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Bacterial lipoproteins: sorting mechanisms and biotechnological applications

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DECLARATION OF AUTHORSHIP

I, Micaela De Santis, hereby certify that this thesis has been composed by me and is based on my own work, unless stated otherwise. No other person's work has been used without due acknowledgement in this thesis. All references and verbatim extracts have been quoted, and all sources of information, including graphs and data sets, have been specifically acknowledged.

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List of abbreviations

Bam	**Barrel assembly machinery complex**
BP	**Backbone protein**
Cm	**Chloramphenicol**
CPs	**Carrier proteins**
FACS	**Fluorescence Activated Cell Sorting**
fH	**Factor H**
fHbp	**Factor H binding protein**
fHbp A	**Factor H binding protein domain A**
fHbp A-B	**Factor H binding protein domains A-B**
fHbp C	**Factor H binding protein domain C**
fHbpΔGLY4	**Factor H binding protein Δ glycine stretch**
fHbpGLY4>ALA4	**Factor H binding protein alanine stretch**
FhuD2	**Ferrichrome-binding protein**
GBS	**Group B Streptococcus**
GBS59 D3	**Group B Streptococcus 59 domain 3**
FITC	**Fluorescein isothiocyanate**
IM	**Inner membrane**
IPTG	**Isopropil-β-D-1-tiogalattopiranoside**
Ig	**Immunoglobulin**
LamB	**Lambda receptor protein**
LB	**Luria Bertani**
Lgt	**Lipoprotein diacylglyceryl transferase**
Lnt	**Apolipoprotein N-acyltransferase**
Lol	**Lipoprotein localization machinery**
Lol C	**Lipoprotein localization machinery component C**
Lol D	**Lipoprotein localization machinery component D**
Lol E	**Lipoprotein localization machinery component E**
Lol A	**Lipoprotein localization machinery component A**

Lol B	**Lipoprotein localization machinery component B**
LP	**Leader peptide**
LPS	**Lipopolysaccharide**
LspA	**Proplipoprotein signal peptidase II**
NHBA	**Neisseria Heparin binding antigen**
NMR	**Nuclear magnetic resonance**
OM	**Outer membrane**
OmpA	**Outer membrane protein A**
OmpC	**Outer membrane protein C**
OmpF	**Outer membrane protein F**
OMV	**Outer membrane vesicles**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PhoE	**Outer membrane phosphoporin protein 3**
PIPE	**Polymerase incomplete primer extension**
PMSF	**Phenylmethylsulfonyl fluoride**
PPs	**Passenger proteins**
SDS-PAGE	**Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis**
Sec	**Sec dependent secretory pathway**
SOC	**Super optimal broth**
Spa DEABC	**Staphylococcal protein A**
Tat	**Twin arginine traslocation system**
TLR2	**Toll like receptor 2**

Executive summary

Bacterial lipoprotein biogenesis, inner membrane translocation and maturation are all events occurring with a common process in all gram negative bacteria. The mechanism responsible for lipoprotein sorting is conserved among bacterial species, generally involving the Lol transport machinery. However, the final destination of lipoproteins may vary among species. In *E. coli*, lipoproteins can be retained in the outer leaflet of the inner membrane, thanks to the presence of specific amino acid residues (retention signals) which prevent lipoproteins from binding to the Lol transport system. In the absence of the retention signals, lipoproteins leave the inner membrane, cross the periplasm or reach the inner leaflet of the outer membrane. In *N. meningitidis*, lipoproteins that reach the outer membrane undergo an additional step, which ultimately leads to their exposition on the cell surface. The mechanism by which lipoprotein exposition occurs remains to be fully elucidated. The involvement of a specific enzyme, a “flippase”, that moves lipoproteins from the inner to the outer leaflet of the outer membrane has been postulated but its existence has never been proved.

In an attempt to shed light on the mechanism of lipoprotein surface exposition, in this work we selected two *N. meningitidis* lipoproteins, fHbp and NHBA, and we first asked the question whether when expressed in *E. coli*, they could reach the outer membrane. The proteins were both efficiently expressed in *E. coli* and inserted in the outer membrane, as determined by the fact that the proteins were found associated to Outer Membrane Vesicles (OMVs), vesicles that are naturally released by gram-negative bacteria through a budding out process of the outer

membrane. However, when their precise localization was investigated they were both found in the membrane but with a different orientation: fHbp was exposed on the surface while NHBA was not. These results suggest that fHbp has specific signals that promote its translocation without the aid of a specific transport machinery. In an attempt to identify such “transport signatures” a number of amino acid deletions/substitutions were created at the N terminus of the protein but none of them affected the capacity of fHbp to be exposed. We next took advantage of the available 3D structure of fHbp to further investigate the sorting mechanism. fHbp is organized in three structural domains, referred to as domain A, B, and C. Therefore we created specific domain deletions and we investigated which of them are potentially involved in the exposition process. Interestingly, based on the results obtained, we hypothesized that domain A carries the information necessary to allow the translocation of fHbp to the cell surface.

The fact that fHbp protrudes out of the cell surface can be biotechnologically exploited to bring foreign proteins on the surface of *E. coli* cells by creating protein chimeras. To test this possibility, we selected three proteins, including the domain 3 of GBS59 pilus protein from *Streptococcus agalactiae*, *Staphilococcus aureus* fhuD2 and *Staphilococcus aureus* spaDEABC and then we fused them to full length fHbp and/or to fHbp domain A.

The results indicate that all proteins were efficiently delivered to the outer membrane. Furthermore, the D3 domain of GBS59 was also exposed on the surface of *E. coli* and *E. coli*-derived OMVs.

Alltogether our data indicate that fHbp can be exploited to direct foreign proteins to the outer membrane.

1) INTRODUCTION

1.1 Organization of Gram-negative envelope

The cell envelope of gram-negative bacteria consists of two membranes, the Inner Membrane (IM) and the Outer Membrane (OM), that are separated by the periplasm containing the peptidoglycan layer (Bos M.P. *et al.,* 2007) (Figure 1). The two membranes have an entirely different structure and composition. Whereas the IM is organized in two phospholipid-containing leaflets, the OM is an asymmetrical bilayer, consisting of phospholipids and lipopolysaccharides (LPS) in the inner and outer leaflets, respectively (Ruiz N. *et al.,* 2009).

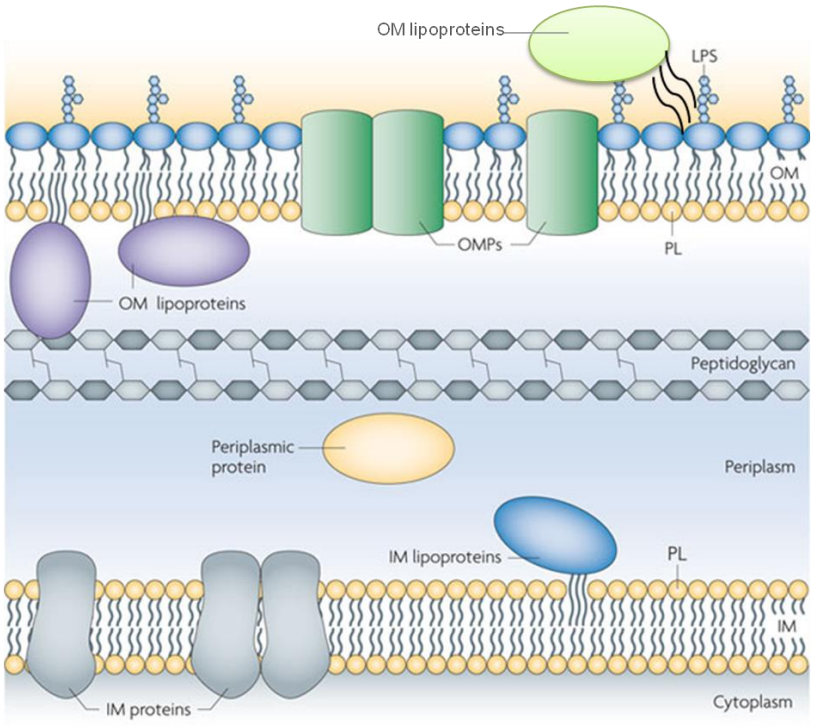


Figure 1 – Schematic representation of Gram negative bacteria envelope (Natividad Ruiz *et al.,* 2009)

IM, OM and periplasm contain a number of proteins with key biological functions. Most of the proteins destined to these compartments are synthesized in the cytoplasm as precursors with N-terminal leader peptides (LP) and are translocated across the inner membrane following two distinct export pathways (Okuda S. *et al.,* 2011). The Sec-dependent (Sec) secretory pathway is the predominant route of protein transport (Driessen A.J.M. *et al.,* 2008). The Sec machinery recognizes LP of secreted proteins during the translation process and transport across the IM occurs while proteins are still in an unfolded conformation. Once translocated through the IM, specific leader peptidases remove LP and folded mature proteins are either released into the periplasmic space or directed to the membrane compartments. By contrast, the more recently discovered Twin arginine traslocation system (Tat) promotes the transport from the cytoplasm to the periplasm of folded and even oligomeric proteins (Sargent F. *et al.,* 2005). The LPs of the proteins translocated via the Tat system are characterized by the presence of an essential twin-arginine motif (Okuda S. *et al.,* 2011).

As said above, once translocated through the IM, proteins are either retained in the periplasm or, in the case of membrane proteins, they reach their final destination using specific transport machineries. There are two main classes of membrane proteins: the integral outer membrane proteins and lipoproteins. Outer-membrane proteins such as OmpA, OmpC, OmpF, LamB and PhoE span the outer membrane with amphipathic β-strands possessing alternating hydrophobic residues. Unlike a typical transmembrane segment comprising a

cluster of hydrophobic residues, amphipathic β-strands do not cause the retention of proteins in the inner membrane and their insertion into the outer membrane is mediated by a dedicated transport system called Bam complex (Pugsley A.P. *et al.,* 1993).

The other class of membrane proteins includes the lipid-modified proteins, or lipoproteins, carrying acyl groups at their N-terminus. Such acyl moieties can penetrate into the external and internal leaflets of the IM and OM, respectively, thus anchoring lipoproteins to the membranes. *Escherichia coli* possesses more than 90 lipoproteins, most of which are located in the outer membrane facing the periplasm, the others being anchored to the inner membrane (Kovacs-Simon A. *et al.,* 2011). Because of the extremely hydrophobic property of the lipid moiety, the release of lipoproteins from IM and destined to the inner leaflet of OM does not occur spontaneously. Instead, it involves the Lol system, which removes lipoproteins from the IM, transports them through the periplasmic space, and finally targets them to the OM.

It has to be pointed that in some gram-negative species, but not in *E. coli*, lipoproteins anchored to the OM do not face the periplasm but rather protrude out of the surface. As described below, the mechanism responsible for surface exposition of lipoproteins is not completely understood (Narita S. *et al.,* 2004) and the attempt to elucidate such mechanism is the main objective of this thesis project.

1.2 Biogenesis of bacterial lipoproteins

Bacterial lipoproteins of both gram-positive and gram-negative bacteria are initially synthesized as preprolipoproteins in the cytosol carrying a LP similar to the signal peptides of other secreted proteins (Inouye S. *et al.,* 1977). However, the LP of lipoproteins is characterized by a specific conserved sequence at its C-terminal region, **Leu-(Ala/Ser)-(Gly-Ala)-Cys**, called lipobox (Kovacs-Simon A. *et al.,* 2011) (Hutchings M.I. *et al.,* 2008) (figure 2).

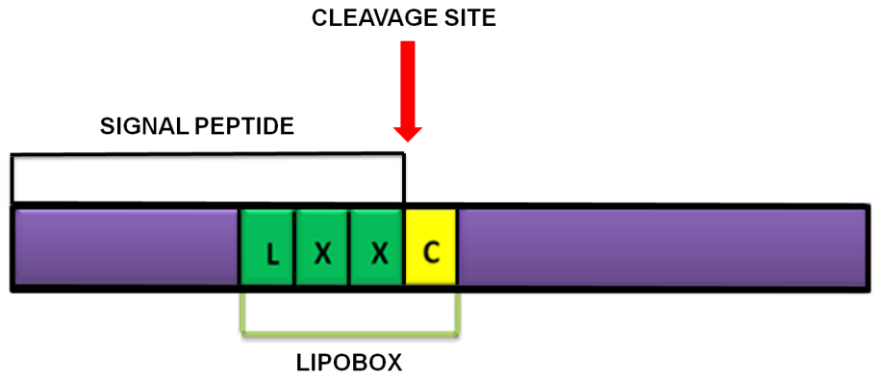


FIGURE 2 - Schematic representation of lipoproteins. Arrows indicates the cleavage site of the leader peptidase

This LP can be recognized by either Sec or Tat system and lipoprotein precursors cross the cytoplasmic membrane as described above (Hutchings M.I. *et al.,* 2008) Processing of preprolipoproteins into their mature forms takes place on the periplasmic side of the inner membrane (Okuda S. *et al.,* 2011) (figure 3 ***a***). The thiol group on the side chain of the cysteine residue is modified by the covalent attachment of a diacylglycerol moiety by a transferase enzyme (Lgt), generating a prolipoprotein (Babu M.M. *et al.,* 2006) (figure 3 ***b***). After lipidation, a peptidase

(LspA) cleaves the LP of the lipidated prolipoprotein and the lipobox cysteine becomes the amino-terminal residue of mature lipoproteins (Tokunaga M. *et al.,* 1982) (figure 3 ***c***).

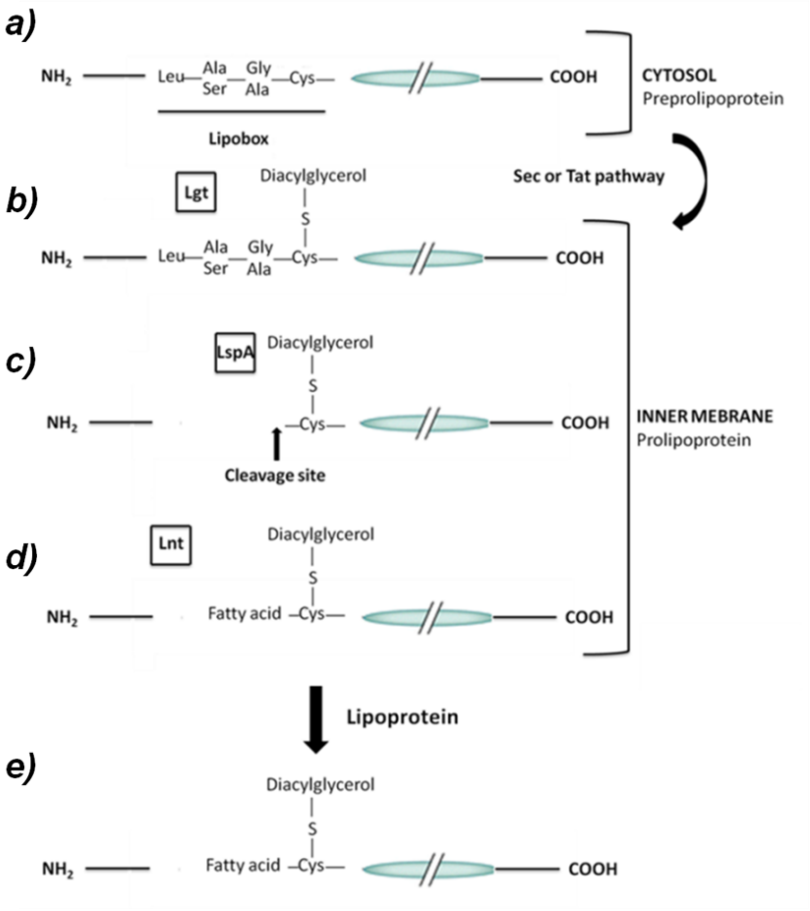


FIGURE 3 - Lipoproteins maturation process-*a)* Lipoproteins are synthesized in the cytosol as a preprolipoprotein. ***b)*** After traslocation across the inner membrane by Sec or Tat pathway, the enzyme Ltg forms a thioether linkage between N-terminal Cys and diacylglycerol. ***c)*** Lspa enzyme cleaves the signal peptide of the prolipoprotein, ***d)*** The N-terminal Cys residue is acylated by Lnt enzyme. ***e)*** The mature lipoprotein can reach the final localization (Okuda S. *et al*., 2011).

In gram-negative bacteria another event occurs: the attachment of an amide-linked acyl group to the N terminal cysteine residue by a transacylase enzyme (Lnt), forming the mature lipoprotein (Kovacs-Simon A. *et al.,* 2011) (figure 3 ***d***). Both the diacylglyceryl group and the amino-terminal acyl group participate to the anchoring of the lipoprotein to the membrane (Hantke K. *et al.,* 1973) (figure 3 **e**).

1.3 Difference in lipoprotein localization between *E. coli* and *N. meningitidis*

In many gram-negative bacteria, such as *E. coli*, mature lipoproteins are localized either in the outer leaflet of IM or in the inner leaflet of OM. Transport of lipoproteins from the IM to the OM is mediated by the lipoprotein localization machinery (Lol) (Tokuda H. 2009). However, in pathogenic spirochetes (Schulze R.J. et al., 2006) and *N. meningitidis* (Bos M.P. *et al.,* 2007) mature lipoproteins reach the outer leaflet of OM by a process that remains to be fully elucidated.

- *Lipoprotein sorting mechanism in E. coli: the Lol system*

Once the lipoproteins are acylated as described above they have two destinies: either they are retained in the IM or they are transported to the OM. The final destination of lipoproteins depends on the amino acids flanking the lipidated cysteine in the mature protein (Bos M.P. *et al.,* 2007). Studies in *E. coli* have demonstrated that the presence at position +2 of an aspartic acid residue prevents lipoproteins from binding to the Lol machinery and therefore this amino

acid represents a critical IM retention signal. Aspartic acid replacement with a serine residue results in Lol-dependent lipoprotein translocation to the outer membrane (Seydel A. *et al.,* 1999). More recently, the importance of amino acid residues at positions +3 and +4 (Tokuda H. *et al.,* 2004) has also been reported: for example lysine is able to weaken the inner membrane retention whereas aspartate or glutamate are able to fortify the retention signal (Seydel A. *et al.,* 1999).

In the absence of retention signals lipoproteins move to the OM. Since the periplasmic space is hydrophilic, lipoproteins must be solubilized to jump from IM to OM. Indeed, lipoproteins are not detected in the periplasm under normal conditions (Tokuda H. 2009).

As said above, lipoprotein trafficking is mediated by the Lol system, consisting in a transmembrane protein complex (LolCDE), an ATP-binding cassette (ABC) transporter, a periplasmic chaperone (LolA) and an outer-membrane receptor (LolB) (Tokuda H. 2009).

The LolCDE complex represents the IM lipoprotein-releasing factor. This three-protein complex is localized in the inner membrane and has similarities with ATP-binding cassettes (ABC) (Yakushi T. *et al.,* 2000). LolC and LolE are integral membrane proteins (figure 4 ***b***), while LolD is an ABC ATPase (figure 4 ***a***). LolCDE is a unique ABC transporter in that it does not catalyze the transmembrane transport of substrates but rather releases substrates from the outer leaflet of the inner membrane.

The release of lipoproteins from LolCDE complex is mediated by LolA. LolA, a protein essential for cell viability (Matsuyama S. *et al.,* 1995), is a lipoprotein-specific molecular chaperone, which forms a soluble one-to-one complex with lipoproteins (Matsuyama S. *et al.,* 1997) (Tajima T. et al., 1998) (figure 4 ***c***). LolA has an unclosed β-barrel and a lid composed of three α-helices, together constituting a hydrophobic cavity that is able to accept the lipid moiety of lipoproteins. Energy seems to be needed to disrupt the hydrogen bonds and promote the formation of the LolA-lipoprotein complex and the stability of the LolA-lipoprotein complex is increased relative to that of the free form of LolA.

The outer-membrane protein LolB, is the factor able to receive lipoproteins from LolA (Matsuyama S. *et al.,* 1997) (Yakushi T. *et al.,* 1998). LolB is itself a lipoprotein and is anchored to the outer membrane (figure 4 ***d***).

Transfer of lipoproteins from LolA to LolB occurs in the absence of energy and is presumably driven by the difference in affinity for lipoproteins between LolA and LolB (Yakushi T. *et al.,* 1998). LolB then transfers lipoproteins to the inner leaflet of the outer membrane through a currently unknown mechanism (figure 4 ***e***). Although the sequence similarity between LolA and LolB is low, the overall structure of LolB is very similar to that of LolA and it has a hydrophobic cavity that seems to be the binding site for the lipid moieties of lipoproteins (Narita S. *et al.,* 2004).

The five LolA-E proteins are all well conserved in gram-negative bacteria, indicating that lipoprotein transport is an essential biological process.

