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Eight stranded β -barrel and related outer membrane proteins: Role in bacterial pathogenesis.

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Abstract:

Gram negative bacteria have evolved many mechanisms of attaching to and invading host epithelial and immune cells. In particular, many outer membrane proteins (OMPs) are involved in this initial interaction between the pathogen and their host. This review focuses on a number of small pore-forming OMPs that are all composed of eight-stranded β -barrel proteins and include members of the OmpA, OmpW and OmpX families of proteins. These proteins, together with the related OmpA-like peptidoglycan associated lipoproteins, are involved in interactions with host cells and are mediators of virulence. In many cases, these proteins interact with host immune cells and can be considered as pathogen associated molecular patterns (PAMPS) due to their ability to signal via Toll like receptor molecules and other pattern recognition receptors. The role of these proteins in pathogenesis is discussed here, together with the potential for these proteins to be used as immunoprophylactic agents to protect against infection.

Keywords: cellular interactions ; host immune response ; Lipoproteins ; Outer membrane proteins; Pathogenesis ; immunoprophylaxis.

Introduction:

Bacterial pathogens have evolved many mechanisms of attaching to and invading host epithelial and immune cells and subverting the host response, including pili and fimbria, type III secretion systems and trimeric autotransporters [1-3]. However, integral outer membrane proteins with barrel structures are also involved in these processes. Gram negative bacteria have evolved three major classes of outer membrane proteins (OMPs) to facilitate in the transport of nutrients into the cell. Many OMPs comprise monomeric or trimeric barrels that are composed of 12 to 22 anti-parallel β -strands. These include the TonB-dependent active transporters, such as FhuA in *Escherichia coli*, which are involved in the uptake of larger molecules, for example, siderophores. The second group, classed as general porins, including OmpF and OmpC, both of which are present in *E. coli* and *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), also form trimeric aqueous channels and are involved in non-specific diffusion of small hydrophilic compounds across the outer membrane. A third class of proteins is involved in substrate-specific transport of nutrients. These form β -barrels that are composed of only eight to ten transmembrane domains and include OmpA, OmpW and OmpX. The classical trimeric bacterial proteins, for example, OmpF, PhoE and OmpC which play roles in the transport of nutrients and other molecules are well described elsewhere [4] and are not the focus of this review. Rather, the eight- stranded β -barrel proteins that link to peptidoglycan will be discussed. These have been shown to have a variety of different functions, including lipid metabolism, structural functions and cellular adhesion. In addition, a number of outer membrane lipoproteins have been identified which also bind to peptidoglycan. These are described as “OmpA-like” and as a result are also included in this review.

There is increasing evidence in recent years that these OMPs and lipoproteins interact with signalling pathways in host cells and play roles in host response and or host evasion. Pathogen associated molecular patterns (PAMPs) are highly conserved structures on microorganisms that are shared by large groups of pathogens and are essential for their survival. These interact with receptors referred to as Pattern recognition receptors (PRRs), including Toll-like receptors and nucleotide

binding and oligomerisation domain-like receptors (NLRs). This review will focus on a selection of these integral eight stranded β -barrel OMPs and lipoproteins with OmpA-like domains and will discuss the role that they play in pathogenesis. The interactions between both of these groups of proteins and host cells will be described and their potential role as vaccine candidates will be highlighted.

OmpA

Outer membrane protein A (OmpA) is a 38kDa protein that exemplifies the β -barrel proteins with eight transmembrane domains. It is an integral component of the outer membrane of Gram-negative bacteria and is highly conserved among Enterobacteriaceae and other γ -Proteobacteria [5] (Table 1). Comparisons between *E. coli* OmpA and *Pasteurella multocida*, PmOmpA, for example, indicated that they share 60% identity [6]. OmpA is also a major antigen which is highly conserved in *Burkholderia pseudomallei*, the causative agent of melioidosis. It is so well conserved that it has been considered a suitable antigen for the serodiagnosis of melioidosis [7]. It is a major OMP of *E. coli* with about 100,000 copies per cell. It interacts specifically with the peptidoglycan layer [8] and its gene has been shown to be highly regulated and environmentally responsive, as reviewed by Smith et al., [9].

