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Identification of new *P. aeruginosa* antigens and characterization of the CdrA adhesin involved in biofilm formation.

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List of Abbreviations

ADPRT	ADP-ribosyltransferase activity
AECOPD	Acute exacerbation of chronic obstructive pulmonary disease
AMPs	Antimicrobial peptides
ASL	Airway surface fluid
BCC	Biotin Carboxyl Carrier
c-di-GMP	Guanosine monophosphate bis-(30-50)-cyclic dimer
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
COPD	Chronic obstructive pulmonary disease
CV	Crystal Violet
eDNA	Extracellular DNA
EPS	Exopolysaccharides
E. Coli	Escherichia coli
FACS	Fluorescence activated cell sorting
FCM	Flow cytometry
FT	Flow-through
IMAC	Immobilized Metal Affinity Chromatography
IQS	Integrated quorum sensing
M	Marker
MDR	Multi drug resistant
MFI	Mean fluorescence intensity
MV	Membrane vesicles
nanoDSF	Scanning nano fluorimetry
Ni2+	Nickel
NTA	Ni-nitrilotriacetic acid
OD	Optical density
Opr	Outer membrane protein
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PDB	Protein data bank
PI	Pre-immune
PLA2	Phospholipase A2

PQS	Pseudomonas Quinolone Signal
P. aeruginosa	Pseudomonas aeruginosa
QS	Quorum Sensing
REP	Repetitive extragenic palindromic
RhoGAP	Rho GTPase activating protein
RU	Resonance unit
SDS-PAGE	Sulphate-poly-acrylamide gel electrophoresis
SPR	Surface Plasmon Resonance
SPR	Surface plasmon resonance spectroscopy
T3SS	Type 3 secretion system
Tm	Melting temperature
TPS	Two-partner secretion system
UK	University Hospital Southampton
VAP	Ventilator-associated pneumonia
WHO	The World Health Organization

INTRODUCTION

1.1 Pseudomonas aeruginosa: history and pathogenesis

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium belonging to the family [1] that normally inhabits the soil and surfaces in aqueous environments but due to its adaptability and high intrinsic antibiotic resistance is able to survive in a wide range of other natural and artificial settings, including surfaces in medical facilities. The bacterium genome is relatively large compared to other sequenced bacteria and encodes a large proportion of factors for metabolism, transportation and efflux of organic compounds. Within the species, the genome size varies between 5.5 and 7 Mbp [2, 3]. This enhanced coding capability of the *P. aeruginosa* genome allows for great metabolic versatility and high adaptability to environmental changes [4, 5]. The genome is composed of a 'core' part, which is found in all *P. aeruginosa* strains which includes all conserved essential genes and of the accessory genome composed by genes varying from strain to strain that generate a wide diversity. This diversity in the accessory genome causes divergences in genome size within species. [6, 7]. The accessory genome is characterized by extrachromosomal elements such as blocks of DNA inserted into the chromosome at various loci and plasmids. The acquisition of these elements, which may be present in various subgroups of the *P. aeruginosa* population as well as in individual strains, appears to occur through horizontal gene transfer from various sources, including other species or genera [8]. Most of the intra- and inter-species genomic diversity in *P. aeruginosa* is therefore dependent on the different composition of the accessory genome. After integration into the host chromosome, they appear as 'foreign' building blocks in the core genome. Therefore, a *P. aeruginosa* chromosome is often described as a mosaic structure of conserved core genome frequently interrupted by the inserted parts of the accessory genome. The continuous acquisition of new foreign DNA, as well as larger or smaller deletion events, single nucleotide mutations and even chromosome inversions, [9-13], all of which have the potential to affect parts of the nucleus and/or accessory genome, continually modify the genome by modulating the phenotype of the *P. aeruginosa* strain and differentiating it from others [2]. It has also been found that the *P. aeruginosa* genome is characterized by mobile genetic elements, which leads to a significant variability in gene location, identity and function, even within strains of a given species. In some bacteria are present also the repetitive extragenic palindromic (REP) elements which are DNA sequences with different length, between 21 and 65 bp, that

are found in extragenic regions [14]. Due to its relatively large genome and flexible metabolic capabilities that this organism is able to exploit different environmental conditions in which to grow and to evade normal immune defenses [15]. *P. aeruginosa* is one of the most common hospital pathogens and is the second most common pathogen detected in patients with ventilator-associated pneumonia (VAP) [16]. The main causes of the high incidence of *P. aeruginosa* in healthcare institutions include the poor health status of patients, the high rate of spread of often multi-resistant strains in hospital wards, and the overuse of broad-spectrum antibiotics [17]. The stages through which the bacterium induces infection in patients are: bacterial attachment and colonization, followed by local invasion and finally spread and systemic disease [18]. Severe *P. aeruginosa* infections are often nosocomial, and almost all are associated with compromised host defenses, such as in neutropenia, severe burns or cystic fibrosis [19]. The pathogen transmission can also occur in the community setting, although rarely, and in people with an underlying immunity defect [20]. Hospitalized patients infected with the bacterium present respiratory diseases, which are often classified into two types, acute or chronic.

1.1.1 Acute lung infections

Usually, acute nosocomial pneumonias are caused by direct trauma, such as damage to the epithelium due to intubation or smoke inhalation [21]. In patients with VAP, insertion of the endotracheal tube, which can be a reservoir for *P. aeruginosa*, often induces epithelium rupture inducing an increased susceptibility to the development of an acute lung infection. This type of respiratory infection can also occur in those who are unable to mount the right immune response to the pathogen, leading to a greater susceptibility to infection, such as in people with advanced age, neutropenia due to chemotherapy, or immunosuppression due to organ transplantation [22].

1.1.2 Chronic lung infections

A chronic infection is the result of an underlying medical condition that does not allow for an effective immune response, such as in the elderly or in individuals with cystic fibrosis (CF) [21]. Indeed, if *P. aeruginosa* is not eradicated during the acute infection phase it can establish itself in the lung environment, leading to the formation of biofilm [23], i.e. a complex aggregate of bacteria enclosed in a self-generated matrix of extracellular polymeric

substances (EPS), resulting in chronic infection. As mentioned above, the target population most affected is the elderly or individuals with CF. The latter usually develop a Pseudomonas lung infection within adolescence and may live with the infection for 20 years or more. The cystic fibrosis disease is caused by mutations in the chromosome 7 encoding a 1480 amino acid polypeptide, named cystic fibrosis transmembrane regulator (CFTR), which functions as a chloride channel in epithelial membranes [23-26]. Mutation of this channel results in the formation of a dehydrated and thickened airway surface fluid (ASL) that impairs mucociliary clearance from the conducting airways. The inhaled bacteria settle into the altered ASL and cause an initial acute infection and vigorous inflammatory response which is compromised by the thickened ASL. At this point, it is the persistent immunological stimulation by the bacteria and/or the host's inability to control the inflammation that causes chronic lung inflammation [22, 27, 28].

1.2 Bacterium virulence factors

Current therapies, including antibiotics that are used to kill or inhibit the growth of this bacterium [29], have been revealed to be ineffective in fact they have been associated with unacceptably high rates of morbidity and mortality. The development of therapeutic agents capable of antagonizing virulence factors is one of the potentially useful approaches for the treatment of serious infections caused by this bacterium. The severity and type of infection that can be induced by the bacterium are closely related to the interaction between the pathogen's virulence factors and the host's immune response.

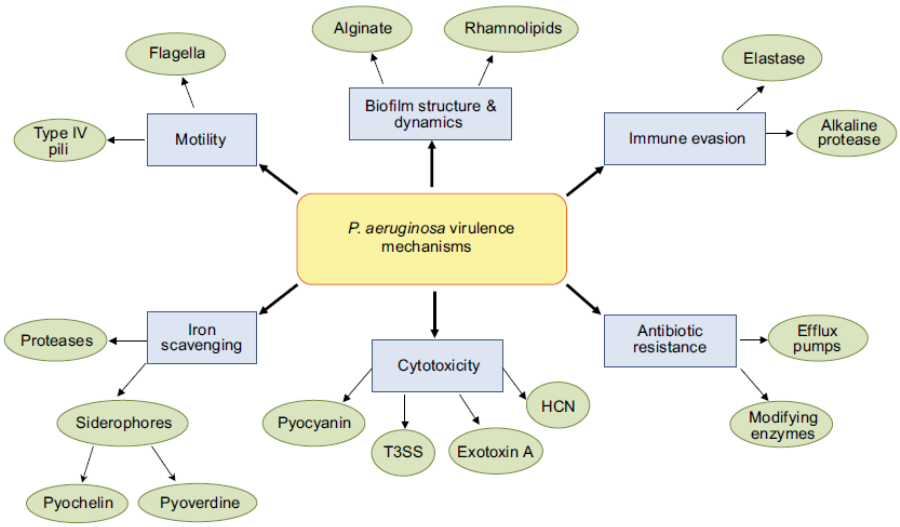


Fig 1: Virulence mechanisms involved in *P. aeruginosa* infections [30].

The outcome of *P. aeruginosa* infections is dependent on virulence determinants such as flagella, pili and lipopolysaccharides, as well as active processes such as toxin secretion, biofilm formation and quorum sensing [27, 31-33]. *P. aeruginosa* has a large number of virulence factors (Table 1, Table 2), here I will focus on four of them, which are the most representative during infections: flagella and type 4 pili, type III secretion system, quorum sensing and biofilm formation.

Table 1: *P. aeruginosa* virulence factors [34].

Cell surface virulence factors	**Biological function**
Lipopolysaccharide (LPS)	Dominant antigenic determinant on cell surface; loss of sugar unit side chains during chronic infection creates "rough" LPS and serum sensitivity
Pili or Fimbriae	Adherence to epithelium
Flagella	Tethering and adhering to epithelial cell
Alginate (mucoide strains)	Adherence to epithelium; barrier to phagocytes and antibiotics; inhibits antibody and complement binding
Secreted virulence factors	
Protease enzymes	Tissue damage; epithelial cell tight junction separation; degrade fibronectin; cleave antibodies creating non-functional blocking antibodies; inactivate α1-antiproteinase, complement components and cytokines; cleave C3b receptors from neutrophils.
Exotoxin A	Cytotoxic by inhibiting protein synthesis; toxic to macrophages; T cell mitogen; inhibits granulocyte and macrophage progenitor cell proliferation
Phospholipase C	Haemolysis; tissue damage; surfactant inactivation
Pigments (Pyocyanin, pyoverdin)	Inhibit ciliary beat; siderophores; toxic to other bacterial species and human cells; enhance oxidative metabolism of neutrophils; inhibit lymphocyte proliferation
Rhamnolipids	Haemolysis; inhibit ciliary beat; stimulate mucus secretion, affect ion transport across epithelium
Lipase	Tissue damage
Histamine	Impair epithelial integrity
Exoenzime S	Adherence to epithelium; cytotoxic
Leukocidin	Cytotoxic to neutrophils and lymphocytes

1.2.1 Flagella and type 4 pili

Each *P. aeruginosa* cell has a single polar flagellum and several shorter type 4 pili, also located at a cell pole. The *P. aeruginosa* flagellum is required for swimming motility but also plays crucial roles in biofilm dispersal and adhesion to the host cell surface [35-37]. These appendages function both as adhesins and as major mechanisms of motility and are also capable of initiating an inflammatory response [38]. Flagella present a primary structural component called Flagellin which is classified into two distinct serotypes, a and b

[39]. Furthermore, the conserved domains of this protein are strongly antigenic and capable to induce a strong NFκB-mediated inflammatory response because they act as a pathogen-associated molecular pattern (PAMP) that is recognized by the 5 Toll-like receptors on the surface of host cells. Thus, the 5 Toll-like receptor is used by the host as a surveillance mechanism to detect invading *P. aeruginosa* bacteria and in turn trigger the immune response by inducing the synthesis of cytokines such as TNF, IL-6 and IL-8 [40-42]. Non-flagellated mutants are defective in acute infection models [43, 44], whereas in chronic infections, a large proportion of isolates exhibit flagellar downregulation or are aflagellated. This indicates that flagella are essential in acute infection, whereas in chronic infection the bacterium actually downregulates flagellin expression, perhaps to evade the host immune response [45, 46]. In CF patients, flagella are considered a virulence factor necessary to induce an early stages of infection even though 40% of CF isolates do not produce them [47]. Together with flagella, pili are involved in the bacterium motility. Type 4 pili are located at one cell pole and are probably the most important adhesins in *P. aeruginosa* involved in contraction motility and biofilm formation. They allow movement of the cell along solid surfaces by a process termed "twitching motility" [31]. Pili are also involved in bacterial aggregation leading to the formation of microcolonies on target tissues, which occurs in the early stages of biofilms and offers the possibility of protecting the bacterium from the host immune system and antibiotics [48-50]. Pili-deficient mutants show reduced virulence in various models. Pili, like flagella, are therefore an interesting target for possible therapy, although the antigenic variability of pili between *P. aeruginosa* strains represents a significant obstacle [31].

1.2.2 Type 3 secretion system (T3SS)

The type 3 secretion system (T3SS), shared by many Gram-negative bacteria, allows the translocation of toxins from the bacterium to the host cell via a pore formed in the host cell membrane. The T3SS of *P. aeruginosa* is reported to be a major determinant of virulence, indeed its expression by the pathogen is usually associated with acute invasive infections leading to increased mortality in infected patients [27, 51]. There are 43 genes that regulate the entire apparatus and they are regulated in a coordinated manner. These genes code for secretion-regulating factors, for components of the secretion apparatus that export toxins to the other side of the bacterial cell, for a translocator that is responsible for injecting these toxins into the host cell and for factors that regulate secretion [52, 53]. Three proteins are

involved in the assembly of a competent translocator: PcrV, PopB and PopD [54, 55]. The secreted toxins, also called effectors, that have been identified are only four: ExoS, ExoT, ExoU and ExoY. The expression of these effectors varies in different strains and isolates. The two main exotoxins exoU and exoS are expressed by almost all strains, but rarely express both [56], while the toxins exoY and exoT, which have minor roles, are expressed by most strains [51]. The ExoS gene is expressed in approximately 70% of clinical isolates [57], and its function, which is closely related to that of ExoT, is to encode for both Rho GTPase activating protein (RhoGAP) activity and ADP-ribosyltransferase activity (ADPRT) [58, 59] which are involved in destroying the actin cytoskeleton of the host cell, blocking phagocytosis and causing cell death [60]. ExoU, which is found approximately in 30% of clinical isolates [57], is the most virulent of the *P. aeruginosa* type III effector proteins. ExoU is estimated to be a cytotoxin 100 times more potent than ExoS which encodes phospholipase A2 (PLA2) activity but only after interaction with a host cell cofactor. Its PLA2 activity leads to rapid death of eukaryotic host cells due to loss of plasma membrane integrity and increased tissue damage, but its phospholipase activity can also stoke the inflammatory fire, during infection, contributing to increase tissue damage and bacterial spread of infectious diseases caused by ExoU-secreting strains. Knowledge of TTSS in the pathogenesis of *P. aeruginosa* infections is crucial for evaluating various strategies to destroy it, such as the development of antibodies and vaccines that target the type III secretion apparatus and therefore prevent secretion [31, 32, 51, 61-63].

Table 2: *P. aeruginosa* toxins involved in the Type 3 secretion system [34].

Toxins	Biological function
ExoS	Involved in destroying the actin cytoskeleton of the host cell, blocking phagocytosis and causing cell death
ExoT	It has effects on the eukaryotic cytoskeleton and it is involved in cytotoxicity
ExoY	An adenylate cyclase of the T3SS
ExoU	Major cytotoxin; Possess phospholipase / lysophospholipase activity; disrupting eukaryotic cell membrane

1.2.3 Quorum sensing

Many pathogens, including *P. aeruginosa*, use an intercellular signaling process called Quorum Sensing (QS) to adapt in a coordinated manner across the bacterial population to environmental changes as well as to the lung environment. This adaptation is mediated by

small compounds called autoinducers that are constitutively produced by each bacterium and released in the environment. When these compounds reach a threshold concentration they are then perceived by neighboring bacteria which are then able to deduce the density of the bacterial population, as the concentration of self-inducers in the medium is directly proportional to the concentration of bacteria, and thus to regulate the transcription of approximately 350 genes (6% of the *P. aeruginosa* genome) resulting in a coordinated response throughout the bacterial population. The genes involved have an impact on various processes including biofilm formation and the production of numerous toxins [64-66]. Genes regulated by the QS system are estimated to account for up to 10% of the genome and more than 20% of the expressed bacterial proteome [64]. The identified systems involved in QS are four: las, rahl, Pseudomonas Quinolone Signal (PQS) and integrated quorum sensing (IQS). Their specific receptors are respectively: LasR, RhlR, and PqsR while receptor for IQS has yet to be fully determined [30, 67-70]. These systems act in a hierarchical manner, in fact PQS is controlled by the las component which also positively regulates rhl, suggesting that the las component acts as a bridge between the two system [71]. IQS production is triggered by both the las system and phosphate starvation, the first acting during normal bacterial growth conditions and the second during bacterial infections [72]. IQS plays an essential role in *P. aeruginosa* virulence and regulates the expression of a large number of genes, many of which are QS-associated genes and virulence factor-related gene. Virulence, cell survival and biofilm formation are controlled by the QS system; thus, strains deficient in any one of the components of this systems demonstrate reduced pathogenicity.

1.2.4 Quorum Sensing (QS) in biofilm development

The Las, Rhl and PQS systems in *P. aeruginosa* play important roles in the production of compounds that are involved in biofilm development. These include rhamnolipid, pyocyanin, Pel polysaccharides, pyoverdine, and lectins. Rhamnolipid is a compound involved in the preservation of pores and channels between microcolonies, ensuring the passage of liquids and nutrients within mature biofilms [73, 74]. Pyocyanin is a secondary metabolite that has a cytotoxic function. It is therefore able to induce cell lysis causing the release of DNA into the extracellular space (eDNA), which is one of the components of the biofilm matrix. This metabolite, interacting with eDNA, has a strong impact on increasing the viscosity of the solution, which in turn plays a key role in the physical-chemical interactions between the biofilm matrices and the surrounding environment, as well as in

cell aggregation [75]. Another biofilm component that can interact with eDNA within the biofilm matrix, using cationic-anionic interactions, is the polysaccharide Pel. This interaction can strengthen the biofilm structure [76]. Pyoverdine is an essential component for biofilm development as it is able to sequester iron in the environment and deliver it to the cell. The availability of iron that the bacterium has access to is directly related to biofilm formation. In an environment where iron is limited, there is increased motility of contraction to the detriment of sessile growth and therefore biofilm formation [77, 78]. Other compounds that contribute to biofilm structure, as well as adhesion to biological surfaces, are Lectins, soluble proteins located in the outer membrane that exhibit adhesive properties that allow and facilitate the retention of cells and exopolysaccharides (EPS) in a growing biofilm. The adhesive properties are carried out by the two forms in which lectins exist: LecA which binds to galactose and its derivatives and LecB which binds to fucose, mannose and oligosaccharides [79, 80]. Collectively, all these compounds lead to the creation of a robust and mature biofilm.

1.2.5 Biofilm

The biofilm is a complex aggregate of bacteria, a highly organized microbial community enclosed in a self-generated matrix of exopolysaccharides, nucleic acids, lipids and proteins [81, 82] and is one of the most important strategies used by the bacterium for species survival when there is an unexpected change in living conditions [28]. The EPS matrix accounts for 50-90% of the volume of the biofilm and is therefore the most distinctive component that provides the chemical and physical robustness of the community to overcome mechanical forces and reduce the penetration of toxic chemicals such as antibiotics [81]. It also acts as a scaffold for adhesion to biotic and abiotic surfaces in fact the biofilm is able to grow on most surfaces such as eukaryotic and prokaryotic cells, surgical implants, endotracheal tubes, catheters or the airways of individuals with CF becoming a medical problem [82]. Moreover, the matrix provides a repertoire of public goods including essential nutrients, enzymes and cytosolic proteins for the biofilm community, acts as a scaffold for encapsulated bacteria, allowing them to evade the host's immune system, and facilitates cell-to-cell communication [83]. Among the polysaccharides involved in surface attachment, formation and the stability of biofilm architecture we can identify Psl, Pel and alginate [84, 85]. The latter, an unbranched linear polymer composed of D-mannuronic acid and L-guluronic acid [86], is mainly produced by mucoid strains of Pseudomonas due to a mutation

in the mucA22 allele. These strains are mainly found in cystic fibrosis isolates and represent the transition point from acute to chronic infection [87, 88]. In cystic fibrosis patients, the impairment of cough clearance in the lung is related to the ratios of mannuronic acid and guluronic acid as they affect the viscoelastic properties of biofilms [89, 90]. Alginate plays a wide range of important functions: it contributes to the structural stability of the biofilm, to the retention of water and nutrients [91], to protection against phagocytosis and opsonization, and to the decreased dissemination of antibiotics [92, 93]. The polysaccharide Psl is a repeating pentasaccharide composed of d-glucose, l-rhamnose and d-mannose [94, 95].This pentasaccharide is critical for the adhesion of sessile cells, which refers to cells attached to a surface, and cell-cell interactions during biofilm initiation of both mucoid and non-mucoid strains [84, 96, 97]. It has been observed that as a result of increased expression of Psl there is an induction in the formation of cell aggregates in liquid culture, a phenotype that has been observed in the sputum of cystic fibrosis patients [98, 99]. Psl is also involved in the production of c-di-GMP (guanosine monophosphate bis-(30-50)-cyclic dimer), which, when present at high levels, induces the formation of a thicker and more robust biofilm [99]. Finally, this exopolysaccharide, protecting the bacteria in the biofilm from phagocytosis by neutrophils [100] and antimicrobials [85], enables the implementation of an effective defense that ultimately leads to a persistent infection. The Pel polysaccharide is a polymer of partially deacetylated N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. The function of this polysaccharide is analogous to the Psl function as it is an essential component of the biofilm matrix in non-mucoid strains and is involved in the initial stages of adhesion as well as in maintaining biofilm integrity [101, 102]. Pel and Psl synthesis are closely related to the response to surrounding conditions and are strain-specific [103]. Another functional component of the biofilm is eDNA, which is released into the environment after cell lysis that can be caused by environmental stress, such as antimicrobial treatment. Lysis can take place both in the initial biofilm phase and in the planktonic phase where bastoncellar bacteria rapidly change to a round shape due to structural damage of the cell wall. It is thanks to membrane fragments from lysed cells that released eDNA, cytosolic proteins and especially RNA are encapsulated in membrane vesicles (MV) [104]. The role attributed to eDNA is to: 1) be a source of nourishment for the bacteria in the biofilm; 2) activate the type VI secretion system, which disseminates virulence factors within the host, through the chelating activity of divalent cations (Mg2+ and Ca2+) located on the outer membrane; 3) maintaining cellular organization and alignment through contraction motility 4) limiting the penetration of antimicrobial agents through the acidification of the biofilm environment and

infection sites that occurs through eDNA deposition 5) influencing the inflammatory process activated by neutrophils [105-108]. Biofilm formation occurs through the phenotypic transition of planktonic cells, which are the freely suspended cells involved mainly during the initial colonization of new surfaces, into sessile cells, which are the surface-attached cells that characterize the biofilm [109]. The biofilm development of *P. aeruginosa* can be divided into five distinct phases:

- Phase I: planktonic bacterial cells reversibly adhere to a surface suitable for growth through the support of cellular appendages such as flagella and type IV pili [35, 110].
- Phase II: bacterial cells switch from reversible to irreversible adhesion.
- Phase III: adherent bacteria switch to a more structured architecture in which there is the formation of microcolonies in the EPS matrix.
- Phase IV: from microcolonies there is a transition to three-dimensional mushroom-shaped structures, which represents the transition to a mature biofilm in which the bacteria cover the entire surface.
- Phase V: the matrix cavity in the center of the microcolony undergoes cell autolysis allowing the dispersion of cells [94] that switch from sessile to planktonic growth mode to induce colonization of other surfaces, thus allowing the biofilm cycle to repeat [111, 112].