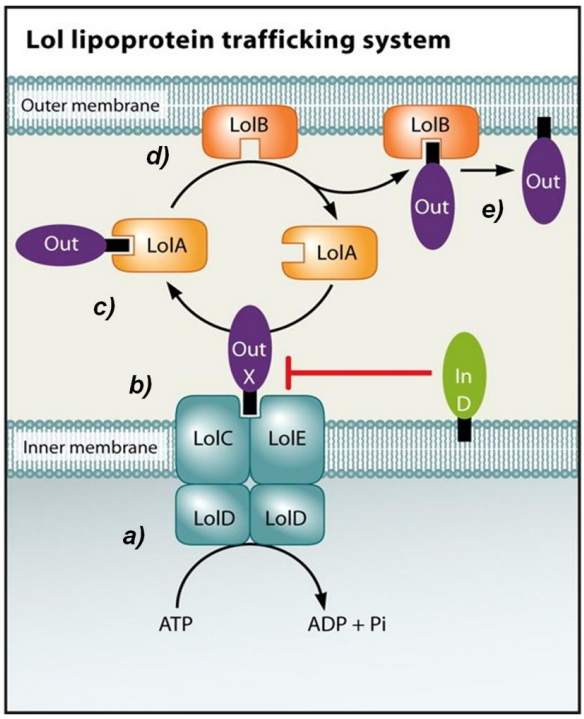


FIGURE 4 – Schematic representation of the Lol-mediated lipoprotein trafficking

a-*b)* Lol CDE is lipoprotein-releasing factor and it is an ATP-binding cassette (ABC) transporter. ***c)*** The LolA-lipoprotein is an intermediate during lipoprotein transfer to the outer membrane (Yakushi T. *et al.,* 1998) able to interact with an ABC transporter called LolCDE. ***d)*** The outer-membrane LolB protein, is the factor able to receive lipoproteins. ***e)*** LolB then transfers lipoproteins to the inner leaflet of the outer membrane through a currently unknown mechanism (Matsuyama S. *et al.,* 1997).

- *Lipoprotein sorting mechanism in Neisseria meningitidis*

While in *E. coli* all known lipoproteins face the periplasm (Tokuda H. 2009), in some gram-negative bacteria, including pathogenic spirochetes (Schulze R.J. et al., 2006) and *Neisseria meningitidis*, lipoproteins are present on the outer leaflet of the OM (Bos M.P. *et al.,* 2007) (figure 5). Little is known about the exact mechanism of lipoprotein translocation across the outer membrane. The

spirochete *Borrelia burgdorferi* lacks a complete Lol system and it has been postulated that this may be one of the reasons why several lipoproteins are localized in the outer leaflet of the outer membrane of this bacterium (Schulze R.J. *et al.,* 2006). More recently, work on lipoproteins of *Borrelia* has led to the proposal that translocation to the outer membrane is mediated by specific signatures (Schulze R.J. *et al.,* 2006). A recent study used nuclear magnetic resonance (NMR) to demonstrate that the amino terminus of the *Neisseria meningitidis* lipoprotein, fHbp, serves as an extended linker to display the protein at the extracellular surface (Mascioni A. *et al.,* 2009 *J Biol Chem*) (Mascioni A., *et al.,*2009 *Biochim et Biophysica Acta*). Finally, the existence in *N. meningitidis* of a "flippase" working in a similar manner as the LPS flippase has been postulated. However, such enzyme has not been discovered yet and it is not known whether specific structural signatures of lipoproteins are required to promote their flippase-mediated transport.

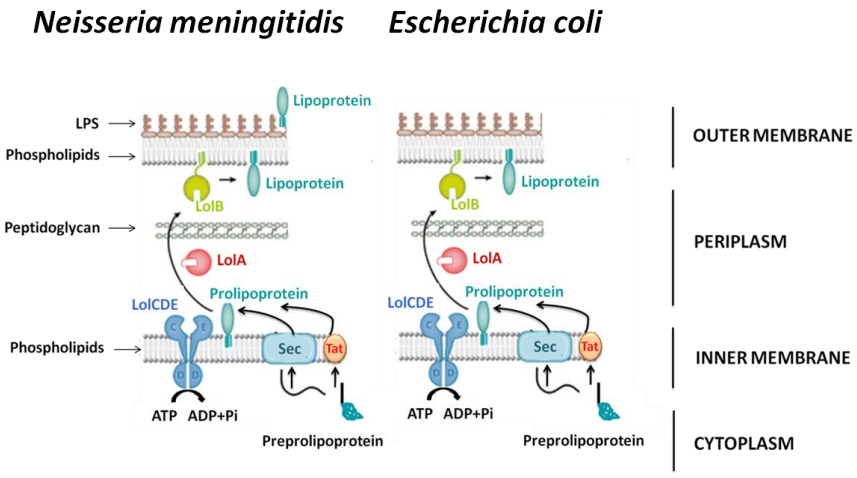


FIGURE 5 - Schematic representation of the difference in lipoprotein sorting between *E. coli* and *N. meningitidis*

Aim of the project

This research project has two main objectives: 1) understanding of the sorting mechanisms of lipoproteins, and 2) development of a lipoprotein-based system to deliver foreign proteins to the surface of bacterial cells.

E coli does not expose lipoproteins to the surface of OM while *Neisseria meningitidis* does. There are two possible explanations for this functional difference. One explanation is that *N. meningitidis* has a specific transport system responsible for the traslocation of lipoproteins once they reach the inner leaflet of the OM. Alternatively, translocation could be due to specific structural properties embedded in *N. meningitidis* lipoproteins and absent in lipoproteins from *E. coli*. In order to establish which of the two mechanisms plays a major role in surface exposition of lipoproteins, two *N. meningitidis* lipoproteins have been expressed in *E. coli* and their fate in cellular compartmentalization has been analyzed. The rationale behind this strategy is that if the export properties are “encoded” in the sequence/structure of lipoproteins, the two selected proteins are expected to be surface-localized in *E. coli* as well. If, on the other hand, a translocation machinery is involved, the two proteins should be retained in the inner side of OM. In this case a shotgun cloning of *N. meningitidis* genes in *E. coli* expressing the two proteins could be used as a tool to identify the gene(s) of the transport system.

The second aim of the project is to investigate whether surface-exposed lipoproteins can be exploited as vehicles to bring heterologous proteins to the

bacterial surface. To this aim, specific heterologous proteins are selected, fused to *N. meningitidis* lipoproteins and their compartmentalization investigated.

2) RESULTS

2.1 Lipoprotein selection

Factor H binding protein (fHbp) and *Neisseria* heparin binding antigen (NHBA) are both surface-exposed lipoproteins from *Neisseria meningitidis* originally identified by reverse vaccinology (Delany I. *et al.,* 2013) (Serruto D. *et al.,* 2012). They are included in a multicomponent vaccine against Serogroup B *Neisseria meningitidis* recently registered in Europe (Serruto D. *et al.,* 2012). fHbp is an important virulence factor enabling the meningococcus to evade the complement system (Piet J.R. *et al.,* 2012). NHBA structure consists of an 8-strand β-barrel that closely resembles the C-terminal domains of fHbp and of transferrin-binding protein B (Serruto D. *et al.,* 2012). This common fold, together with additional structural similarities, suggests a common ancestor for these important antigens and a role of the β-barrel fold in inducing immunogenicity against *N. meningitidis* (Esposito V. *et al.,* 2011).

2.1.1_*Neisseria meningitidis* factor H binding protein

Factor H binding protein (fHbp) is a 28 kDa surface-exposed lipoprotein of *Neisseria meningitidis* (Cantini F. *et al.,* 2005). fHbp is able to bind human complement factor H (fH), the central regulator of the alternative complement pathway (Cendron L. *et al*., 2011), and in this way it down-regulates complement activation (Lo H. *et al.,* 2009). The interaction between fHbp and human Factor H prevents complement-mediated lysis (Madico G. et al., *2006;* Schneider M.C. *et*

al., 2009). fHbp is present on the surface of all meningococcal strains (Fletcher L.D. *et al*., 2004) and high levels of fHbp expression have been found in hyper-virulent meningococcal strains (Schneider M.C. *et al.,* 2009).

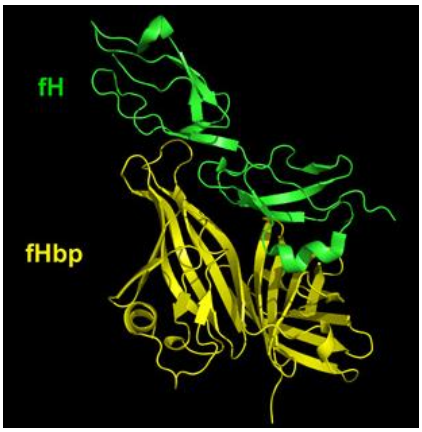


FIGURE 6 - 3D of fHbp binding to human Factor H - The short consensus repeat region 6 (SCR6) of Factor H is mostly the site bound by fHbp. Binding was mediated by charged amino acid residues in the fHbp structure, which mimicked portions of sugar molecules that were previously identified to bind to fH SCR 6. (Based on coordinates published by Schneider M.C. *et al.,* 2009) (McNeil L.K. *et al.,* 2013)

fHbp is synthesized as a precursor carrying at its N- terminal, a canonical signal peptide of 19 amino acids including a lipobox. Like all lipoproteins, the first amino acid of matured fHbp is a cysteine.

Structural studies have shown that fHbp is organized in three domains, called A, B, C (Cantini F. *et al*., 2005). Domain A encompasses amino acid 27 to 119, Domain B starts from amino acid 120 and ends at residue 183, and finally Domain C spans from amino acid 184 to the C-terminal end (amino acid 274)

(figure 7). The protein structure consists of eight-stranded antiparallel β-barrel (157-274) topped by a single α-helix (157-160) and a flexible tail (120-156), which links Domain A to Domain B (Cantini F. *et al*., 2006) (figure 7). The Factor H binding site is located in Domain A and it has been shown that a single amino acid replacement (R41S) is sufficient to impair fH binding (Beernink P.T. *et al.,* 2012).

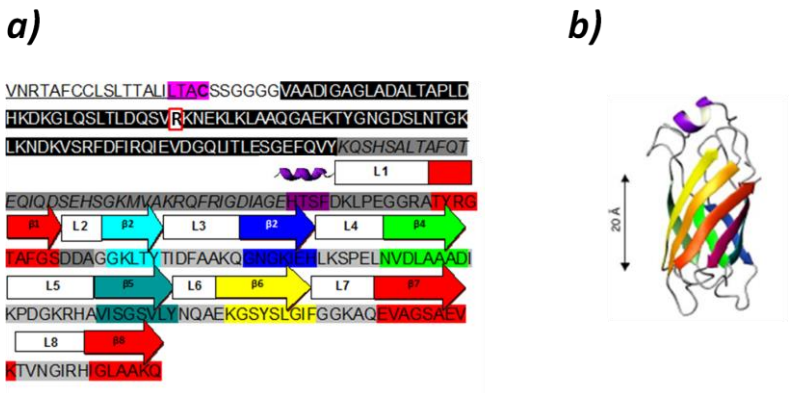


FIGURE 7 – Structural organization of Domains B and C of fHbp. ***a)*** Amino acid sequence of fHbp. Location of β-barrels and α-helix are highlighted in colors and schematized under the sequence. ***b)*** The crystal structure of domains B and C have been characterized and the analysis showed the presence of eight-stranded antiparallel β-barrel topped by a single α-helix and a flexible tail (Beernink P.T. *et al.,* 2012).

2.1.2_Neisseria Heparin Binding antigen (NHBA)

Neisseria meningitidis heparin-binding antigen (NHBA) is a 49-kDa surface-exposed lipoprotein. Its amino acid sequence carries an arginine-rich region responsible for heparin binding, which correlates with an increased survival of *N. meningitidis* in human serum (Serruto D. *et al.,* 2010). The 3D structure of the C-terminal domain of NHBA has been solved. This domain independently folds and

is organized in an 8-strand β-barrel, resembling the very well characterized C-terminal domains of *N. meningitidis* fHbp (figure 8). As already pointed out, these structure similarities suggest a common ancestor for these important antigens (Esposito V. *et al.,* 2011).

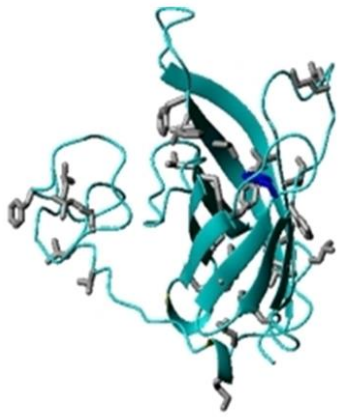


FIGURE 8 - Crystal structure of *Neisseria meningitidis* heparin binding antigen (Esposito V. *et al.,* 2011).

2.2 Expression of fHbp and NHBA in *E. coli*

fHbp and NHBA genes were amplified by PCR from *N. meningitidis* strain MC58 and the amplification products were cloned into the pET21b plasmid, by using the PIPE method (Klock H.E. *et al*,. 2009). The derived pET21_fHbp and pET21_NHBA plasmids were used to transform BL21D3(*ΔompA*) strain, obtaining the recombinant strains (pET21_fHbp)BL21D3(*ΔompA*) and (pET21_NHBA)BL21D3(*ΔompA*). Both strains were grown at mid-log phase and then antigen expression was induced for two hours by addition of 1mM IPTG (as described in Materials and Methods). Figure 9 shows the SDS-polyacrilamide gel

electrophoresis (SDS-PAGE) of total lysates before and after IPTG induction. fHpb appeared to be well expressed in *E. coli*, as indicated by the presence of a new protein band with the expected molecular mass in the cell extracts from induced cultures (figure 9 ***a***). As far as NHBA protein is concerned, its expression could not be clearly deduced from the SDS-PAGE because of its co-migration with endogenous protein species. However, an intensification of a specific band suggested that NHBA was also successfully expressed, even though at a level inferior to the one of fHbp (Figure 9 ***b***). These conclusions were corroborated by Western Blot analysis using anti-fHbp and anti-NHBA specific antibodies (Figure 10 ***a*** and 10 ***b***, first two lanes). Interestingly, for both proteins two bands were visible, the ones of higher molecular mass are likely to correspond to the protein precursors still carrying the LP.

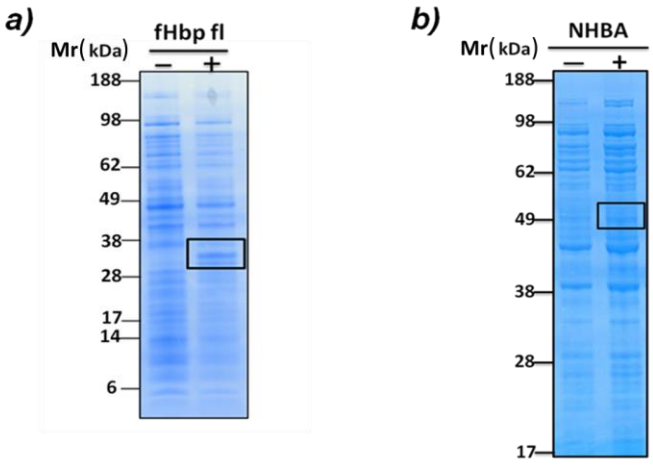


FIGURE 9 - Expression of fHbp and NHBA antigens in *E.coli*. *a)* SDS-PAGE of total cell extracts of *E. coli* BL21 Δ*ompA* strain expressing fHbp shows a clear expression after induction of lipoprotein. The lighter band represents prolipoprotein form, that could be located both in cytoplasm or on the inner membrane (Kovacs-Simon A. *et al.*, 2011); the heavier band corresponding to mature form (Inouye S. *et al.,* 1977). Both of them are boxed. ***b)*** SDS-PAGE of total cell extracts of *E. coli* BL21 Δ*ompA* strain expressing NHBA shows weak lipoprotein expression after induction. The feeble band is boxed.

2.3 Cellular compartmentalization of fHbp and NHBA in *E. coli*

We next investigated whether fHbp and NHBA reached the outer membrane of *E. coli*. To address this question we took advantage of the property of BL21D3(*ΔompA*) to release Outer Membrane Vesicles (OMVs) in the culture supernatant. OMVs are small spherical particles 20-250 nm in diameter constitutively secreted by all gram-negative bacteria as a result of a "budding out" process of the outer membrane (Klock H. *et al*., 2009). Since OMVs are constituted by OM (LPS, OM proteins and lipids), peptidoglycan and periplasmic proteins, (Ellis T.N. *et al.*, 2010), if fHbp and NHBA were expressed either intracellularly or anchored to the inner membrane, they should not be found in OMVs. On the other hand, if the two proteins are localized in the periplasm or attached to the outer membrane they should be part of the OMV proteome.

OMVs were purified from the supernatants of (pET21_fHbp)BL21D3(*ΔompA*) and (pET21_NHBA)BL21D3 (*ΔompA*) cultures after 2 hour induction with IPTG. Culture supernatants were collected by centrifugation at 5.000 x g for 15 minutes and subjected to a final step of ultracentrifugation (see Materials and Methods). Pellets containing OMVs were analyzed by Western Blot using the fHbp or NHBA specific polyclonal antibodies. As a negative control, OMVs purified from *E. coli* BL21(Δ*ompA*) transformed with pET21 empty vector were used. As shown in figure 10, both fHbp and NHBA proteins were found in OMVs. Interestingly, only the lower molecular mass band of fHpb reached the OMVs, supporting the hypothesis that the other band represents the protein precursor still carrying the LP. Less clear is the situation for NHBA for which two immune-reactive bands

were visible in OMVs, the lower band having the expected molecular mass of the mature form.

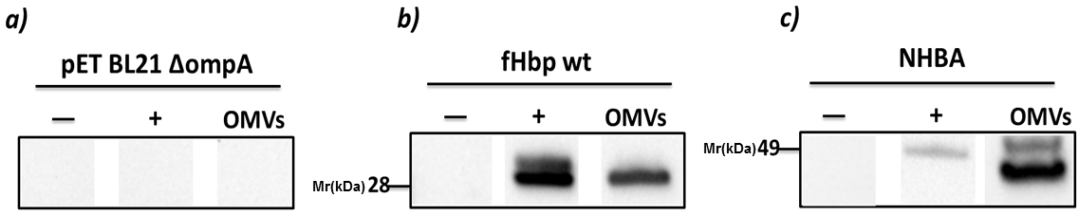


FIGURE 10 - fHbp full length and NHBA expression in total extracts and OMVs evaluated by Western Blot analysis- ***a)*** Double band corresponding both to the precursor and mature lipoprotein in bacterial cells can be compared to mature form presented in a single band in OMVs (Inouye S. *et al.,* 1977) (Kovacs-Simon A. *et al*., 2011). ***b)*** NHBA expression is evident in both total lysates and OMVs even if the different number of bands cannot be appreciated.

Finally, we investigated whether the two proteins were exposed on the surface of *E. coli*, as it occurs in *N. meningitidis*, or rather they remained anchored to the internal leaflet of OM. To address this question we used two experimental approaches. The first approach consisted in the “shaving” of purified OMVs with proteinase K. The proteolytic treatment removes all proteins protruding out of the OMV surface while the luminal compartment is protected. As shown in Figure 11, fHbp was completely digested by proteinase K treatment indicating that the protein was exposed on the OMV surface. On the contrary, NHBA digestion occurred only upon SDS treatment of OMVs.

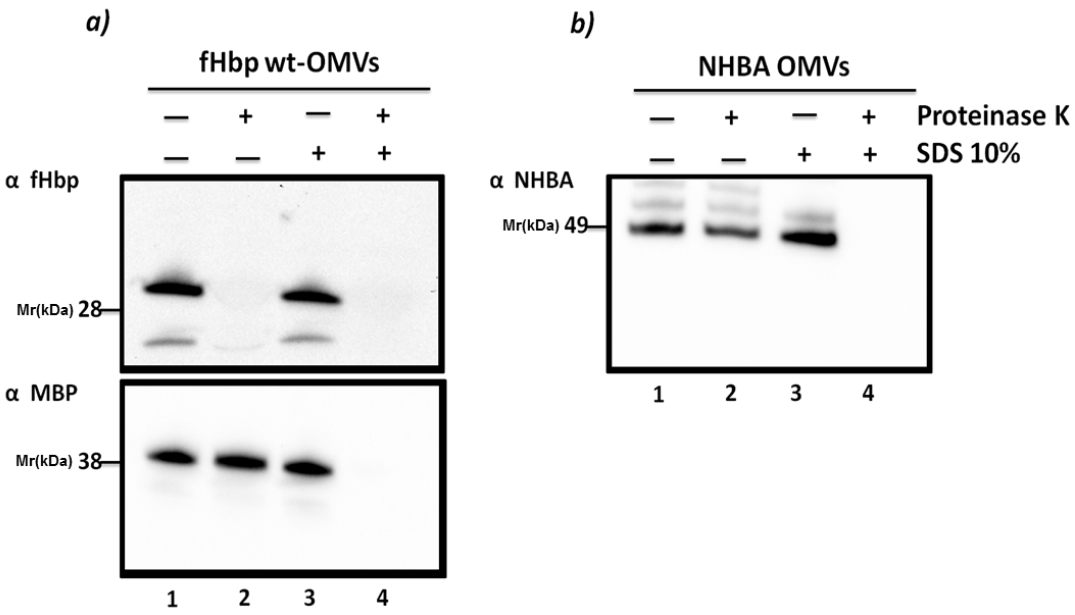


FIGURE 11 - Analysis of antigens localization and orientation in OMVs- Purified OMVs expressing the heterologous protein were incubated with and without Proteinase K for 10 min at 37°C in the presence or absence of a pre-treatment with 1% SDS to destroy OMV integrity. Data indicate the fHbp localization on the outer membrane of OMVs and orientation to the external environment (***a***). The mbp-control shows the integrity of the OMVs (***a1***). The NHBA protein signal is still present after treatment with protease: this is an evidence of the non-digestion of NHBA ***(b)***. The protection conferred by the presence of the outer membrane makes possible hypothesize the localization of NHBA in the periplasm or anchored to the OM but oriented to the lumen of OMVs.

