.coli* TolC channel. Therefore, we decided to use a proprietary carrier developed at IBMC (Porto, Portugal) and evaluate the immunogenic capacity of the fusion carrier-TolC 282-298. For this we used a SLIC cloning method as described in the methods section to obtain a carrier-TolC 282-298 fusion as well as a carrier-APRc presenting a Rickettsia epitope for control purposes. Both were generated with a C-terminal 6xHis tag that allowed to monitor potential truncated expression or loss of target epitope by protease cleavage. In this line, our results clearly indicate that both carrier-TolC and control fusions can be expressed and present the full target epitope as conformed by western blot analysis using an anti-His antibody.

Purification of the carrier-TolC 282-298 fusion was performed taking advantage of two features: 1) the 6x His tag presented in the C-terminal and 2) the presence of a carrier with high affinity to nucleotides. The first one allowed the purification with an IMAC which yielded a very good amount of protein however it does required another step of purification. On the other hand, the presence of the carrier allowed the purification with a commercial resin with immobilized nucleotides which resulted in lower yields but with a significantly better purity grade, strikingly not requiring any further purification step. Samples purified with the resin with immobilized nucleotides were further characterized by DSC. A single peak melting profile with a transition temperature at 62°C revealed a high robustness and stability as previously described for this carrier indicating that the length and residue composition of the TolC epitope did not significantly compromised the stability of the carrier.

Obtaining highly pure immunogens allowed us to move forward towards the immunization protocol. ELISA titers of the produced hyperimmune eggs revealed good TolC-specificity for both immunization protocols. Since we were targeting a bacterial protein, specificity of the produced antibodies could have been increased if we utilized adjuvants free of bacterial contents nevertheless, we did not observe cross-reactivity issues with bacterial contaminants namely with western blot assays.

Antibodies were successfully purified from egg yolk with two different methods: 1) a standard antibody purification intended to purify the whole bulk of IgY presented in the egg yolk, and 2) an immunogen-affinity purification intended to purify only the fraction that is specific to the extracellular domain of TolC channel.

Regarding the standard purification yields shown in Table 7, we believe that the discrepancy in results was due to the time that passed since the egg laying until their processing, which was higher for the eggs from the TolC 1-450 protocol. This aging of the eggs stored at room temperature before processing, could have compromised the yield of the process.

On the other hand, immunogen-affinity purification of the anti-TolC 1-450, showed very good yielding. Since this protocol made use of the egg yolks from the TolC 1-450 protocol, and the immunogen used for the purification was the TolC 282-298, this

result revealed not only the good immunogenicity of the extracellular domain of this protein but also the good efficiency of this type of purification.

Anti-TolC antibodies produced and purified as previously described were further characterized in terms of target-specificity and reactivity by western blot and ELISA assays.

Western blot analysis clearly revealed the specificity of the produced antibodies. Both anti-TolC 1-450 did not recognize the control carrier-APRc thus confirming their specificity. Moreover, anti-TolC 1-450 was able to recognize the carrier-TolC 282-298 fusion indicating that a fraction of the polyclonal antibodies produced is able to recognize the extracellular epitope. On the other hand, anti-TolC 282-298 were also able to recognize the full-length protein indicating that the designed strategy based on the linear epitope was also successful for the targeting of the extracellular

ELISA characterization of the produced antibodies revealed good reactivity against the trimeric form of TolC. Normalizing these results we obtained the effective concentration used to detect the TolC: 24,25ng/mL for the anti-TolC 1-450 standard purified; 7,81ng/mL for the anti-TolC 1-450 immuno-affinity purified; and 44,79 ng/mL for the anti-TolC 282-298 standard purified. This result revealed that the immunogen-affinity purification was able to enrich the fraction that specifically bind to the TolC channel.

Concluding, in this work we were able to efficiently express, purify and characterize three forms of *E.coli* TolC channel. The purified proteins, were then used as immunogens to produce IgY antibodies in a newly experimental facility of antibody production in quails. Produced antibodies were purified from egg yolk which revealed good target-specificity against the *E.coli* TolC channel.

This work confirmed that the *E.coli* TolC is suitable for immunotargeting particularly the extracellular region of the channel.

Future perspectives

Produced anti-TolC antibodies revealed good specific-activity against the recombinant *E.coli* TolC channel. Nevertheless, it is imperative to further characterize these antibodies from a functional perspective, meaning to evaluate and characterize their capacity to modulate the extrusion activity in assays using the native TolC system. Moreover, immunization protocols generated hyperimmune birds which could be used as a starting point for the production of monoclonal antibodies in the future. Those birds contain an immunological repertoire against the *E.coli* TolC channel from where we can extract the genetic information that codifies for specific antibodies that bind the extracellular domain of the TolC channel. Identifying the sequence of such monoclonal antibodies would allow to further analyze the structural basis of their immuno-blocking activity for example by crystallographic studies Fab fragments (antigen-binding arms of the antibody). Indeed, as shown in Figure 23, antibody binding to the extracellular region of TolC channel, will most likely occur through a single Fab arm given the relative overall dimension of the antibody when compared to the channel. Functional assays will also help to further characterize these Fab fragments, identifying those capable of binding to the extracellular region and impair efflux. Ultimately the binding and blocking properties of these antibodies/fragments could be improved by protein engineering approaches, resulting in potential therapeutic candidates to fight antimicrobial resistance [62].

The immunotargeting approach explored herein against *E.coli* TolC channel is likely to be reproduced against other major Gram-negative bacterial strains involved in life-threating infections. If proven to be effective such specific antibodies could become powerful adjuvants to be used in combination with antibiotics increasing their therapeutic or preventive potency to combat multidrug resistance.

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Annexes

Annex 1 – Anti-TolC 23-450 WSPF titer.

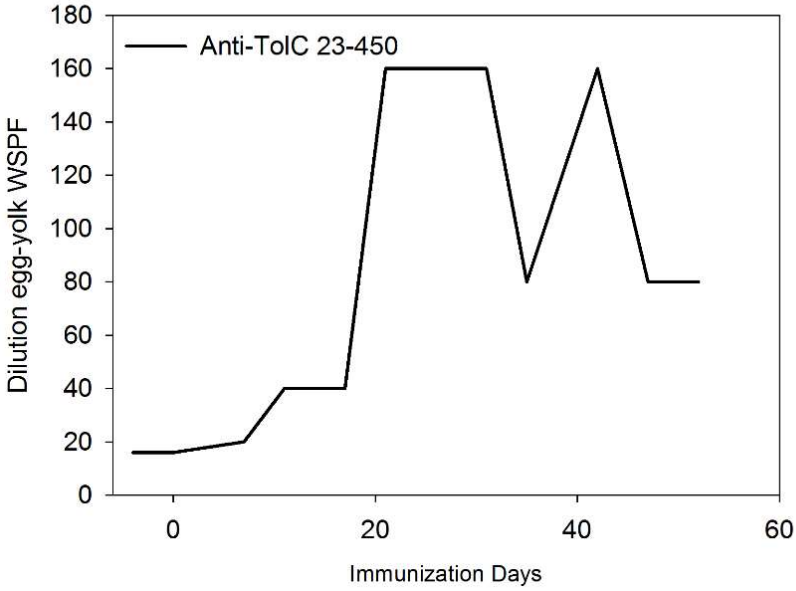

Figure 24 –Monitoring Anti-TolC titer in hyperimmune eggs. *Titers obtained for immunization procedure using TolC 23-450. Low antibody titer were obtained, thus no antibody purification was performed.*