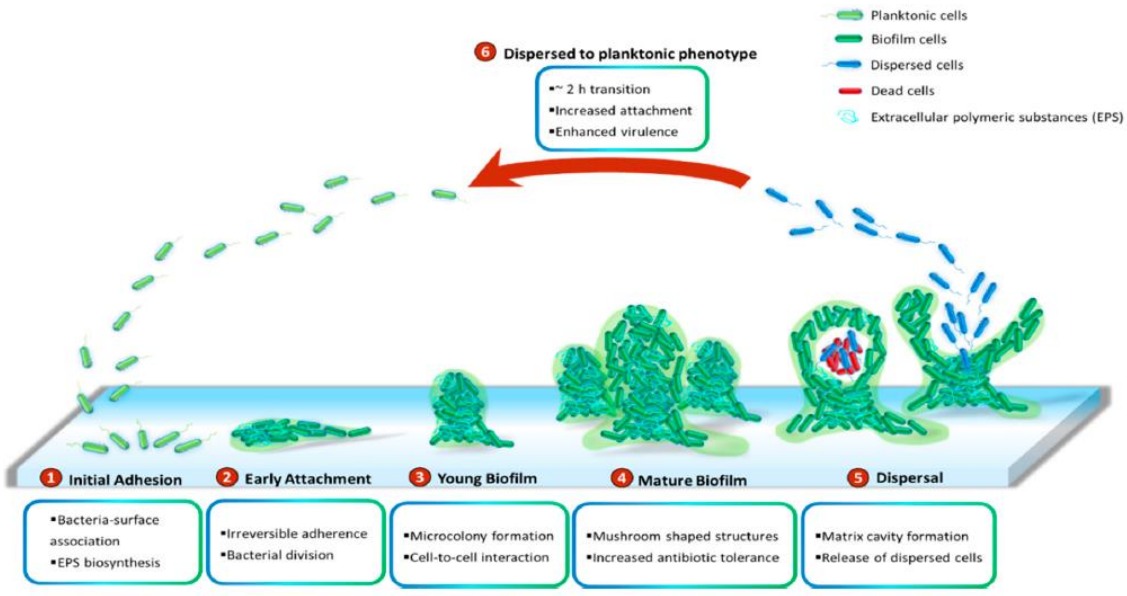


Fig 2: Stages of *P. aeruginosa* biofilm formation [109].

The structure of the biofilm at the beginning of development is composed of highly motile bacteria, whereas when the structures of mushroom-shaped micro-colonies occur, cells with low motility are present. The degree of motility is nutrient specific [60]. The physiological difference between biofilm cells and planktonic cells is well known while not much is known about the intermediate events in which the attached forms switch to a free-swimming lifestyle, generating highly virulent detached cells. This transitional phase from cell detachment to the presence of planktonic cells could represent a further stage in the development of the biofilm (phase VI), as the cells in this specific phase have a distinct physiology from planktonic and sessile cells. Cells generated in this phase switch to a planktonic state after remaining in a 2-h lag phase in a low intracellular c-di-GMP and pyoverdine environment [61,62]. It has been observed, in *in vitro* and *in vivo* experiments, that free cells are more sensitive to iron depletion and highly cytotoxic to macrophages compared to planktonic cells [63].

1.2.6 The CdrA adhesin and its role in the biofilm development

The role and impact that exopolysaccharides have on the *P. aeruginosa* biofilm has been extensively studied [113], while the different roles that proteins may play in the biofilm matrix are less clear [114]. The first protein to be identified in the *P. aeruginosa* biofilm matrix was CdrA, an adhesin that plays a structural role in biofilm aggregates [115]. Indeed, it is hypothesized that CdrA promotes biofilm stability through its interaction with the exopolysaccharide Psl [115], conferring protection from proteolytic degradation, or through the CdrA-CdrA interaction, protecting the biofilm from mechanical destruction [116]. Computational analysis indicates that this protein is long, with a molecular weight of 220 kDa, rod-shaped and has a β-helical motif characterizing a repeat region, as well as an N-terminal signal sequence and an N-terminal prerepeat region. This adhesin is part of a two-partner secretion system (TPS) encoded by the cdrAB operon (19) and can be found either on the cell membrane or released into the supernatant as it has a C-terminal TAAG cut site that can be processed by the periplasmic protease LapG, under conditions of low cyclic di-GMP, allowing CdrA to be exported from the periplasm to the cell surface via CdrB, which acts as an outer membrane pore. The protein released from the cell surface has a molecular weight of 150 kDa despite the fact that the CdrA gene encodes a 220 kDa protein [115-118].

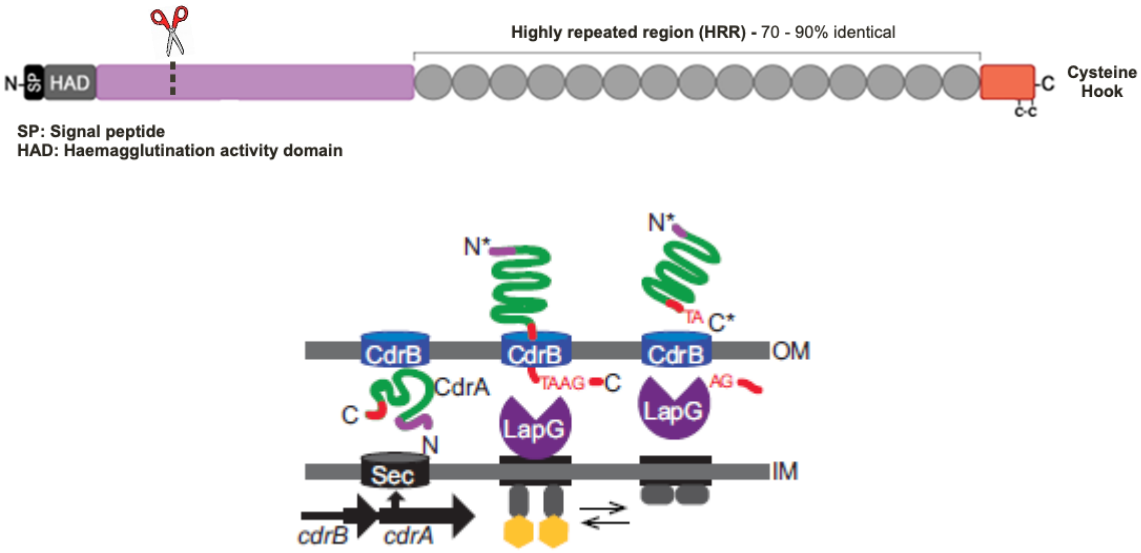


Fig 3: The two-partner secretion system encoded by the cdrAB operon. Processing of CdrA by the protease LapG [116].

This protein also presents a second N-terminal proteolytic site identified between residues 437 and 438. Previously, due to the CdrA ability to bind Psl, it was thought that CdrA was only important for strains that produced a Psl-rich biofilm matrix, but new studies have shown that this is not the situation as this protein can interact with a number of biomolecules in addition to Psl [115].

1.3 Therapy and antibiotic resistance

P. aeruginosa is able to resist most of the antibiotics currently available on the market [119] and this makes the treatment of its infections difficult to treat, in fact The World Health Organization (WHO) has identified this pathogen as one of the three bacterial species, resistant to carbapenems, for which there is an urgent need to develop new treatments [120]. What makes the situation even more critical is the overuse of antibiotics, used during treatments, leading to the development of multi-resistant *P. aeruginosa* strains [121]. As mentioned above, *P. aeruginosa* plays a crucial role in nosocomial infection where it forms biofilms on the surface of medical instruments and devices as well as on patients' implants [122]. Extensive antibiotic resistance is acquired by the pathogen through the inter- and intra-transfer of resistance genes, a phenomenon that occurs most efficiently during the biofilm, where numerous mutations occur, which can also lead to changes in the antibiotic

target [123]. Antibiotics to which *P. aeruginosa* is resistant include aminoglycosides, quinolones and β-lactams [124]. Despite the widespread resistance to antibiotics exhibited by *P. aeruginosa*, the methods used to prevent and combat infections are: prudent prescribing methods, early but aggressive antibiotic treatment, which has improved treatment outcomes for some diseases such as cystic fibrosis, strict hygiene protocols and separation of susceptible but uninfected patients from infected ones [125]. Bacteria that are tolerant to an antibiotic are selected and establish in the absence of competition for environmental resources such as nutrients. An alternative class of antibiotics can overcome specific antibiotic resistance. However, bacteria have developed some effective defense mechanisms that lead to multi drug resistant (MDR) species [126].

1.3.1 P. aeruginosa resistance mechanisms

A multiplicity of mechanisms have led to antimicrobial resistance in microorganisms. Resistance mechanisms can be classified into intrinsic, acquired and adaptive resistance mechanisms. Intrinsic antibiotic resistance refers to the innate ability of a bacterium to reduce the efficacy of a specific antibiotic through intrinsic structural or functional characteristics. In contrast, acquired resistance is based on the acquisition of new functions. Adaptive resistance is known to emerge as a consequence of concentration gradients, as well as contact with subinhibitory concentrations of antibiotics, both of which are known to occur in human patients and livestock. [127] When bacteria become resistant to one or more initially effective antibiotics, they are termed multi-resistant and often referred to as "superbugs". [128]. This can occur through mutational changes or acquisition of resistance genes through horizontal gene transfer. Horizontal gene transfer is not only common between microorganisms of the same genus, but also occurs between bacteria that are evolutionarily distant. *P. aeruginosa* is innately resistant to many antibiotics; the intrinsic nearly impermeable cell wall, outer membrane protein (Opr) channels (limiting substrate entry by size and hydrophobicity), and multi-drug efflux pumps confer a basal level of resistance to certain antibiotics. Higher therapeutic doses and continuous administration of antibiotics will eventually lead to full resistance [126, 129-133]. Bacteria can deal with the action of antibiotics by exploiting several mechanisms based on: (i) hydrolysis or structural modification of the drug leading to its inactivation, (ii) lower membrane permeability or overexpression of efflux pumps preventing the access to the target, (iii) mutation or post-translational modifications of the antibiotic targets [134-136]. Lastly, adaptive resistance is

the ability of a bacterium to tackle antibiotics through transient alterations in gene expression in response to specific stimuli. The acquired phenotype is reversible and when the stimulus is removed the inherent bacterial sensitivity is restored [127]. The main mechanisms of this type of resistance are the formation of biofilm and the generation of persister cells.

1.3.2 Biofilm-induced resistances

An important feature of bacterial biofilms is their resistance to antimicrobial substances and to the action of the host's immune system. It has been shown that bacteria growing in the sessile form can achieve levels of resistance to antibiotic therapy up to 1000 times higher than the same microorganism grown in the sessile form [139]. Biofilm resistance is multifactorial [140]:

- **Reduced penetration of antimicrobial substances:** the EPS has a role as a chemical-physical barrier, reducing the penetration of several chemically reactive substances, cationic antibiotics and antimicrobial peptides (AMPs). The limited diffusion of biocidal substances may reduce the amount of certain agents transported within the biofilm but does not play a decisive role in long-term exposure, as the limited transport depends on the binding of the molecules to reactive sites in the biofilm. Once these sites are saturated, antimicrobial activity can resume.

- **Physiological gradient within the biofilm:** bacteria located further out in the biofilm have greater access to nutrients, water and oxygen, while those located in the deep layers must adapt to living in limiting conditions. This difference in the availability of nutrients and oxygen leads to a diversified bacterial population with different metabolic activity and sensitivity to antimicrobials. So-called 'slow-growing' or stationary phase bacteria will be insensitive to the will be insensitive to antibiotics such as β- lactams or tetracycline. lactams or tetracyclines, whose efficacy depends on the growth and replication rate of the bacteria. replication rate of the bacteria.

- **Biofilm-specific phenotype:** Following adhesion, bacterial cells can undergo physiological, metabolic and phenotypic changes. One example is algC, a gene required for alginate synthesis in *P. aeruginosa* biofilms, which has been shown to be up-regulated as early as 15 minutes after surface adhesion. The regulation of protein expression also changes in sessile cells: changes in proteins involved in resistance to

oxidative damage are seen during biofilm development, exopolysaccharide production and metabolism.

- **Efflux pumps:** the increase in the extrusion of an antibiotic plays a critical role in bacterial resistance. Efflux pumps are capable of extruding drugs, toxic metal ions, organic solvents and other ligands: exposure to non-lethal doses of a large number of antibiotics promotes their expression.

- **Persisters cells:** Several studies, most notably that of Brooun, Liu, and Lewis 2000, have shown that even when concentrations of a given antibiotic are increased considerably, there is always a subpopulation within the biofilm, known as a persister, that resists biocidal action. These variants have an inefficient programmed cell death (PCD) system, which does not trigger cell apoptosis even after cell damage and allows them to survive even at high doses of antibiotic.

In summary, in the early stages of biofilm development there are changes in gene expression following surface adhesion, leading to a biofilm-specific phenotype and increased resistance. Subsequent production of extracellular matrix contributes to cell survival by delaying the entry of antimicrobial substances. Maturation of the biofilm and increased cell density create nutrient and oxygen gradients with reduced metabolic activity and growth rate; general stress response and increased expression of efflux pumps are also activated. Environmental conditions induce or select persisters resistant to high antibiotic concentrations [140].

AIM OF THE STUDY

Pseudomonas aeruginosa is an opportunistic pathogen of animals and humans causing medical complications in burns, wounds, and cystic fibrosis. *P. aeruginosa* is efficient at adapting its virulence phenotype depending on the site of infection. It is because of its relatively large genome and flexible metabolic capabilities that this organism is able to exploit different environmental conditions in which to grow and to evade normal immune defenses [15]. *P. aeruginosa* is one of the most common hospital pathogens and is the second most common pathogen detected in patients with ventilator-associated pneumonia (VAP) [16]. The main reasons for the high incidence of this bacterium in healthcare institutions include the poor health status of patients, the high rate of spread of often multi-resistant strains in hospital wards, and the overuse of broad-spectrum antibiotics [17]. Emerging multi-drug resistant strains, also due to the biofilm that confers resistances, and the limited number of effective anti-pseudomonal antibiotics make *P. aeruginosa* infections extremely difficult to treat. To address this need, this work considers the production and the biochemical and functional characterization of the CdrA protein, which is considered to be one of the most important protein involved in the biofilm stabilization, and the identification of new antigens involved in *P. aeruginosa* infections, in order to use them as vaccine candidates and therapeutic targets.

MATERIALS AND METHODS

3.1 Production of recombinant CdrA protein constructs in E. coli

Genomic DNA isolated from the *Pseudomonas aeruginosa* strain PAO 1 was used as template for cloning all the recombinant proteins. The gene fragments coding for the different constructs of CdrA were cloned using PIPE method [141] in pET15 (N-term 6x His tag) and pET21(C-term 6xHis tag) vectors and using *E. coli* Stellar (Takara) strain. The identity of the resulting vectors was checked by sequencing. Vectors containing the different CdrA constructs where further transformed in *E. coli* BL21DE3 cells for protein expression. Colonies were picked from agar plates and inoculated into 10 ml of LB-medium and Ampicillin 100ug/ml and growth at 37°C, 180 rpm. Pre-cultures were diluted 1:100 into 75ml of HTMC medium (15g/L Glycerol, 30g/L Yeast extract, 0.5 g/L $MgSO_4$, 20 g/L K_2HPO_4, 5 g/L KH_2PO_4) and 75 µl of Ampicillin 100 µg/ml and the flasks were growth ON at 20°C and 160 rpm. Protein expression was induced by adding 75 µl of IPTG 1 M solution to each flask and culture were left in the shaker for 24h at 20°C. The cells pellet was subjected to chemical lysis in B-Per buffer (containing lysozyme and DNAse) and a sample of the total proteins fraction was collected. After centrifugation cell debris were removed and soluble proteins fraction was collected. To assess if the CdrA protein constructs were expressed and present in the soluble fraction, 12 µl of each sample were loaded in a sodium dodecyl sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE). Samples were denatured 5' at 95°C and then loaded in to NuPAGE 4-12% Bis-Tris 1.00mm Mini Protein Gel, with Protein standard and the gel was run at 180V, 40minutes in MES buffer 1x. Protein detection was performed by Coomassie staining.

3.2 Proteins solubilization from inclusion bodies

E. coli Cells expressing the CdrA constructs were harvested by centrifugation and resuspended in IB wash buffer (25mMTris-HCl [pH 7.6], 100 mM NaCl, 5 mM β mercaptoethanol, and 2 mM EDTA) supplemented with 0.5 mg/ml lysozyme and incubated for 15 min, at which time 1% (vol/vol) Triton X-100 was added and cells were lysed by sonication. Inclusion bodies were pelleted by centrifugation at 16,000g for 10 min, washed twice with IB buffer containing 1% Triton X-100, and washed two more times with IB buffer without detergent. Washed inclusion bodies were solubilized in denaturing buffer (100 mM Tris-HCl [pH 7.8], 8 M urea, and 5 mM β-mercaptoethanol) for 1 h at room temperature at

1000rpm. Solubilized inclusion bodies were filtered through a 0.22 μm filter and loaded onto an Ni-nitrilotriacetic acid (NTA) His-Trap column (GE Healthcare). The column was washed with 50 column volumes of 25mMTris-HCl (pH 7.6), 150 mM NaCl, 8 M urea, 5 mM imidazole, and 5 mM β-mercaptoethanol. The CdrA protein was refolded on the Ni-NTA column with a linear gradient from 8 M to 0 M urea. The final elution step was performed with 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 300 mM imidazole. Imidazole was removed with a PD-10 desalting column (GE Healthcare), and the presence of the soluble protein in the elution fraction was evaluated with a sodium dodecyl sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE).

3.3 Design of recombinant CdrA domains

To perform antigen design and to identify the CdrA domains for the expression in mammalian system, the entire protein sequence was used to perform 3D homology modelling (PDB-BLAST, Swiss Model and PyMOL). Stuctural analyses were combined with literature-derived information to design the final sequences to be ordered for protein production.

3.4 Production of recombinant CdrA proteins in mammalian cells

The 2 truncated forms of CdrA corresponding to the aminoacidic residues 438-904 and 438-2060 were cloned using PIPE method [141] in pcDNA 3.1 (+) vector. The synthetic genes optimized for mammalian expression coding for the different constructs of CdrA, with additional N-terminal IgK secretion sequence and N-terminal His tag were cloned into pCDNA3.1 vector by Geneart (Life Technologies). Clones were transformed in *E. coli* STELLAR (Takara) strain for DNA amplification and the DNA was recovered for protein production in Mammalian expression systems using a midi-plasmid DNA extraction kit. In order to obtain high-yield of CdrA in soluble form, the mammalian EXPI 293 GnTi cell line (ThermoFisher Scientific) were transfected with the DNA encoding for the two different constructs of the CdrA protein according to the User Guide protocol: Briefly, 100 μg of expressing vector was used for transfection of 100 ml cells culture containing 300 x 10^6 Expi293F GnTI cells using ExpiFectamine 293 Reagent. Cells were incubated at 37°C, 120 rpm, 8% CO2 and after 24 h, ExpiFectamine 293 Transfection enhancer 1 and 2 were added. Cells were further incubated at 37°C for 72h and 144h after collecting the supernatants and resuspending the cells in fresh expi-medium. After that, the supernatants were collected after

centrifugation at 300g for 10 minutes and the CdrA-containing supernatants were harvested, pooled, clarified by centrifugation, filtered through a 0.22 μm filter, and stored at 4°C until purification. Affinity chromatography with Ni2+ was used to purify the constructs from culture supernatants using affinity chromatography columns, HisTrap FF crude (Takara). Fractions of interest were pooled and were concentrated by using 10 kDa cutoff spin concentrators (Millipore Amicon Ultra); sodium dodecyl sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE) was performed to check purity. Samples were mixed with 1X NuPage LDS loading buffer and 1X NuPage Sample reducing agent (Life Technologies), were heated at 98°C for 10 min and 12 μl were loaded onto a 4–12% NuPAGE Bis-Tris gel (Life Technologies). For western blotting analysis the proteins from the gel were electro-transferred onto nitrocellulose membranes with iBlot 2 Dry Blotting System (Life Technologies). The membranes were blocked for 60 min with PBS + 0.1% Tween 20 + 3% milk and then incubated for 1 h with mouse anti-His-tag HRP antibody (Merck) diluted 1:1000 in blocking buffer. Membranes were washed thrice with T-PBS and then incubated with Opti-4CN™ Colorimetric Kits (Bio-Rad).