To confirm the external localization of fHbp, Fluorescence Activated Cell Sorting (FACS) analysis was performed on (pET21_fHbp)BL21D3 (*ΔompA*) strain and on (pET21)BL21D3 (*ΔompA*) strain as control using anti-fHbp antibodies (Figure 12). Cultures were grown to $OD_{600=}$ 0.5 and subsequently induced by addition of 1mM IPTG. Cells from 1 ml of each culture were collected by centrifugation at 10.000 x g for 5 min at 4°C, and pellets were re-suspended in PBS to obtain a suspension of $2x10^7$/ml cells. Cells (50 µl) were dispensed in 96-well plate with round bottom

and three different concentrations of anti-fHbp polyclonal antibodies were added followed by incubation with a secondary antibody labeled with FITC (see Material and Methods). Finally cells were re-suspended in 4% formalin and analyzed by FACS. As shown in Figure 13, anti-fHbp antibodies specifically recognized (pET21_fHbp)BL21D3 (*ΔompA*) cells, confirms the results obtained by Proteinase K treatment of OMVs.

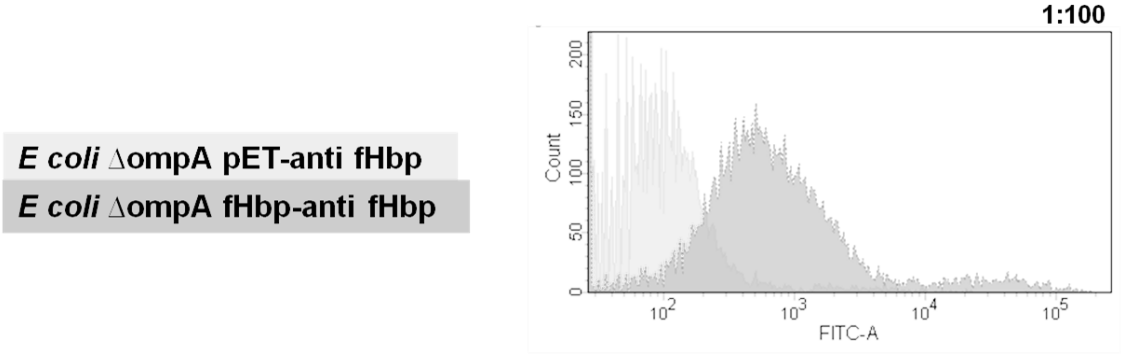


FIGURE 12 - The evidence of the external orientation of fHbp confirmed by Fluorescence Activating Cell Sorting analysis_ Bacteria cells were analyzed by FACS using the antigen-specific polyclonal antibody. Peaks that came out from the ΔompA pET21empty vector strain labeled with fluorescein isothiocyanate (***a***) were compared to those produced by ΔompA expressing fHbp strain (***b***) at the same concentration. The overlapping of the two cell populations (***c***) shows an evident shift of the peaks corresponding to the strain able to express the lipoprotein.

2.4_Quantification of fHbp in OMVs

Finally, we investigated the amount of fHbp present in OMVs. Such quantification was carried out using Western Blot analysis. Increasing amounts of OMVs were separated by SDS-PAGE together with different amount of purified recombinant fHbp. After separation, proteins were transferred to nitrocellulose filter by electro-blotting and stained with anti-fHbp antibodies. As shown in Figure 13, 0.5 μg of

OMVs extracted proteins carried approximately 100 ng of fHbp, corresponding to approximately 20% of total OMV proteins.

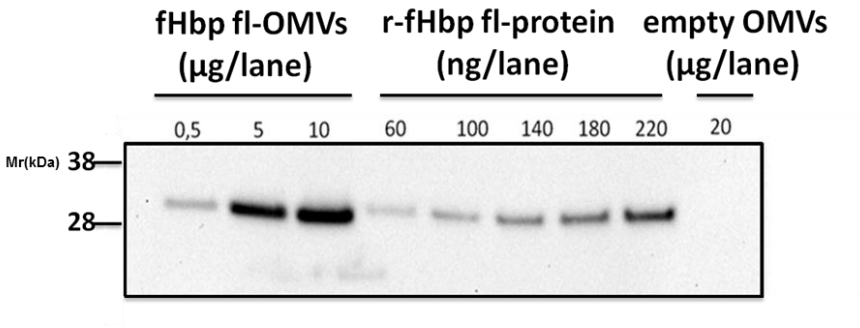


FIGURE 13 – Quantification of OMV-associated fHbp by Western Blot - Different quantities of purified recombinant protein and OMVs expressing fHbp were separated by SDS-PAGE, transferred to nitrocellulose filters and incubated with fHbp-specific antibodies. From the comparison of band intensities it was estimated that 1 µg of OMV carries approximately 200 ng of fHbp, corresponding to 20% of total OMV proteins.

2.5 Elucidation of structural features promoting fHbp exposition on bacterial surface

The results described above indicate that fHbp, but not NHBA, reaches the surface of *E. coli*, the same way it does in *N. meningitidis*. This interesting observation leads to the hypothesis that the "flipping out" mechanism, which mediates the transfer of lipoproteins from the inner leaflet of OM to the external LPS-containing leaflet, does not require the intervention of specific transport systems but rather is dependent on the presence of structural signatures embedded in lipoproteins. The data also suggest that such signatures can differ from lipoprotein to lipoprotein and are not universally recognized by all gram-

negative species, thus giving an explanation why NHBA is not surface-localized when expressed in *E. coli*.

In the attempt to shed light on the structural signatures that might be involved in promoting the transfer of fHbp to the bacterial surface, we created a number of fHbp mutations and we investigated in which way such mutations could affect fHbp translocation.

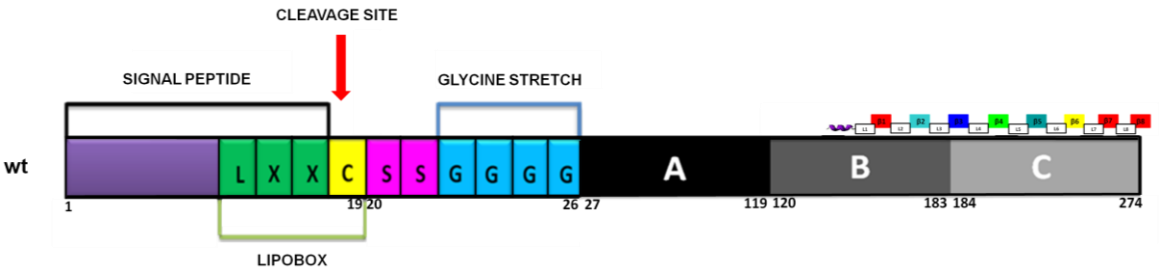


FIGURE 14 – Schematic representation of fHbp structural organization

We first focused our attention to the fHbp region immediately adjacent to the N-terminal acylated cysteine. The sequence analysis of this region reveals the presence of a stretch of four glycine residues. Such glycine-rich box is relatively conserved in lipoproteins, as deduced from the sequence analysis of different gram negative lipoproteins (figure 15).

To test whether the glycine residues are involved in the translocation process, two different mutated forms of fHbp were created (figure 16). In one mutant, the four glycine residues were removed, while in the second mutant the four glycine residues were replaced with alanine.

LIPOPROTEIN NAME	BACTERIAL SPECIES	PROTEIN SEQUENCE
fHbp	*N. meningitidis*	ALILTACSS**GGGG**V
NHBA	*N. meningitidis*	FALSAC**GGGGGG**S
TbpB	*N. meningitidis*	PVFLLSACL**GGGGG**S
Lactoferrrin-binding protein B	*N. meningitidis*	LPLLLASCI**GG**N
Lip	*N.gonhorreae*	LLSLALAAC**GG**E
GNA1946	*N.gonhorreae*	ALALILAAC**GG**Q

FIGURE 15- Schematic list of lipoproteins with conserved region glycine stretch

In order to generate both fHbp mutants, pET21_fHbp wt plasmid was used as template of a PCR-mediated site-directed mutagenesis (see Materials and Methods for details). The resulting pET21_fHbpΔGLY_4 and pET21_fHbpGLY_4>ALA_4 plasmids were used to transform *E. coli* BL21(*ΔompA)* strain. The derived strains were grown at mid-log phase and then fHbpΔGLY_4 and fHbpGLY_4>ALA_4 expression was induced for two hours by addition of 1mM IPTG. Before and after IPTG addition bacterial cells from 1 ml culture were collected by centrifugation and analyzed by SDS-PAGE. Furthermore, OMVs were purified from culture supernatants after IPTG induction (see Materials and Methods for details of OMV purification). As shown in figure 17 ***a-b***, both fHbpΔGLY_4 and fHbpGLY_4>ALA_4 were expressed to a similar level with respect to the wild type protein and were compartmentalized in OMVs. Also similarly to what observed in wild type fHbp, two protein species were present in total extracts, while OMVs

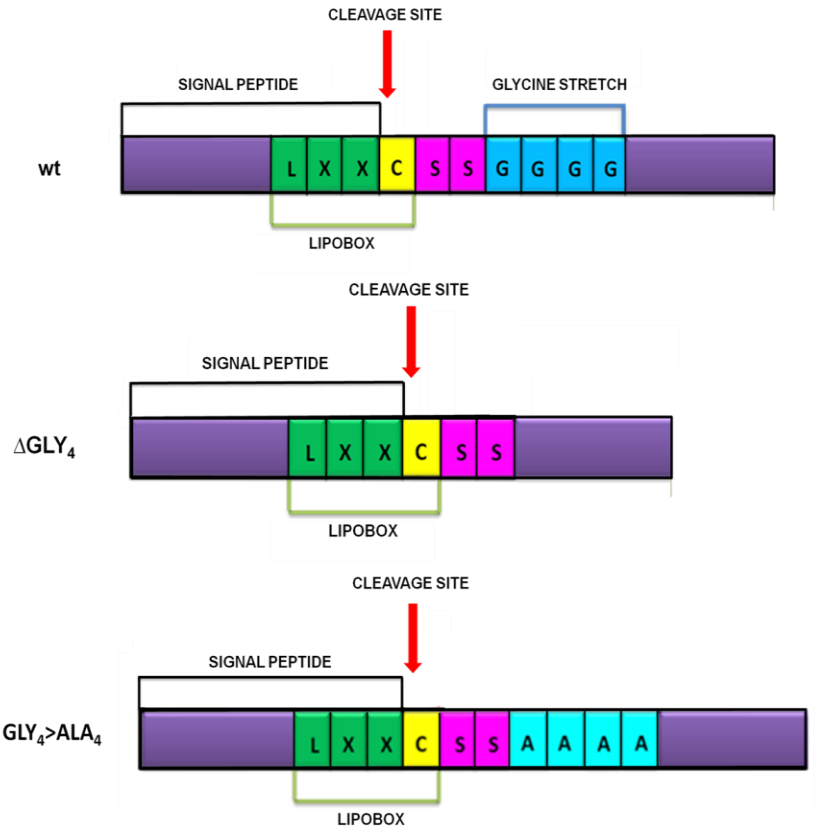


FIGURE 16 – Schematic representation of the fHbp mutants targeting the four N-terminal glycine residues

carried a single species, most likely representing the processed, LP-less mature forms.

Having demonstrated that both mutant forms of fHbp were expressed and reached the OMVs, we next asked the question whether the mutations have somehow affected their compartmentalization in the membrane. To this aim, the Proteinase K assay was performed, in which OMVs expressing fHbpΔGLY$_4$ and fHbpGLY$_4$>ALA$_4$ were treated with protease in presence or absence of SDS and then analyzed by Western Blot. As shown in Figure 18, neither the removal of the

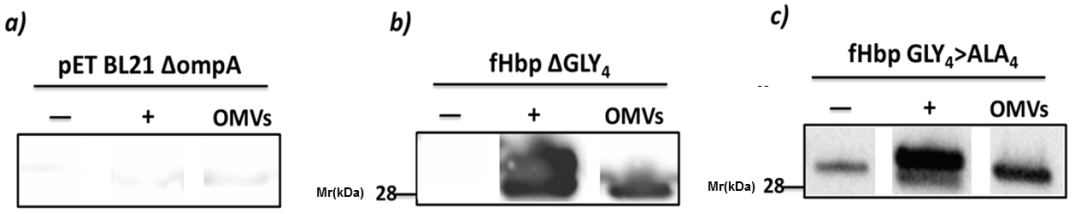


Figure 17 - Western Blot analysis of fHbpΔGLY$_4$ and fHbpGLY$_4$>ALA$_4$ expression in bacterial total lysates and OMVs –

glycine stretch nor its replacement with the four alanine residues prevented fHbp from being exposed on the surface of OMVs, indicating that despite its conservation this motif is not involved in lipoprotein flipping out mechanism.

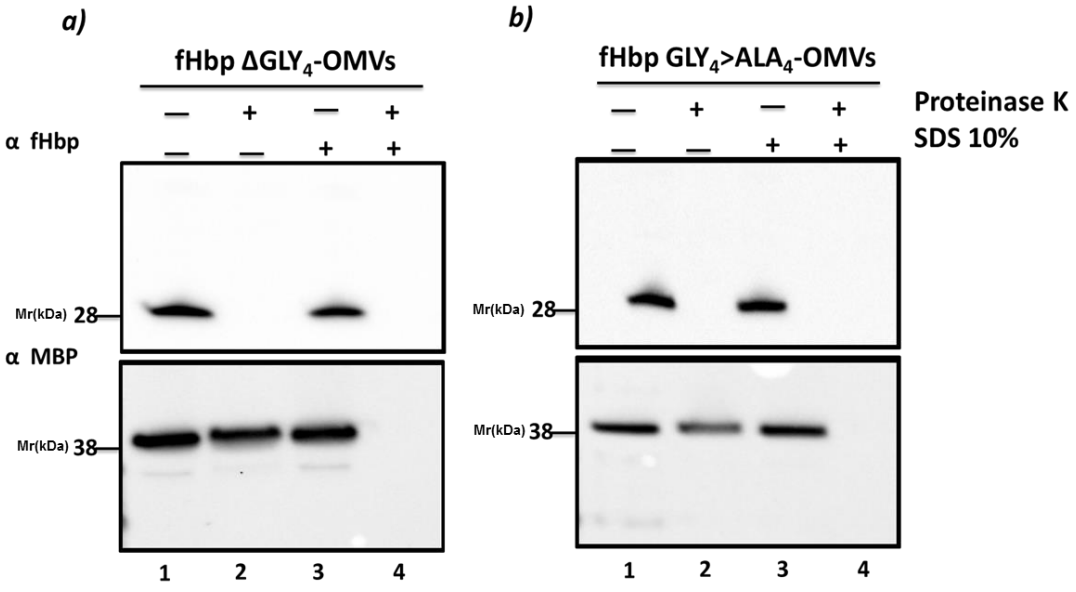


FIGURE 18 – Localization of fHbpΔGLY$_4$ and fHbpGLY$_4$>ALA$_4$ in OMVs. The Proteinase K assay in the presence or absence of detergent was used to establish protein compartmentalization. See Text for details.

We next focused our attention on the three structural domains in which fHbp protein is organized. As already anticipated (see also Figures 7 and 14), the 3D structure of fHbp has revealed that the protein is composed of three main structural domains that have been previously shown to fold independently when expressed separately in *E. coli* (Giuliani M.M. *et al.,* 2004). The main question we wanted to address was whether any of these domains was specifically involved in the flipping out process. To answer this question, the three fHbp mutants depicted in Figure 19 were generated. In one mutant the C-terminal C domain was removed, while in a second mutant both B and C domains were simultaneously eliminated. As far as the third mutant is concerned, domain A and B were deleted so that the C domain was juxtaposed next to the glycine stretch. The three mutants were generated using the same approach described for the construction of fHbpΔGLY_4 and fHbpGLY_4>ALA_4 (Klock H. *et al*., 2009). The three derived plasmids carrying the mutated forms of fHbp, pET21_fHbpA, pET21_fHbpA-B, pET21_fHbpC, were used to transform *E.coli ⊿ompA* strain. The resulting strains were grown at mid-log phase and then fHbpA, fHbpA-B and fHbpC expression was induced for two hours by addition of 1mM IPTG. Total cell extracts of bacterial cells were prepared by collecting cells from 1 ml culture and by re-suspended the cell pellets directly in SDS-PAGE sample loading buffer. Figure 20 shows the Western Blot analysis of total extracts of the recombinant strains expressing the three fHbp mutants. fHbpA and fHbpA-B were both expressed even though the amount of proteins appeared to be lower than what observed in the lysate of the strain expressing the full length fHbp (figure 20 **a-b**).

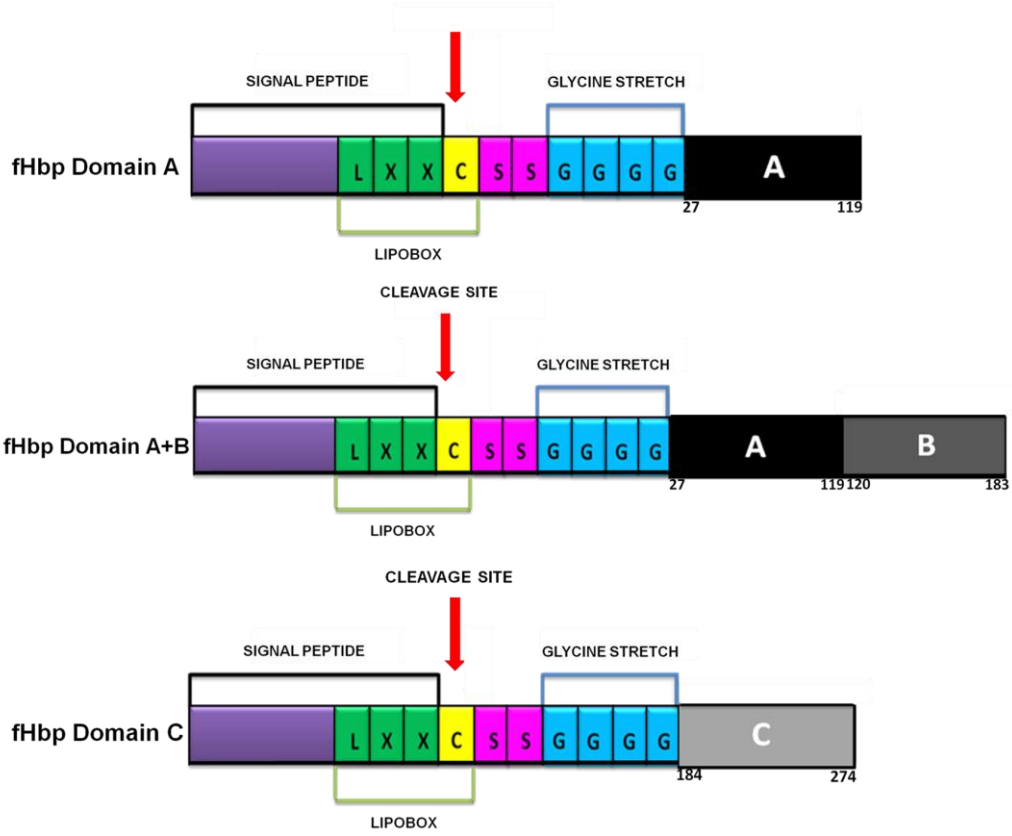


FIGURE 19 – Schematic representation of the fHbp domain deletion mutants

By contrast, no immune-reactive band corresponding to the domain C fused to the LP could be observed, suggesting that the protein was not expressed (figure 20 **c**). It has to be pointed out that the polyclonal antibodies used in Western Blot were raised against the whole fHbp and therefore it cannot be excluded that domain C is poorly recognized by these antibodies. Antibodies specificity with respect to the different fHbp domains should also be taken into account before drawing conclusions on the level of expression of fHbpA and fHbpA-B with respect to the wild type protein.

To evaluate the expression and localization in OMVs of fHbpA and fHbpA-B, Proteinase K assay was carried out. As shown in figure 21, fHbpA-B and at least

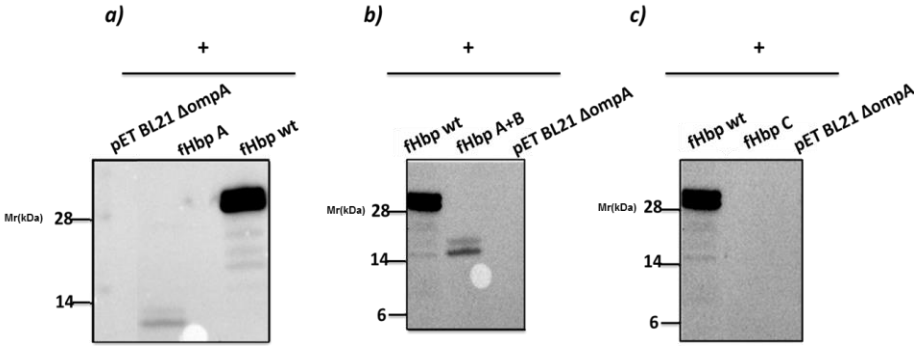


FIGURE 20 - Expression of fHbpA, fHbpA-B and fHbpC in *E. coli* total cell extracts.

partially fHbpA were digested by protease treatment even in the absence of detergent. This result strongly suggests that both deleted proteins still carry the structural signatures required for the flipping out mechanism and such signatures reside, at least in part, in the N-terminal A domain.