OmpA Structure: The structure of OmpA has been well studied and compared with other porins showing an overall β -barrel structure. Its eight transmembrane domains are connected by short periplasmic turns and with four longer extracellular loops (figure 1). Although early work predicted a pore structure, subsequent studies predicted that OmpA was unlikely to form a pore as no continuous channel could be detected [10]. More recently, it has been shown that OmpA can function as a gating channel. Salt bridges between Glu 52 and Arg 138 can switch to form alternative bridges with other amino acids and function as an ion gate allowing survival of bacteria under osmotic

stress [11]. Furthermore, two temperature dependent conformers of OmpA have been identified: the majority of OmpA is observed as a small “closed” pore form which has eight transmembrane regions and a globular domain at the C-terminus and a minority of molecules which are in a larger “open” pore form and have 16 transmembrane regions [12].

Although conserved across species, diversity has been observed among hypervariable regions within the molecule, for example, high variation exists in the surface exposed region of OmpA across the 15 serovars of *Haemophilus parasuis* with hypervariable regions, which did not seem to affect antigenicity [13]. In addition, the identification of amino acid differences in OmpA between invasive and non-invasive strains of meningitic strains of *E. coli*, together with recent studies using mutants where individual loops had been deleted, demonstrated that this variation contributes to invasive potential and pathogenesis [14-16].

Physiological function: A diverse range of functions that have been attributed to OmpA include participating in biofilm formation, acting as both an immune target and in immune evasion, serving as a receptor for several bacteriophages, and playing a part in bacterial adherence to host tissues [6, 17, 18]. The N-terminal part of the molecule anchors the protein with the outer membrane, while the C-terminal portion resides in the periplasm and interacts with peptidoglycan. This physical link between the peptidoglycan layer and the outer membrane suggests that OmpA plays a role in integrity of the bacterial surface. Indeed, over thirty years ago, double mutants of OmpA and another peptidoglycan binding lipoprotein (Lpp) led to the formation of spherical cells which showed extensive blebbing of the outer membrane and evidence of free-floating murein [19]. OmpA is upregulated in *E. coli* biofilms [20] and increases biofilm formation on abiotic surfaces via repression of cellulose production [21]. OmpA also acts as a receptor for bacteriophages and for bacteriocins [22, 23]. It has also been shown that *E. coli* mutants of OmpA were type L colicin resistant and the OmpA expressed could no longer function as a phage receptor [24].

Role of OmpA in virulence of Enterobacteria: Many studies on the virulence of OmpA have been carried out on strains of *E. coli* strain K1, the causative agent of meningitis in neonates; however,

OmpA family proteins are also associated with a wide range of Gram negative bacteria from opportunistic respiratory pathogens to organisms that colonise the urinary tract [14, 25]. Some of the earliest studies on virulence of OmpA were carried out by Prasadarao et al., [14] who showed that OmpA is essential for the invasion of meningitic strains of *E. coli* into human brain microvascular endothelial cells (HBMEC). More recently, it has been demonstrated that OmpA Loops1, 2, and 3 contribute to binding and invasion of *E. coli* K1 strain RS218 into HBMEC, while Loop 4 does not play a significant role. Loops1 and 2 of OmpA attached to a HSP90 homologue on HBMECs (gp96) and subsequently triggered cytosolic phospholipase A2 α (cPLA2 α) activation which is involved in invasion of HBMECs [15]. Inhibition of HSP90 resulted in decreased invasion of bacteria and reduced cPLA2 α activation, both events orchestrated by Loops1 and 2 [15]. *E. coli* OmpA also allows survival of the pathogen within macrophages and monocytes, OmpA mutants do not survive in macrophages [26]. Furthermore, OmpA expression in *E. coli* K1 strain also prevented dendritic cells from maturation [27]. Recent mutant studies by Mittal et al., have shown that loops 1 and 2 are important to the survival inside neutrophils and dendritic cells, while loops 1 and 3 are essential for survival in macrophages [28]. It was recently demonstrated that mice that had been depleted of macrophages were resistant to meningitis caused by *E. coli* K1. In addition, the Fc γ receptor (Fc γ R1) on the surface of macrophages was identified as the ligand to which OmpA binds and it was shown that this plays a key role in the development of meningitis [29]. Furthermore, OmpA binding to Fc γ R1 induced novel tyrosine kinase signaling patterns which it was suggested may allow *E. coli* expressing OmpA to avoid macrophage antimicrobial responses. OmpA has also been shown to confer serum resistance via binding to complement regulator protein C4bp and it has been recently demonstrated that certain regions of loops 1, 2 and 4 that are responsible for this mechanism [28]. These studies identified that Loop 4 mutants showed higher virulence, recruited more microglia, B cells, macrophages and granulocytes in the brains of infected mice and elicited higher production of pro-inflammatory cytokines which is likely to be associated with these mutants avoiding serum complement activity [28].