3.5 Peptide - N - glycosidase F (PNGase F) treatment

Removal of N-Linked Glycans from the recombinant protein samples were performed using the enzyme PNGase F. The samples were first incubated with a denaturing buffer at 100°C for 10 minutes. A mixture of reaction buffer, 10% of NP-40 and PNGase F was then added to the samples and incubated at 37°C for 30 minutes. The deglycosiylated proteins were analyzed by SDS-PAGE.

3.6 NanoDSF (scanning nano fluorimetry) analysis

To assess the fluorescence-monitored unfolding of the 2 truncated forms of CdrA and to evaluate their binding to the exopolysaccharide Psl, a nanoDSF analysis was performed. For the evaluation of CdrA-Psl binding, CdrA protein was mixed with the polysaccharide at a 1:3 ratio, according to Reichhardt *et al* protocol [116] and incubated overnight in a shacking condition (450 rpm). Samples were manually loaded into nanoDSF grade standard capillaries in triplicates and transferred to a Prometheus NT.48 nanoDSF device. For intrinsic tryptophan fluorescence measurements, the excitation wavelength of 280 nm was used, and the emission of tryptophan fluorescence was measured at 330 nm, 350 nm, and their ratios (350 nm/330 nm). Data were analyzed with Prometheus PR. Control software

(NanoTemper Technologies) and plotted using the fluorescence ratio against the temperature.

3.7 ThermoFluor analysis

The ThermoFluor assay is a quick, temperature-based assay to assess the stability of proteins [142]. In this method each sample is diluted to a final concentration of 0,2 mg/ml and then are added 4 µl of the SYPRO Orange dye 1000X (Molecular Probes) in order to reach a final volume of 40 µl using the protein buffer and the mix was added to the wells of a 96-well thin-wall PCR plate (Bio-Rad). Water was added instead of test compound in the control samples. Each sample was analyzed in triplicate. The plates were sealed with Optical-Quality Sealing Tape (Bio-Rad) and heated in an iCycler iQ Real Time PCR Detection System (Bio-Rad) . As the temperature increases and the protein unfolds, it is possible to monitor the increment in fluorescence and determine the melting temperature of the sample. Data are plotted correlating the derivative of fluorescence and temperature using GraphPad program.

3.8 SPR (Surface Plasmon Resonance) analysis

The shortest construct of CdrA 438-904 was diluted to reach a concentration of 0,5 ug/ml with running buffer HBS-EP+ (0.01 M HEPES, 0.15 M NaCl, 0.003 M EDTA and 0.05% v/v Surfactant P20) and captured on the surface of a sensor chip NTA that was previously activated by injecting a 0.5 mM solution of Ni^{2+} ions and washed with 3mM of EDTA. 0,16 ug/ml of the Psl and the anti-CdrA antibodies (1:500) were injected, separately, in the Biacore T200 and the binding signals monitored. The NTA surface was then regenerated by a pulse of 350 mM of EDTA.

3.9 CdrA Protein immunization and Luminex binding assay

All animal experiments were performed in accordance with Institutional Animal Care and Use Committee protocols. In this immunization scheme, 5-week-old CD-1 female mice were immunized 3 times 28 days apart with the two CdrA constructs at a concentration of 7.5 µg/mouse adjuvanted with ALUM in 100 µl of total volume intramuscularly. Mice were bled before the first immunization (pre-immune sera), a few days before the third immunization and 14 days after the third immunization to collect, respectively, POST-II and POST-III sera that were then used in the *in vitro* biofilm inhibition assay **(Fig. 4)**.

			DAY 0	DAY 1	DAY 28	DAY 55	DAY 58	DAY 72
ADMIN	IMMUN			**29**	**29**		**29**	
SAMPLING	BLEED	SERU	**29**			**29**		
	FINBL	SERU						**29**

Fig. 4: CdrA scheme immunization.

To assess the presence of specific anti-CdrA antibodies in mouse sera derived from immunization, a Luminex binding assay was performed. In this assay, 5 µl of Luminex beads (MC12XXX; MagPlex) were coupled with 5 µl of the CdrA recombinant purified protein. The mouse sera diluted 1:500 were added to the beads and all the mix was incubated for 1h in agitation (1200 x g) at room temperature. Beads were washed 3 times with PBS 0,05% + Tween20 (Sigma-Aldrich) and incubated for 45 minutes with 25 µl of the AffiniPure Donkey Anti-Mouse IgG (H+L) (Jackson Immunoresearch) in agitation (1200 x g) at room temperature. After 3 washes, beads were resuspended in PBS 0,05% + Tween20 (Sigma-Aldrich) and signals are acquired by BioPlex 3D suspension array system (#BioPlex3D; BIO-RAD). Data are plotted using GraphPad program.

3.10 Flow cytometric assay and western blotting analysis to evaluate the CdrA protein expression

In order to isolate single colonies, *P. aeruginosa* strain PA01 was plated into TSA agar plates and plates were incubated ON at 37°C, 5% CO2. The following day, single colonies were resuspended in 4ml of PBS 1X + BSA1% filtered (Sigma- Aldrich) and 300 µl of the culture at OD 0.1 were diluted in 20ml of TSB medium. The flask was incubated at 37°C,180rpm. At different time points of the growth curve, the OD of 2ml of bacterial culture was evaluated and 1ml was centrifuged at 3700 rpm for 5 minutes. The supernatant was discarded and the pellet resuspended with 1ml of PBS 1X + BSA1% filtered (Sigma- Aldrich). For FACS assay 50 µl of bacterial suspension were incubated with 50 µl of the serum dilution (1:100) for 1h at room temperature. The sera analyzed were the PAO1 inactivated whole cell mouse serum, as positive control, the pre-immune sera, as negative control and the construct #7

(438-904) and #8 (438-2060) derived sera. After a centrifugation step of 3 minutes at 13400 rcf, the supernatant was discarded and the pellet resuspended for 30 minutes at room temperature with 100 µl of the Goat anti-Mouse IgG Secondary Antibody, Alexa Fluor 488 (ThermoFisher) diluted 1:500. To separate the pellet from the supernatant a second centrifugation step, as above, was performed and then each bacterial pellet was resuspended for 30 minutes with 1ml of formaldehyde solution 4% (Sigma-Aldrich). Finally, the analysis of each sample was performed using the FACS Canto II instrument. To assess the presence of the CdrA protein in the culture medium as secreted protein, culture medium samples were analyzed by western blot. For the first, samples were mixed with 1X NuPage LDS loading buffer and 1X NuPage Sample reducing agent (Life Technologies), were heated at 98°C for 10 min and 12 µl were loaded onto a 4–12% NuPAGE Bis-Tris gel (Life Technologies). The proteins were electro-transferred from the gel onto nitrocellulose membranes with iBlot 2 Dry Blotting System (Life Technologies). The membranes were blocked for 60 min with PBS + 0.1% Tween 20 + 3% milk and then incubated for 1 h with sera listed above diluted 1:1000 in blocking buffer. Membranes were washed thrice with T-PBS and then incubated firstly with the anti-CdrA mouse sera of the construct #7 and #8 (Group 1 and Group 2) and then with a Goat anti-Mouse IgG (H+L) Secondary Antibody-HRP conjugated (ThermoFisher) and then with Opti-4CN™ Colorimetric Kits (Bio-Rad).

3.11 Flow cytometric assay to evaluate the biofilm formation inhibition

P. aeruginosa PAO1 wild type and ΔCdrA strains kindly provided by Professor Imperi from La Sapienza University, both known as biofilm-forming strains [115, 143] , were plated on TSA plates and growth at 37 °C, 8% CO2. The strains are subcultured on TSA medium until an OD = 0.1 was reached and then 300 µl of the bacterial culture were plated on 96 well black plate (ibidi) presenting a hydrophilic tissue culture-treated surface which makes the surface adhesive to all cell types. For each experiment three replicates were performed and the un-inoculated medium was included as a blank. Bacteria growth was stopped at different time points (24h, 48h, 72h, 120h) with a medium change every 12 hours. At the desired time point, 3 wash steps with PBS 1X were executed to remove planktonic cells. To detach the mature biofilm from the well mechanical techniques, scraping, with a cell scraper, and rinsing with a 2-mL syringe, were used. In the first experiment, sonication of the samples was also performed to avoid the formation of bacterial aggregates, using a 40 Hz sonicator bath (Sonicator 60) and it was compared with cells vortexing to assess which technique was

more effective and less time consuming. Vortexing was selected as the preferred technique for the following experiments. After the biofilm detachment, 200 µl of the TO – PRO 3 iodide dye (ThermoFisher - T3605) were added to each sample and incubated for 20 minutes in dark environment. After centrifugation of the samples for 5 minutes at 3500 rpm, the supernatant was discarded and the pellet was resuspended with 1X PBS. Two steps of centrifugation, at 3500 rpm for 5 minutes, were performed. With the first step, the supernatant was removed and the pellet was resuspended with 200 µl of 4 % formaldehyde solution (Sigma -Aldrich), to fix the bacterial samples. With the second step, the formaldehyde solution (Sigma -Aldrich) was eliminated from the pellet which is further resuspended with 300 µl of PBS 1X. Before the FACS reading, 50 µl of the CountBright absolute counting beads (Molecular Probes,OR, USA) were added to 300 the samples to determine the bacteria number. Finally, the FACS Canto II equipped with a red diode laser (λex=635 nm) and a bandpass filter measuring the red (653-669 nm) was used to analyze the bacterial suspensions.

3.12 Crystal Violet (CV) assay

The PAO1 wt and ΔCdrA strains were plated on TSA plates maintained at 37 °C, 8% CO2. Then the strains were subcultured on TSA medium until an OD=0.1. 300 µl\well of the bacterial suspension were added in a 96 well black plate presenting a hydrophilic, tissue culture-treated surface which makes the surface adhesive to all cell types. For each experiment 3 replicate wells were used. Bacteria were grown at different time points (24h, 48h, 72h, 120h) with a medium change every 12 hours. At the desired time point, 3 wash steps with PBS 1X were executed to remove planktonic cells. To each well 125 µl of a 0.1 % of Gram's crystal violet (CV) solution (Sigma-Aldrich) were added and the plate was incubated at room temperature for 15 minutes. To remove the excess of dye, 3 wash steps with sterile dH2O were performed. The plate was dried overnight at room temperature. To solubilize the Crystal Violet, 125 µl of 30% acid acetic were added to each well and the plate was incubated for 15 minutes at room temperature. 125 µl of the solubilized CV were transferred to a new flat-bottomed plate and the absorbance values were read at 590 nm. 30% acetic acid in water was used as a blank.

3.13 Human samples from AERIS clinical trial

Human samples were collected from the AERIS study (https://clinicaltrials.gov/ct2/show/NCT01360398), a longitudinal epidemiological study to assess how changes in the COPD airway microbiome contribute to the incidence and severity of acute exacerbation of chronic obstructive pulmonary disease (AECOPD). In this trial 152 chronic obstructive pulmonary disease (COPD) patients aged 40-85 years were followed monthly for 2 years and reviewed within 72 hours of the onset of AECOPD symptoms. Exacerbations were recorded using daily electronic diary cards. Blood, sputum, nasopharyngeal and urine samples were collected at set times. Diagnostic and molecular typing techniques were used to describe the dynamics of airway infection during AECOPD and stable disease, and associations with clinical outcome.

3.4 Protein array analysis

The protein arrays, generated by Sengenics, are composed by full-length, correctly folded proteins which present at the N or C termini a Biotin Carboxyl Carrier (BCC). The proteins of interest are immobilized on the array through Streptavidin tetramers covalently bound to hydrogel layer presents on the glass slides. The protein arrays with the spotted proteins were incubated at room temperature for 1 hour with 300 µl of BlockIt Blocking Buffer (ArrayIt, Thompson Place Sunnyvale, CA, USA). The BlockIt™ Blocking Buffer excess was removed from each slide using the appropriate paper towels to dry the slide; The arrays were incubated with mice sera, diluted 1:100 and 1:200, and with patients derived primary antibodies, diluted 1:100 for two hours at room temperature, in the dark, horizontally in wet chamber. Three washing steps with PBS 1x and Tween20 (P1379; Sigma-Aldrich) were performed. Donkey Anti-Mouse IgG antibodies, Cy3-conjugated (Sigma - Aldrich) and Cy3 AffiniPure Goat Anti-Human IgG (H+L) antibodies were diluted 1:800 using BlockIt™ Blocking Buffer and 300µl/well were added to the arrays and incubated for 1h at RT, in the dark, horizontally in a wet chamber. Three wash steps with PBS 1x and Tween20 (Sigma-Aldrich) were performed and the chemiluminescence signal was detected by Power Scanner (Tecan Trading AG, Switzerland). The chemiluminescence values were processed with the ImaGene software and the values obtained were analyzed with the R studio software.

RESULTS

4.1 CdrA recombinant constructs are expressed but insoluble when produced in E. coli expression system

CdrA has been identified as a key protein structural component of the *P. aeruginosa* EPS matrix [144]. Studies conducted on *P. aeruginosa* and CdrA knock-out strains have shown that CdrA is secreted outside the cell and binds to the Psl polysaccharide promoting auto-aggregation in liquid culture and biofilm formation on surfaces [115]. However, the 3D structure of this protein is not available in the protein data bank (PDB) and the production of CdrA as recombinant protein is not available in the literature. In order to perform a structural, biochemical and functional characterization of CdrA, several attempts were done to obtain the protein in the *E. coli* expression system. Since CdrA is a very large protein, approximately 220 kDa [118], different constructs coding for different fragments of CdrA were generated and tested according to the material and method section (**Fig. 5).**

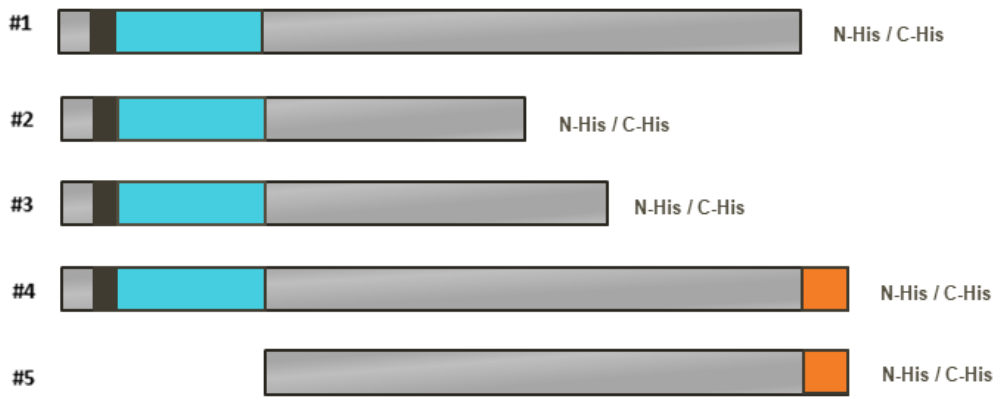


Fig. 5: CdrA constructs expressed in E. coli BL21(DE3) cells. The constructs are characterized by an HIS-tag at the N or C terminus, an hemagglutination site (in black), a pre-repeat region (in light blue), a highly repeated region (in light grey) and a Cysteine Hook region (in orange).

All constructs have an HIS-tag at the N or C terminus and differ in the portion of the full-length protein involved. **Constructs #1** (aminoacidic domain 43-2060), **Construct #2** (aminoacidic domain 43-904) and **Construct #3 (**aminoacidic domain 43-1109) present the hemagglutination site (in black), the pre-repeat region (in light blue) and a highly repeated region (in light grey) and the domain length of which changes from construct to construct.

Construct #4 (aminoacidic domain 43-2154), in addition to all the regions contained in the constructs #1, #2 and #3, also consists of the Cysteine Hook region (in orange), which is involved in the attachment of CdrA to the plasma membrane [117]. Finally, **Construct #5** (aminoacidic domain 861-2060) consists only of the high repeat region and the Cysteine Hook region. The results shown in **Fig.6** demonstrate that all the constructs were successfully expressed in *E. coli* but the protein fragments resulted to be present only in the insoluble fraction. Some of the constructs were selected also for a refolding test. To obtain these constructs in a soluble form, an extraction from the inclusion bodies was performed in denaturing conditions and the affinity purification was performed using a Urea gradient from 8M to 0M to refold the proteins. However, the constructs were not present in the 0M Urea fraction **(Fig. 7)** suggesting that in the absence of the denaturing agent the proteins were not soluble, resulting in a not successful refolding process. In order to achieve a soluble production of CdrA recombinant proteins, an alternative expression system was selected.

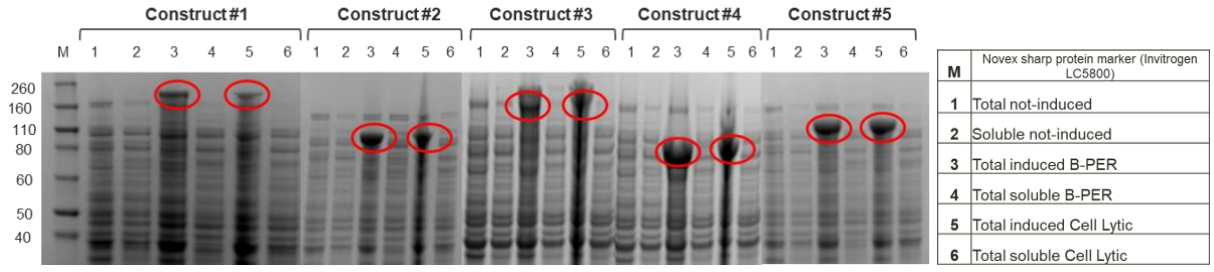


Fig. 6: SDS-Page analysis of total and soluble extracts of E. *coli* expressing CdrA constructs.

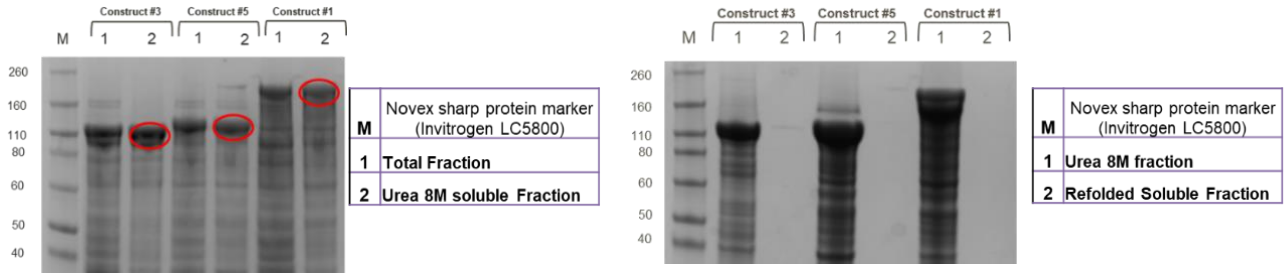


Fig. 7: Protein constructs extracted from the inclusion bodies and the His-tag purification under refolding conditions.

4.2 CdrA recombinant constructs can be successfully expressed as secreted proteins in Expi293F mammalian expression system

To overcome the solubilization problems observed in the *E. coli* expression system, the expression of the CdrA constructs in mammalian cells was tested (**Fig. 8)**. As the structure is unknown, to identify the most promising domains for expression in the mammalian system we used a literature-based information approach and a structure-based design strategy in which, using a combination of software such as PDB-BLAST, Swiss Model and PyMOL, we investigated the sequence homology of CdrA proteins with protein sequences with known 3D structure (**Fig. 9**). The decided domains are the **Construct #2** (aminoacidic domain 43-904) which is the same as the one expressed in *E. coli* cells, with the difference that it has, like the other constructs expressed in this system, a leader sequence specific for mammalian cells; the **Construct #6** (aminoacidic domain 43-471) presenting the hemagglutination site (in black), the pre-repeated region (in light blue) and a highly repeat region (in light grey); the **Construct #7** (aminoacidic domain 438-904) characterized by the pre-repeated region (in light blue) and a highly repeat region (in light grey) and the **Construct #8** (aminoacidic domain 438-2060) that in addition to the regions present in construct #6 also has the Cysteine Hook region (in orange).

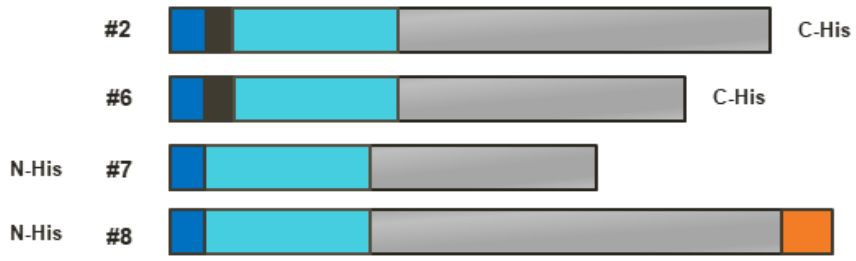


Fig. 8: CdrA constructs expressed in Mammalian Expi293/GnTi cells. The constructs are characterized by an HIS-tag at the N or C terminus, an hemagglutination site (in black), a pre-repeat region (in light blue), a highly repeat region (in light grey) and a Cysteine Hook region (in orange).