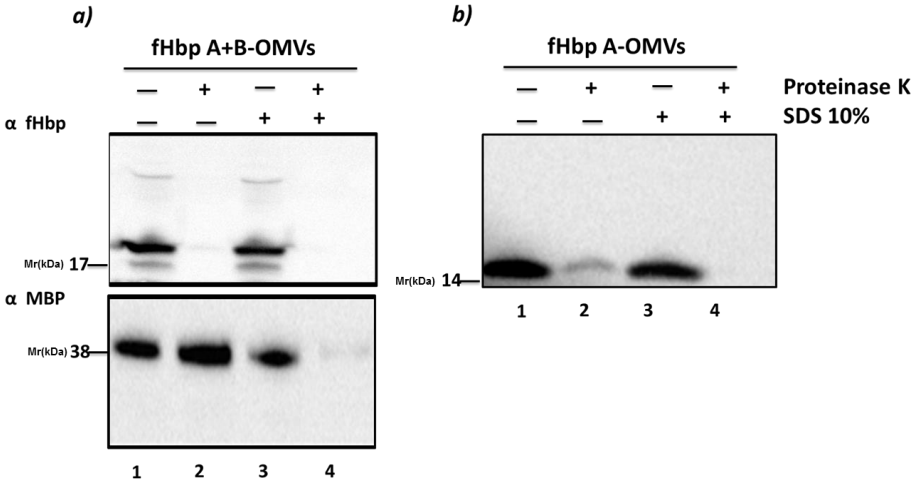


FIGURE 21 - Expression and localization analysis of fHbpA and fHbpAB in OMVs- The Protease K assay described in Figure 18 was used for the analysis of protein compartmentalization. The digestion of the periplasmic maltose binding protein was followed as control.

2.6 fHbp as delivery system of heterologous proteins to bacterial surface

In addition to shed light on the mechanisms of lipoprotein transport in bacteria, the data reported above might pave the way to potentially interesting biotechnological applications.

The ability to decorate the bacterial surface with heterologous proteins has been the object of intense investigation (Chen W *et al.,* 2002) (Georgiou G. *et al.,* 1997)(Lee S.Y. *et al.,* 2003) (Pozzi G. et al., 1992) (Nguyen T.N. *et al.,* 1993). Several applications can be envisaged, including the possibility to develop bioreactors which carry out important chemical reactions catalyzed by surface associated enzymes, and to create bacteria with novel immunogenicity properties for vaccine applications.

A number of surface delivery systems have been already described, many of them exploiting the capacity of proteins naturally destined to the surface ("carrier proteins (CPs)") to act as chaperones of heterologous proteins ("passenger proteins (PPs)") (Wentzel A. *et al.,* 2001). PPs are fused either to the N-terminus or to the C-terminus of CPs so that they can reach, and get anchored to, the cell surface. Although the strategy has been successfully applied in several cases, CPs do not work universally and the optimal CP required for efficient translocation of a specific PP cannot be predicted *a priori*. For these reasons, there is a need of novel CPs capable of exporting otherwise recalcitrant proteins.

On the basis of the above considerations we decided to test whether fHbp could be exploited as a delivery system for hererologous antigens in *E. coli*. We focused our attentions on three promising vaccine candidates: the backbone

protein of pili from *Group B Streptococcus* (GBS), the iron transport protein FhuD2 and Protein A, both from *Staphylococcus aureus*.

- *fHbp-mediated membrane delivery of GBS59 pilus backbone protein*

GBS is a Gram-positive pathogen that causes life-threatening pneumonia, sepsis, and meningitis in newborn and young infants (Johri A.K. *et al.,* 2006) (Dramsi S. *et al.,* 2006) Indeed, GBS is responsible for over 80% of all bacterial infections in infants below four months of age. Because children delivered from women with high titers of anti-GBS opsonophagocytic antibodies, are protected from GBS infections (Baker C.J. *et al.,* 1976) (Lin F.Y. *et al.,* 2004) (Maione D. *et al.,* 2005), vaccination of women in childbearing age or pregnant women appears to be the ideal long-term solution to newborn infections.

Although major efforts have been made in the development of multivalent capsular conjugate vaccines, currently there is no vaccine against GBS. To overcome serotype-specific immunity and the increasing number of non-typeable isolates, vaccines based on conserved protective proteins are highly desirable. The most promising protein-based vaccine candidates selected so far are the structural subunits of pili that are long filamentous structures protruding from the bacterial surface (Nuccitelli A. *et al.,* 2011). Pili are constituted by three structural proteins, one of which, the backbone protein (BP), is highly protective. Extensive analysis of a large panel of GBS isolates has revealed the existence of eight non-cross-protective BP variants (Margarit I. *et al., 2009*). Although they differ in

amino acid sequence, their 3D structure is conserved, being organized in four structural domains (Nuccitelli A. *et al.,* 2011). Interestingly, one of these domains, domain D3, is immunologically relevant and the target of most functional antibodies (Nuccitelli A. *et al.,* 2011).

We decided to create two chimeric proteins in which Domain 3 of GBS59, the backbone protein of pilus variant 2b, was fused to either full length fHbp or to fHbp Domain A (fHbpA).

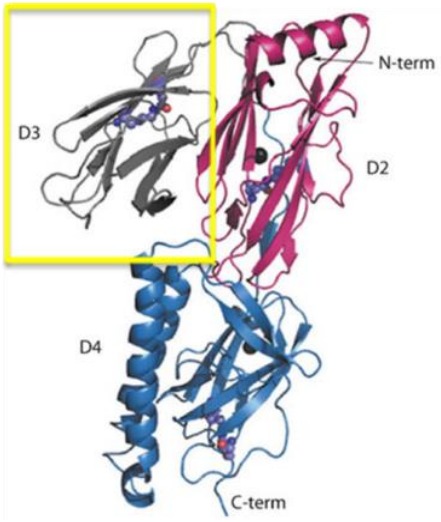


FIGURE 22 - 3D structure of GBS59 pilus protein from *Streptococus agalactiae*. The small immunogenic Domain 3 of the protein is boxed in yellow. (Nuccitelli A*. et al.,* 2011)

GBS59 Domain 3 coding sequence was amplified by PCR from chromosomal DNA of *Streptococcus agalactiae* strain 515 and the amplified product was

inserted into pET21_fHbp and pET21_fHbpA plasmids (Figure 23) using the PIPE method (Klock H. *et al*., 2009).

The derived recombinant plasmids pET21_fHbp-GBS-Dom3 and pET21_fHbpA-GBS-Dom3 plasmids were used to transform *E.coli* BL21(DE3) *ΔompA* strain.

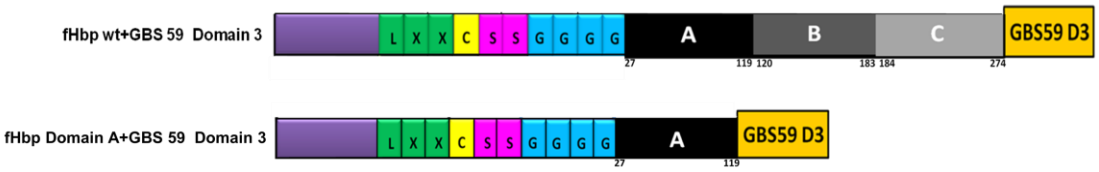


FIGURE 23 - Schematic representation of the two fHbp chimeric proteins carrying Domain 3 of GBS59 pilus backbone protein.

The resulting strains were grown in LB at mid-log phase and then antigens expression was induced for two hours by addition of 1mM IPTG. Before and after induction, 1 ml of culture was collected and bacterial total lysates were prepared by re-suspending bacterial cells in SDS-PAGE sample buffer. Aliquots of total lysates were run on SDS-polyacrylamide gel and transferred to nitrocellulose filter for Western Blot analysis using anti-GBS59 antibodies. As shown in Figure 24, both fusion proteins were expressed, as indicated by the appearance of two immune-reactive bands in cell extracts. The reason why two protein species were resolved on the gel remains to be experimentally investigated. One possible explanation is that both chimeras are cleaved by an endogenous protease at a specific position either at the N-terminus of the fusions or within the GBS59 D3 domain.

Having demonstrated that the fusions expressed in *E. coli*, we asked the question whether the proteins reached the outer membrane and were eventually exposed on the bacterial surface. To address this question, once again, we exploited the OMV overproducing phenotype of the strains. After 2 hours induction with IPTG, OMVs were purified from the culture supernatants and analyzed by Western Blot. As shown in figure 25 ***a-b***, both recombinant proteins could reach the vesicular compartment, indicating fHbp and fHbpA could work as PP for GBS59Dom3.

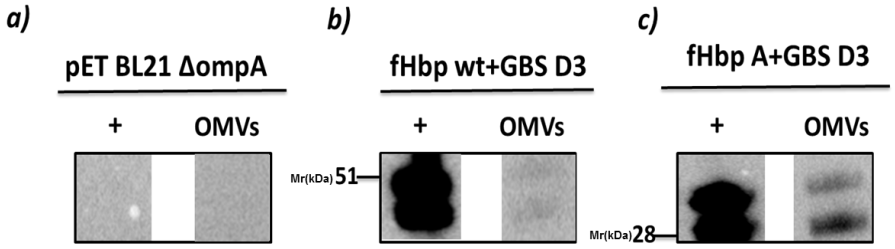


FIGURE 24 - Expression and compartmentalization of fHbp-GBS59Dom3 and fHbpA-GBS59Dom3 chimeras in *E. coli* – Aliquots of total cell extracts and OMVs from recombinant *E.coli* BL21(DE3) *ΔompA* strains expressing fHpb-GBS59Dom3 and fHbpA-GBS59Dom3 were analyzed by Western Blot using GBS59Dom3-specific antibodies

To analyze the localization of the chimeras in OMVs we treated the recombinant OMVs with Proteinase K in the presence or absence of detergent and we subsequently analyzed the samples by Western Blot. The results of this experiment indicate that both proteins were at least partially exposed on the OMV surface in that a fraction of the chimeric proteins was digested even in the absence of SDS (Figure 25).

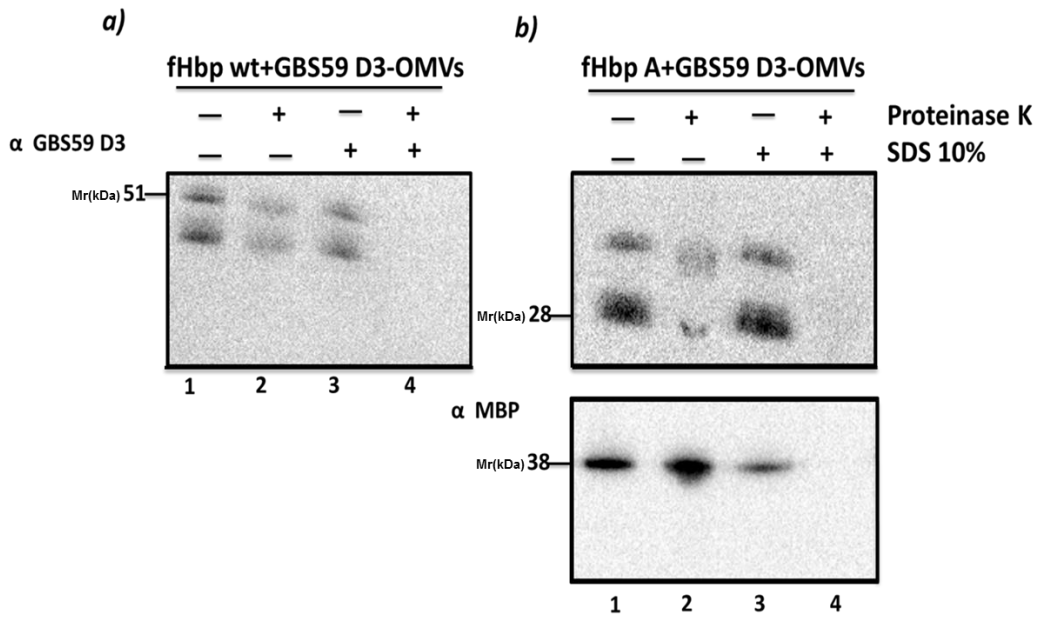


FIGURE 25 – Analysis of localization of fHbp-GBS59Dom3 and fHbpA-GBS59Dom3 in OMVs -OMVs were purified from the culture supernatants of *E.coli* BL21(DE3) *ΔompA* strains expressing fHpb-GBS59Dom3 and fHbpA-GBS59Dom3 and digested with Proteinase K in the presence or absence of 1% SDS. After digestion, the samples were analyzed by Western Blot using a GBS59Dom3-specific antibodies. As control of the integrity of the OMVs in the absence of detergent, aliquots of OMVs carrying fHbpA-GBS59Dom3 were also analyzed by Western Blot using polyclonal antibodies against MBP, a protein present in the lumen of OMV.

- *fHbp-mediated membrane delivery of S. aureus FhuD2 and SpA_DEABC*

On the basis of the promising results obtained with GBS59 Domain 3, we asked the question whether in addition to GBS59Dom3 other proteins could be delivered to the membrane of *E. coli* using fHbp as chaperone. We focused our attention on two *S. aureus* virulence factors, fhuD2 and Protein A, which have been proposed as promising vaccine candidates (Mariotti P. *et al.,* 2013) (Etz H.

et al., 2002). fhuD2 is a lipoprotein involved in iron-hydroxamate uptake, belonging to the core genome of *S. aureus*. The protein is highly conserved and is expressed in a wide variety of epidemiologically relevant *S. aureus* strains (Mishra R.P.N. *et al.,* 2012) (Mariotti P. *et al.,* 2013). The rationale of using FhuD2 as vaccine component is to induce antibodies that inhibit FhuD2-mediated iron up-take in *S. aureus*. In addition, FhuD2- specific antibodies bind to the protein naturally localized on the surface of *S. aureus*, thus mediating opsonophagocytosis.

Protein A is a cell-wall anchored protein expressed in the majority of *S. aureus* isolates and representing the most abundant surface protein of this pathogen. Its major function is to bind the Fc domain (and the VH3 domain) of immunoglobulins (Sasso E.H. *et al.,* 1989) and in so doing exerts two main effects: from one hand it impairs antibody-mediated opsonization and phagocytosis, and on the other hand it stimulates apoptosis of B cells by binding to their receptors (Kim H.K. *et al*., 2010) (Forsgren A. *et al.,*1974) (Goodyear C.S. *et al.,* 2004). The mechanism of action of a SpA-based vaccine involves the elicitation of antibodies that prevents SpA binding to the immunoglobulin Fc domain, thus avoiding SpA toxic activity. In this work we cloned and expressed a particular mutated and truncated form of SpA in which the C-terminal domain has been removed and the Fc binding motif present in each of the five Ig binding domains was modified to prevent Fc binding. When used as vaccine this mutated form is still capable of eliciting fully functional neutralizing antibodies (Kim H.K. *et al*., 2010).

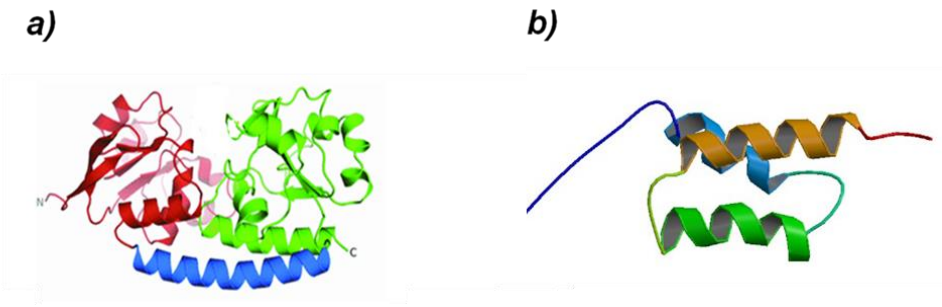


FIGURE 26 – 3D structural models of FhuD2 (*a*)(Mariotti P.et al 2013**) and SpA_DEABC (*b*)** (Gouda H. et al., 1992)

fhuD2 and spa_DEABC coding sequences were amplified from plasmids pET21-fhuD2 AND pET21-spaDEABC and cloned into plasmids pET21-fHbp and pET21fHbpA, respectively by using PIPE method. In so doing, FhuD2 was fused to the C- terminus of full length fHbp while Spa_DEABC to the A domain of fHbp (Figure 27).

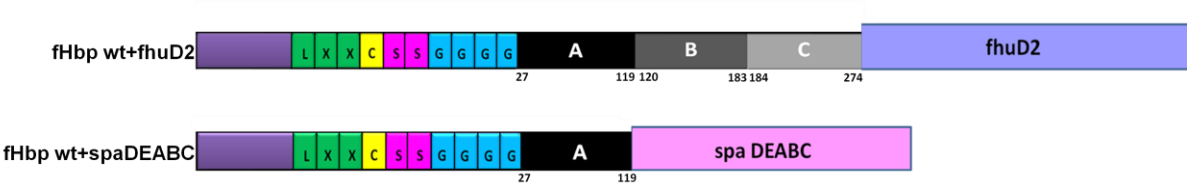


FIGURE 27 - Strategies to clone fhuD2 and spaDEABC proteins to C terminal region of fHbp full length and fHbp domain A respectively

pET21-fHbp-fhuD2 and pET21-fHbpA-spaDEABC plasmids were used to transform *E. coli ⊿ompA* strain. The derived recombinant strains were grown at mid-log phase and total cell extracts were prepared from bacteria collected before and after IPTG addition. In parallel, OMVs were collected from the supernatants of both cultures after IPTG-induced protein expression. Both total cell extracts and OMVs preparations were analyzed by Western Blot using anti-FhuD2 and anti-SpaDEABC antibodies. As shown in Figure 28, both chimeric proteins were not only well expressed but also compartmentalized in the vesicle fraction.

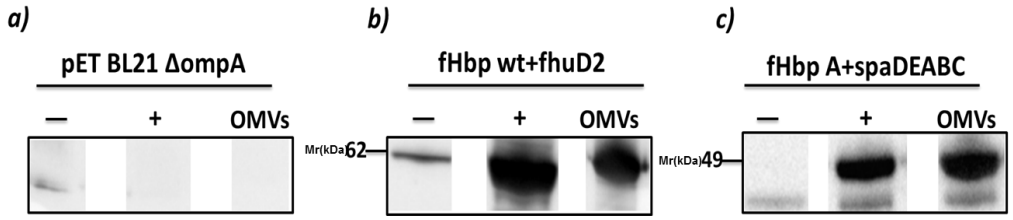


FIGURE 28 - fHbp fl+fhuD2 and fHbp A+spaDEABC chimeric protein expression in bacterial total lysates and in OMVs tested by Western Blot technique- Bands corresponding to the expression of both fusion proteins are evident (***b***),(***c***) suggesting an high level of expression.

Finally we analyzed whether the two proteins were exposed on the surface of the outer membrane using the Proteinase K assay already described. As shown in Figure 29 chimeric proteins were digested only in the presence of detergent, suggesting that they are associated to the outer membrane but oriented toward the periplasmic space.

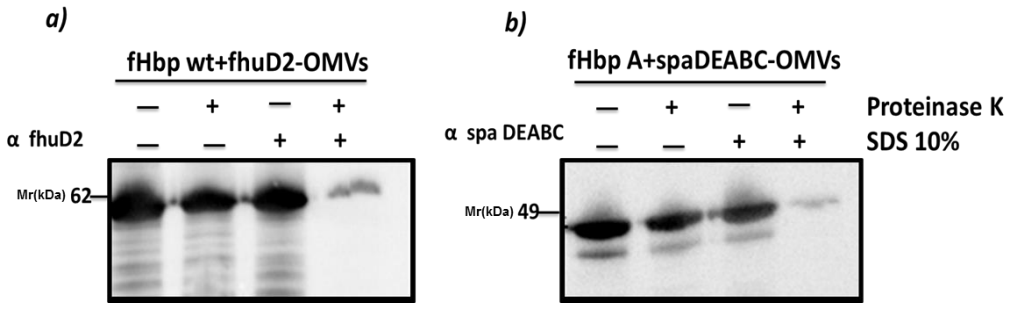


FIGURE 29 – Analysis of fHbp-fhuD2 and fHbpA-spaDEABC compartmentalization in OMVs- Protease K assay was carried out in the presence and absence of detergent (1% SDS) as already reported in Figure 25.

3) DISCUSSION

The two main objectives of this project were 1) to contribute to the understanding of the reason why outer membrane-anchored lipoproteins are surface –exposed in some bacterial species while in others they face the periplasmic space, and 2) to explore the possibility of exploiting surface-exposed lipoproteins as “vehicles” to deliver heterologous proteins to the bacterial surface.

Both objectives have been achieved.