OmpA also mediates adhesion of another *E. coli* strain, enterohemorrhagic *E. coli* (EHEC). Torres et al, have demonstrated that increased expression of OmpA contributes to the adherence of EHEC to HeLa cells and were the first to demonstrate the role of OmpA in EHEC binding [18]. While intimin had previously been identified in the late steps of EHEC adhesion, OmpA appears to be involved in the initial step. The *tdc* operon which controls the genes responsible for transport and anaerobic degradation of threonine also controls OmpA expression in EHEC. In contrast, OmpA does not appear to be involved in either attachment to, or invasion of, uropathogenic *E. coli* (UPEC) into bladder epithelial cells. Rather, it appears to be upregulated post-infection of bladder tissue *in vivo* where it facilitates the development of intracellular bacterial communities post-invasion and also plays a role in persistence [30].

PmOmpA of *Pasturella multocida* is another eight stranded β -barrel protein which has high homology with *E. coli* OmpA (60% identity over 30 N-terminal amino acid residues) and has been shown to attach to extracellular matrix components, including heparin and fibronectin [6]. It has been demonstrated that PmOmpA on this bovine pathogen bound to the surface proteins of Madin Darby canine kidney cells (MDCK) cells and also to fibronectin and heparin. Anti-fibronectin antibodies inhibited binding of PmOmpA to MDCK surface proteins suggesting that *P. multocida* OmpA may use extracellular matrix components to bridge to host cells [6].

OmpA (together with another eight stranded β -barrel protein, OMPX, discussed later) were found to be essential for invasion of *Cronobacter sakazakii* into human epithelial cells [31]. This pathogen (originally classified as *Enterobacter sakazakii*) is also associated with outbreaks of meningitis and necrotizing enterocolitis in premature infants. Although both proteins interacted with gastrointestinal epithelial cells, OmpA, but not OmpX was also shown to be important for attachment to basolateral surface of Caco-2 cells, suggesting that its receptor is in the basolateral side of the cell. Invasion of *C. sakazakii* into HMBEC cells was dependent on microtubule condensation, which was

mediated by OmpA, and involved both protein kinase C α and phosphoinositide-3 kinase signaling [32].

The OmpA of respiratory and urinary tract pathogen, *Klebsiella pneumoniae*, which shows 85% identity with *E. coli* Omp A (297 of the first 350 amino acids, Blastp[33]) binds to and activates dendritic cells and macrophages, triggering cytokine production and subsequent dendritic cell maturation [34]. This work on purified OmpA contrasted with more recent work using OmpA mutants of clinical *K. pneumoniae* strains, which demonstrated that OmpA mutants induced higher levels of IL-8, IL-6 and TNF- α than the clinical wild type strains and were attenuated in the pneumonia mouse model [35]. *K. pneumoniae* OmpA has also been shown to protect bacteria against host antimicrobial peptides [36]. Taken together, however, these data support the suggestion that OmpA of *K. pneumoniae* should be considered as a PAMP [35].

OprF – the OmpA ortholog in *P. aeruginosa*:

Major outer membrane protein OmpA of *Escherichia coli* and major outer membrane protein, OprF of *P. aeruginosa* are orthologs with significant amino acid similarity (56%) and identity (39%) in their C-terminal domains. Even though the N-terminal fragments are considerably less similar (15% identity), based on secondary structure predictions, a model for the N-terminal fragment of OprF has been suggested that was constructed using homology modeling of the primary sequence onto the experimentally determined crystal structure of the N-terminal domain of OmpA [37]. Classified as a major non-specific porin of *Pseudomonas* species, OprF is also involved in attachment [25].