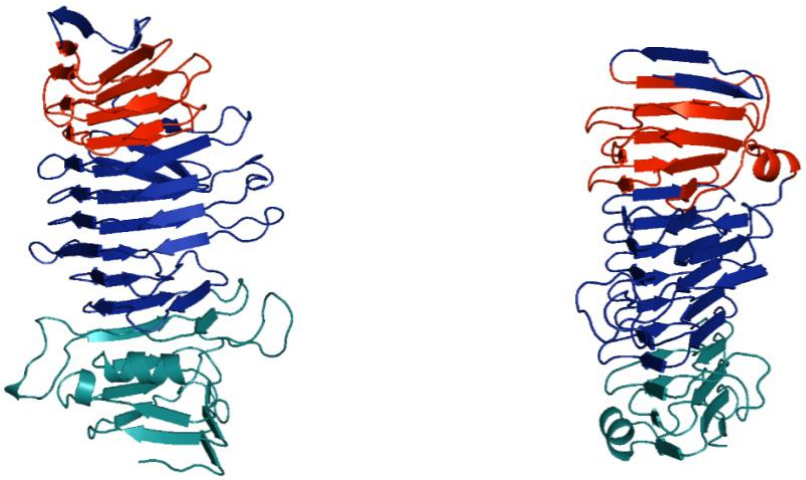


Fig. 9: CdrA mammalian constructs modelling structure. The global model of CdrA predicts a β-helix dominated structure with several exposed loops containing ά-helices. The haemagglutination domain (aa 45-153) is shown in red and a putative sugar-binding domain (332-477) in blue. Protein models were generated using PyMOL software [145].

The CdrA domains were fused to an artificial N-terminal domain composed of a secretion murine Ig-κ chain leader sequence and a 6xHis-tag. For high yield production, Expi293GnTi mammalian cells, which induce reduced glycosylation, were transfected with the pCDNA3.1 plasmids coding for CdrA fragments. The accumulation of secreted CdrA constructs was assessed by checking their expression in culture supernatants at 24 h, 48 h, 72 h and 144 h post-transfection by WB in reducing condition (**Fig. 10).** The western blot analysis was performed using an anti-His antibody, recognizing the His-tag localized at the N-term of the protein. The recognized bands in the WB suggest that the constructs are expressed at all time points. The expression of the recombinant CdrA constructs increased over time and reached a plateau between 72 h and 96 h post-transfection.

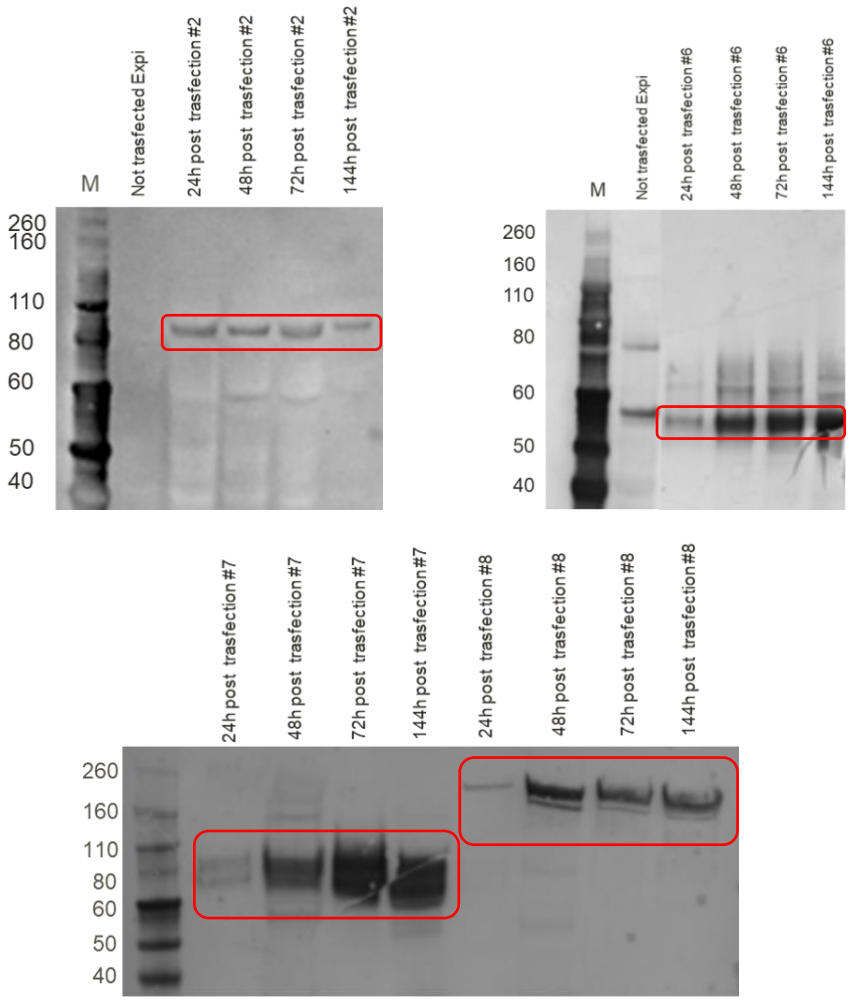


Fig. 10: Western Blot of CdrA constructs expressed in mammalian cells.

Secreted CdrA constructs were purified from 72 h and 144 h pooled culture media using anti-HIS affinity chromatography, as shown in **Fig. 11**, all the constructs were present in the elution fraction but the construct #2 resulted to be degraded.

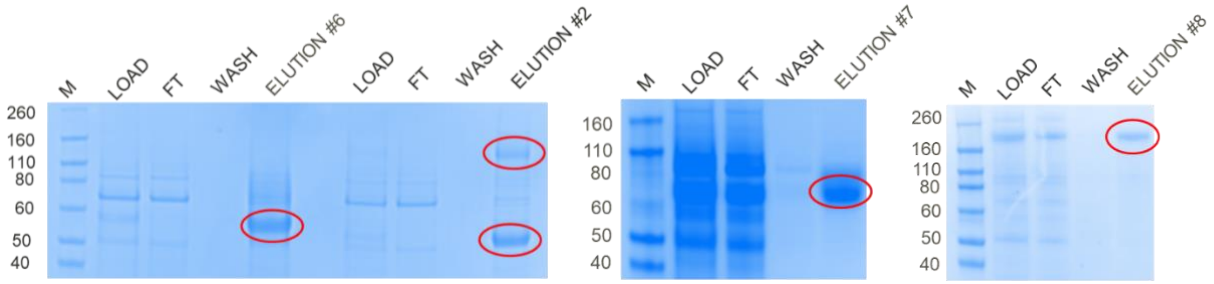


Fig. 11: IMAC (Immobilized Metal Affinity Chromatography) purifications of the CdrA forms, expressed in the mammalian system.

4.3 CdrA constructs expressed in mammalian cells are glycosylated

The recombinant soluble and purified forms of CdrA showed a slightly higher molecular weight than expected. To assess whether this was due to glycosylation, which normally occurs in mammalian cells, we performed a glycosylation sites prediction using the NetNGlyc - 1.0 program (**Fig. 12**) and a de-glycosylation assay using the PNGase enzyme that is able to remove all types of N-linked glycosylation (**Fig. 13**).

Construct #2

```
METDTLLLWVLLLWVPGSTGDAAQPARRARRTKLALGSSHHHHHHHHGSGAAPTGAQFNPNEIKISQQGKTTLIDQSTQR      80
AIINWKGFDVSADEAVRFNQPGVTSSTLNRVTAGQESVIAGRISAPGQVIIYNSNGVVFSGSAKVDVGSLITTTANISDE     160
HFRQGKLIFDQPGNPDARIVNDGSISVAEKGLAAFVAPSVANNGVINARLGTVAMAAGNAATIDLYGDGLVSIAVTDPVT     240
RKPQDAQALVSNGGAIQADGGSVLITAEQASRVVDNAVNLSGVILARGTEVREGSVALVSKSGDIQIAGKIDVSGPKNGG     320
DVLVSGQQVALASTASIDARGTAQGGSVRIGGDFQGRGELPRAKNATLAKGASIDVSATGKGNGGLAVVWSDGNTRMDGR     400
ILARGGAQGGNGGLVETSGKVNLSIADSAYVSVAAPYGNGGTWLLDPTTLRIVASGGTSGSVGGANGASGDATVNASVVT     480
GALAGGKVTLSASDRLSVEAPLITSNLGGASRGLELIATGPAGAVDISAPILFRNGSLAIRAGGNINFLSGGTPQTSGIV     560
DLGSGTLWMQTSTAGKISQQAGTALIAANLAGRAGSIDLASWDNYAGNLALQTFNGTLKYRQSNATGVTTSGTVFDPFIN     640
QSMTGTAQNIVSSVGTRILEANSVGTTGNYTLTADGNSEFDRLVFTALPYRRVSGSASFPTNDSSDYLVTNLRYQVNGSN     720
VTATPNGGAPSGFTVAAGNGSVTTWTGNWGTSWGVKGFGGVIGVTDELQYDVGTGLTEELIFGLGGKTSRVDTRLDLFMR     800
EGAFNSFAERAQVEMFKTTTTAGDILSRQQTATLTANDATRVYGDVNPTLTATMSGINAIDAYVNSQFNDLYQATASTTA     880
TQASNVGQYAITGNANGSEYFSQRYQLVRQDGR
```

Construct #6

```
METDTLLLWVLLLWVPGSTGDAAQPARRARRTKLALGSSHHHHHHHHGSGAAPTGAQFNPNEIKISQQGKTTLIDQSTQR      80
AIINWKGFDVSADEAVRFNQPGVTSSTLNRVTAGQESVIAGRISAPGQVIIYNSNGVVFSGSAKVDVGSLITTTANISDE     160
HFRQGKLIFDQPGNPDARIVNDGSISVAEKGLAAFVAPSVANNGVINARLGTVAMAAGNAATIDLYGDGLVSIAVTDPVT     240
RKPQDAQALVSNGGAIQADGGSVLITAEQASRVVDNAVNLSGVILARGTEVREGSVALVSKSGDIQIAGKIDVSGPKNGG     320
DVLVSGQQVALASTASIDARGTAQGGSVRIGGDFQGRGELPRAKNATLAKGASIDVSATGKGNGGLAVVWSDGNTRMDGR     400
ILARGGAQGGNGGLVETSGKVNLSIADSAYVSVAAPYGNGGTWLLDPTTLRIVASGGTSGSVGGANGASGDATVNASVVT     480
```

Construct #7

```
METDTLLLWVLLLWVPGSTGDAAQPARRARRTKLALGSSHHHHHHHHPTTLRIVASGGTSGSVGGANGASGDATVNASVV      80
TGALAGGKVTLSASDRLSVEAPLITSNLGGASRGLELIATGPAGAVDISAPILFRNGSLAIRAGGNINFLSGGTPQTSGI     160
VDLGSGTLWMQTSTAGKISQQAGTALIAANLAGRAGSIDLASWDNYAGNLALQTFNGTLKYRQSNATGVTTSGTVFDPFI     240
NQSMTGTAQNIVSSVGTRILEANSVGTTGNYTLTADGNSEFDRLVFTALPYRRVSGSASFPTNDSSDYLVTNLRYQVNGS     320
NVTATPNGGAPSGFTVAAGNGSVTTWTGNWGTSWGVKGFGGVIGVTDELQYDVGTGLTEELIFGLGGKTSRVDTRLDLFM     400
REGAFNSFAERAQVEMFKTTTTAGDILSRQQTATLTANDATRVYGDVNPTLTATMSGINAIDAYVNSQFNDLYQATASTT     480
ATQASNVGQYAITGNANGSEYFSQRYQLVRQDGR
```

Construct #8

```
METDTLLLWVLLLWVPGSTGDAAQPARRARRTKLALGSSHHHHHHHHGSGPTTLRIVASGGTSGSVGGANGASGDATVNA      80
SVVTGALAGGKVTLSASDRLSVEAPLITSNLGGASRGLELIATGPAGAVDISAPILFRNGSLAIRAGGNINFLSGGTPQT     160
SGIVDLGSGTLWMQTSTAGKISQQAGTALIAANLAGRAGSIDLASWDNYAGNLALQTFNGTLKYRQSNATGVTTSGTVFD     240
PFINQSMTGTAQNIVSSVGTRILEANSVGTTGNYTLTADGNSEFDRLVFTALPYRRVSGSASFPTNDSSDYLVTNLRYQV     320
NGSNVTATPNGGAPSGFTVAAGNGSVTTWTGNWGTSWGVKGFGGVIGVTDELQYDVGTGLTEELIFGLGGKTSRVDTRLD     400
LFMREGAFNSFAERAQVEMFKTTTTAGDILSRQQTATLTANDATRVYGDVNPTLTATMSGINAIDAYVNSQFNDLYQATA     480
STTATQASNVGQYAITGNANGSEYFSQRYQLVRQDGRLTVTPAQLIVSADAKTKVYGDADPTLTYQVSGLKNSDTAAGVL     560
SGNLGRVAGENVGNYGILQGGLGLNTANYTLSYVGNDLRITPAQLNVIADAKTKVYGDLDPALTYQVSGLKRGDTAGAVL     640
NGGSLSRVAGENVGVYGINQGGLGLVSSNYTLNYQGNNLTITKALLNVIADAKTKVYGDADPALTYQVSGLKNGDTAGAV     720
LNGGSLSRVAGENVGVYGINQGGLGLLSANYDLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGS     800
ILTGGLNRAAGENVGVYGINQGDLALNSGNYDLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGA     880
VLNGGGLVRVSGENVGNYAIQQGGLGLVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDSAG     960
SILTGGLNRAAGENVGVYGINQGDLALNSGNYDLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAG    1040
AVLNGGGLVRVSGENVGNYAIQQGGLGLVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTA    1120
GAVLNGGSLSRVAGENVGVYGINQGDLALNSGNYDLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDT    1200
AGAVLNGGGLVRVSGENVGNYAIQQGGLGLVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGD    1280
SAGSILTGGLNRAAGENVGVYGINQGDLALNSGNYDLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGD    1360
TAGAVLNGGGLVRVSGENVGNYAIQQGGLGLVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNG    1440
DTAGAVLNGGSLSRVAGENVGVYGINQGGLGLVSGNYDLAYQGNNLTITKALLNVIADGKTKVYGDADPSLTYQVSGLKN    1520
GDSAGSILTGGLNRDAGENVGVYGINQGGLVLTSGNYDLAYQGNDLTITKALLNVFADAKSKQVGTADPALTYQVSGLKN    1600
GDSAGQVLAGGLGRVGGEAVGQYDILQGGLALTSGNYQLNYQGNLLSILPLPVTPGDLGQLAALSDLRELQKG
```

Fig. 12: Glycosylation sites prediction of the CdrA sequences expressed in the mammalian system. Asn-Xaa-Ser/Thr sequons in the sequence output are highlighted in blue. Asparagines predicted to be N-glycosylated are highlighted in red. Predictions were obtained with the program NetNGlyc - 1.0 [146].

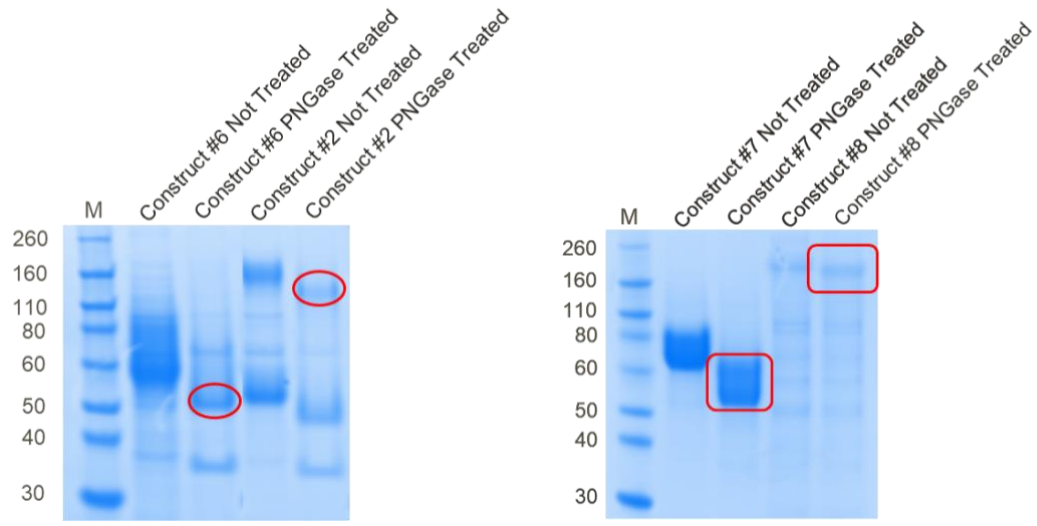


Fig. 13: De-glycosylation assay with PNGase F to remove the glycosylation moieties.

After the treatment, all the constructs reached the expected molecular weight proving that the CdrA constructs produced as recombinant protein in mammalian cells are glycosylated.

4.4 Soluble recombinant CdrA constructs are folded

Based on data collected, on information available in the literature and on the sequence homology of the proteins under study with proteins with known structure we decided to focus on constructs #7 and #8. In order to evaluate their thermal stability a differential scanning nano fluorimetry (nanoDSF) and a Thermofluorimetry assay were performed. The nanoDSF is a label-free method used for thermal unfolding experiments to monitor and quantify the structural stability of proteins. This technique is based on monitoring the intrinsic tryptophan fluorescence of proteins which is strongly dependent on the 3D structure as it is highly sensitive to the neighborhood of tryptophan residues and changes during thermal unfolding. Intrinsic tryptophan fluorescence is measured at 330nm and 350nm wavelength which allows the measurement of even minor differences in fluorescence intensity and fluorescence emission peaks. In this assay the Fluorescence emission increases with increasing temperature, giving rise to a sigmoidal curve which represents a typical thermal denaturation profile of a recombinant protein as shown in **Fig. 14**. Changes in the intensity of the fluorescence reflect the inflection points of the protein corresponding to the midpoint of the unfolding transition Tm (°C), which is determined after plotting the fluorescence ratio against the temperature [147-148]. The obtained value represents the point

at which half of the protein is unfolded. **Fig.15** depicts the constructs 7 and 8, investigated for this analysis, that showed 2 different trends. On the one hand construct 7 shows a sigmoid trend, given by two inflection temperatures, reflecting a correct folding, on the other hand construct 8 showed a thermal transition that was slightly detected (inflection temperature at 69.5 °C), possible due to the low concentration of the sample.

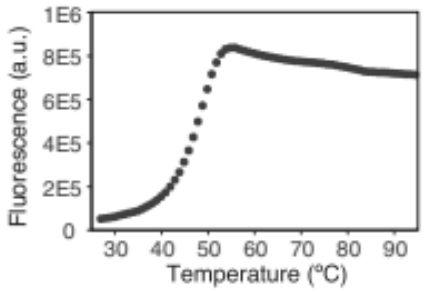
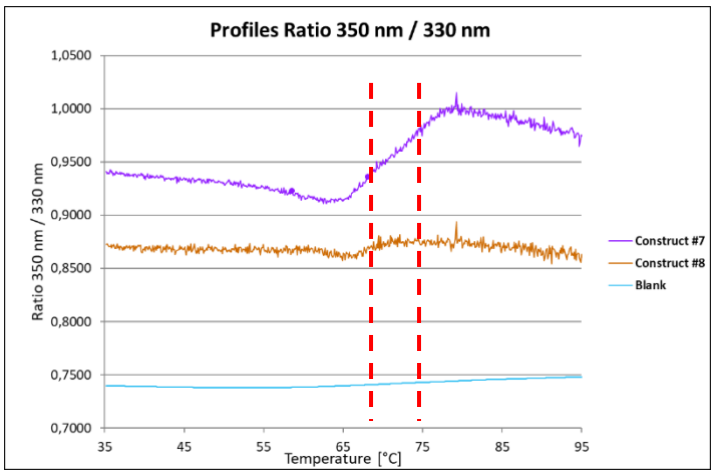


Fig 14: Typical thermal denaturation profile of a recombinant protein. Low fluorescence at room temperature indicates a well-folded protein. Fluorescence emission increases with increasing temperature, giving rise to a sigmoidal curve that represents cooperative unfolding of the protein [148].



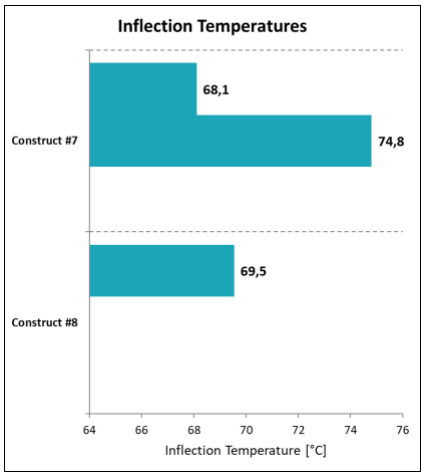


Fig. 15: Thermal unfolding curves and unfolding inflection temperatures of the CdrA construct #7 and #8. The data suggest that construct #7 shows a sigmoidal trend characterized by two inflection temperatures indicating proper folding on the other hand construct 8 shows a similar trend to the blank characterized by a thermal transition that was slightly detected and is characteristic of a non-folded protein.

To further determine the protein stability of these constructs, a Thermofluor analysis was performed. The experiment was executed with the fluorescent dye Sypro Orange which is highly fluorescent in nonpolar environments with low dielectric constants such as hydrophobic sites in proteins. When a protein starts to unfold, the dye binds to exposed hydrophobic parts of the protein, resulting in a significant increase in fluorescence emission. The fluorescence intensity reaches a maximum and then starts to decrease, probably due to precipitation of the complex of fluorescent probe and denatured protein. The stability curves and their mean value (melting temperature, Tm) were obtained by gradually increasing the temperature to unfold the protein and measuring the first derivative of the fluorescence emission, as a function of temperature (−dF/dT) at each point. In this case the Tm is represented as the lowest part of the curve, as it is depicted in the **Fig. 16** [147-148]. Data obtained with this technique (**Fig. 17**) reveal that construct #7 exhibits a Tm around 68 degrees while construct #8 exhibits a similar trend to the blank reflecting the pattern of an unfolded protein. These data confirm those obtained with the nanoDSF.