As far as the mechanism of lipoprotein sorting is concerned, we expressed two *N. meningitidis* lipoproteins, fHbp and NHBA, in *E. coli* and we asked the question whether they could reach the cell surface or rather they were attached to the outer membrane but facing the cytoplasmic space. Since in *N. meningitidis* both proteins are surface-exposed, their appearance on the *E. coli* surface would indicate that the final step of lipoprotein sorting involving their “flipping out” from the inside to the outside of OM occurs *via* intrinsic structural properties. On the other hand, should the proteins be found inside the cells, it would suggest that specific “flippases” are involved in the sorting process.

The results of our work indicate that specific transport systems are not strictly required, at least for some lipoproteins. In fact, fHbp was efficiently transported to the surface of *E. coli* cells. Therefore, at least some lipoproteins, including fHbp, carry structural features sufficient to bring them to the bacterial surface.

However the sorting mechanism appears to be more complicated than expected. In fact, NHBA, another lipoprotein that in *N. meningitidis* protrudes out of the surface, is not surface-exposed in *E. coli*. As already pointed out, to reconcile

these apparently contradictory results we postulate that the “flipping out” mechanism, which mediates the transfer of lipoproteins from the inner leaflet of OM to the external LPS-containing leaflet, does not require the intervention of specific transport systems but rather is dependent on the presence of structural signatures embedded in lipoproteins. However, such signatures can differ from lipoprotein to lipoprotein and are not universally recognized by all gram-negative species.

In an attempt to characterize such signatures we generated a number of mutations in fHbp and we followed the effect of such mutations on surface compartmentalization.

Five fHbp mutants were produced. The first two mutants involved the four-glycine stretch located immediately downstream from the lipobox. Since such glycine stretch is present in other lipoproteins, we tested whether it could play a role in lipoprotein sorting. However, neither the deletion of the four glycine residues nor their replacement with alanine affected the property of fHbp to reach the *E. coli* surface.

We next removed specific structural domains of fHbp. The crystal structure of fHbp has revealed that the protein is organized in three domains (Domain A, B and C), which can fold independently when expressed in *E. coli*. We therefore generated a mutant deprived of the C-terminal C domain (fHbpAB), and a mutant in which both Domain C and B are deleted. Finally, we designed a mutant deprived of Domains A and B in which Domain C is directly fused to fHbp LP and lipobox. When the expression and location of these mutant proteins was

analyzed, we found that Domain C could not be expressed while both fHbpA and fHbpAB were surface-associated. From these data two main conclusions could be drawn. First, that the C Domain is not involved in fHbp sorting. Second, that the A Domain carries most of the “information” needed to deliver fHbp to the OM and to expose it to the extracellular milieu.

Having demonstrated that fHbp (and its Domain A and AB) can travel from the cytoplasm where it is synthesized all the way to the bacterial surface even if it is expressed in *E. coli*, we decided to investigate whether fHbp could work as a surface chaperone for heterologous proteins.

For this study, which represents the second project objective, we focused our attention on three protein antigens which are vaccine candidates against two important human pathogens, *Group B Streptococcus* (GBS) and *Staphylococcus aureus*. One protein is the immunogenic D3 domain of GBS59, the main component of the pili present in all GBS isolates and representing an important virulence factor. It has been previously shown that GBS59 D3 domain induces potent opsonophagocytic antibodies which protect mice from GBS lethal challenge (Nuccitelli *et a.l*, 2011). The other two proteins, FhuD2 and a mutated form of Protein A, are two *S. aureus* antigens with protective activity in different mouse models. The successful delivery of these antigens to the surface of *E. coli* could pave the way to the development of easy-to-produce multi-valent vaccines.

The three proteins were fused either to the full length fHbp or to Domain A and we investigated whether they could be delivered to the OM and eventually could get exposed to the bacterial surface.

As far as the first question is concerned, our data clearly show that the chimeric proteins can indeed reach the outer membrane. This was demonstrated by showing that the proteins could be incorporated into OMVs. This is an important achievement since it has been recently shown that OMVs carrying heterologous antigens can induce potent, antigen-specific, functional antibodies even if the antigens are located in the lumen of OMVs (Fantappiè *et al.,* 2014). In addition, it is known that lipoproteins are particularly immunogenic in that their lipid moiety exerts an adjuvant effect by interacting with TLR2 of antigen presenting cells (APCs) (Hefty *et al*., 2002). Therefore, although it remains to be experimentally demonstrated, because of their being fused to a lipoprotein (fHbp or fHbpA) , GBS59, FhuD2 and Spa_DEABC could elicit good immunological responses.

With respect to the second question as to whether when fused to fHbp or fHbpA the three antigens reach the bacterial surface, the data seem to indicate that this is not always the case. GBS59 was surface-exposed while the other two proteins appear to be predominantly inserted into the OM but facing the periplasmic compartment. The reasons why the three proteins behave differently remain to be investigated. One possible explanation is that the structural nature of the passenger protein plays a role in determining its final destination. However, it has to be pointed out that our conclusion on the localization of the three heterologous antigens in the OM is based on a single experimental approach, namely the effect of Proteinase K treatment of OMVs expressing the three antigens. Other confirmatory experiments such as FACS analysis and mass spectrometry would

be needed to unequivocally determine the localization and orientation of the proteins.

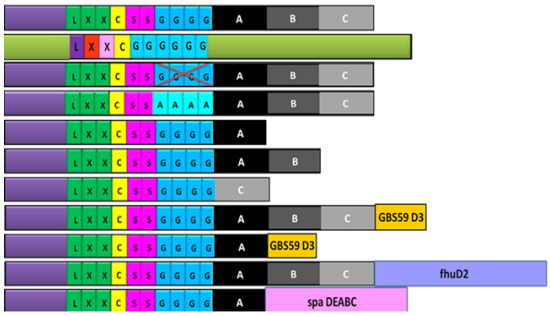
NAME	CONSTRUCTS	ORIENTATION	MW
fHbp full lenght		external environment	30 kDa
NHBA		internal environment	54 kDa
fHbp Δgly		external environment	30 kDa
fHbp ALA		external environment	30 kDa
fHbp domain A		external environment	13 kDa
fHbp domain A+B		external environment	20 kDa
fHbp domain C		not expressed	13 kDa
fHbp fl+GBS59 D3		internal environment	49 kDa
fHbp A+GBS59 D3		external environment	30 kDa
fHbp fl+fhuD2		internal environment	61 kDa
fHbp A+spa DEABC		internal environment	47 kDa

Table 1 - Summary of all constructs generated in this study

4) CONCLUSIONS

It is our opinion that the experimental work described in this document brings an important contribution to the understanding of the sorting mechanisms of lipoproteins in bacteria. Differently from what previously believed, lipoproteins have an intrinsic capacity to reach the surface of the outer membrane and they appear not to require specific sorting machineries. However, the structural signatures that allow lipoproteins to become surface-exposed appear, at least in some cases, species-specific in that when expressed in heterologous systems some lipoproteins remain anchored to the inner leaflet of the outer membrane. This is the case for instance of *N. meningitidis* NHBA, which, when expressed in *E. coli*, is not surface-exposed. Future work is required to shed light of this interesting species-specific process.

The second important contribution of this experimental work is the discovery that fHbp can work as chaperone to expose heterologous proteins on the bacterial surface. This observation can lead to numerous industrial applications, from biocatalysis to immunotherapy and vaccines. However, not all heterologous proteins are properly exposed on the bacterial surface when fused to fHbp. The understanding of the structural constrains that determine the final destination of chimeras should be the object of future experimental work.

5) MATERIALS AND METHODS

5.1_Bacterial strains and culture conditions

E. coli strains BL21-(DE3) and HK-100 (Table 3) were routinely grown in LB broth at 37°C and used for cloning and expression experiments, respectively. Stock preparations of strains in LB + 15% glycerol were stored at -80°C. Each bacterial manipulation was started using an overnight culture of a frozen stock. When required ampicillin or chloramphenicol were added to final concentration of 100μg/ml and 30μg/ml, respectively.

5.2_Construction of plasmids and knockouts

DNA manipulations were carried out routinely using standard laboratory methods (Sambrook, J. *et al*., 1989). The *ΔompA* mutant was produced by replacing *ompA* coding sequence with a chloramphenicol resistance cassette. Briefly the upstream and downstream regions of the *ompA* gene were amplified from BL21 (DE3) genomic DNA with the specific primer pairs ompA-1/ ompA-2 and ompA-3/ompA 4 (Table 2). In parallel, the Cm cassette was amplified from pKD3 plasmid (Datsenko K.A. *et al.,* 2000) using the primers CMR-for/CMR-rev. Finally the three amplified fragments were fused together by mixing 100 ng of each in a PCR containing the 1/4 primers. The linear fragment obtained, in which the antibiotic resistance gene was flanked by the upstream and downstream regions, was used to transform the recombination-prone BL21(DE3) *E. coli* strain (made electro-competent by three washing steps in cold water), and mutants were

selected by plating transformed bacteria on Luria-Bertani (LB) plates containing 30μg/ml Cm. Recombination-prone BL21 (DE3) cells were produced by using the highly proficient homologous recombination system (*red* operon) (Murphy K.C. 1998). Briefly, electro-competent bacterial cells were transformed with 5μg of plasmid pAJD434 by electroporation (5.9 ms at 2.5 kV). Bacteria were then grown for 1 h at 37 °C in 1 ml of SOC broth and then plated on LB plates containing trimethoprim (100μg/ml). Expression of the *red* genes carried by pAJD434 was induced by adding 0.2% L-arabinose to the medium. The gene deletion of the *ompA* gene was confirmed by PCR genomic DNA amplification using primers pairs ompA-1/CMR-rev and CMR-for/ompA-4.

Plasmids **pET21_fHbp wild type, pET21_NHBA, pET21_fHbp-Δgly, pET21_ fHbp-ALA, pET21_ fHbp A, pET21_ fHbp A-B, pET21_ fHbp C, pET21_ fHbp wt-GBS59 D3, pET21_ fHbp A-GBS59 D3, pET21_ fHbp wt-fhuD2**, **pET21_ fHbp A-spa_DEABC** were generated as follows. Briefly, *fHbp* and *nhba* wild type genes were amplified by PCR from *Neisseria meningitidis* serogroup B strain MC58 genome using primers fHbp-ss-F/fHbp R and NHBA ss F/NHBA rev (Table 2) respectively. Primers fHbp-ss-F and NHBA ss F were design to amplify the genes with their own leader sequence and lipobox for secretion and anchoring to the membrane. The polymerase incomplete primer extension (PIPE) cloning method (Klock H. *et al.,* 2009) was used to insert the PCR products into plasmid pET21b which was amplified using primers pet-rev/nohisflag (Table 2). In so doing plasmid pET21_fHbp full length and pET21_NHBA were generated. To generate pET21_fHbp-Δgly and pET21_fHbp-ALA, site-directed mutagenesis was

performed on pET21_fHbp wild type plasmid using primers fHbp ∆gly F/fHbp ∆gly R and fHbp ALA F/fHbp ALA R (Table 2), in order to exclude the glycine stretch located downstream of the lipobox or to substitute this region with an alanine stretch, respectively. pET21_fHbp domain A, pET21_fHbp domain A-B and pET21_ fHbp domain C plasmids were generated amplifying pET21_fHbp full lenght plasmid using primers pairs: nohis flag/fHbp A rev, nohisflag/fHbp B rev, fHbp C for/fHbp lipo rev in order to exclude the domains B+C, the domain C and the domains A+B respectively. To generate pET21_ fHbp fl-GBS59 D3, pET21 fHbp A-GBS59 D3 plasmids, which contain *Streptococcus agalactiae* GBS59 domain 3 fused to fHbp full length or domain A respectively, *GBS59 domain 3* was amplified by PCR from *S. agalactiae* strain 515 genome using GBS dom C/GBS dom rev STOP and GBS dom-A/GBS dom rev STOP primers respectively and cloned into pET21_fHbp full lenght and pET21_fHbp domain A plasmids respectively using PIPE method. To generate pET21_ fHbp fl-fhuD2, pET21_ fHbp A-spa_DEABC plasmids which contain S*taphylococcus aureus fhuD2* gene fused to fHbp full length and *spa_DEABC* gene fused to fHbp domain A respectively, *fhuD2* and *spa_DEABC* genes were amplified from pET21-fhuD2 and pET21-spaDEABC plasmids containing *fhu_D2* and *spa_DEABC* genes, using primers fhu_D2 dom C for/fhu_D2 rev and spa_DEABC dom A for/spa_DEABC rev respectively and cloned into pET21_fHbp full lenght and pET21_fHbp domain A plasmids respectively using PIPE method.

5.3_ Expression of the heterologous proteins in BL21(DE3)Δ*ompA E. coli* strains and OMVs preparation

Plasmid containing the genes of interest and pET21b empty vector as negative control were transformed into BL21(DE3) *ΔompA* strain for protein expression. Recombinant clones were grown in 200 ml LB medium (starting OD_{600}=0.05) and, when the cultures had reached an OD_{600} value of 0.5, recombinant protein expression was induced by addition of 1 mM IPTG After 2 hours, OMVs were collected from culture supernatants by filtration through a 0.22 mm pore size filter (Millipore) and by high-speed centrifugation (200,000 x g for 2 hours). Pellets containing OMVs were finally re-suspended in PBS. Total bacterial lysates were prepared by suspending bacterial cells from 1 ml cultures (centrifuged at 13,000 X g for 5 minutes) and re-suspended in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, heated at 100°C for 5 minutes and loaded onto a 4-12% or 10% polyacrylamide gel (Invitrogen). Polyacrylamide gels were run in MES or MOPS buffer (Invitrogen) and stained with Coomassie Blue.

5.4_Western blot analysis

Total lysates were prepared from bacteria grown in LB broth. Liquid cultures were pelleted in a bench-top centrifuge and suspended in SDS-PAGE loading buffer in an appropriate volume to normalize cell density to a final OD_{600} of 10. About 10 ml of each sample was then separated on a 4-12% SDS-PAGE (Invitrogen). For

quantification of fHbp heterologous protein expression 20 µg recombinant OMVs were loaded onto 4-12 % SDS-polyacrylamide gels along with increasing concentration of the corresponding purified recombinant protein used as standard. Empty OMVs were also loaded as a negative control. The polyacrylamide gels were then transferred onto nitrocellulose filter by standard methods. The filters were blocked overnight at 4°C by agitation in blocking solution (10% skimmed milk and 0.05% Tween in PBS), followed by incubation for 90 minutes at 37°C with a 1:1000 dilution of the required immune sera in 3% skimmed milk and 0.05% Tween in PBS. After three washing steps in PBS-Tween, the filters were incubated in a 1:2000 dilution of peroxidase-conjugated anti-rabbit immunoglobulin (Dako) in 3% skimmed milk and 0.05% Tween in PBS for an hour, and after three washing steps, the resulting signal was detected by using the SuperSignal West Pico chemiluminescent substrate (Pierce).

5.5_Proteinase K protection assay

To investigate heterologous proteins localization in the OMVs 100 µg/ml proteinase K (Fermentas) was added to 30 µg intact and solubilized (in 1% SDS) OMVs expressing the heterologous proteins, and the mixture was then incubated at 37°C for 10 minutes. After proteinase K deactivation with 10mM phenylmethylsulfonyl fluoride (PMSF; Sigma Aldrich) samples were loaded on a 4-12% or 10% polyacrylamide gel and Western blot analysis was performed with the required antibody to detect the presence of the heterologous proteins.

5.6_ Fluorescence-Activated Cell Sorting (FACS) analysis

15 ml of Luria-Bertani medium was inoculated at OD_{600}= 0.05 starting from an overnight culture of each transformant. Cultures were then grown until the $OD_{600=}$ 0.5 (2.5 $X10^8$ CFU/mL) and expression of the recombinant proteins was induced by addition of 1mM IPTG and further incubation for 2 hours. BL21 *E. coli* strain transformed with pET21b empty vector was used as negative control. 1 ml of bacterial cells was harvested by centrifugation at 10,000 X g for 5 minutes at 4°C and re-suspendedin PBS + 1% BSA dilution buffer in order to obtain $2x10^7$ CFU/ml cells. 50 µl were then dispensed in a 96 well plate with round bottom.

Primary antibodies against heterologous proteins were diluted at three different concentrations (1:50, 1:100, 1:200) and 5 µl of each dilution were added to the wells containing he bacteria suspension and incubated 1 h on ice. Each well was then washed with 100 µl PBS + 1% BSA buffer. 20 µl of commercial FITC labeled anti-rabbit secondary antibody diluted 1:200 in dilution buffer were added in each wells and incubated 1 h in ice. Each well was then washed with 100 µl PBS + 1% BSA buffer and the plate was centrifuged at 4000 X g for 5 min. Samples were then re-suspended in formalin solution 4% , incubated 20 min a 4°C and then centrifuged at 4000 X g for 5 min. Samples were re-suspended in 130 µl of PBS and data were acquired by using BD FACS Canto II cell analyzer.

NAME	SEQUENCE (5'-3')	REFERENCE
nohisflag pet-rev	TAACATCACCATCACCATCACGATTACAAAGA CATATGTATATCTCCTTCTTAAAGTTAAAC	
ompA-1	GATCGGTTGGTTGGCAGAT	Fantappiè et al, 2014
ompA-2	CACCAGGATTTATTTATTCTGCGTTTTTGCGCCTCGTTATCAT	Fantappiè et al, 2014
ompA-3	TACTGCGATGAGTGGCAGGCGCAGGCTTAAGTTCTCGTC	Fantappiè et al, 2014
ompA-4	AAAATCTTGAAAGCGGTTGG	Fantappiè et al, 2014
CMR-for	CGCAGAATAAATAAATCCTGGTG	Fantappiè et al, 2014
CMR-rev	CCTGCCACTCATCGCAGTA	Fantappiè et al, 2014
fHbp ∆gly R	GGCGACGCTGCTGCAGGCGGTCAG	This study
fHbp ALA F	GCGGCCGCGGCCGTCGCCGCCGACATCGGTG	This study
fHbp ALA R	GGCCGCGGCCGCGCTGCTGCAGGCGGTCAG	This study
fHbp lipo rev	ACCACCCCCTCCGCTGCTGC	This study
fHbp C for	GCGGAGGGGGTGGTGGAAAACTGACCTACACCA	This study
fHbp rev GBS	TTGCTTGGCGGCAAGGC	This study
fHbp A rev GBS	TTGTTTGTATACTTGGAACTCTCCACTCTC	This study
GBS dom C	CTTGCCGCCAAGCAAGCAACAACCCAACAATTGAA	This study
GBS dom A	CCAAGTATACAAACAAGCAACAACCCAACAATTGAA	This study
GBS dom rev STOP	GTGATGGTGATGTTATTAAGCTTTTTCTGCATCTGTTGC	This study
fHbp A rev	GTGATGGTGATGTTACACCTCTCAAGGTTCATATGTTTGTT	This study
fHbp B rev	GTGATGGTGATGTTATCCGCCGGCATCGTCTG	This study
nhba ss F	GGAGATATACATATGTTTAAACGCAGCGTAATC	This study
nhba rev	GTGATGGTGATGTTATCAATCCTGCTCTTTTTTG	This study
Fhu_D2 dom C for	CTTGCCGCCAAGCAAGGGAACCAAGGTGAAAAAAATAACAAAG	This study
Fhu_D2 rev	GTGATGGTGATGTTATTATTTTGCAGCTTTAATTAATTTTTCTTTTA AATCTTTAC	This study
Spa DEABC dom A for	CCAAGTATACAAACAAGCACAGCATGATGAAGCCAAAAAA	This study
Spa DEABC rev	GTGATGGTGATGTTATTATTTAGGTGCCTGTGCGTCGTT	This study

Table 2 - Oligonucleotide primers used in this study

BACTERIAL STRAINS	DESCRIPTION	REFERENCE
E. coli		
BL21-(DE3)		
Δ*ompA*	BL21-(DE3) with *ompA* deleted	Fantappiè et al, 2014
HK-100		Klock et al, 2009
N.meningitidis		
MC58	Wild-type	Pizza et al., 2000
S. agalactiae		
515	Wild-type	Maione et al., 2005
PLASMIDS	**DESCRIPTION**	**REFERENCE**
pET21	Amp^r, cloning vector	Novagen
pET21_fHbp full length	pET21 carrying the full length *fHbp* gene from *N. meningtidis* MC58 strain	This study
pET21_NHBA	pET21 carrying the full length *nhba* gene from *N. meningtidis* MC58 strain	This study
pET21_fhuD2	pET21_ carrying fhuD2 gene	Mishra et al., 2012
pET21_spaDEABC	pET21_ carrying spa_DEABC	Kim et al., 2010
pET21_fHbp A	pET21_carrying fHbp domain A	This study
pET21_fHbp A-B	pET21_ carrying fHbp domain A and B	This study
pET21_fHbp C	pET21_ carrying fHbp domain C	This study
pET21_fHbp -GBSD3	pET21_fHbp full length with GBS59 domain 3 fused at the C-terminus	This study
pET21_fHbp A-GBSD3	pET21_fHbp A with GBS59 domain 3 fused at the C-terminus of the fHbp domain A	This study
pET21_fHbp –fhuD2	pET21_fHbp full length with fhuD2 fused at the C-terminus	This study
pET21_fHbp A-spa_DEABC	pET21_fHbp A with spa_DEABC fused at the C-terminus of the fHbp domain A	This study

Table 3 – Strains and plasmids used in this study

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Appendix

Publications, Contributions and Abstracts

During this period, I also had the occasion to provide a contribution to other Project, mostly in collaboration with Dr. Laura Fantappie (Professor Guido Grandi research group, CIBIO, University of Trento) where I performed gene cloning and Western blot analysis of some of the heterologous protein selected to be expressed in *E coli* OMVs periplasm compartment. (Cornelis P., 2000)

- *Paper*

  Fantappie L., De Santis M., Chiarot E., Carboni F., Bensi G., Jousson O., Margarit I. and Grandi G.. Antibody-mediated immunity induced by engineered Escherichia coli OMVs carrying heterologous antigens in their lumen (2014). *Journal of Extracellular Vesicles*, 3: 24015

- *Abstract*

  De Santis M., Fantappiè L., Tani C., Norais N., Jousson O and Grandi G. Characterization of *Escherichia coli* mutants that over-produce Outer Membrane Vesicles (OMVs)

Journal of Extracellular Vesicles

COACTION PUBLISHING

ORIGINAL RESEARCH ARTICLE

Antibody-mediated immunity induced by engineered *Escherichia coli* OMVs carrying heterologous antigens in their lumen

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Background: Outer membrane vesicles (OMVs) from Gram-negative bacteria are gaining increasing attention as vaccine platform for their built-in adjuvanticity and for their potential use as carriers of heterologous antigens. These 2 properties offer the opportunity to make highly effective, easy to produce multi-valent vaccines. OMVs can be loaded with foreign antigens by targeting protein expression either to the outer membrane or to the periplasm of the OMV-producing strain. Periplasmic expression is simple and relatively efficient but leads to the accumulation of recombinant antigens in the lumen of OMVs and the ability of OMVs carrying internalized antigens to induce antigen-specific antibody responses has been only marginally investigated and is considered to be sub-optimal.