Structure and function of OprF: Modelling studies carried out by Brinkman et al., [38] showed that OprF lacks the salt bridges associated with closure of the OmpA channel allowing for a larger channel, relative to OmpA. More recently, it has been shown that both OmpA and OprF exist in two conformers, the majority of molecules exist in an eight barrel structure in a closed channel with the C-terminus domain in the periplasm interacting with peptidoglycan [39]. In contrast, in the minority

more open channel conformer, the C-terminus is exposed on the cell surface. The conservation of the salt bridge gate function of OmpA has been identified mainly in phylogenetically similar organisms, but does not appear to be universal among OmpA homologs present in other gamma-proteobacteria, such as *P. aeruginosa* [39].

In order to determine whether OprF functioned as a porin and to examine the relatively slow solute diffusion that has been observed through the channel, Sugawara *et al.*, carried out elegant studies using reconstituted proteoliposomes whereby liposomes containing OprF which was in the open channel conformation could be distinguished from those without open channels on the basis of density due to sucrose uptake [39]. They showed that the fraction of open channel conformer in the OprF population was 5%. This led them to suggest that although OprF and OmpA both function as porins; their main function was to use the N terminal barrel to insert in the OM, thereby connecting the OM to the peptidoglycan via the C- terminus.

Role of OprF in virulence: When *P. aeruginosa* chronically colonises the CF lung the production of mucin, flagella and pili are lost. In addition several virulence-associated traits and immunostimulatory components of *P. aeruginosa* are turned off [40]. In contrast, an upregulation of outer membrane protein OprF is essential for optimal microaerobic growth allowing *P. aeruginosa* to adapt to the anaerobiosis found within mucus plugs in CF airways [40]. In a proteomic and transcriptomic analysis of sequential isogenic isolates from three cystic fibrosis patients, Hoboth *et al.*, [41] have shown that OprF expression is increased in lung-selected *P. aeruginosa* isolates. Furthermore, it is indispensable for growth in anaerobic *P. aeruginosa* biofilms [42].

Interferon gamma binds to OprF, resulting in the upregulation of a number of virulence factors, including the *lecA* gene, a *P. aeruginosa* adhesin, which is quorum-sensing (QS)-dependent [43]. More recently, it has been shown that OprF is involved a range of virulence characteristics in *P. aeruginosa*, including adhesion to host cells, secretion of toxins through the Type III secretion system, production of virulence factors such as pyocyanin, elastase and exotoxin A, in addition to Lectin PA-1L [25]. It has been shown that in the absence of OprF, mutants showed impaired adhesion to animal cells, a lack of secretion of ExoT and ExoS toxins through the type III secretion system (T3SS), and

reduced production of the QS-dependent virulence factors pyocyanin, elastase, lectin PA-1L and exotoxin A. In addition, the production of a key signaling molecule, 30-c12-HSL was substantially reduced in OprF mutants. It was suggested that OprF is a host immune system sensor modulating QS to enhance virulence when bacteria are in contact with the host [25].

OmpA in other Gammaproteobacteria

Another OmpA homolog, Omp5 or p5 fimbria of Non-typeable *Haemophilus influenzae* (NTHi) has been shown to adhere to intercellular adhesion molecule 1 (ICAM-1) of respiratory epithelial cells, resulting in the upregulation of expression of ICAM [17]. ICAM also acts as a receptor for human rhinoviruses and coxsackieviruses, however, in the case of NTHI, not only does it exploit ICAM as a receptor, but it also up-regulates the expression of its own cell surface receptor. *Acinetobacter baumannii* is another gamma-proteobacterium which is an opportunistic pathogen, causing pneumonia and urinary tract infections. The outer membrane protein A (AbOmpA) which has 24% identity with *E. coli* OmpA across the entire 362 aa sequence (blastP [33]), is a potential virulence factor that induces epithelial cell death and death of dendritic cells due to the production of reactive oxygen species [44].

The role of OmpA in adhesion is not exclusive to pathogens, a high frequency of OmpA variants of *Bacteroides vulgatus*, a predominant organism of the gut microflora, were recently observed among isolates of the colons of ulcerative colitis patients. The variation in OmpA was a factor in causing an increase in the adherence of the bacterium [45].