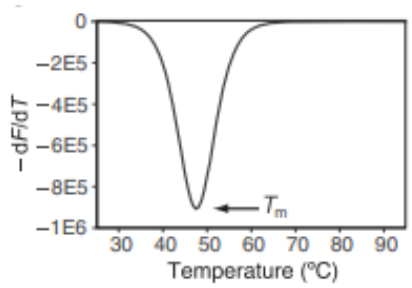


Fig 16: Tm identified by plotting the first derivative of the fluorescence emission as a function of temperature (−dF/dT). Here, Tm is represented as the lowest part of the curve [148].

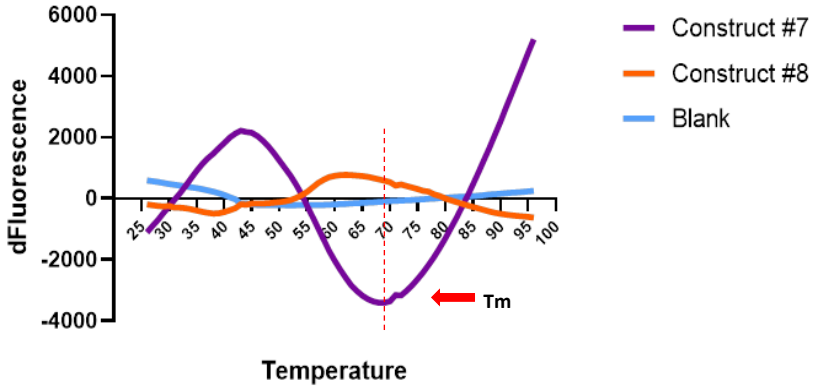


Fig. 17: Thermal shift assay result for the constructs #7 and #8. A significant difference in the shape of the curves could be observed between the construct #7 and the construct #8. Construct #7 exhibits a Tm around 68 degrees while construct #8 exhibits a similar trend to the blank reflecting the pattern of an unfolded protein.

4.5 Recombinant CdrA interacts with exopolysaccharide Psl

In the literature it is hypothesized that CdrA promotes biofilm stability through its interaction with the exopolysaccharide Psl [115], conferring protection from proteolytic degradation. Given the importance that this interaction could have on biofilm stability, the *in vitro* interaction of construct #7 of purified recombinant CdrA and purified Psl was evaluated using two different techniques, nanoDSF and surface plasmon resonance spectroscopy (SPR). Construct #8 was not considered since it appears to be unfolded from previous analyses. Using nanoDSF, described above, we investigated whether the presence of the polysaccharide Psl influenced the thermal stability of the CdrA protein, indicating an interaction between the two molecules. In the presence of the polysaccharide Psl (kindly provided by the Vaccines chemistry team, GSK Siena) the construct #7 undergoes a change in the inflection temperature point compared to that obtained with the construct alone, further supporting the evidence that Psl interacts with the protein and this interaction induces greater stabilization of the protein, which unfolds at higher temperature (**Fig. 18**).

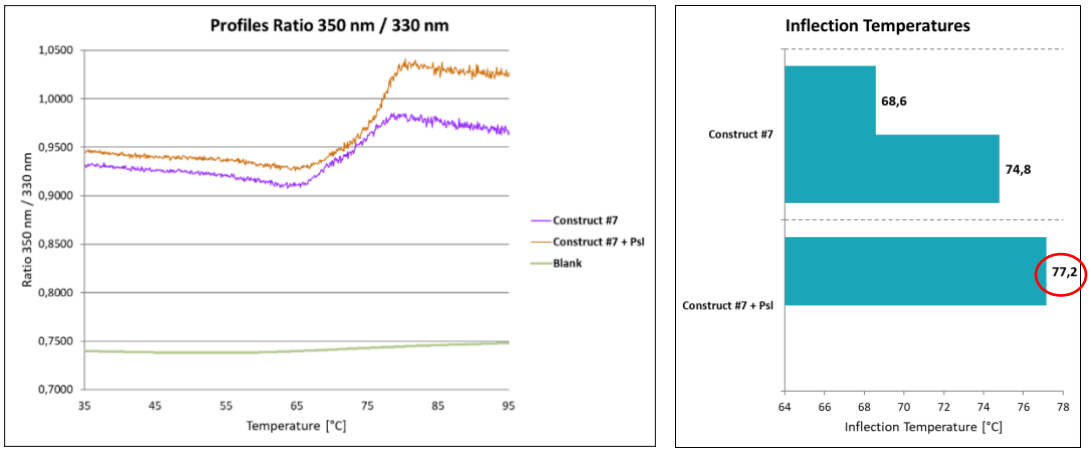


Fig. 18: Thermal unfolding curves and unfolding inflection temperatures of the CdrA construct #7 alone and with the exopolysaccharide Psl which induces a change in the inflection temperature of the protein #7.

Another method, used to assess the binding between the protein and the Psl, is the Surface plasmon resonance (SPR) spectroscopy which investigates biomolecular interactions in real time. The output signal obtained in this analysis is measured in resonance units (RU) where 1000 RU is equal to 1 ng of mass per 2 mm^2. The construct #7, containing the His - tag at the N-terminus, was attached on the sensor surface trough the capture of the histidine-tag,

achieved via nickel (Ni2+) chelation of nitrilotriacetic acid (NTA). After the immobilization step, the Psl was added to the chip to assess its interaction with the CdrA protein. Results showed that the Psl, interacting with the protein, induced its detachment from the sensor chip, as indicated by the green line corresponding to the bind between the two molecules, which appeared to have a lower resonance unit (RU) than the red control line representing the HBS-EP+ running buffer (**Fig. 19**).

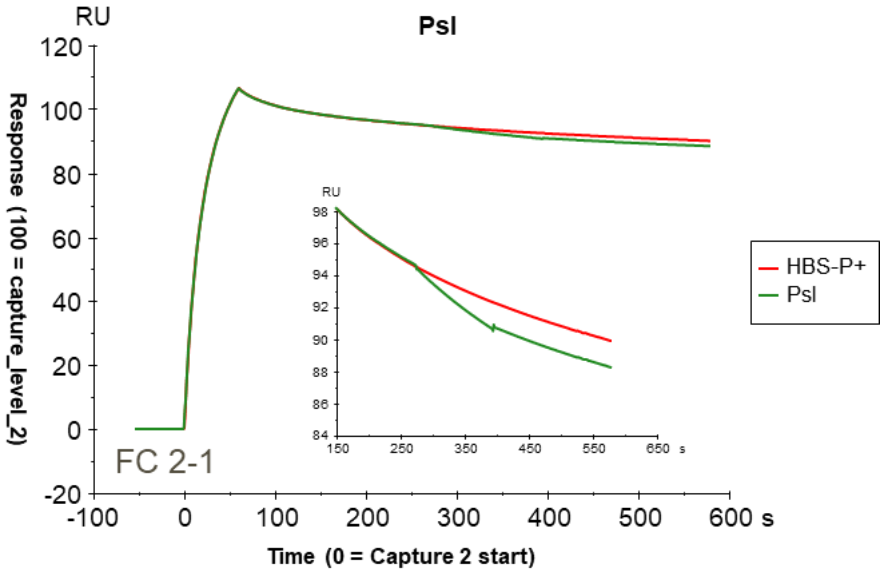


Fig. 19: Evaluation of the binding between CdrA and the exopolysaccharide Psl. This latter induces the detachment of the protein from the sensor chip as reported by the green line which is lower than the red control line (enlargement in the graph).

The binding of the protein to specific anti-CdrA antibodies was also assessed with the same SPR experiment. The results showed that the protein is present and correctly immobilized on the chip, since a stable binding between the protein and its specific antibodies is observed, as indicated by the green line. The red line indicates the running buffer used as a control. (**Fig 20**). These preliminary results highlight the presence of a bond between the CdrA protein and the Psl, although further analysis is required to better understand this interaction.

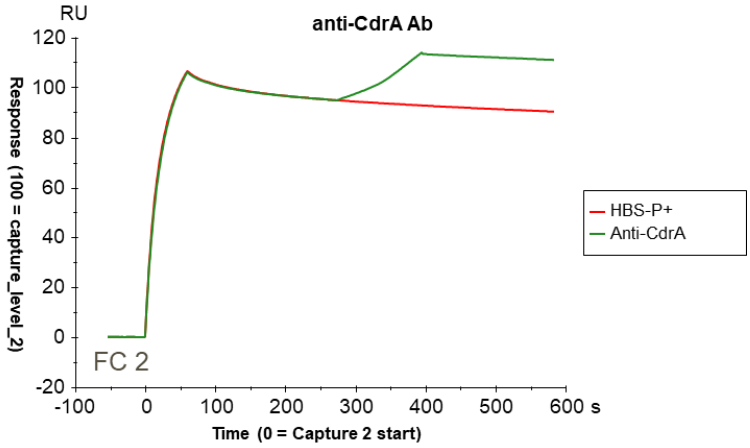


Fig. 20: Evaluation of the binding between CdrA and the anti-CdrA antibodies to confirm the presence of the protein on the sensor chip. The green line indicates the presence of a stable binding between the protein and its specific antibodies.

4.6 Recombinant CdrA protein is immunogenic

As described in the material and method section, two different forms of CdrA (construct #7 and #8) were used to immunize mice and the sera obtained were tested in an *in vitro* biofilm model of *P. aeruginosa* to assess their ability to inhibit bacterial adhesion **(Fig. 21)**.

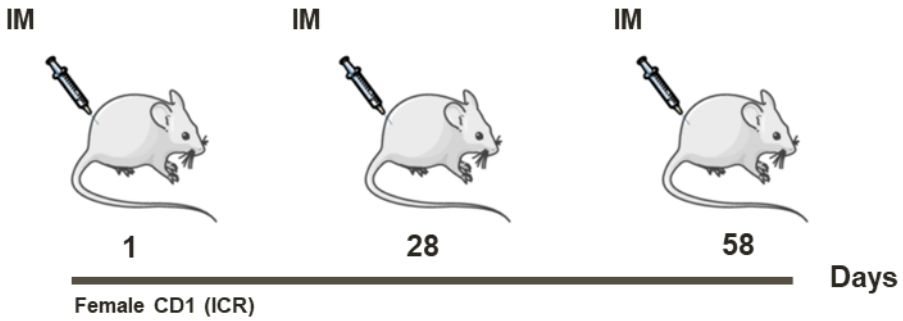


Fig. 21: CdrA immunization scheme.

To assess the presence of specific anti-CdrA antibodies after the third immunization (post III sera) a Luminex binding assay was performed. In this assay, color-coded superparamagnetic beads coated with the CdrA antigens were incubated with the specific anti-CdrA antibodies. The mean fluorescence intensity (MFI) signal was detected using a cocktail of biotinylated detection antibodies and a streptavidin-phycoerythrin conjugate. The high MFI values indicate the presence of specific anti-CdrA antibodies in the sera derived from the recombinant CdrA constructs immunization (**Fig. 22).**

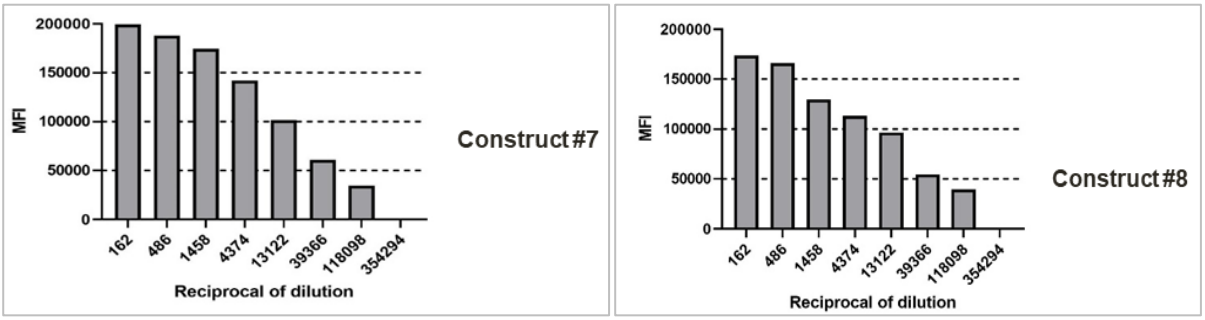


Fig 22: Luminex assay to evaluate the presence of specific anti-CdrA antibodies. The MFI values indicates the presence of specific anti-CdrA antibodies in the sera derived from immunization.

4.7 Anti-CdrA antibodies raised against recombinant CdrA are able to recognize native P. aeruginosa protein

CdrA is a protein present on the cell membrane that is subsequently released into the culture medium as a result of the action of a periplasmic protease that processes the TAAG cutting site present in the protein portion of the cysteine Hook [118]. In order to understand at which step of the pathogen growth the protein is released, the presence of CdrA was determined in bacterial pellet of the WT PAO1 strain harvested at different optical density (OD) points. The results show that the protein is in the total extract sample only at OD = 0.1, suggesting that at higher ODs CdrA is released into the culture medium (**Fig. 23**).

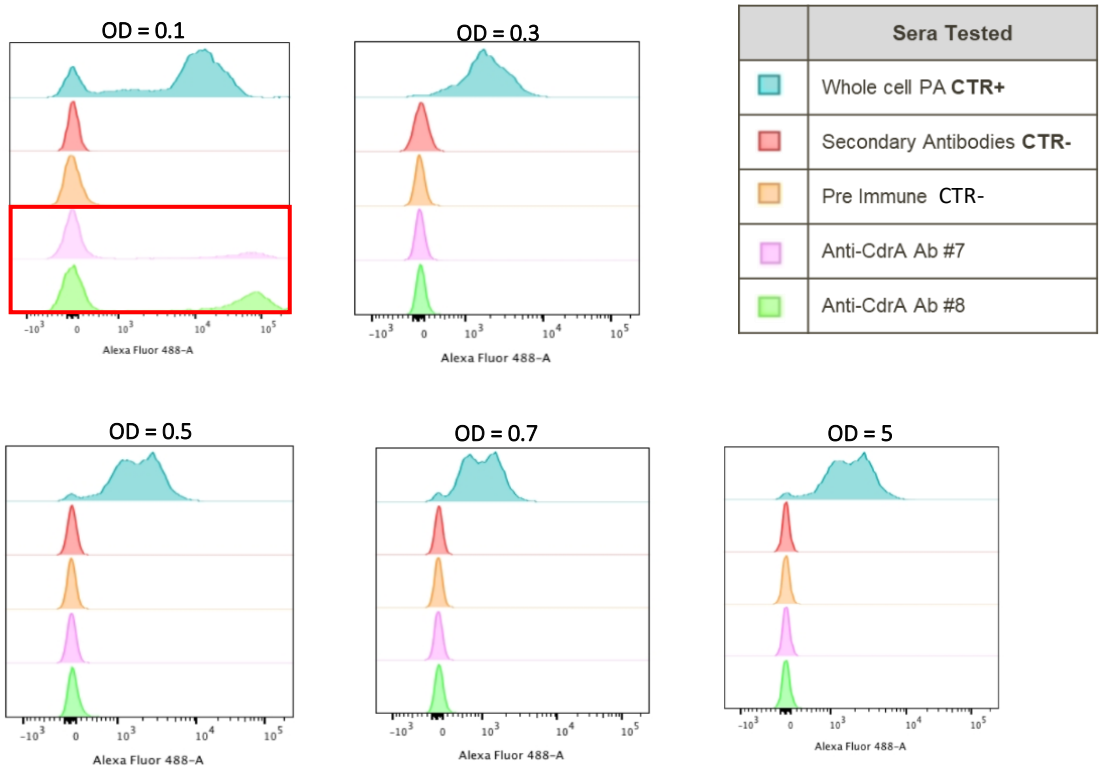


Fig. 23: FACS analysis to evaluate the recognition of the CdrA protein from the specific anti-CdrA antibodies, at different OD. The Protein is present on the bacterial membrane only at the optical density of 0.1.

To confirm this result, the medium of the cultures collected at different ODs were analyzed in WB with the anti-CdrA antibodies which detected the presence of the protein at ODs greater than 0.1, indicating its release into the culture medium (**Fig. 24**). With higher ODs the amount of CdrA detected decreased indicating protein degradation. Protein degradation is also detected in the whole total cells extracts, indicating that the protein is further proteolyzed in many fragments and subdomains.

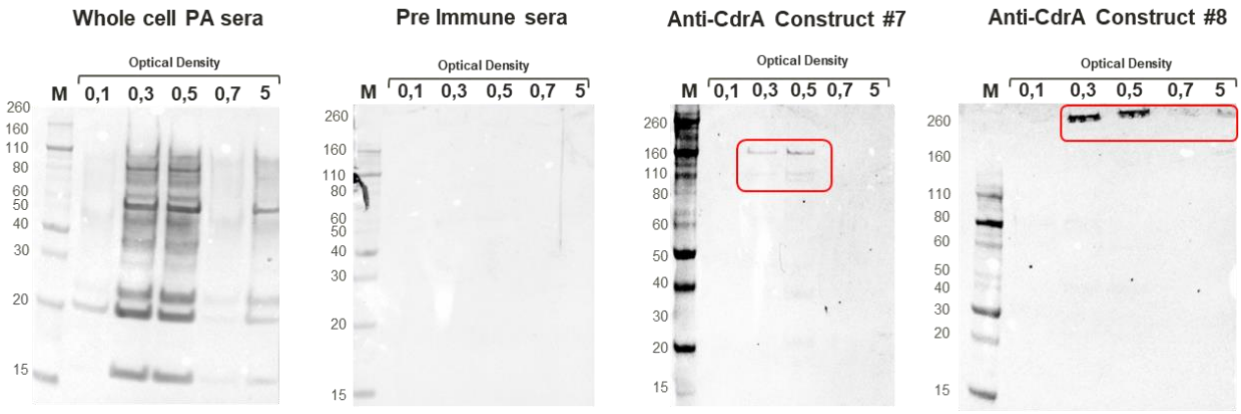


Fig. 24: Recognition of the native CdrA protein from the anti-CdrA antibodies in the P. aeruginosa culture supernatants. Anti-CdrA antibodies recognize the presence of CdrA protein in the supernatants.

4.8 Anti-CdrA sera inhibit biofilm formation and reduce biofilm biomass

Most of the currently available techniques do not allow the enumeration of the viable cells fraction within the biofilm and are often time consuming to be performed [149]. A fast and precise alternative method was set up to determine the number of viable cells within the biofilm using a flow cytometry (FCM) technique using the single-stain viability dye TO-PRO-3 iodide which is a carbocyanine monomer nucleic acid stain with red fluorescence, which is a nuclear counterstain and dead cell indicator. This method, adapted from Kerstens *et al* [149] was set up to assess the number of live cells in the *P. aeruginosa* biofilm after treatment with anti-CdrA antibodies derived from the Construct #7 in immunization. The approach allows to generate a single-cell suspension for FCM analysis and the experimental procedure used consists of the following steps: growth and harvest of the biofilm at different time points from 24h to 120h. At each defined time point, the removal of the planktonic cells is performed by a washing step and then, the remotion of the biofilm cells from the wells of the 96 well black plate is achieved through a combination of scraping and rinsing steps. Scraping is used to detach biofilm in the center of the well, while rinsing with a syringe is used to remove biofilm near the edges. For the optimization of the dissociation of the biofilm aggregates we compared the efficacy of sonication and vortexing procedures using the PAO1 WT strain. As shown in the **Fig. 25**, there is no significant difference between the two techniques, so subsequent experiments were carried out using vortexing as it less time consuming.

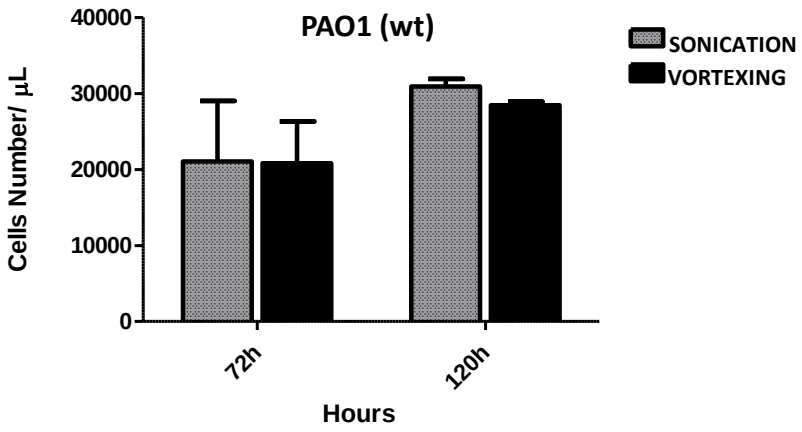


Fig. 25: Comparison of sonication and vortexing procedures to assess which technique is more effective useful for the dissociation of the biofilm aggregates. As shown in the graph, there is no significant difference between the two techniques.

Finally, the biofilm cells were stained with TO-PRO 3 iodide and fixed for FACS (fluorescence activated cell sorting) reading. Only two washing steps are required for the sample preparation, presenting an additional advantage over other techniques involving the addition and subsequent removal of fixatives and staining solutions in which several wash steps are required that may increase the possibility of biofilm detaching from the abiotic surface. The results obtained demonstrate that it is possible with this technique to monitor biofilm formation and maturation, the additional advantage is that with this approach it is possible to determine cell viability and number. Indeed, we observed that cells in the biofilm remain viable for up to 5 days (120h) and increase in number at different time points. A slight decrease in the number of cells at 120h was observed and this may be due to the part of bacteria which returns to the stage V (as indicated in the **Fig. 2** in the introduction section) of the biofilm where there is more formation of planktonic cells, removed by the washing step (**Fig. 26**).