Methods: We have systematically analyzed in qualitative and quantitative terms antibody responses induced by OMVs carrying different heterologous antigens in their lumen. *Group A Streptococcus* (GAS) Slo, SpyCEP, Spy0269 and *Group B Streptococcus* (GBS) SAM_1372 were fused to the OmpA leader sequence for secretion and expressed in *Escherichia coli*. OMVs from the recombinant strains were purified and tested for immunogenicity and protective activity.

Results: All proteins were incorporated into the OMVs lumen in their native conformation. Upon mice immunization, OMVs induced high functional antibody titers against the recombinant proteins. Furthermore, immunization with Slo-OMVs and SpyCEP-OMVs protected mice against GAS lethal challenge.

Conclusions: The efficiency of antigen delivery to the vesicular lumen via periplasmic expression, and the surprisingly high immunogenicity and protective activity of OMVs carrying internalized recombinant antigens further strengthens the potential of OMVs as vaccine platform.

Keywords: *outer membrane vesicles*; *vaccines*; *heterologous antigens*; *periplasmic expression*; Group A Streptococcus; Group B Streptococcus

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Outer membrane vesicles (OMVs) are closed spheroid particles of heterogeneous size (10–300 nm in diameter) released by all Gram-negative bacteria and generated through the "budding out" of the bacterial outer membrane. The production of OMVs was originally described as an essential step for rapid adaptation to environmental changes, but a multitude of other functions have now been attributed to OMVs, including delivery of toxins and virulence factors to host cells, inter-species and intra-species cell-to-cell cross-talk, biofilm formation, genetic transformation and defence against innate and adaptive host immune responses (1–3).

OMVs purified from several pathogens, including *Neisseria, Salmonella, Pseudomonas, Vibrio cholerae, Burkholderia*, and *Escherichia coli*, induce potent protective immune responses against the pathogens they derive from, and highly efficacious anti-*Neisseria* OMV-based vaccines are already available for human use (4–8). Such remarkable protection is attributed to 3 key features of OMVs. First, they carry surface-associated antigens, usually including protective antigens. Second, they are

readily phagocytosed by professional antigen-presenting cells, allowing efficient presentation of OMV-derived peptides. This results in efficient elicitation of antibodies and extracellular antigen mediated CD4+ T cell response against OMV proteins. For instance, mice immunized with *Salmonella* OMVs develop robust *Salmonella*-specific B- and T-cell responses, and OMVs also stimulate IFN-γ production by a large proportion of CD4+ T cells in mice previously infected with *Salmonella*, indicating that OMVs are an abundant source of antigens recognized by *Salmonella*-specific CD4+ T cells (4). Third, OMVs carry many pathogen-associated-molecular patterns (PAMPs) which, by binding to pathogen recognition receptors (PRRs), play a key role in stimulating innate immunity and promoting adaptive immune responses (9–11). An additional key feature of OMVs is the possibility to manipulate their protein content by genetic engineering. Kesty and Kuehn (12) demonstrated that *Yersinia enterocolitica* outer membrane protein Ail assembled on OMVs' surfaces when expressed in *E. coli*, and that the GFP fluorescence protein fused to the "twin-arginine transport" (Tat) signal sequence was incorporated in the OMV lumen. Furthermore, several heterologous antigens have been successfully exported to the surface of OMVs when fused to the β-barrel forming auto transporter AIDA and to the haemolysin ClyA, 2 proteins that naturally compartmentalize in *E. coli* OMVs (13–15).

For a full blown development of OMVs as vaccine platform, a number of issues are yet to be addressed. Among them there are 2 relevant questions. First, although strategies to load OMVs with recombinant antigens have been described, their general applicability remains to be demonstrated. Second, recombinant antigens can theoretically be engineered (a) to reach the lumen of OMVs, either as soluble proteins or associated to the inner leaflet of the membrane and (b) to be associated to the membrane facing the external milieu. However, how protein compartmentalization in OMVs affects antibody responses deserves further investigation.

In this work, we addressed the question whether compartmentalization of recombinant antigens in the lumen of *E. coli*-derived OMVs represents a broadly applicable approach to induce protective antibody responses. To this aim, we selected a group of heterologous antigens and we show that all of them can be incorporated in the lumen of *E. coli*-derived OMVs in a functional conformation. Furthermore, we show that mouse immunization with engineered OMVs induces functional antibodies and protective immune responses. Altogether our data strengthen the potential of OMVs as vaccine platform in that they demonstrate that complex cloning strategies to deliver recombinant antigens to the OMV surface are not strictly necessary to promote effective immunological responses.

Materials and methods

Bacterial strains and culture conditions

HK-100 and BL21(DE3) *E. coli* strains were routinely grown in Luria-Bertani (LB) broth at 37°C and used for cloning and expression experiments, respectively. When required, Ampicillin or Chloramphenicol were added to a final concentration of 100 μg/ml and 30 μg/ml, respectively.

Construction of plasmids and knockout mutants

DNA manipulations were carried out using standard laboratory methods (16).

BL21(DE3) *ΔompA* mutant was produced by replacing the *ompA* coding sequence with a Chloramphenicol (Cm) resistance cassette. Briefly, the upstream and downstream regions of the *ompA* gene were amplified from BL21 (DE3) genomic DNA with the specific primer pairs ompA-1/ompA-2 and ompA-3/ompA-4 (Table I). In parallel, the Cm cassette was amplified from pKD3 plasmid (17) using the primers CMR-for/CMR-rev. Finally, the 3 amplified fragments were fused together by mixing 100 ng of each in a PCR reaction containing primers ompA-1 and ompA-4. The obtained linear fragment, in which the antibiotic resistance gene was flanked by the upstream and downstream regions of *ompA* gene, was used to transform BL21(DE3) *E. coli* (made electro-competent by 3 washing steps in cold water). *ΔompA* mutant colonies were selected by plating transformed bacteria on LB plates containing 30μg/ml Cm. Recombination-prone BL21 (DE3) cells were obtained by the highly proficient homologous recombination system (*red* operon) (18). Briefly, electro-competent bacterial cells were transformed with 5 μg of plasmid pAJD434 by electroporation (5.9 ms at 2.5 kV). Bacteria were then grown for 1 hour at 37°C in 1 ml SOC broth and then plated on LB plates containing Trimethoprim (100 μg/ml). Expression of the *red* genes carried by pAJD434 was induced by adding 0.2% L-arabinose to the medium. Deletion of the *ompA* gene was confirmed by genomic DNA amplification using the primer pairs ompA-1/CMR-rev and CMR-for/ompA-4 (Table I).

Plasmids pET21_bla, pET21_slo, pET21_slo-dm, pET21_spycep, pET21_spy0269 and pET21_sam1372 were generated as follows. *slo, spycep* and *spy_0269* genes were amplified by PCR from M1 3348 *Group A Streptococcus* (GAS) strain (19) using primers GAS25-F/GAS25-R, spyCEP-F3/spyCEP-R3 and Spy0269-F/Spy0269-R (Table I), respectively. The gene encoding inactive Slo was cloned from plasmid pET24-slo-dm (20) using GAS25-F/GAS25-R primers. The beta-lactamase gene was amplified using pET-21b plasmid DNA as template and primers bla-omp-F and bla-omp-R. Finally, amplification of *Streptococcus agalactiae sam_1372* gene from the CJB111 genome was conducted using primers

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Table I. Oligonucleotide primers used in this study

Name	Sequence
slo-F	ACCGTAGCGCAGGCCAACAAACAAAACACTGCTAGTACAG
slo-R	GTGATGGTGATGTTACTACTTATAAGTAATCGAACCATATG
SpyCEP-F3	ACCGTAGCGCAGGCCGCAGCAGATGAGCTAAGCACAATGAGCGAACC
SpyCEP-R3	GTGATGGTGATGTTATTAGGCTTTTGCTGTTGCTGAGGTCGTTGACTTGGTTGG
Bla-omp-F	ACCGTAGCGCAGGCCCGGTAAGATCCTTGAGATTTTTCG
Bla-omp-R	GTGATGGTGATGTTATTACCAATGCTTAATCAGTGAGGC
omprev	GGCCTGCGCTACGGTAGCGAAA
nohisflag	TAACATCACCATCACCATCACGATTACAAAGA
ompA-1	GATCGGTTGGTTGGCAGAT
ompA-2	CACCAGGATTTATTTATTCTGCGTTTTTGCGCCTCGTTATCAT
ompA-3	TACTGCGATGAGTGGCAGGCGCAGGCTTAAGTTCTCGTC
ompA-4	AAAATCTTGAAAGCGGTTGG
CMR-for	CGCAGAATAAATAAATCCTGGTG
CMR-rev	CCTGCCACTCATCGCAGTA
Spy0269-F	ACCGTAGCGCAGGCCGATGATAGAGCCTCAGGAGAAACG
Spy0269-R	GTGATGGTGATGTTATCACTTAGATTCCTTACGGAACC
SAM_1372-F	ACCGTAGCGCAGGCCGACGACGCAACAACTGATAC
SAM_1372-R	GTGATGGTGATGTTAGGTTACTTTTTTGTTTTGAACTTGTTGGG
Pet-rev	CATATGTATATCTCCTTCTTAAAGTTAAAC
Slo-F-no	GGAGATATACATATGAACAAACAAAACACTGCTAGTACAG
Spycep-F-no	GGAGATATACATATGGCAGCAGATGAGCTAAGCACAATGAGCGAACC
Spy_0269-F-no	GGAGATATACATATGGATGATAGAGCCTCAGGAGAAACG
SAM_1372-F-no	GGAGATATACATATGGACGACGCAACAACTGATAC

SAM_1372-F and SAM_1372-R. Primers were designed to amplify the genes without their natural leader sequence for secretion and, in the case of SpyCEP and SAM_1372, the C-terminal cell wall–anchoring domains (aa 1614–1647 and aa 620–674, respectively) were also omitted. The polymerase incomplete primer extension (PIPE) cloning method (21) was used to insert all PCR products into plasmid pETOmpA, a pET21 derivative carrying the sequence encoding the leader peptide for secretion of *E. coli* OmpA, which was amplified using primers omprev/nohisflag (Table I). In so doing, all recombinant proteins were expressed fused to the OmpA leader sequence. Cytoplasmic expression of the proteins was accomplished by cloning the amplified genes into a pETOmpA derivative plasmid in which the *ompA* leader sequence was removed using the primers pet-rev/nohisflag (Table I) for PIPE amplification. The plasmids encoding the genes without signal peptide were named pET21_spycep-noLS, pET21_spy0269-noLS, pET21_slo-noLS and pET21_sam1372-noLS.

Expression of the heterologous proteins in BL21(DE3)*ΔompA E. coli* strain and OMVs preparation

pET plasmid derivatives containing the genes of interest were transformed into BL21(DE3) *ΔompA* strain. Recombinant clones were grown in 200 ml LB medium (starting $OD_{600} = 0.05$) and, when the cultures had reached an OD_{600} value of 0.5, recombinant protein expression was induced by addition of 1 mM IPTG. After 2 hours, OMVs were collected from culture supernatants by filtration through a 0.22 μm pore size filter (Millipore) and by high-speed centrifugation (200,000 × g for 2 hours). Pellets containing OMVs were finally suspended in PBS. Total bacterial lysates were prepared by suspending bacterial cells from 1 ml cultures (centrifuged at 13,000 × g for 5 minutes) in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, heated at 100°C for 5 minutes and loaded onto a 4–12% polyacrylamide gel (Invitrogen). Polyacrylamide gels were run in MES buffer (Invitrogen) and stained with Coomassie Blue.

Western blot analysis

Total lysates were prepared from bacteria grown in LB broth. Liquid cultures were pelleted in a bench-top centrifuge and suspended in SDS-PAGE loading buffer in an appropriate volume to normalize cell density to a final OD_{600} of 10. About 10 μl of each sample was then separated on a 4–12% SDS-PAGE (Invitrogen). For quantification of heterologous protein expression, 30 μg of OMVs were loaded onto 4–12% SDS-polyacrylamide gels along with increasing concentrations of the corresponding purified recombinant protein used as standard.

The gels were then transferred onto nitrocellulose filters by standard methods (16). The filters were blocked overnight at 4°C by agitation in blocking solution (10% skimmed milk and 0.05% Tween in PBS), followed by incubation for 90 minutes at 37°C with a 1:1,000 dilution of the required immune sera in 3% skimmed milk and 0.05% Tween in PBS. Anti-Bla and anti-Mbp antibodies were obtained from Abcam, while antibodies against Slo, SpyCEP, Spy_0269 and SAM_1372 were produced by immunizing mice with 3 doses of recombinant proteins (10 μg/dose) formulated in Alum. After 3 washing steps in PBS-Tween, the filters were incubated in a 1:2,000 dilution of peroxidase-conjugated anti-mouse immunoglobulin (Dako) in 3% skimmed milk and 0.05% Tween in PBS for 1 hour, and after 3 washing steps, the resulting signal was detected using the Super Signal West Pico chemo-luminescent substrate (Pierce).

Laser scattering analysis

The size distribution profile of OMVs was determined by dynamic light scattering based on laser diffraction method employing Malvern Zetasizer (Version 6.0, Malvern, UK). The OMV diameter was determined after dilution in PBS (1/100) and by measuring the back scattering intensity (175°) at 25°C. Three measurements (15 experimental runs per measurement) were averaged to determine the vesicles size.

Lipopolysaccharide quantification

Lipopolysaccharide (LPS) content was determined using the limulus amoebocyte lysate (LAL) assay (Thermo Scientific). Briefly, OMVs were diluted in pyrogen-free water and 50 μl were combined with 50 μl LAL reagent and incubated at 37°C for 10 minutes prior to addition of a chromogenic substrate. Absorbance was then measured at 405 nm. LPS concentration was estimated from a standard curve prepared with *E. coli* endotoxin standard.

Proteinase K protection assay

To confirm that heterologous proteins were expressed in the lumen of OMVs, proteinase K (Fermentas) was added to 15 μg of intact or solubilized (in 1% SDS) OMVs at a final concentration of 100 μg/ml, and the mixture was then incubated at 37°C for 10 minutes. After proteinase K deactivation with 10 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Aldrich), samples were loaded onto a 4–12% polyacrylamide gel and Western blot analysis was performed as described above.

Functional assays

To measure SpyCEP hydrolytic activity on IL-8, OMVs expressing SpyCEP were permeabilized with 1% Triton X-100 at room temperature for 20 minutes and subsequently incubated at different concentrations with human IL-8 (50 μg/ml; Peprotech) at 37°C for 2 hours. Hydrolysis of IL-8 was followed by SDS-PAGE (18%).

Slo haemolytic activity was tested by incubating OMVs expressing the wild type form of the toxin with sheep blood erythrocytes as follows. About 50 μl of serial dilutions (in PBS + 0.5% BSA) of OMVs were dispensed into 96-well plates with U-shaped bottom. Erythrocytes were collected by centrifugation (3,000 × g for 5 minutes) from 1 ml of sheep blood, washed 3 times in PBS and finally suspended in 5 ml of PBS. About 50 μl of this suspension were added to each well and incubated at 37°C for 30 minutes. As positive control, erythrocytes were added to wells containing 50 μl of water (under these ipotonic conditions erythrocytes completely lyse) while, as negative control, blood cells were incubated with permeabilized OMVs prepared from BL21(DE3) *ΔompA* strain carrying pETOmpA vector plasmid (from now on defined as "empty OMVs" to indicate that they do not carry any heterologous antigen). Plates were then centrifuged for 5 minutes at 1,000 × g, the supernatants were transferred to 96-well flat-bottomed plates and absorbance was measured at 540 nm.

OMVs expressing β-lactamase (Bla) were incubated with the chromogenic substrate nitrocefin and the Bla activity was measured as follows. OMVs were permeabilized in 1% Triton X-100 at room temperature for 20 minutes and different aliquots were incubated with nitrocefin (0.5 mg/ml; Oxoid, Thermo Scientific, Cambridge, United Kingdom) for 30 minutes at 37°C in the dark. Hydrolysis was determined at OD_{485} and Bla concentration was calculated using a standard curve obtained with purified Bla (VWR).

Mice immunization

Five-week old CD1 female mice were immunized intraperitoneally (i.p.) on days 0, 21 and 35 with 25 μg of OMVs and, unless otherwise specified, with 20 μg or 0.5 μg of recombinant proteins formulated in 2 mg/ml Alum hydroxide as adjuvant. Mouse sera were collected before the first immunization (pre-immune sera) and 2 weeks after each immunization. In challenge studies, 3 weeks after the third immunization mice were given $2–3 \times 10^6$ colony forming unit (CFU) of M1 3348 GAS strain, via i.p. Mice survival was monitored daily for a 7-day period. All procedures were approved by the National Health Institution and Novartis Vaccines Animal Care and Ethical Committee and for humanitarian reasons animals were sacrificed at symptoms of sickness as recommended by 3Rs rules ("Refinement, Reduction, Replacement" policy towards the use of animals for scientific procedures – 99/167/EC, Council Decision of 25/1/99).

ELISA

Ninety six-well Maxisorp plates (Nunc, Thermo Fisher Scientific) were coated with 3 μg/ml of purified recombinant antigens in PBS. Plates were incubated for 2 hours at room temperature, then washed 3 times with TPBS (0.05% Tween 20 in PBS, pH 7.4) and blocked with

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250 µl/well of 2% BSA (Sigma–Aldrich) for 1 hour at room temperature. Each incubation step was followed by triple TPBS wash. Serum samples were initially diluted 1:100,000 in 2% BSA in TPBS, transferred to coated-blocked plates (200 µl) and serially 2-fold diluted followed by 2 hours incubation at 37°C. Then 100 µl/well of 1:2,000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma-Aldrich) or IgM, or IgG1, or IgG2A (Southern Biotechnologies) were added and left for 2 hours at 30°C. Bound alkaline phosphatase was visualized by adding 100 µl/well of 3 mg/lL para-nitrophenyl-phosphate disodium hexahydrate (Sigma–Aldrich) in 1 M diethanolamine buffer (pH 9.8). After 10 minutes at room temperature, plates were analyzed at 405 nm in a microplate spectrophotometer and antibody titers (expressed as EU/ml) were calculated using a reference calibration curve.

Opsonophagocytosis assay

The opsonophagocytosis assay (OPA) was performed using *Group B Streptococcus* (GBS) strain CJB111 as target bacteria and HL-60 cell line (ATCC; CCL-240) differentiated into granulocyte-like cells by adding 100 mM N, N Dimethylformamide (Sigma) to the growth medium for 4 days. Mid-exponential bacteria were incubated at 37°C for 1 hour in the presence of phagocytic cells, 10% baby rabbit complement (Cederlane) and heat-inactivated mouse antisera. Negative controls consisted of reactions either with pre-immune sera, or without HL-60, or with heat-inactivated complement. For each serum sample, 6 serial dilutions (1:2) were tested. The reaction plates were incubated for 1 hour at 37°C and 300 rpm (Eppendorf Thermomixer) and the number of CFUs at the time zero (T0) and after 1 hour (T60) were compared. Bacterial killing was calculated as follows: killing (%) = [(mean CFU at T0 - mean CFU at T60)/mean CFU at T0]*100. OPKA titers were expressed as the reciprocal serum dilution leading to 50% killing of bacteria.