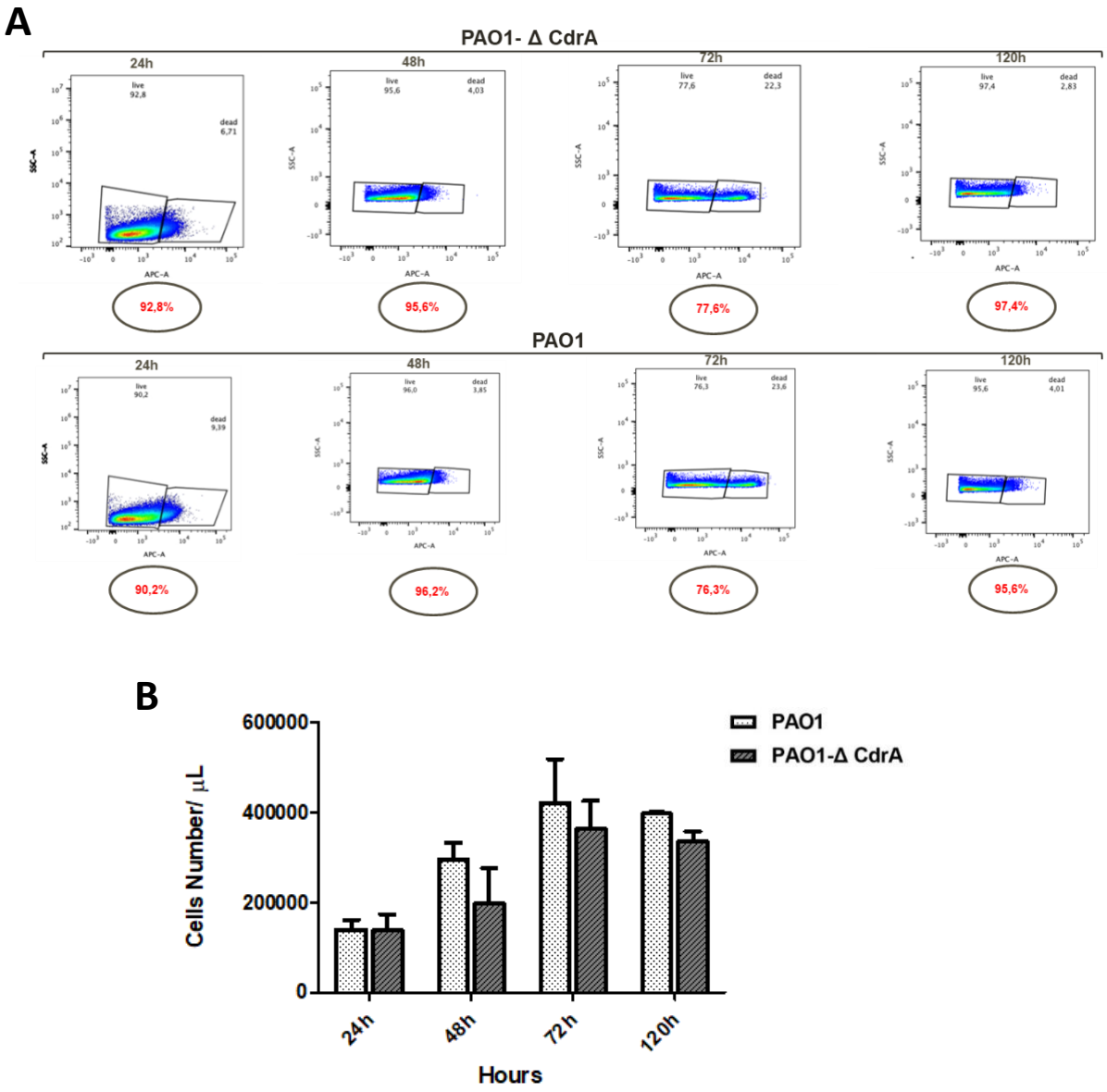


Fig. 26: FACS analysis to determine the enumeration of the viable cell fraction within the biofilm. Biofilm cells remain viable for up to 5 days (120h) **(A)** and increase in number at different time points with a slight decrease in the number of cells at 120h **(B)**.

After setting up this method and assessing its reproducibility, the effect of anti-CdrA antibodies and the corresponding preimmune (PI) sera on the number of live cells in the *P. aeruginosa* biofilm was evaluated. From the results it can be observed that, at all-time points, the anti-CdrA antibodies do not have a relevant effect on the biofilm bacterial vitality (**Fig. 27**), as it may be expected, but they can reduce the number of bacteria trapped in the biofilm of the PAO1 WT strain without affecting the number of bacteria of the ΔCdrA strain **(Fig. 28**).

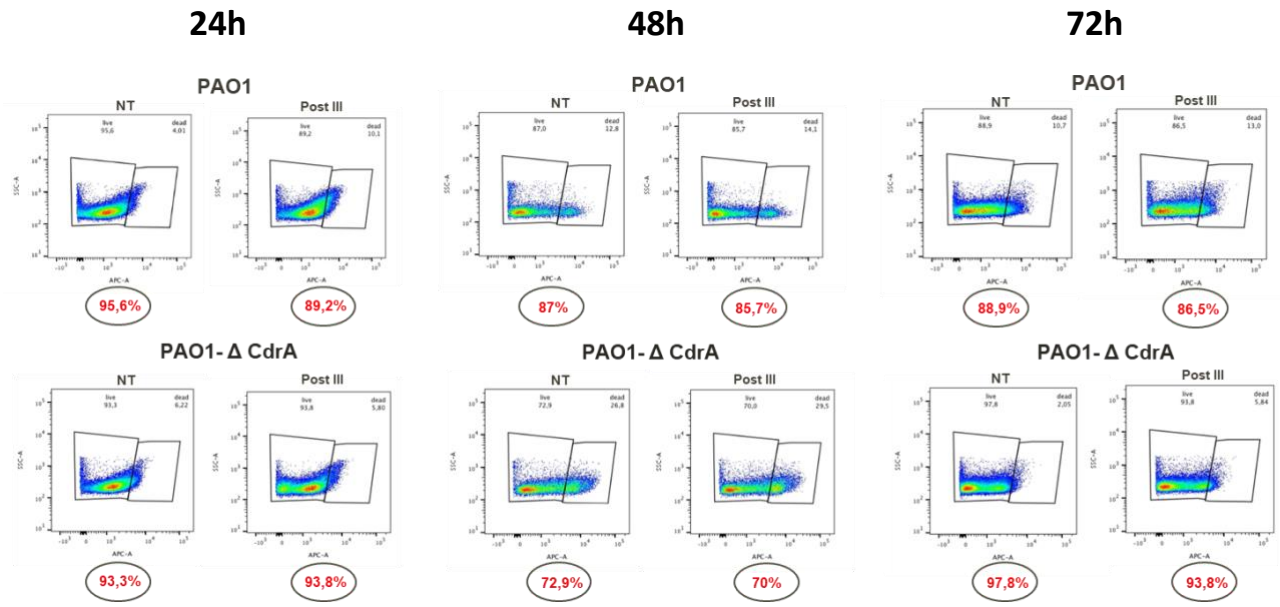


Fig. 27: Bacteria vitality evaluation after the use of specific anti-CdrA sera. This latter has not relevant effect on the bacteria vitality of the PAO1 and ΔCdrA strains.

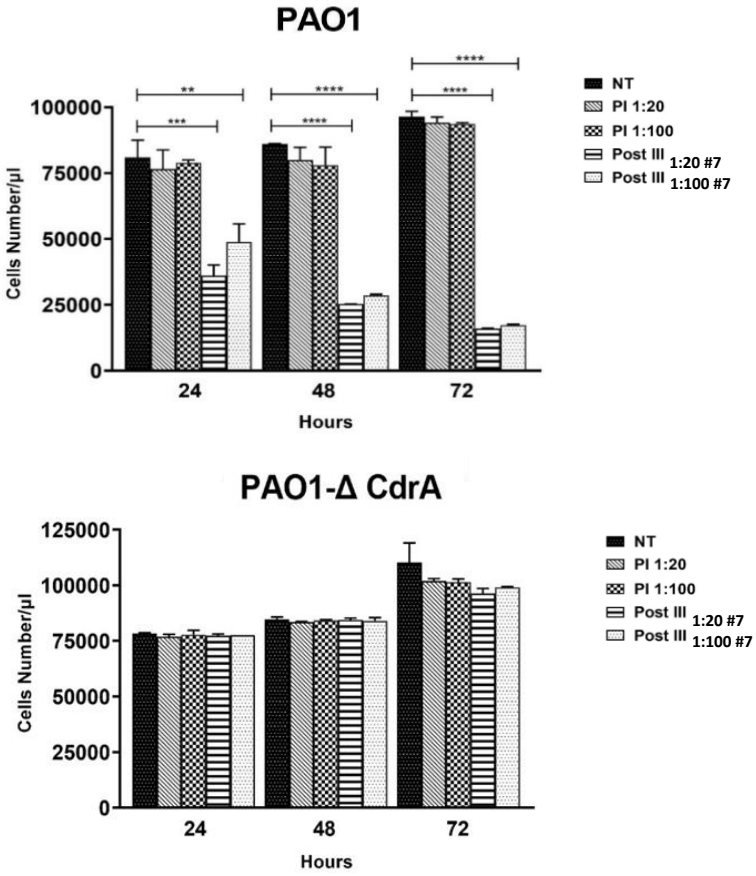


Fig. 28: Determination of the biofilm bacterial numbers as a result of the use of the anti-CdrA antibodies**.** A reduction in the number of bacteria trapped in the biofilm of the PAO1 wt strain is observed while the number of bacteria of the ΔCdrA strain are not affected.

To assess whether anti-CdrA antibodies also have an effect on the formation of the biofilm biomass, an assay employing the use of the crystal violet (CV) was applied [150]. Crystal violet is a dye which binds negatively charged molecules and thus stains both the bacteria and the surrounding biofilm matrix. As in the previous experiment, the biofilm cells are grown and harvested at different time points (from 24h to 120h) and the planktonic cells were removed after washing steps. CV staining was then performed and followed by further washing steps to remove the excess of dye. The absorbance readings at 590 nm indicates the biofilm biomass is formed at the different time points and that it is not affected by the preimmune sera, while after treatment with anti-CdrA antibodies, showed a marked reduction in the biofilm of the treated samples compared to the untreated one (**Fig. 29**). No effects were observed on ΔCdrA strains treated with preimmune or anti-CdrA serum.

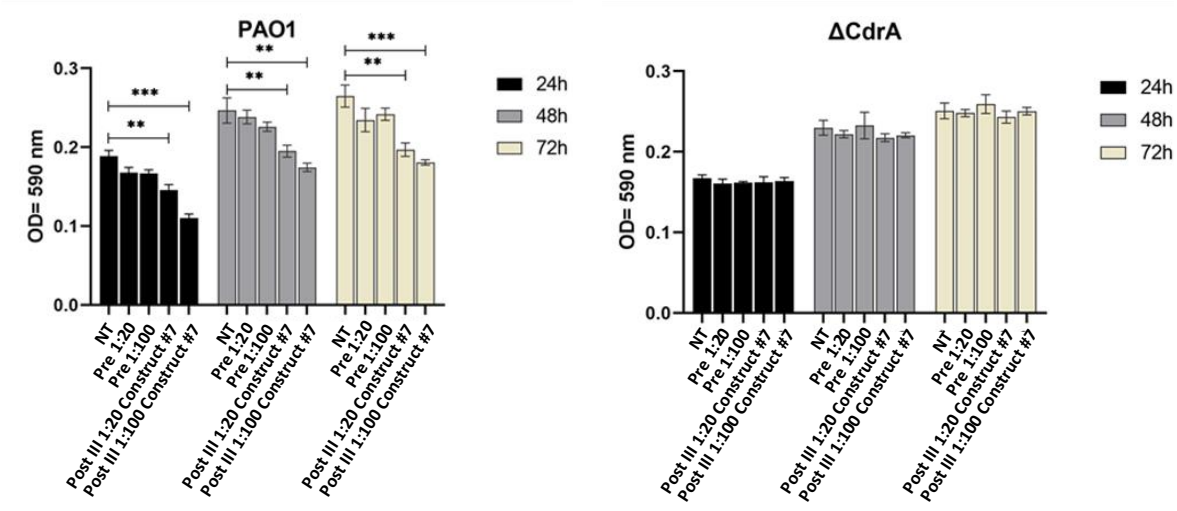


Fig. 29: Evaluation of the Biofilm biomass reduction after the treatment with the anti-CdrA serum. The specific antibodies induce a marked reduction in the biofilm of the treated samples compared to the untreated one.

4.9 Novel P. aeruginosa antigens are recognized by P. aeruginosa anti-whole cell antibodies and by patient- derived sera

Since the treatment of *P. aeruginosa* infections has become a major challenge due to the ability of this bacterium to resist many of the currently available antibiotics [119], the development of new antibiotics or alternative preventive/therapeutic strategies for the treatment of *P. aeruginosa* infections is urgently required. In this perspective, the discovery of new antigens potentially involved in the pathogenesis of *P. aeruginosa* is crucial to develop new vaccines to fight and prevent infections caused by this pathogen [151]. To this purpose, to evaluate new potential vaccine candidates, for this thesis we have exploited the

use of an innovative protein microarrays generated in collaboration with Sengenics. 60 *P. aeruginosa* proteins, including the CdrA protein, were printed on the microarray slides. The arrays were used to test anti-*P. aeruginosa* whole cell mouse sera and human sera from a clinical trial in which COPD patients positive for the pathogen and relative negative control patients were followed for 2 years. The microarray chips, developed in collaboration with the company Sengenics company, present full-length, correctly folded proteins. The Sengenics technology utilizes Biotin Carboxyl Carrier Protein (BCCP) folding marker which is cloned in-frame with the gene encoding the protein of interest. The BCCP acts not only as a protein folding marker but also as a protein solubility enhancer. The proteins on the chip are purified from cell lysates and immobilized in a single step by the high binding affinity of the carboxylated biotin to the streptavidin, coating on the solid support. This happen because proteins are expressed as fusions to the folding marker BCCP which becomes biotinylated only when the protein is correctly folded. Conversely, misfolded proteins drive the cotranslational misfolding of BCCP to such an extent that it becomes catalytically inactive, and is unable to become biotinylated. Hence, misfolded proteins no longer have a way of attaching to the streptavidin-coated solid support. Therefore, BCCP allows monitoring of protein folding by measuring the extent of biotinylation.

The proteins spotted on the chips can be divided into 4 groups (**Fig. 30**):

1. **BCCP N- terminus not secreted proteins**
2. **BCCP C- terminus not secreted proteins**
3. **Hypothetical proteins:** Proteins produced by the pathogen but whose function is not known.
4. **Secreted proteins:** Of the secreted proteins, 10 have the BCCP tag at both the N- and C-terminus and this will be useful in the serum recognition evaluation.

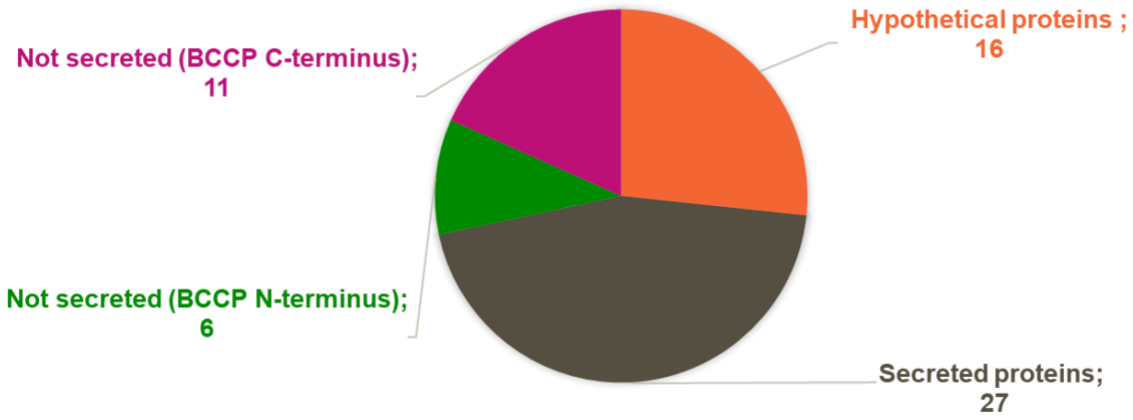


Fig. 30: *P. aeruginosa* proteins spotted on the array.

Initially, *P. aeruginosa* whole cell mouse sera were tested on the arrays at two different dilutions (1:100, 1:200), to assess which serum concentration was most suitable for analysis. Using a serum dilution of 1:100, which has been shown to be the most appropriate, as demonstrated from mouse serum 4, 18/60 spotted antigens were recognized by the *P. aeruginosa* anti-whole cell antibodies. The results also show that the position of the tag is crucial for the recognition of the protein by the sera, as in the case of proteins 22 and 47, and that all sera recognize with high affinity the protein 41, a hypothetical protein, which might be an interesting antigen for further investigations (**Fig. 31**).

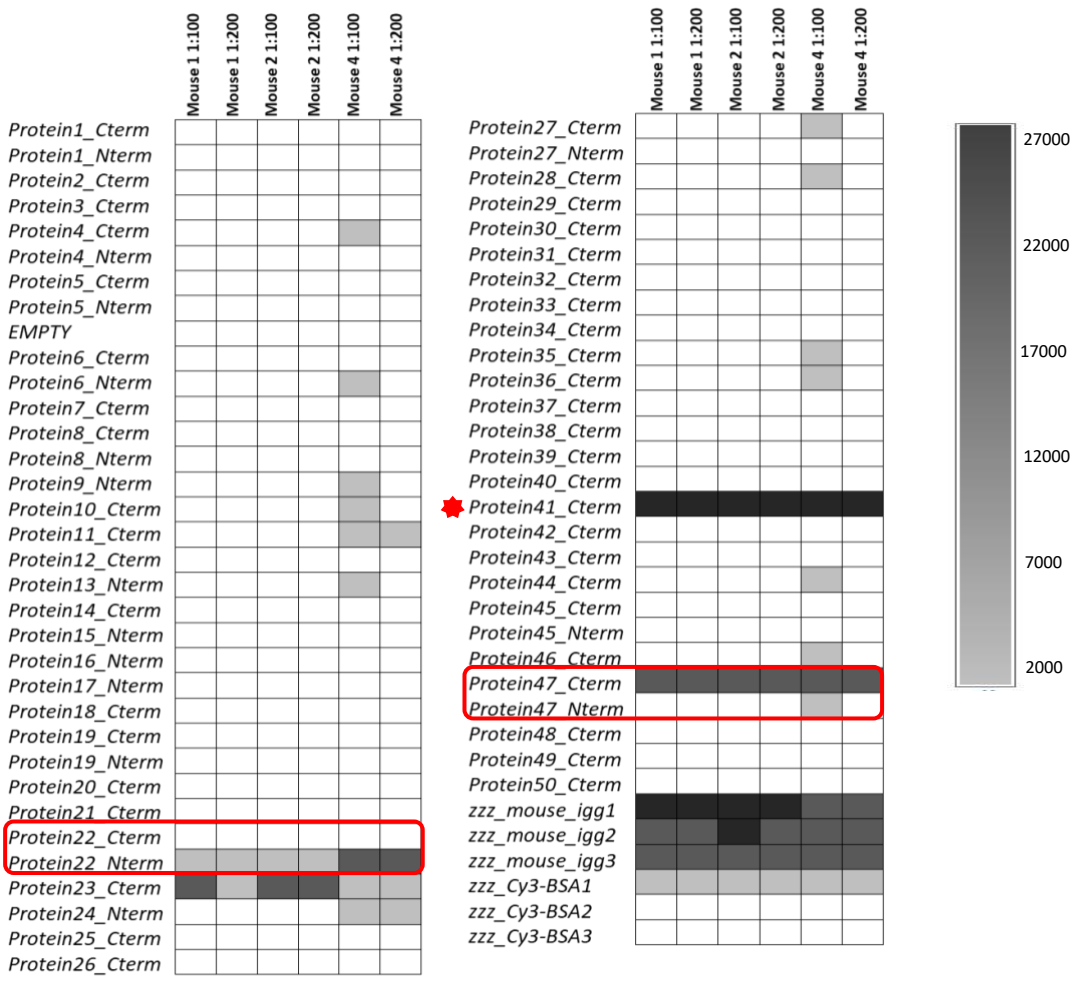


Fig. 31: Analysis of P. *aeruginosa* protein array tested with mouse derived anti - *P. aeruginosa* antibodies. The recognized proteins are 18/60, in particular the protein 41 is recognized with high affinity by all mouse sera (red dot). The position of the BCCP tag affects the protein recognition by sera (red rectangle).

The experiment was replicated using human sera from a clinical trial in which 152 patients were enrolled and tested for being positively colonized for a variety of pathogens, including *P. aeruginosa.* Among all subjects, only the serum of 17 of them resulted *P. aeruginosa* positive at all visits. Sera from these patients, as well as the serum of two *P. aeruginosa* negative patients, used as a comparator, were tested on the microarray at the dilution of 1:100. The proteins recognized by human sera amounted to 44 out of 60. Even in this case it was observed that the position of the tag affects the protein recognition by the sera as well as the fact that protein 41 is recognized, not only by all mouse sera, but also by almost all human sera underlining how this protein may be an interesting antigen to evaluate as vaccine

candidate (**Fig. 32**). The protein microarray analysis, that allowed to identify the protein 41 and other proteins that resulted to be immunogenic in COPD patients colonized by the pathogen, is resulted to be a good approach to select potential vaccine candidates.