Results

Heterologous antigens expressed in the E. coli periplasm are incorporated into OMVs

To test whether the delivery of antigens into the OMV lumen by periplasmic expression is broadly applicable, we selected 3 proteins from GAS (the cell wall–anchored protein SpyCEP (22), the secreted toxin Streptolysin O (23) and the putative surface exclusion protein Spy0269 (19)), 1 protein from GBS (the cell-wall anchored pilus subunit SAM_1372 (24)) and 1 from *E. coli* (the periplasmic TEM1 β-lactamase, a protein shown to be incorporated into the OMVs of *Pseudomonas aeruginosa* and *Moraxella catarrhalis* (25,26)) (Table II).

Antigen selection was based on 3 criteria: their belonging to different cellular compartments, their having a measurable functional activity and their capacity to induce antibody-mediated protective immune responses in animal models. The first criterion addresses whether specific categories of proteins are more or less prone to reach the periplasm and be delivered to the OMVs. The second criterion was selected to exploit functional activity as a surrogate for antigen folding. Finally, the third criterion allows evaluating the quantity and quality of antibody responses elicited by the recombinant antigens once incorporated inside the OMVs.

All genes were cloned into the pETOmpA plasmid, a derivative of pET21b carrying the coding sequence of the *E. coli* OmpA signal peptide downstream from the T7 inducible promoter, thus allowing N-terminal fusion of the *E. coli* periplasmic secretion sequence to the protein of interest. Because of the presence of the OmpA leader sequence, all proteins (except TEM1 beta-lactamase) were cloned without their natural secretory signal. Furthermore, the C-terminal cell-wall anchoring domains of SpyCEP and SAM_1372 (amino acids 1,614–1,647 and amino acids 620–674, respectively) were also eliminated. In summary, 5 plasmids were generated, pET21_bla, pET-21_slo, pET21_spycep, pET21_spy0269 and pET21_sam1372, which were used to transform the OMV overproducer strain *E. coli* BL21(DE3) *ΔompA*. The derived strains were grown to mid-log phase and antigen expression was induced for 2 hours by addition of 1 mM IPTG. Figure 1a shows SDS-PAGE analysis of total lysates of cultures obtained with or without IPTG induction. As shown in the figure, all antigens appeared to be expressed after induction.

We next asked the question whether the proteins reached the OMV compartment. To this aim, after 2 hours' induction with IPTG, OMVs were purified from bacterial culture supernatants as described in Materials

Table II. Antigens used for expression in OMVs

Antigen	Pathogen	Functional assay	Protective immune responses
SpyCEP	*S. pyogenes*	IL-8 cleavage	Neutralizing Ab and mouse challenge
Streptolysin O	*S. pyogenes*	Haemolysis	Neutralizing Ab and mouse challenge
Spy0269	*S. pyogenes*	Not available	Mouse challenge
SAM_1372	*S. agalactiae*	Not available	Opsonophagocytic activity
R-TEM b-lac	*E. coli*	Hydrolysis of Nitrocefin	Not available

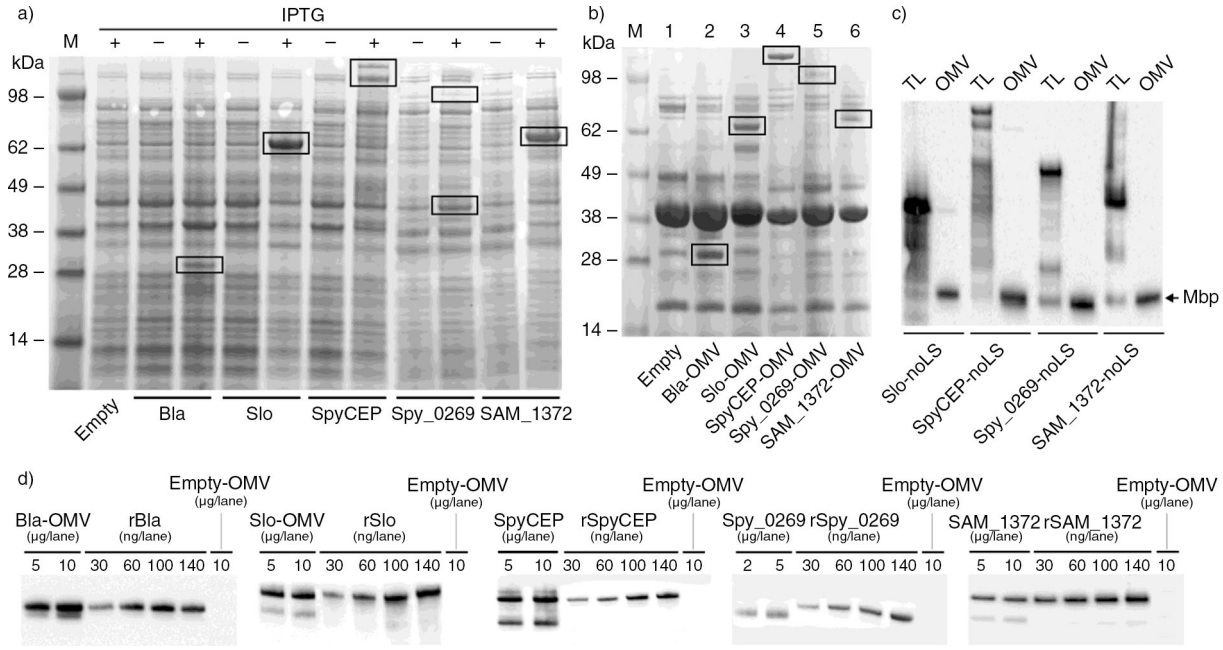

Fig. 1. Expression of heterologous antigens in OMVs – (a) SDS-PAGE analysis of *E. coli* BL21Δ*ompA* total cell extracts expressing selected antigens. Bacteria were grown to mid-log phase and recombinant protein expression induced (+) or not induced (−) with IPTG; 1 ml aliquots were collected by centrifugation, suspended in 100 µl of SDS-PAGE loading buffer and 25 µl aliquots were loaded in each lane. Bands corresponding to recombinant antigens are boxed. (b) SDS-PAGE analysis of OMV from *E. coli* BL21Δ*ompA* expressing selected antigens. OMVs were purified by ultracentrifugation of 200 ml bacterial culture supernatants and pellets were suspended in 200 µl of PBS. Aliquots corresponding to 30 µg of total OMV proteins were added with SDS-PAGE loading buffer and loaded to each lane. Bands corresponding to recombinant antigens are boxed. (c) Western Blot analysis of total cell extracts and OMVs from *E. coli* BL21Δ*ompA* expressing the selected antigens in their cytosol. Bacteria were grown to mid-log phase and protein expression was induced with IPTG. OMVs were purified from the culture supernatants by ultracentrifugation; antigen expression was assessed in total cell lysates (TL) and in the purified OMV fraction using antigen-specific polyclonal antibodies. Polyclonal antibodies against the periplasmic Maltose binding protein (Mbp) were used as a control for OMV quantitation and integrity. (d) Estimation of the amount of heterologous proteins incorporated into OMVs. Different quantities of purified recombinant proteins and OMVs expressing the protein of interest were analyzed by Western Blot. From the comparison of band intensities, the following percentages (µg of antigen expressed in OMVs/µg of total proteins $\times 100$) were estimated: Bla: 5%; Slo: 5%; SpyCEP: 10%; Spy0269: 0.5–1%; SAM_1372: 0.5%.

and Methods and analyzed by SDS-PAGE. As shown in Fig. 1b, all proteins were found in the OMVs at levels ranging from 0.5% to approximately 10% of total OMV proteins, as judged by Western Blot protein quantitation analysis in which different amounts of purified recombinant antigens were used as standard controls (Fig. 1d). The proteins that showed the lowest level of expression in OMVs were SAM_1372 and Spy0269, both representing approximately 0.5% of total OMV proteins. Bla, Slo and SpyCEP represented between 5 and 10% of total OMV proteins. Antigen fusion to the OmpA leader sequence was indispensable for protein delivery to the OMVs. In fact, when the proteins were not fused to the OmpA leader sequence (see Material and Methods), all antigens accumulated in the cytoplasm and were not found in OMVs, as judged by Western Blot analysis (Fig. 1c).

In order to assess the size and the homogeneity of the purified vesicles, they were analyzed by light scattering (see Materials and Methods). As shown in Fig. 2, vesicle size ranged from 20 to 300 nm, and the majority of vesicles had a diameter of 80–140 nm. Vesicle dimension was not affected by the presence of different recombinant antigens, and size distribution of vesicles stored at -20°C did not vary over a period of 1 month (data not shown).

Although recombinant antigens should reside in the internal space of the vesicles, the unexpected evidence that HtrA, a *Chlamydia trachomatis* protein we recently expressed in OMVs (27), was at least partially exposed on the OMV surface, prompted us to further investigate the location of the 5 antigens in OMVs. To this aim, purified OMVs were treated with proteinase K either in the presence or in the absence of 1% SDS. As shown in Fig. 3, recombinant antigens were digested by the protease only in the presence of detergent, indicating that they became protease sensitive only upon disruption of the OMV membrane.

Taken together these results demonstrate that antigen fusion to a sec-dependent leader sequence for secretion is sufficient to guarantee antigen targeting to the internal

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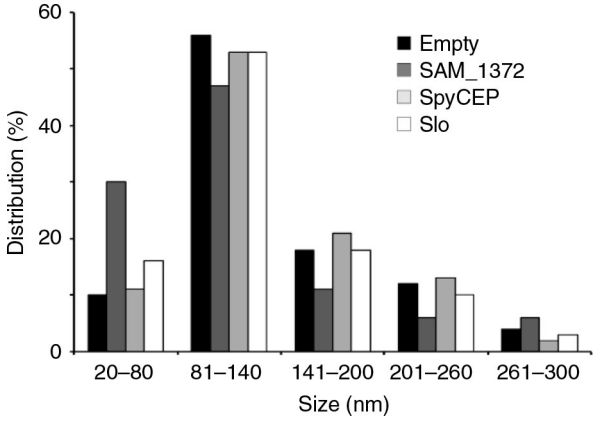

Fig. 2. Analysis of OMV dimension by light scattering – The size of OMVs released from *E. coli* BL21Δ*ompA* strain expressing SAM_1372, SpyCEP, Slo, or no antigens was determined by dynamic light scattering, measuring the back scattering intensity (175°) at 25°C. The graph represents the average size distribution from 3 independent measurements.

space of OMVs, suggesting that no major selection processes take place to determine which periplasmic protein should or should not be taken up by OMVs during vesiculation.

Heterologous antigens expressed in the lumen of OMVs preserve their native conformation

We next investigated whether recombinant antigens expressed in the lumen of OMVs had a 3D structure similar to their native conformation. This is an important prerequisite to guarantee that, when OMVs are used in immunization, the antigens can elicit functional antibody responses. As surrogate of antigen folding, we measured the functional activity of Slo, SpyCEP and R-TEM β-lactamase. In particular, Slo haemolytic activity was tested by incubating increasing concentrations of Slo-OMVs with sheep blood erythrocytes. As shown in Fig. 4a, 100% haemolysis was observed when 50 μg of OMVs (total protein content) were added to the reaction mixture. Considering that this amount of OMVs corresponds to approximately 2 μg of Slo, the kinetics of haemolysis was in line with that previously reported using purified recombinant Slo (20). The enzymatic activity of OMVs-containing SpyCEP was measured by incubating human IL-8 (50 μg/ml) with Triton-permeabilized OMVs and following the hydrolysis of IL-8 by SDS-PAGE. As shown in Fig. 4b (lane 3), IL-8 was completely cleaved after 30 minutes' incubation with 4.5 μg of OMVs (corresponding to approximately 450 ng of SpyCEP). As in the case of Slo, the specific activity of OMV-internalized SpyCEP appeared to be similar to recombinant SpyCEP purified from the *E. coli* cytoplasm (28). Finally, to determine the β-lactamase activity in OMVs, the hydrolysis of the chromogenic substrate nitrocefin was investigated after incubation with vesicles (Fig. 4c). Again, estimating that β-lactamase corresponded to approximately 5% of total OMV proteins, the specific activity of the enzyme in OMVs was similar to the one of purified R-TEM β-lactamase. Interestingly, assuming that the specific activity of R-TEM in OMVs is comparable to the specific activity of the purified enzyme, we estimated that 23 ng of β-lactamase were present in 1 μg of OMV, corresponding to 2.5% of total proteins. This number is in line with our estimation of enzyme concentration based on Western Blot analysis.

Taken together these results indicate that heterologous antigens expressed in the lumen of *E. coli* OMVs preserved their native conformation and biological activity.

Heterologous antigens expressed in the lumen of OMVs are immunogenic

Having demonstrated that the conformation of antigens delivered to the lumen of OMVs is similar to their native structure, at least as judged by functional activity, we next investigated whether, despite the fact that they are localized inside the vesicles, those antigens are capable of inducing specific antibody responses. To this aim, mice were i.p. immunized 3 times at 2-week intervals with 25 μg of OMVs and antigen-specific antibody titers were measured collecting blood samples 2 weeks after the last dose. For these experiments, the immunogenicity of Slo_{dm}-OMV, SpyCEP-OMV and SAM_1372-OMVs was investigated. Slo_{dm} is a mutated form of Slo, carrying 2 point mutations which inactivate the enzymatic activity of the antigen without affecting its immunogenic properties (20). As shown in Fig. 5, the 3 recombinant OMVs induced antigen-specific antibody responses which, in the case of Slo_{dm}-OMVs and SpyCEP-OMVs, were in the

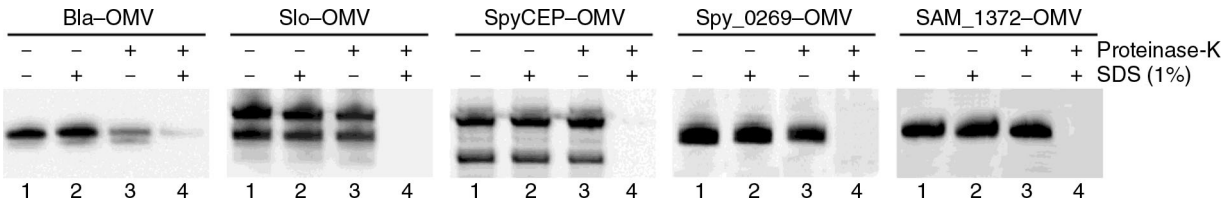

Fig. 3. Analysis of antigen localization in OMVs. – Purified OMVs expressing heterologous proteins were incubated with and without proteinase K in the presence or absence of 1% SDS. Samples were then subjected to SDS-PAGE and Western Blot analysis using antigen-specific polyclonal antibodies. The data indicate that all recombinant antigens are localized in the lumen of OMVs.

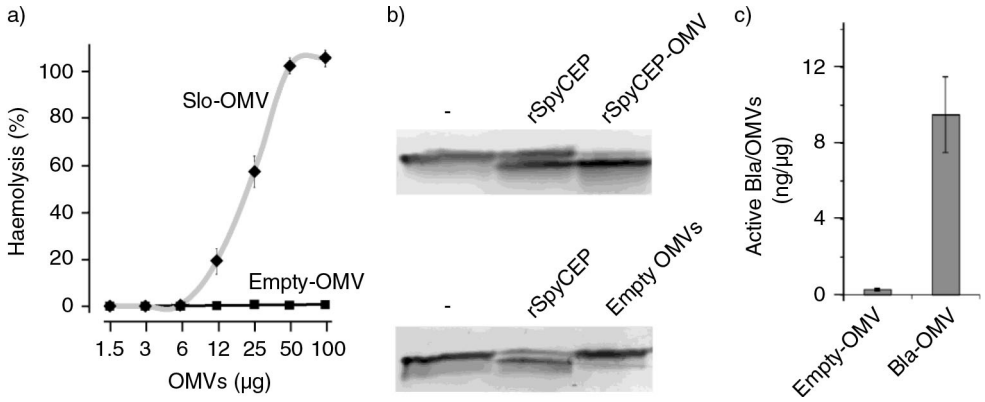

Fig. 4. Analysis of the functional activities of protein antigens expressed in the lumen of OMVs – (a) Haemolytic activity of Slo-OMVs. Aliquots of purified OMVs were incubated with sheep erythrocytes and absorbance at OD_{540} was measured from the supernatant of each sample. Haemolytic activity is expressed as percentage of OD values over the OD values obtained incubating erythrocytes with water (100% haemolysis). (b) Proteolytic activity of SpyCEP-OMVs. 10 µg of SpyCEP-OMVs and "empty" OMVs were permeabilized with Triton X-100 and subsequently incubated with IL-8. IL-8 hydrolysis was followed by Western Blot analysis using anti-IL-8 specific antibodies. (c) β-lactamase activity of Bla-OMVs – Bla-OMVs and "empty" OMVs were permeabilized with Triton X-100, and β-lactamase activity was monitored by following the hydrolysis of nitrocefin at OD_{485}. β-lactamase concentration in OMVs (ng of active enzyme/µg OMVs) was estimated using a standard curve obtained with purified β-lactamase as determined by spectrophotometry.

same range as the titers elicited by 20 µg doses of the corresponding recombinant antigens formulated in Alum. When the 20 µg doses of Slo_{dm} and SpyCEP were mixed together with 25 µg of "empty OMVs," an appreciable increase (approximately 5-fold) in antibody titers was observed highlighting the strong adjuvanticity of the vesicles. As far as SAM_1372-OMVs are concerned, they induced SAM_1372 antibody titers that were approximately 10-fold lower than those elicited by vaccination with Alum-formulated recombinant SAM_1372 (10 µg/dose). This might reflect the relatively poor expression of this antigen in OMVs, estimated to be in the range of 0.5% of total OMV proteins (Fig. 1d). No specific antibody titers were detected when mice were immunized with empty OMVs or Alum alone as controls.

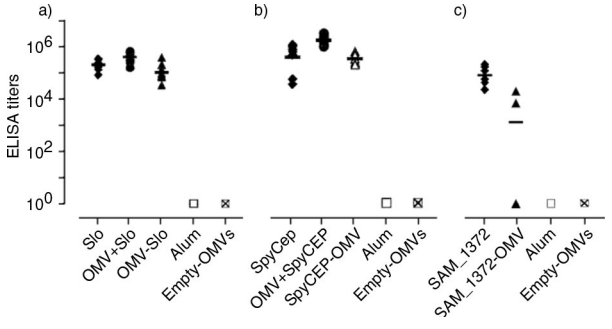

Fig. 5. IgG titers elicited in mice immunized with Slo-OMVs (a), SpyCEP-OMVs (b) and SAM_1372-OMVs (c). Groups of 6 CD1 female mice were immunized i.p. 3 times at 2-week intervals with the following immunogens formulated in Alum: 25 µg OMVs expressing the recombinant antigens (Slo-OMVs, SpyCEP-OMVs, SAM_1372-OMVs), 20 µg of purified recombinant antigen (Slo, SpyCEP, SAM_1372), 25 µg empty OMVs combined with 20 µg of purified recombinant antigen (OMVs + Slo, OMVs + SpyCEP), adjuvant alone (Alum). Sera were collected 2 weeks after the third immunization and IgG titers were analyzed by ELISA, using plates coated with the corresponding recombinant antigen (1 µg/well). Each symbol represents the ELISA value of an individual mouse. Horizontal bars represent the geometric mean values within each group.

One interesting property of OMVs is their built-in adjuvanticity and their ability to skew immune responses towards a Th1/Th17 profile (4,9–11,29). Since the measurement of IgG1/IgG2A ratio is routinely used in mice as a surrogate to establish the type of immune response induced (high and low IgG1/IgG2A ratios indicating a TH2-type and a TH1-type of response, respectively (30,31)), we analyzed the isotype of antigen-specific antibodies upon immunization of mice with recombinant OMVs carrying Slo and SpyCEP. The isotype profiles were compared to the ones obtained immunizing animals with Alum-formulated recombinant proteins. As shown in Fig. 6, while purified proteins induced antibodies predominantly belonging to IgG1 isotype, IgG2A was the predominant isotype of antigen-specific antibodies obtained after immunization with recombinant OMVs. As expected for immunization regimes involving 3 doses, no antigen-specific IgM antibodies were detectable in sera from mice immunized with either purified antigens or recombinant OMVs.