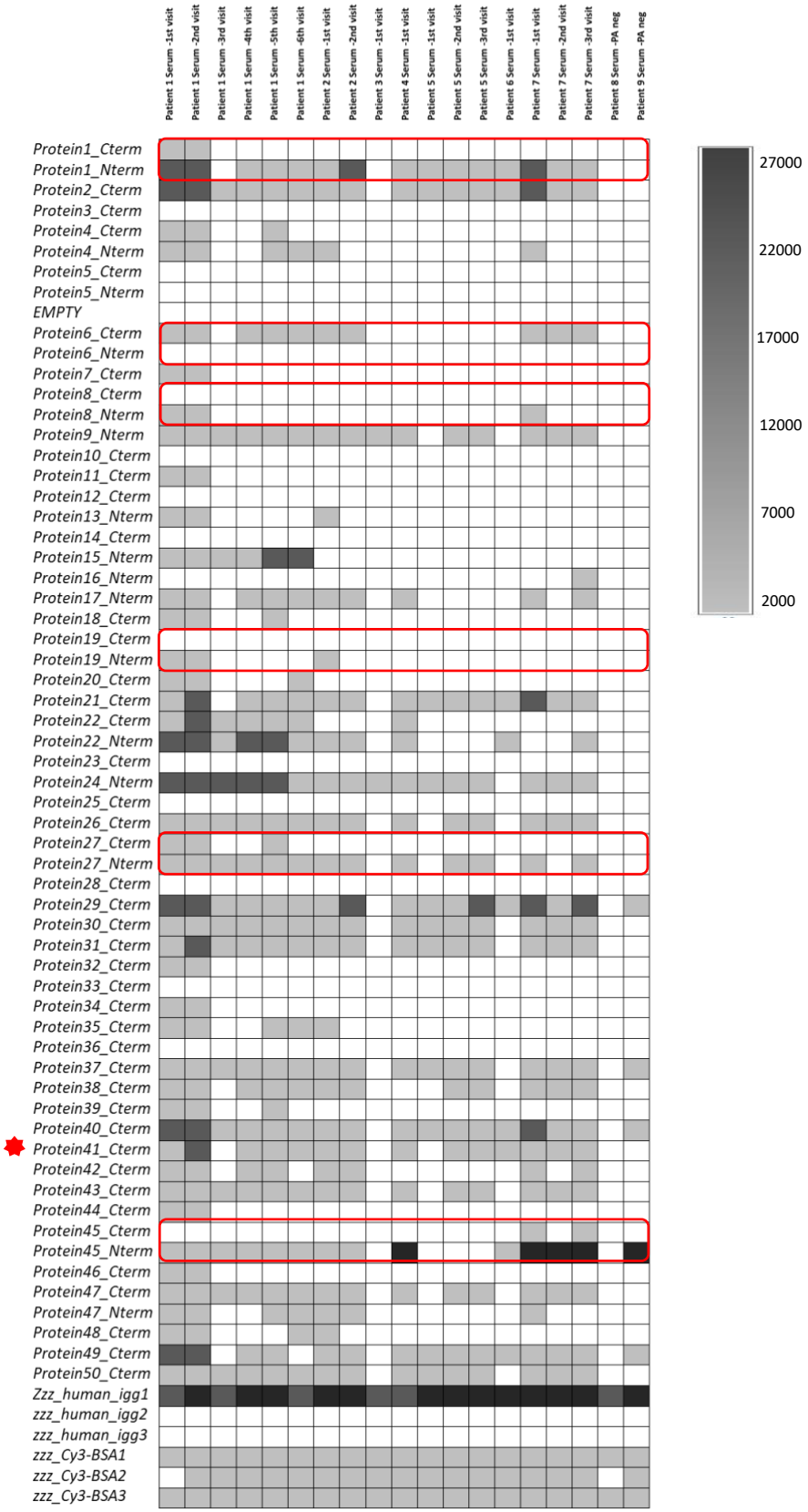


Fig. 32: Analysis of human sera derived antibodies on the *P. aeruginosa* protein array. Sera recognized 44/60 proteins spotted on the array and almost all of them recognized with high affinity the protein 41 (red dot). The position of the BCCP tag influences the protein recognition by the human sera (red rectangle).

DISCUSSION AND CONCLUSION

The problem of antimicrobial drug-resistant microorganisms is widespread globally. Decades after the first treatment with an antimicrobial substance, bacterial infections have once again become a threat due to the evolution and adaptation of microorganisms [152, 153]. A as a result of spontaneous and/or drug-induced mutations, bacteria can genotypically and phenotypically change to evade antibiotic strategies of action, adapt to their environment and survive for prolonged periods of time causing infections that are impossible to eradicate and which can lead to the host death. This is complicated by the ability of bacteria to grow in the sessile form, enclosed in the biofilm matrix, where susceptibility to antibiotics is dramatically lower than in the same cells grown in the planktonic form. This reduced susceptibility is a multifactorial process due, besides the acquisition of mobile gene factors for resistance, also to the reduced diffusion or the uptake of antimicrobial substances by the matrix and the different replicative phases of the cells within the biofilm that make antibiotics active only on specific bacterial sub-populations and thus not completely eliminating the problem [154]. The continuous increase in resistant bacterial strains to conventional antibiotics has prompted worldwide researchers to search for new strategies to defeat them, including the development of new vaccines and/or the use of target therapies. The research, development and refinement of new substances and strategies that prevent the development of superbug infections are essential and compulsory steps in the fight against what has become an alarming global phenomenon representing by the infections caused by multi-resistant bacteria that, only in Europe, cause 25,000 deaths every year [155]. In this thesis work, first, a key antigen of *Pseudomonas aeruginosa* was successfully obtained for the first time as soluble and folded recombinant protein. CdrA has been largely studied in the literature since it was the first protein identified in the *P. aeruginosa* biofilm matrix that plays a structural role in biofilm aggregates [115]. In fact, it has been shown that this protein induces biofilm stability and biofilm protection from mechanical destruction [115, 116]. However, CdrA has never been obtained as recombinant protein, thus preventing its testing as vaccine candidate and as target to generate therapeutic antibodies. CdrA is a bacterial protein and usually the main system used for the production of recombinant bacterial protein is the *E. coli* system, that has several advantages, including fast and cost-effective protocols. However, due to the complexity of this protein, including the presence of several disulphide bridges, make this antigen particularly tricky to be obtained in *E. coli*. The possibility to obtain CdrA in the mammalian expression system is a new approach applied for production

of complex bacterial proteins. The purified proteins showed the presence of glycosylation, as predicted by the presence of putative glycosylation sites in the sequence. However, the glycosylation was not detrimental for the folding or immunogenicity of the antigen which resulted to be immunogenic when tested in vivo. The CdrA protein is located on the cell membrane and only after the action of a protease is released into the culture medium. In this thesis we were able to evaluate that the passage of the protein from the cell surface to the culture medium occurs in the early stages of growth of the pathogen. Based on these results, the CdrA constructs can be now used for further structural studies to better elucidate the function and the molecular features of this protein, as well as its interaction with the exopolysaccharide Psl. Another innovative aspect of this thesis project was the development of a method to assess the viability and number of bacteria enclosed in *P. aeruginosa* biofilms using a flow cytometric approach. The positive aspect of using this method, unlike the others, is that with a single reproducible and fast analysis it is possible to obtain information on both the vitality and the number of bacteria in the biofilm. With this method it is possible to keep the biofilm cells viable for up to 5 days (120h). Moreover, from the results obtained, it is possible to observe that the number of bacteria increases at different time points even if around 120h can be observed a slight decrease in the number of cells, probably due to the fact that a part of bacteria in the biofilm re-enters in the stage V of the biofilm characterized by the formation of planktonic cells that detach from the biofilm to colonize new surfaces. This reduction is not observed in the experiments in which the anti-CdrA sera are tested because the formation of biofilm is evaluated up to 72h as we want to assess whether there is inhibition of adhesion that normally occurs in the early stages. In the experiments of biofilm inhibition and biomass reduction induced by the anti-CdrA sera, the untreated biofilm of the ΔCDrA strain did not show a clear difference in cell number and OD value because in this strain a thinner and less structured biofilm was formed but it did not differ significantly from that of the PAO1 WT strain [115]. However, it is possible to observe that anti-CdrA sera are able to inhibit biofilm formation by reducing the number of bacteria trapped in the biofilm of the PAO1 WT strain without affecting the number of bacteria in the ΔCdrA strain and to induce a reduction in biofilm biomass in the PAO1 WT strain that is not observed in the ΔCdrA strain. These results indicate that anti-CdrA sera represent a potential tool to counteract biofilm formation in the early stages, when it is not yet a structured biofilm. Finally, since the identification of new antigens is a key step in the development of new vaccines that can be used to counteract bacterial resistances, in this thesis, using a microarray technology, new interesting antigens have been identified that may

be involved in the pathogenesis of *P. aeruginosa* and on which further analysis will be performed. An interesting thing that could be observed is that the position of the BCC tag on the protein has effects on its recognition by the sera underlining how the addition of the tag at the N or C-terminus is a determining factor that should be considered for future experiments. Among the antigens spotted on the microarrays we can find the CdrA protein with the BCC tag at both the N- and C-terminus. Interestingly, also this protein is recognized by both some mouse and human sera. It would be interesting to analyze various domains of this protein to assess whether the reactivity of the sera changes, considering that, as observed in this thesis, depending on the portion of the protein that is taken into account, different properties can be observed at the level of solubility, folding and immunogenicity. The identification of new candidate vaccines as well as the production and characterization of new therapeutic targets are key tools to fight multi-resistant infections carried by resistant bacteria such as *P. aeruginosa*, since, up to now, there are no authorized vaccines and/or mAbs for infections caused by this pathogen due to the suboptimal levels of protection observed or limited coverage [156].

STATEMENTS

6.1 Transparency statement & conflict of interest

This work was sponsored by GlaxoSmithKline Biologicals SA. Giada Antonelli is a PhD student at the Siena University and participates in a post graduate studentship program at GSK.

6.2 Animal ethical statement

All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EEC and the GSK policy on the Care, Welfare and Treatment of Animals.

6.3 Human samples ethical statement

AERIS clinical trial is conducted in the University Hospital Southampton (UK) in accordance with the Declaration of Helsinki and Good Clinical Practice and has been approved by the institutional ethics and review board. The use of samples was performed upon written informed consent obtained from participants before the study-specific procedures [157].

ANNEXES

7.1 CdrA domains expressed in E. coli BL21 (DE3) cells

Construct #1 (amminoacidic domain 43-2060)	N-His	MGSSHHHHHHENLYFQGAPTGAQFNPNEIKISQQGKTTLIDQSTQRAIINWKGFD VSADEAVRFNQPGVTSSTLNRVTAGQESVIAGRISAPGQVIIYNSNGVVFSGSAKV DVGSLITTTANISDEHFRQGKLIFDQPGNPDARIVNDGSISVAEKGLAAFVAPSVA NNGVINARLGTVAMAAGNAATIDLYGDGLVSIAVTDPVTRKPQDAQALVSNGG AIQADGGSVLITAEQASRVVDNAVNLSGVILARGTEVREGSVALVSKSGDIQIAGKI DVSGPKNGGDVLVSGQQVALASTASIDARGTAQGGSVRIGGDFQGRGELPRAK NATLAKGASIDVSATGKGNGGLAVVWSDGNTRMDGRILARGGAQGGNGGLVE TSGKVNLSIADSAYVSVAAPYGNGGTWLLDPTTLRIVASGGTSGSVGGANGASG DATVNASVVTGALAGGKVTLSASDRLSVEAPLITSNLGGASRGLELIATGPAGAVD ISAPILFRNGSLAIRAGGNINFLSGGTPQTSGIVDLGSGTLWMQTSTAGKISQQAG TALIAANLAGRAGSIDLASWDNYAGNLALQTFNGTLKYRQSNATGVTTSGTVFDP FINQSMTGTAQNIVSSVGTRILEANSVGTTGNYTLTADGNSEFDRLVFTALPYRRV SGSASFPTNDSSDYLVTNLRYQVNGSNVTATPNGGAPSGFTVAAGNGSVTTWT GNWGTSWGVKGFGGVIGVTDELQYDVGTGLTEELIFGLGGKTSRVDTRLDLFMR EGAFNSFAERAQVEMFKTTTTAGDILSRQQTATLTANDATRVYGDVNPTLTATM SGINAIDAYVNSQFNDLYQATASTTATQASNVGQYAITGNANGSEYFSQRYQLVR QDGRLTVTPAQLIVSADAKTKVYGDADPTLTYQVSGLKNSDTAAGVLSGNLGRVA GENVGNYGILQGGLGLNTANYTLSYVGNDLRITPAQLNVIADAKTKVYGDLDPAL TYQVSGLKRGDTAGAVLNGGSLSRVAGENVGVYGINQGGLGLVSSNYTLNYQG NNLTITKALLNVIADAKTKVYGDADPALTYQVSGLKNGDTAGAVLNGGSLSRVAG ENVGVYGINQGGLGLLSANYDLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTY QVSGLKNGDTAGSILTGGLNRAAGENVGVYGINQGDLALNSGNYDLSYQGNNLT ITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGAVLNGGGLVRVSGENV GNYAIQQGGLGLVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQV SGLKNGDSAGSILTGGLNRAAGENVGVYGINQGDLALNSGNYDLSYQGNNLTITK ALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGAVLNGGGLVRVSGENVGNY AIQQGGLGLVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGL KNGDTAGAVLNGGSLSRVAGENVGVYGINQGDLALNSGNYDLSYQGNNLTITKA LLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGAVLNGGGLVRVSGENVGNYA IQQGGLGLVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLK NGDSAGSILTGGLNRAAGENVGVYGINQGDLALNSGNYDLSYQGNNLTITKALLN VIADAKTKVYGDADPSLTYQVSGLKNGDTAGAVLNGGGLVRVSGENVGNYAIQQ GGLGLVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGD TAGAVLNGGSLSRVAGENVGVYGINQGGLGLVSGNYDLAYQGNNLTITKALLNVI ADGKTKVYGDADPSLTYQVSGLKNGDSAGSILTGGLNRDAGENVGVYGINQGGL VLTSGNYDLAYQGNDLTITKALLNVFADAKSKQVGTADPALTYQVSGLKNGDSAG QVLAGGLGRVGGEAVGQYDILQGGLALTSGNYQLNYQGNLLSILPLPVTPGDLG QLAALSDLRELQKGRDPDTPGDAVYRTTTLENPFLENPFLRAYALGMDVSDPNLL PATAAGPAEDASAKRVGQFTDRPLRAEAESGAGCSNQSYLADYWSCFNKPLNF
Construct #1 (amminoacidic domain 43-2060)	C-His	MAPTGAQFNPNEIKISQQGKTTLIDQSTQRAIINWKGFDVSADEAVRFNQPGVTS STLNRVTAGQESVIAGRISAPGQVIIYNSNGVVFSGSAKVDVGSLITTTANISDEHF RQGKLIFDQPGNPDARIVNDGSISVAEKGLAAFVAPSVANNGVINARLGTVAMA AGNAATIDLYGDGLVSIAVTDPVTRKPQDAQALVSNGGAIQADGGSVLITAEQAS RVVDNAVNLSGVILARGTEVREGSVALVSKSGDIQIAGKIDVSGPKNGGDVLVSG QQVALASTASIDARGTAQGGSVRIGGDFQGRGELPRAKNATLAKGASIDVSATGK GNGGLAVVWSDGNTRMDGRILARGGAQGGNGGLVETSGKVNLSIADSAYVSV AAPYGNGGTWLLDPTTLRIVASGGTSGSVGGANGASGDATVNASVVTGALAGG KVTLSASDRLSVEAPLITSNLGGASRGLELIATGPAGAVDISAPILFRNGSLAIRAGG NINFLSGGTPQTSGIVDLGSGTLWMQTSTAGKISQQAGTALIAANLAGRAGSIDL ASWDNYAGNLALQTFNGTLKYRQSNATGVTTSGTVFDPFINQSMTGTAQNIVSS VGTRILEANSVGTTGNYTLTADGNSEFDRLVFTALPYRRVSGSASFPTNDSSDYLVT NLRYQVNGSNVTATPNGGAPSGFTVAAGNGSVTTWTGNWGTSWGVKGFGGV IGVTDELQYDVGTGLTEELIFGLGGKTSRVDTRLDLFMREGAFNSFAERAQVEMF KTTTTAGDILSRQQTATLTANDATRVYGDVNPTLTATMSGINAIDAYVNSQFNDL YQATASTTATQASNVGQYAITGNANGSEYFSQRYQLVRQDGRHHHHHH
Construct #2 (amminoacidic domain 43-904)	N-His	MGSSHHHHHHENLYFQGAPTGAQFNPNEIKISQQGKTTLIDQSTQRAIINWKGF DVSADEAVRFNQPGVTSSTLNRVTAGQESVIAGRISAPGQVIIYNSNGVVFSGSAK VDVGSLITTTANISDEHFRQGKLIFDQPGNPDARIVNDGSISVAEKGLAAFVAPSV ANNGVINARLGTVAMAAGNAATIDLYGDGLVSIAVTDPVTRKPQDAQALVSNG GAIQADGGSVLITAEQASRVVDNAVNLSGVILARGTEVREGSVALVSKSGDIQIAG KIDVSGPKNGGDVLVSGQQVALASTASIDARGTAQGGSVRIGGDFQGRGELPRA KNATLAKGASIDVSATGKGNGGLAVVWSDGNTRMDGRILARGGAQGGNGGLV ETSGKVNLSIADSAYVSVAAPYGNGGTWLLDPTTLRIVASGGTSGSVGGANGASG DATVNASVVTGALAGGKVTLSASDRLSVEAPLITSNLGGASRGLELIATGPAGAVD ISAPILFRNGSLAIRAGGNINFLSGGTPQTSGIVDLGSGTLWMQTSTAGKISQQAG TALIAANLAGRAGSIDLASWDNYAGNLALQTFNGTLKYRQSNATGVTTSGTVFDP FINQSMTGTAQNIVSSVGTRILEANSVGTTGNYTLTADGNSEFDRLVFTALPYRRV SGSASFPTNDSSDYLVTNLRYQVNGSNVTATPNGGAPSGFTVAAGNGSVTTWT GNWGTSWGVKGFGGVIGVTDELQYDVGTGLTEELIFGLGGKTSRVDTRLDLFMR EGAFNSFAERAQVEMFKTTTTAGDILSRQQTATLTANDATRVYGDVNPTLTATM SGINAIDAYVNSQFNDLYQATASTTATQASNVGQYAITGNANGSEYFSQRYQLVR QDGR
Construct #2 (amminoacidic domain 43-904)	C-His	MAPTGAQFNPNEIKISQQGKTTLIDQSTQRAIINWKGFDVSADEAVRFNQPGVTS STLNRVTAGQESVIAGRISAPGQVIIYNSNGVVFSGSAKVDVGSLITTTANISDEHF RQGKLIFDQPGNPDARIVNDGSISVAEKGLAAFVAPSVANNGVINARLGTVAMA AGNAATIDLYGDGLVSIAVTDPVTRKPQDAQALVSNGGAIQADGGSVLITAEQAS RVVDNAVNLSGVILARGTEVREGSVALVSKSGDIQIAGKIDVSGPKNGGDVLVSG QQVALASTASIDARGTAQGGSVRIGGDFQGRGELPRAKNATLAKGASIDVSATGK GNGGLAVVWSDGNTRMDGRILARGGAQGGNGGLVETSGKVNLSIADSAYVSV AAPYGNGGTWLLDPTTLRIVASGGTSGSVGGANGASGDATVNASVVTGALAGG KVTLSASDRLSVEAPLITSNLGGASRGLELIATGPAGAVDISAPILFRNGSLAIRAGG NINFLSGGTPQTSGIVDLGSGTLWMQTSTAGKISQQAGTALIAANLAGRAGSIDL ASWDNYAGNLALQTFNGTLKYRQSNATGVTTSGTVFDPFINQSMTGTAQNIVSS VGTRILEANSVGTTGNYTLTADGNSEFDRLVFTALPYRRVSGSASFPTNDSSDYLVT NLRYQVNGSNVTATPNGGAPSGFTVAAGNGSVTTWTGNWGTSWGVKGFGGV IGVTDELQYDVGTGLTEELIFGLGGKTSRVDTRLDLFMREGAFNSFAERAQVEMF KTTTTAGDILSRQQTATLTANDATRVYGDVNPTLTATMSGINAIDAYVNSQFNDL YQATASTTATQASNVGQYAITGNANGSEYFSQRYQLVRQDGRHHHHHH

Construct #3 (amminoacidic domain 43-1109)	N-His	MGSSHHHHHENLYFQGAPTGAQFNPNEIKISQQGKTTLIDQSTQR AIINWKGFDVSADEAVRFNQPGVTSSTLNRVTAGQESVIAGRISAP GQVIIYNSNGVVFSGSAKVDVGSLITTTANISDEHFRQGKLIFDQPG NPDARIVNDGSISVAEKGLAAFVAPSVANNGVINARLGTVAMAAG NAATIDLYGDGLVSIAVTDPVTRKPQDAQALVSNGGAIQADGGSV LITAEQASRVVDNAVNLSGVILARGTEVREGSVALVSKSGDIQIAGK IDVSGPKNGGDVLVSGQQVALASTASIDARGTAQGGSVRIGGDFQ GRGELPRAKNATLAKGASIDVSATGKGNGGLAVVWSDGNTRMD GRILARGGAQGGNGGLVETSGKVNLSIADSAYVSVAAPYGNGGT WLLDPTTLRIVASGGTSGSVGGANGASGDATVNASVVTGALAGG KVTLSASDRLSVEAPLITSNLGGASRGLELIATGPAGAVDISAPILFRN GSLAIRAGGNINFLSGGTPQTSGIVDLGSGTLWMQTSTAGKISQQ AGTALIAANLAGRAGSIDLASWDNYAGNLALQTFNGTLKYRQSNA TGVTTSGTVFDPFINQSMTGTAQNIVSSVGTRILEANSVGTTGNYT LTADGNSEFDRLVFTALPYRRVSGSASFPTNDSSDYLVTNLRYQVN GSNVTATPNGGAPSGFTVAAGNGSVTTWTGNWGTSWGVKGFG GVIGVTDELQYDVGTGLTEELIFGLGGKTSRVDTRLDLFMREGAFN SFAERAQVEMFKTTTTAGDILSRQQTATLTANDATRVYGDVNPTL TATMSGINAIDAYVNSQFNDLYQATASTTATQASNVGQYAITGNA NGSEYFSQRYQLVRQDGRLTVTPAQLIVSADAKTKVYGDADPTLTY QVSGLKNSDTAAGVLSGNLGRVAGENVGNYGILQGGLGLNTANY TLSYVGNDLRITPAQLNVIADAKTKVYGDLDPALTYQVSGLKRGDT AGAVLNGGSLSRVAGENVGVYGINQGGLGLVSSNYTLNYQGNNL TITKALLNVIADAKTKVYGDADPALTYQVSGLKNGDTAGAVLN
Construct #3 (amminoacidic domain 43-1109)	C-His	MAPTGAQFNPNEIKISQQGKTTLIDQSTQRAIINWKGFDVSADEA VRFNQPGVTSSTLNRVTAGQESVIAGRISAPGQVIIYNSNGVVFSG SAKVDVGSLITTTANISDEHFRQGKLIFDQPGNPDARIVNDGSISVA EKGLAAFVAPSVANNGVINARLGTVAMAAGNAATIDLYGDGLVSI AVTDPVTRKPQDAQALVSNGGAIQADGGSVLITAEQASRVVDNA VNLSGVILARGTEVREGSVALVSKSGDIQIAGKIDVSGPKNGGDVL VSGQQVALASTASIDARGTAQGGSVRIGGDFQGRGELPRAKNATL AKGASIDVSATGKGNGGLAVVWSDGNTRMDGRILARGGAQGG NGGLVETSGKVNLSIADSAYVSVAAPYGNGGTWLLDPTTLRIVASG GTSGSVGGANGASGDATVNASVVTGALAGGKVTLSASDRLSVEA PLITSNLGGASRGLELIATGPAGAVDISAPILFRNGSLAIRAGGNINFL SGGTPQTSGIVDLGSGTLWMQTSTAGKISQQAGTALIAANLAGRA GSIDLASWDNYAGNLALQTFNGTLKYRQSNATGVTTSGTVFDPFI NQSMTGTAQNIVSSVGTRILEANSVGTTGNYTLTADGNSEFDRLV FTALPYRRVSGSASFPTNDSSDYLVTNLRYQVNGSNVTATPNGGA PSGFTVAAGNGSVTTWTGNWGTSWGVKGFGGVIGVTDELQYDV GTGLTEELIFGLGGKTSRVDTRLDLFMREGAFNSFAERAQVEMFKT TTTAGDILSRQQTATLTANDATRVYGDVNPTLTATMSGINAIDAYV NSQFNDLYQATASTTATQASNVGQYAITGNANGSEYFSQRYQLVR QDGRLTVTPAQLIVSADAKTKVYGDADPTLTYQVSGLKNSDTAAG VLSGNLGRVAGENVGNYGILQGGLGLNTANYTLSYVGNDLRITPA QLNVIADAKTKVYGDLDPALTYQVSGLKRGDTAGAVLNGGSLSRV AGENVGVYGINQGGLGLVSSNYTLNYQGNNLTITKALLNVIADAKT KVYGDADPALTYQVSGLKNGDTAGAVLNHHHHHH
Construct #4 (amminoacidic domain 43-2154)	N-His	MGSSHHHHHHENLYFQGAPTGAQFNPNEIKISQQGKTTLIDQSTQ RAIINWKGFDVSADEAVRFNQPGVTSSTLNRVTAGQESVIAGRISA PGQVIIYNSNGVVFSGSAKVDVGSLITTTANISDEHFRQGKLIFDQP GNPDARIVNDGSISVAEKGLAAFVAPSVANNGVINARLGTVAMAA GNAATIDLYGDGLVSIAVTDPVTRKPQDAQALVSNGGAIQADGGS VLITAEQASRVVDNAVNLSGVILARGTEVREGSVALVSKSGDIQIAG KIDVSGPKNGGDVLVSGQQVALASTASIDARGTAQGGSVRIGGDF QGRGELPRAKNATLAKGASIDVSATGKGNGGLAVVWSDGNTRM DGRILARGGAQGGNGGLVETSGKVNLSIADSAYVSVAAPYGNGG TWLLDPTTLRIVASGGTSGSVGGANGASGDATVNASVVTGALAG GKVTLSASDRLSVEAPLITSNLGGASRGLELIATGPAGAVDISAPILFR NGSLAIRAGGNINFLSGGTPQTSGIVDLGSGTLWMQTSTAGKISQ QAGTALIAANLAGRAGSIDLASWDNYAGNLALQTFNGTLKYRQSN ATGVTTSGTVFDPFINQSMTGTAQNIVSSVGTRILEANSVGTTGNY TLTADGNSEFDRLVFTALPYRRVSGSASFPTNDSSDYLVTNLRYQV NGSNVTATPNGGAPSGFTVAAGNGSVTTWTGNWGTSWGVKGF GGVIGVTDELQYDVGTGLTEELIFGLGGKTSRVDTRLDLFMREGAF NSFAERAQVEMFKTTTTAGDILSRQQTATLTANDATRVYGDVNPT LTATMSGINAIDAYVNSQFNDLYQATASTTATQASNVGQYAITGN ANGSEYFSQRYQLVRQDGRLTVTPAQLIVSADAKTKVYGDADPTLT YQVSGLKNSDTAAGVLSGNLGRVAGENVGNYGILQGGLGLNTAN YTLSYVGNDLRITPAQLNVIADAKTKVYGDLDPALTYQVSGLKRGD TAGAVLNGGSLSRVAGENVGVYGINQGGLGLVSSNYTLNYQGNN LTITKALLNVIADAKTKVYGDADPALTYQVSGLKNGDTAGAVLNGG SLSRVAGENVGVYGINQGGLGLLSANYDLSYQGNNLTITKALLNVI ADAKTKVYGDADPSLTYQVSGLKNGDTAGSILTGGLNRAAGENVG VYGINQGDLALNSGNYDLSYQGNNLTITKALLNVIADAKTKVYGDA DPSLTYQVSGLKNGDTAGAVLNGGGLRVSGENVGNYAIQQGGLG LVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSG LKNGDSAGSILTGGLNRAAGENVGVYGINQGDLALNSGNYDLSYQ GNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGAVL NGGGLVRVSGENVGNYAIQQGGLGLVSGNYDLAYQGNNLTITKA LLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGAVLNGGSLSRV AGENVGVYGINQGDLALNSGNYDLSYQGNNLTITKALLNVIADAK TKVYGDADPSLTYQVSGLKNGDTAGAVLNGGGLVRVSGENVGNY AIQQGGLGLVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDAD PSLTYQVSGLKNGDSAGSILGGLNRAAGENVGVYGINQGDLALNS GNYDLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKN GDTAGAVLNGGGLVRVSGENVGNYAIQQGGLGLVSGNYDLAYQ GNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGAVL NGGSLSRVAGENVGVYGINQGGLGLVSGNYDLAYQGNNLTITKAL LNVIADGKTKVYGDADPSLTYQVSGLKNGDSAGSILTGGLNRDAG ENVGVYGINQGGLVLTSGNYDLAYQGNDLTITKALLNVFADAKSK QVGTADPALTYQVSGLKNGDSAGQVLAGGLGRVGGEAVGQYDIL QGGLALTSGNYQLNYQGNLLSILPLPVTPGDLGQLAALSDLRELQK GRDPDTPGDAVYRTTTLENPFLENPFLRAYALGMDVSDPNLLPAT AAGPAEDASAKRVGQFTDRPLRAEAESGAGCSNQSYLADYWSCF NKPLNF

Construct #4 (amminoacidic domain 43-2154)	C-His	MAPTGAQFNPNEIKISQQGKTTLIDQSTQRAIINWKGFDV SADEAVRFNQPGVTSSTLNRVTAGQESVIAGRISAPGQVII YNSNGVVFSGSAKVDVGSLITTTANISDEHFRQGKLIFDQP GNPDARIVNDGSISVAEKGLAAFVAPSVANNGVINARLGT VAMAAGNAATIDLYGDGLVSIAVTDPVTRKPQDAQALVS NGGAIQADGGSVLITAEQASRVVDNAVNLSGVILARGTEV REGSVALVSKSGDIQIAGKIDVSGPKNGGDVLVSGQQVAL ASTASIDARGTAQGGSVRIGGDFQGRGELPRAKNATLAK GASIDVSATGKGNGGLAVVWSDGNTRMDGRILARGGA QGGNGGLVETSGKVNLSIADSAYVSVAAPYGNGGTWLLD PTTLRIVASGGTSGSVGGANGASGDATVNASVVTGALAG GKVTLSASDRLSVEAPLITSNLGGASRGLELIATGPAGAVDI SAPILFRNGSLAIRAGGNINFLSGGTPQTSGIVDLGSGTLW MQTSTAGKISQQAGTALIAANLAGRAGSIDLASWDNYAG NLALQTFNGTLKYRQSNATGVTTSGTVFDPFINQSMTGT AQNIVSSVGTRILEANSVGTTGNYTLTADGNSEFDRLVFTA LPYRRVSGSASFPTNDSSDYLVTNLRYQVNGSNVTATPNG GAPSGFTVAAGNGSVTTWTGNWGTSWGVKGFGGVIGV TDELQYDVGTGLTEELIFGLGGKTSRVDTRLDLFMREGAF NSFAERAQVEMFKTTTTAGDILSRQQTATLTANDATRVY GDVNPTLTATMSGINAIDAYVNSQFNDLYQATASTTATQ ASNVGQYAITGNANGSEYFSQRYQLVRQDGRLTVTPAQLI VSADAKTKVYGDADPTLTYQVSGLKNSDTAAGVLSGNLG RVAGENVGNYGILQGGLGLNTANYTLSYVGNDLRITPAQL NVIADAKTKVYGDLDPALTYQVSGLKRGDTAGAVLNGGS LSRVAGENVGVYGINQGGLGLVSSNYTLNYQGNNLTITKA LLNVIADAKTKVYGDADPALTYQVSGLKNGDTAGAVLNG GSLSRVAGENVGVYGINQGGLGLLSANYDLSYQGNNLTIT KALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGSILT GGLNRAAGENVGVYGINQGDLALNSGNYDLSYQGNNLTI TKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGAVL NGGGLVRVSGENVGNYAIQQGGLGLVSGNYDLAYQGN NLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDSA GSILTGGLNRAAGENVGVYGINQGDLALNSGNYDLSYQG NNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDT AGAVLNGGGLVRVSGENVGNYAIQQGGLGLVSGNYDLA YQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKN GDTAGAVLNGGSLSRVAGENVGVYGINQGDLALNSGNY DLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGL KNGDTAGAVLNGGGLVRVSGENVGNYAIQQGGLGLVSG NYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQV SGLKNGDSAGSILTGGLNRAAGENVGVYGINQGDLALNS GNYDLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQ VSGLKNGDTAGAVLNGGGLVRVSGENVGNYAIQQGGLG LVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSL TYQVSGLKNGDTAGAVLNGGSLSRVAGENVGVYGINQG GLGLVSGNYDLAYQGNNLTITKALLNVIADGKTKVYGDAD PSLTYQVSGLKNGDSAGSILTGGLNRDAGENVGVYGINQ GGLVLTSGNYDLAYQGNDLTITKALLNVFADAKSKQVGTA DPALTYQVSGLKNGDSAGQVLAGGLGRVGGEAVGQYDIL QGGLALTSGNYQLNYQGNLLSILPLPVTPGDLGQLAALSD LRELQKGRDPDTPGDAVYRTTTLENPFLENPFLRAYALGM DVSDPNLLPATAAGPAEDASAKRVGQFTDRPLRAEAESG AGCSNQSYLADYWSCFNKPLNFHHHHHH
Construct #5 (amminoacidic domain 861-2060)	N-His	MGSSHHHHHHENLYFQGDLYQATASTTATQASNVGQYAI TGNANGSEYFSQRYQLVRQDGRLTVTPAQLIVSADAKTK VYGDADPTLTYQVSGLKNSDTAAGVLSGNLGRVAGENVG NYGILQGGLGLNTANYTLSYVGNDLRITPAQLNVIADAKT KVYGDLDPALTYQVSGLKRGDTAGAVLNGGSLSRVAGEN VGVYGINQGGLGLVSSNYTLNYQGNNLTITKALLNVIADA KTKVYGDADPALTYQVSGLKNGDTAGAVLNGGSLSRVAG ENVGVYGINQGGLGLLSANYDLSYQGNNLTITKALLNVIA DAKTKVYGDADPSLTYQVSGLKNGDTAGSILTGGLNRAA GENVGVYGINQGDLALNSGNYDLSYQGNNLTITKALLNVI ADAKTKVYGDADPSLTYQVSGLKNGDTAGAVLNGGGLV RVSGENVGNYAIQQGGLGLVSGNYDLAYQGNNLTITKAL LNVIADAKTKVYGDADPSLTYQVSGLKNGDSAGSILTGGL NRAAGENVGVYGINQGDLALNSGNYDLSYQGNNLTITKA LLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGAVLNG GGLVRVSGENVGNYAIQQGGLGLVSGNYDLAYQGNNLTI TKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGAVL NGGSLSRVAGENVGVYGINQGDLALNSGNYDLSYQGNN LTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAG AVLNGGGLVRVSGENVGNYAIQQGGLGLVSGNYDLAYQ GNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGD SAGSILTGGLNRAAGENVGVYGINQGDLALNSGNYDLSY QGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNG DTAGAVLNGGGLVRVSGENVGNYAIQQGGLGLVSGNYD LAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLK NGDTAGAVLNGGSLSRVAGENVGVYGINQGGLGLVSGN YDLAYQGNNLTITKALLNVIADGKTKVYGDADPSLTYQVS GLKNGDSAGSILTGGLNRDAGENVGVYGINQGGLVLTSG NYDLAYQGNDLTITKALLNVFADAKSKQVGTADPALTYQV SGLKNGDSAGQVLAGGLGRVGGEAVGQYDILQGGLALTS GNYQLNYQGNLLSILPLPVTPGDLGQLAALSDLRELQKG

7.2 CdrA domains expressed in Mammalian cells

Construct #2 (amminoacidic domain 43-904)	N-His	METDTLLLWVLLLWVPGSTGDAAQPARRARRTKLALGSSHHHHHHHHGSGAAPTGAQFNPNEIKISQQGKTTLI DQSTQRAIINWKGFDVSADEAVRFNQPGVTSSTLNRVTAGQESVIAGRISAPGQVIIYNSNGVVFSGSAKVDVGSLI TTTANISDEHFRQGKLIFDQPGNPDARIVNDGSISVAEKGLAAFVAPSVANNGVINARLGTVAMAAGNAATIDLYG DGLVSIAVTDPVTRKPQDAQALVSNGGAIQADGGSVLITAEQASRVVDNAVNLSGVILARGTEVREGSVALVSKSG DIQIAGKIDVSGPKNGGDVLVSGQQVALASTASIDARGTAQGGSVRIGGDFQGRGELPRAKNATLAKGASIDVSAT GKGNGGLAVVWSDGNTRMDGRILARGGAQGGNGGLVETSGKVNLSIADSAYVSVAAPYGNGGTWLLDPTTLRI VASGGTSGSVGGANGASGDATVNASVVTGALAGGKVTLSASDRLSVEAPLITSNLGGASRGLELIATGPAGAVDIS APILFRNGSLAIRAGGNINFLSGGTPQTSGIVDLGSGTLWMQTSTAGKISQQAGTALIAANLAGRAGSIDLASWDN YAGNLALQTFNGTLKYRQSNATGVTTSGTVFDPFINQSMTGTAQNIVSSVGTRILEANSVGTTGNYTLTADGNSEF DRLVFTALPYRRVSGSASFPTNDSSDYLVTNLRYQVNGSNVTATPNGGAPSGFTVAAGNGSVTTWTGNWGTSW GVKGFGGVIGVTDELQYDVGTGLTEELIFGLGGKTSRVDTRLDLFMREGAFNSFAERAQVEMFKTTTTAGDILSRQ QTATLTANDATRVYGDVNPTLTATMSGINAIDAYVNSQFNDLYQATASTTATQASNVGQYAITGNANGSEYFSQ RYQLVRQDGR
Construct #6 (amminoacidic domain 43-471)	C-His	METDTLLLWVLLLWVPGSTGDAAQPARRARRTKLALGSSHHHHHHHHGSGAAPTGAQFNPNEIKISQQGKTTLI DQSTQRAIINWKGFDVSADEAVRFNQPGVTSSTLNRVTAGQESVIAGRISAPGQVIIYNSNGVVFSGSAKVDVGSLI TTTANISDEHFRQGKLIFDQPGNPDARIVNDGSISVAEKGLAAFVAPSVANNGVINARLGTVAMAAGNAATIDLYG DGLVSIAVTDPVTRKPQDAQALVSNGGAIQADGGSVLITAEQASRVVDNAVNLSGVILARGTEVREGSVALVSKSG DIQIAGKIDVSGPKNGGDVLVSGQQVALASTASIDARGTAQGGSVRIGGDFQGRGELPRAKNATLAKGASIDVSAT GKGNGGLAVVWSDGNTRMDGRILARGGAQGGNGGLVETSGKVNLSIADSAYVSVAAPYGNGGTWLLDPTTLRI VASGGTSGSVGGANGASGDATVNASVVT
Construct #7 (amminoacidic domain 438-904)	N-His	METDTLLLWVLLLWVPGSTGDAAQPARRARRTKLALGSSHHHHHHHHPTTLRIVASGGTSGSVGGANGASGDA TVNASVVTGALAGGKVTLSASDRLSVEAPLITSNLGGASRGLELIATGPAGAVDISAPILFRNGSLAIRAGGNINFLSG GTPQTSGIVDLGSGTLWMQTSTAGKISQQAGTALIAANLAGRAGSIDLASWDNYAGNLALQTFNGTLKYRQSNA TGVTTSGTVFDPFINQSMTGTAQNIVSSVGTRILEANSVGTTGNYTLTADGNSEFDRLVFTALPYRRVSGSASFPTN DSSDYLVTNLRYQVNGSNVTATPNGGAPSGFTVAAGNGSVTTWTGNWGTSWGVKGFGGVIGVTDELQYDVGT GLTEELIFGLGGKTSRVDTRLDLFMREGAFNSFAERAQVEMFKTTTTAGDILSRQQTATLTANDATRVYGDVNPTL TATMSGINAIDAYVNSQFNDLYQATASTTATQASNVGQYAITGNANGSEYFSQRYQLVRQDGR
Construct #8 (amminoacidic domain 438-2060)	N-His	METDTLLLWVLLLWVPGSTGDAAQPARRARRTKLALGSSHHHHHHHHGSGPTTLRIVASGGTSGSVGGANGAS GDATVNASVVTGALAGGKVTLSASDRLSVEAPLITSNLGGASRGLELIATGPAGAVDISAPILFRNGSLAIRAGGNIN FLSGGTPQTSGIVDLGSGTLWMQTSTAGKISQQAGTALIAANLAGRAGSIDLASWDNYAGNLALQTFNGTLKYRQ SNATGVTTSGTVFDPFINQSMTGTAQNIVSSVGTRILEANSVGTTGNYTLTADGNSEFDRLVFTALPYRRVSGSASF PTNDSSDYLVTNLRYQVNGSNVTATPNGGAPSGFTVAAGNGSVTTWTGNWGTSWGVKGFGGVIGVTDELQYD VGTGLTEELIFGLGGKTSRVDTRLDLFMREGAFNSFAERAQVEMFKTTTTAGDILSRQQTATLTANDATRVYGDVN PTLTATMSGINAIDAYVNSQFNDLYQATASTTATQASNVGQYAITGNANGSEYFSQRYQLVRQDGRLTVTPAQLI VSADAKTKVYGDADPTLTYQVSGLKNSDTAAGVLSGNLGRVAGENVGNYGILQGGLGLNTANYTLSYVGNDLRIT PAQLNVIADAKTKVYGDLDPALTYQVSGLKRGDTAGAVLNGGSLSRVAGENVGVYGINQGGLGLVSSNYTLNYQ GNNLTITKALLNVIADAKTKVYGDADPALTYQVSGLKNGDTAGAVLNGGSLSRVAGENVGVYGINQGGLGLLSAN YDLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGSILTGGLNRAAGENVGVYGINQGDLAL NSGNYDLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGAVLNGGGLVRVSGENVGNYAIQ QGGLGLVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDSAGSILTGGLNRAAGENVG VYGINQGDLALNSGNYDLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGAVLNGGGLVRV SGENVGNYAIQQGGLGLVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTAGAVLNG GSLSRVAGENVGVYGINQGDLALNSGNYDLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGLKNGDTA GAVLNGGGLVRVSGENVGNYAIQQGGLGLVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGDADPSLTYQVSGL KNGDSAGSILTGGLNRAAGENVGVYGINQGDLALNSGNYDLSYQGNNLTITKALLNVIADAKTKVYGDADPSLTY QVSGLKNGDTAGAVLNGGGLVRVSGENVGNYAIQQGGLGLVSGNYDLAYQGNNLTITKALLNVIADAKTKVYGD ADPSLTYQVSGLKNGDTAGAVLNGGSLSRVAGENVGVYGINQGGLGLVSGNYDLAYQGNNLTITKALLNVIADGK TKVYGDADPSLTYQVSGLKNGDSAGSILTGGLNRDAGENVGVYGINQGGLVLTSGNYDLAYQGNDLTITKALLNV FADAKSKQVGTADPALTYQVSGLKNGDSAGQVLAGGLGRVGGEAVGQYDILQGGLALTSGNYQLNYQGNLLSIL PLPVTPGDLGQLAALSDLRELQKG

7.3 Publication

Antonelli, G., et al., *Strategies to Tackle Antimicrobial Resistance: The Example of Escherichia coli and Pseudomonas aeruginosa. Int J Mol Sci*, 2021. **22**(9): p. 4943.

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