Overall, these data seem to confirm the capacity of OMVs to direct the immune response towards a TH1 profile. However, to establish the precise nature of immunity induced, a direct measurement of cytokines and the characterization of T cell subpopulations will be required. In particular, it will be interesting to analyze

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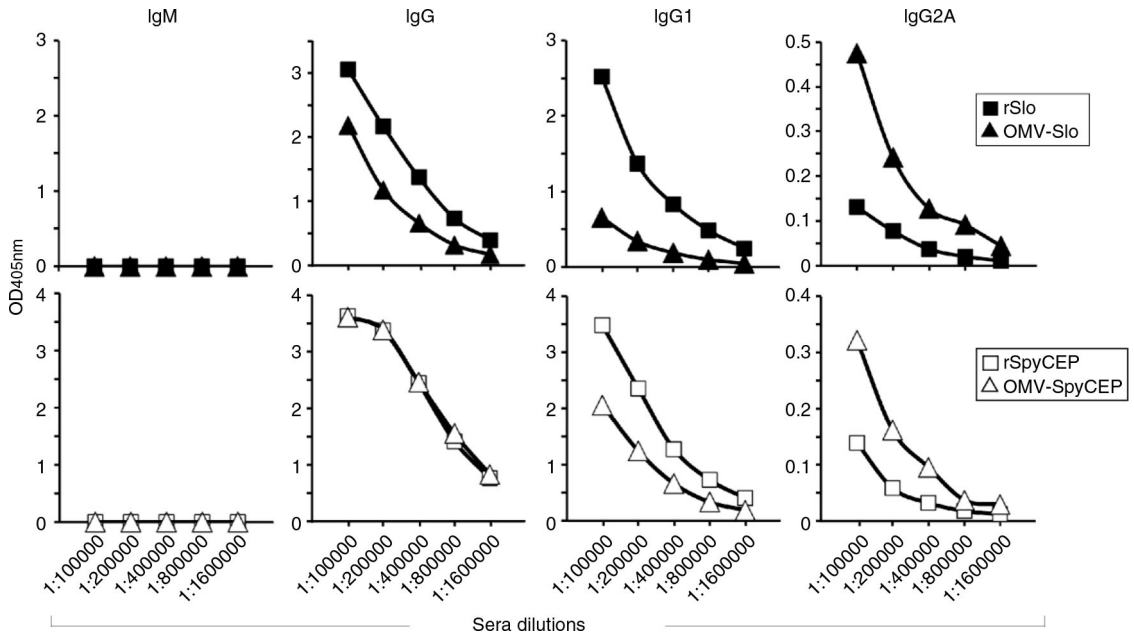

Fig. 6. Isotype analysis of antibodies elicited in mice immunized with Slo-OMVs and SpyCEP-OMVs – Sera from mice immunized i.p. 3 times with 25 μg OMVs expressing Slo or SpyCEP antigen (triangles) and with 20 μg of purified recombinant Slo or SpyCEP (squares) were serially diluted and Ig isotypes were analyzed by ELISA using plates coated with the corresponding recombinant antigens. Alkaline phosphatase-conjugated goat anti-mouse IgG, IgM, IgG1 and IgG2A were used as secondary antibodies. Graphs represent the OD values obtained for each serum dilution.

the presence of $CD4_{TH17}$ cell subset, as recently elegantly determined by Kim and co-workers (29).

Immunization with OMVs carrying recombinant antigens in their lumen induces antigen-specific functional antibodies

Having demonstrated that immunization with recombinant vesicles elicited antigen-specific antibodies, we next investigated whether these antibodies displayed functional activity. Since recombinant Slo, SpyCEP and SAM_1372 induce antibodies which neutralize Slo haemolytic activity, inhibit IL-8 cleavage and promote complement-dependent opsonophagocytosis, respectively (19,24), we analyzed whether sera from mice immunized with recombinant OMVs had similar functions. To this aim, recombinant SpyCEP was incubated with different dilutions of sera from mice immunized with either 20 μg of recombinant SpyCEP, or with 20 μg of recombinant SpyCEP+25 μg of "empty" OMVs or with 25 μg of SpyCEP-OMVs, and purified IL-8 was subsequently added to each mixture. As shown in Fig. 7a panel (i), sera from mice receiving 3 doses of each vaccine similarly inhibited IL-8 cleavage in a dose-dependent manner. Interestingly, when sera from mice immunized with 2 doses of vaccines were tested, mice given SpyCEP-OMVs had higher titers of functional antibodies with respect to mice that received either recombinant SpyCEP or recombinant SpyCEP+"empty" OMVs (Fig. 7a panel (ii)). Similarly, purified recombinant Slo was pre-incubated with different dilutions of sera from mice immunized with 3 doses of OMV-Slo and subsequently goat erythrocytes were added to the mixtures. As shown in Fig. 7b, haemolysis was effectively inhibited at serum dilutions up to 1:250 and inhibition levels were at least equal to those obtained with sera from mice immunized with 0.5 μg of recombinant Slo, a dose close to that estimated for 25 μg of OMVs (approx. 1 μg of Slo). Finally, OPK titers of sera from mice immunized with 3 doses of SAM_1372-OMVs were determined and compared to those from animals immunized with 10 μg/dose of recombinant SAM_1372. While the antibody titers induced by SAM_1372-OMVs were relatively low with respect to those induced by the purified recombinant antigen (Fig. 5), engineered vesicles elicited 5-fold higher bactericidal antibody titers compared to the recombinant antigen (Fig. 7c).

In conclusion, not only OMVs carrying heterologous antigens in their lumen induced antigen-specific antibodies but also such antibodies had potent functional activities which could exceed, in terms of either time of elicitation or potency of the immune response, those obtained with the corresponding recombinant antigens

Immunization with Slo-OMVs and SpyCEP-OMVs protects mice against S. pyogenes infection

We have previously shown that immunization with SpyCEP and Slo protected mice from GAS lethal challenge (19). Therefore, we tested whether, in addition

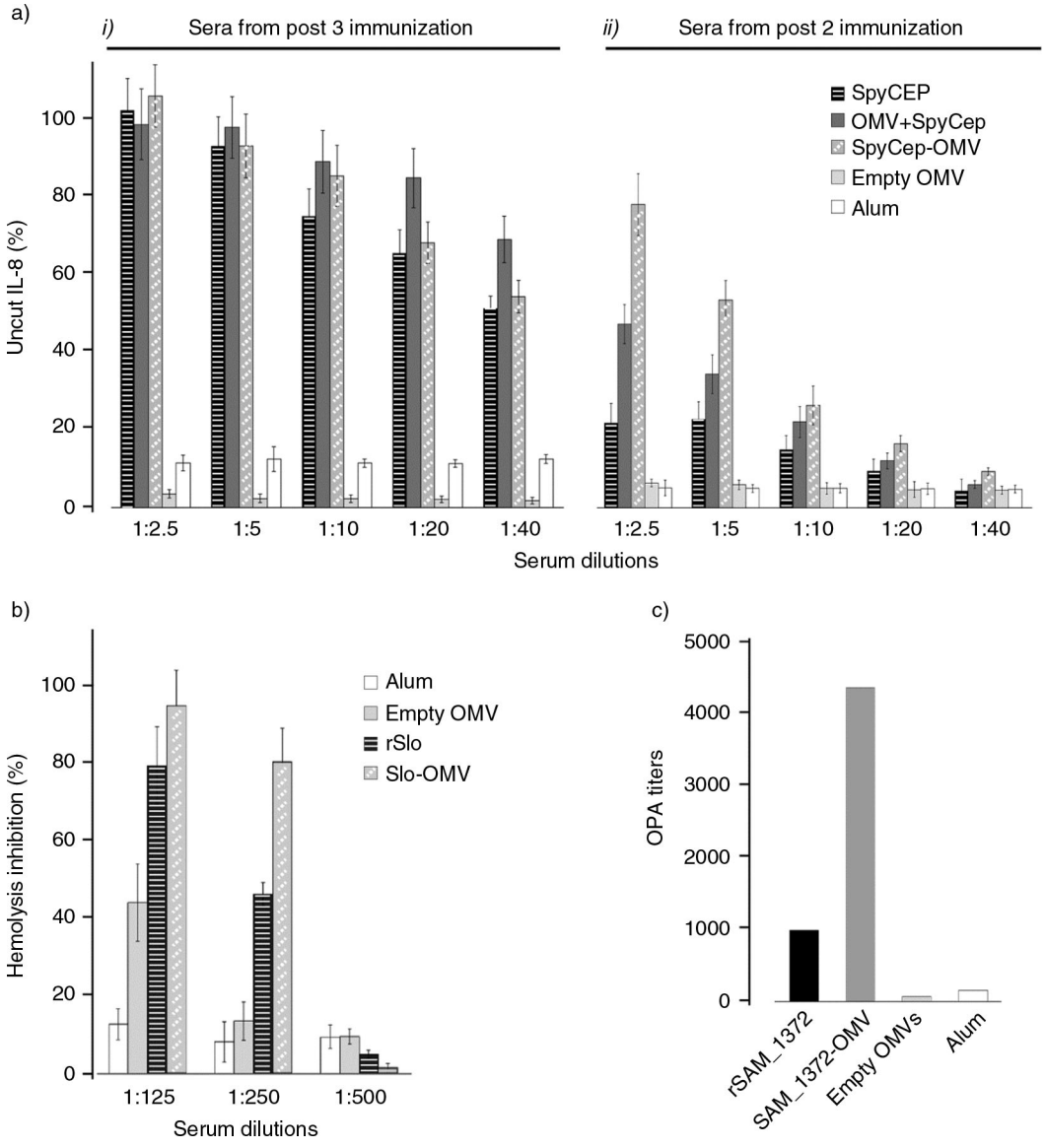

Fig. 7. Analysis of functional antibodies induced by immunization with Slo-OMVs, SpyCEP-OMVs and SAM_1372-OMVs – (a) Inhibition of IL-8 hydrolysis by sera from mice immunized with SpyCEP-OMVs. IL-8 hydrolysis was followed as described in Materials and Methods in the presence of serial dilutions of sera from mice immunized with 3 (i) or 2 (ii) doses of the different immunogens. Graphs show the percent of residual IL-8 measured after hydrolysis. Sera from each group were pooled and bars represent the geometric mean values from 3 independent experiments. (b) Inhibition of haemolytic activity of sera from mice immunized with Slo-OMVs. Haemolysis of sheep erythrocytes was measured in the presence of increasing dilutions of sera from mice immunized with the different immunogens. Haemolytic activity is expressed as percentage of OD values over the OD values obtained incubating erythrocytes with water (100% haemolysis). Sera from each group were pooled and bars represent the geometric mean values of 3 independent experiments. (c) Opsonophagocytosis activity of sera from mice immunized with SAM_1372-OMVs. OPK was carried out as described in Materials and Methods using the pools of sera from mice immunized as described in Fig. 5. Titers are expressed as serum dilutions resulting in 50% bacterial killing.

to eliciting functional antibodies, vaccination of mice with vesicles carrying Slo and SpyCEP could induce protective immunity. To this aim, mice were i.p. immunized with 3 doses of either Slo-OMVs or SpyCEP-OMVs (25 μg/dose) and subsequently i.p challenged with a lethal dose of M1-3348 GAS strain. In this sepsis model, death mainly occurs during the first 2 days, and survival is reported after day 7. As shown in Fig. 8, both OMVs induced robust protection against bacterial challenge and protection levels were at least equal to those observed by immunization with 20 μg/dose of recombinant proteins.

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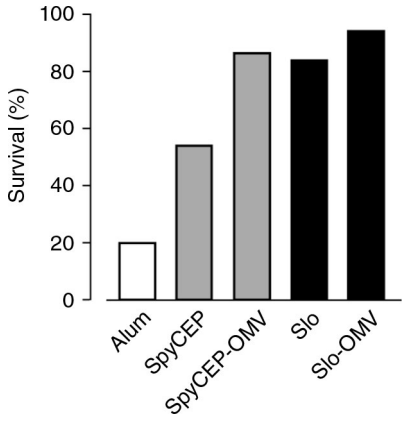

Fig. 8. In vivo protective activity of Slo-OMVs and SpyCEP-OMVs – Groups of 16 mice were immunized with 3 doses of Alum-formulated OMVs carrying heterologous antigens (25 μg total proteins), Alum-formulated purified recombinant proteins (20 μg) or Alum alone. After 2 weeks, mice were challenged intraperitoneally with a LD_{80} dose of M1 3348 GAS (2×10^6 CFUs) and survival was followed over a period of 7 days.

Discussion

Kesty and Kuehn (12) have been the first to demonstrate that the protein content of bacterial OMVs can be manipulated by targeting heterologous proteins to the outer membrane and/or to the periplasmic space of Gram-negative bacteria. In light of the potent adjuvant property of OMVs, which stimulate cell-mediated and humoral immune responses when given both systemically and mucosally, the pivotal observation by Kesty and Kuehn pointed towards OMVs as a potentially ideal platform to develop highly effective, easy to produce multi-valent vaccines.

However, for OMVs to become broadly applicable, a number of questions remain to be addressed. Among them, it is important to demonstrate the robustness of the genetic engineering strategies for antigen delivery to the OMVs and to identify the most appropriate strategy for inducing the desired immune responses (cell-mediated immunity and/or antibody responses).

Theoretically, the best way to elicit antigen-specific antibody responses using engineered OMVs is to localize recombinant antigens on the vesicle surface, thus providing the best antigen accessibility for B cell receptor binding. However, targeting proteins to bacterial outer membrane can be challenging and usually requires the construction of chimera between antigen and endogenous outer membrane proteins (13–15). The success of such fusion strategies usually inversely correlates with the size of the passenger protein. An easier approach to deliver recombinant antigens to OMVs would be to express them in the bacterial periplasm. Since periplasmic proteins get trapped in the OMV lumen during vesicle formation (2), the addition of a sec-dependent leader sequence to the N-termini of antigens should be sufficient to guarantee their incorporation into OMVs, provided that no selective periplasmic protein inclusion/exclusion occurs during vesiculation. However, luminal localization of antigens could be a sub-optimal way to induce an antigen-specific antibody response, unless membrane integrity is partially lost at the site of OMVs injection with concomitant exposure of their cargo to B cells.

In this work, we focused our attention on antigen delivery to the OMVs with the aim of establishing in a systematic manner (a) how efficiently heterologous protective antigens can be incorporated into the lumen of the vesicles and (b) to what extent antigens localized in the OMV lumen can elicit functional, antigen-specific antibody responses.

As far as the first question is concerned, our data demonstrate that all antigens selected in this study could be delivered to the *E. coli* vesicles by hooking them to the *E. coli* OmpA leader sequence for secretion. In addition, 3 heterologous antigens were previously reported to reach the OMVs via periplasmic expression (12,32,33), and we have recently achieved OMV delivery of 5 additional protective antigens from gram-positive pathogens (unpublished). In light of the foregoing results, we conclude that most bacterial antigens eliciting antibody-mediated protection can be successfully incorporated into *E. coli* OMVs via the sec-dependent pathway of periplasmic expression. This conclusion also implies that, differently from that proposed for at least some outer membrane proteins (34), vesiculation does not use a selective process for periplasmic protein incorporation in OMVs.

The high rate of success in targeting recombinant antigens to the lumen of OMVs (in essence, all heterologous antigens we have tested so far were successfully incorporated into bacterial vesicles) could appear surprising. Indeed, it has been demonstrated that binding of the leader peptide to the ATP motor protein SecA is necessary but not sufficient for protein secretion through the sec-dependent pathway and that concomitant SecA binding by conserved motifs in mature proteins is also required (35,36). This is why many cytoplasmic proteins that do not carry SecA-binding motifs cannot reach the periplasm even when fused to leader peptides. To explain the efficiency of our OMV protein delivery, it has to be pointed out that we focused our attention on heterologous bacterial antigens inducing protective antibody responses. Many of these antigens, such as those selected for this study, belong to the category of proteins that are naturally secreted or surface-exposed by the sec-dependent pathway. Therefore, these proteins are the ideal candidates to be expressed in the *E. coli* periplasm as long as an *E. coli*-compatible leader sequence is fused to their N-termini. It is in fact known that leader

sequences can be species-specific, and in particular leader sequences of Gram-negative bacteria differ from those of gram-positive species (37). In this study, we have used the OmpA leader sequence for antigen secretion, but other leader sequences are expected to work as well and indeed to achieve optimal secretion levels we are currently testing each antigen with a battery of different *E. coli* leader sequences.

Antigens inducing antibody-mediated protective activity include members of the integral membrane family and proteins that are secreted via alternative secretion pathways. Although not tested in this work, such proteins are likely to be less prone to OMV delivery through fusion to leader sequences, and other strategies might be required to achieve their expression in OMVs.

The second question we addressed in this study is whether immunization with OMVs carrying antigens in their lumen can induce antigen-specific antibodies. Theoretically speaking, localizing antigens inside the vesicles should prevent antigen-specific B cell binding and the consequent proper stimulation of antibodies. To the best of our knowledge, only 2 groups have so far analyzed immune responses against luminal recombinant antigens (32,33). Both groups have reported the elicitation of antigen-specific antibodies, but titers were relatively low and the authors reached the conclusion that, for practical vaccine application, antigens should be localized on the OMV surface. In the case of pneumococcal PspA expression in Salmonella OMVs, the authors presented evidence that the protein was released into the growth culture supernatants in addition to being localized in the periplasm/OMV lumen. Therefore, the observed PspA-specific antibodies could have been at least partially generated by the secreted protein "contaminating" the OMV preparation.

Our data unequivocally demonstrate that recombinant antigens delivered to the lumen of OMVs do induce effective antibody responses. Indeed, antibody titers were often close to those obtained with 20 μg of recombinant proteins, corresponding in some cases to 1 log higher antigen amounts than those carried in the 25 μg of OMVs doses used for mouse immunization. Most important, antibodies were characterized by a functional activity which could be greater than the one observed with sera from animals immunized with the corresponding recombinant proteins. This is particularly evident for SAM_1372-OMVs: despite the fact that the protein induced low ELISA titers (most likely because of its poor expression in OMVs) antibodies had an excellent functional activity in terms of bacterial opsonophagocytic killing.

Elicitation of high-quality antibodies translated into good protection *in vivo*, as judged by the ability of animals immunized with SpyCEP-OMVs and Slo-OMVs to survive GAS lethal challenges. At least 2 important factors contribute to such potent functional responses. First, all antigens used in this work preserved their native conformation when expressed in the lumen of OMVs, as deducible from the fact that the antigens had fully enzymatic/toxic activity. This guaranteed the elicitation of conformational antibodies capable of binding and neutralizing the antigen toxic activities. Second, and particularly important, recombinant OMVs have the intrinsic ability to co-deliver antigen and adjuvant to the same antigen-presenting cells, thus allowing the optimal activation of antigen-specific B- and T-cells (38). The relevance of antigen/adjuvant co-delivery can be appreciated when the inhibition of SpyCEP proteolytic activity by antibodies induced by 2 injections of different SpyCEP-containing vaccine formulations was compared (Fig. 7). Sera from SpyCEP-OMVs immunized animals showed a higher neutralization activity with respect to both Alum-formulated recombinant SpyCEP and SpyCEP mixed with "empty" OMVs. While the antigen-adjuvant co-delivery concept can explain how APCs taking up SpyCEP-OMVs optimally stimulate the generation of SpyCEP-specific plasma cells and memory B cells, the presentation of a luminal protein to naïve B cells necessarily requires that the integrity of OMVs is partially compromised in order to expose the protein to B cell receptors. Based on the foregoing, we propose that OMVs are taken up at the site of injection by APCs, which present the antigen-specific peptides to T cells. At the same time, a fraction of OMVs are degraded before being phagocytosed by APCs and this allows antigen presentation to naïve B cells. Antigen-specific B cells are finally stimulated to become effector and memory B cells by interaction with antigen-specific Helper T cells generated by those APCs that have taken up intact vesicles. According to this model, the ratio between intact and degraded OMVs at the site of injection is expected to be important for the generation of a sufficiently large population of antigen-specific T- and B-cells and, ultimately, a good antibody response. The extent of vesicle degradation should be determined by a number of factors, including site of immunization, formulation and nature of OMVs. In this respect, it has to be pointed out that the strains used for vesicle production and engineering usually carry specific mutations that confer an OMV-overproducing phenotype. Many of these mutations have a direct effect on the integrity of the outer membrane or on the interaction between outer and inner membranes (39). In this study we used a BL21 *E. coli* strain carrying the *ompA* gene deletion, which results in the removal of one of the major membrane proteins in *E. coli*. It is possible that the OMVs derived from this mutant are sufficiently robust/fragile to induce the good antigen-specific antibody responses we observed in our work. Other mutations could generate vesicles with different chemical–physical

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properties and this could ultimately affect immune responses both qualitatively and quantitatively.

The reactogenicity of OMVs and the implication of such reactogenicity in OMV-based vaccine development deserve one last comment. It is well known that OMVs carry large amount of LPS. We measured the LPS content in our OMV preparations and determined an LPS:protein ratio of approximately 0.5 (50 ng of LPS/100 ng of OMV proteins). This number is consistent with that recently reported by Park and co-workers (40). Such LPS concentrations are toxic in mice and 20 μg of *E. coli* OMVs injected intraperitoneally have been shown to be sufficient to rapidly kill animals (40). This is the reason why, to decrease OMV reactogenicity, throughout this work OMV immunization was carried out in the presence of Alum, which absorbs LPS and prevents its systemic dissemination. However, for human use, OMVs must be detoxified. This can be achieved in at least 2 ways. One possibility is to remove LPS using detergent extraction. For instance, the meningococcal OMV-based vaccines available on the market are produced by deoxycholate treatment of whole bacteria. A second approach is to use OMVs deriving from mutant strains that carry detoxified variants of LPS. Mutations that produce detoxified OMVs have been described for *Neisseria meningitidis* (41) and *E. coli* (42). Interestingly, a synthetic biology methodology has recently been reported that allows modulating the innate immune response of LPS by engineering the LPS biosynthetic pathway of *E. coli* (43). We are pursuing similar approaches to design OMVs with TLR4 agonist activities tailored on the basis of specific vaccine needs.

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