Immunoprophylactic potential of OmpA: As discussed above, OmpA interacts with host immune cells and behaves as a PAMP in certain pathogens, such as *K. pneumoniae*. OmpA is among a number of low molecular weight immunogenic proteins in *Salmonella*-induced reactive arthritis [46]. In typhoid fever, the immune response is also directed against porins and OmpA [47-49]. This immunogenicity has application in the use of OmpA as a vaccine candidate in the protection against pathogens where OmpA plays a role in attachment and virulence. *S. typhimurium* OmpA induced

functional and phenotypic maturation of dendritic cells and the activation of ERK1/2 and p38 MAPK via TLR4; activating T cells via a Th1 response [50]. In addition to the potential use of OmpA protein as an immunoprophylactic agent [34], the *ompA* gene was one of two components of a DNA vaccine that protected against *Klebsiella pneumoniae* in mice [51].

The immunoprotective potential of OmpA is not universal among Gram negative bacteria. Immunisation of mice with recombinant *P. multocida* OmpA (PmOmpA) stimulated potent serum IgG responses but did not protect against subsequent bacterial challenge [52]. Indeed, administration of PmOmpA together with unpurified OMPs reduced the protective response obtained when mice were immunized with OMPs alone (survival rates of 88% protection reduced to 53%) indicating that purified PmOmpA had a deleterious effect on the protective response. *P. multocida* is a facultative intracellular pathogen and it is likely that the potent serum antibody indicative of a prominent Th2 response was not sufficient to protect against an intracellular pathogen such as this. Intracellular pathogens generally require a mixed Th1/Th2 response for protection. It is clear that immunogenicity of OmpA alone is not sufficient to protect against pathogens and more research on potential adjuvants is required to fully exploit the prophylactic potential of OmpA.

OmpW family

A different family of eight-stranded β -barrel proteins have also been associated with host-bacterial interactions. The OmpW family is comprised of small OM proteins and is widespread among Gram negative bacteria. While they do not share sequence homology, they have some structural and functional similarities with OmpA. There are 461 homologs of this family listed in the KEGG database [53]. This protein has been identified in bacteria that colonise the gastrointestinal tract, including *Vibrio* species, *Salmonella*, *E. coli*, and in the respiratory pathogen *P. aeruginosa*, where its equivalent, is OprG. It is a major OMP of *P. aeruginosa* [53] and of many *Vibrio* species [54, 55] and is expressed in all known strains of *V. cholerae* [56]. In contrast, it is considered to be a minor OMP protein in *E. coli* [57].

OmpW Structure: Like OmpA, the structure of the OmpW of *E. coli* also comprises an eight-stranded β -barrel with pore-forming properties (Figure 1), which in the case of OmpW form a long, narrow hydrophobic channel [58]. This deep hydrophobic binding pocket distinguishes OmpW from the channels of other OMPs which typically have hydrophilic interiors [58]. The homolog in *P. aeruginosa*, OprG, has 49% identity with that of OmpW and a high number of hydrophobic residues; it is considered that this also acts as a porin [53]. OmpW has been shown to have a heat-modifiable structure resulting in an increase in its apparent molecular weight on SDS-PAGE gels from 19 kDa to 21kDa. This is thought to be due to unfolding of the β -barrel to an α -helix structure [54]. OprG also has been shown to have a heat-modifiable character, albeit with a larger increase in MW from 20kDa to 25kDa [53].

Touw *et al.*, [59] compared the OmpW orthologs from 5 different species (*P. aeruginosa*, *V. cholerae*, *E. coli*, *P. putida* and *Aeromonas hydrophila*) and showed that the similarity between these proteins was greatest in the barrel regions, but that less than 15 residues were absolutely conserved across all species. Two proline residues were conserved adjacent to the lateral opening in the barrel wall and are likely to be responsible for the lateral opening. It was suggested that the conservation of these proline residues indicates that the lateral opening is present in all OmpW family members. It was proposed that OmpW family proteins are involved in the uptake of small hydrophobic molecules across the OM [59].

OmpW Function: To date, very little direct evidence has been provided for the function of OmpW. It acts as a receptor for Colicin S4 in *E. coli* [57] and also has a role in osmoregulation [55]. Its expression in *V. cholerae* is affected by a range of environmental factors, salinity, temperature, availability of nutrients, such as simple sugars, and oxygen [54]. Recently, it has been shown that the OmpW gene in *S. typhimurium* has a class1 SoxS-dependent promoter suggesting that OmpW is involved in the response to oxidative damage [60]. In a stress-sensitive variant of an EHEC strain, oxidative stress induced a viable but non-culturable state in which OmpW expression increased by 2100-fold [61]. It was later shown that this phenotypic change was induced as a genetic alteration

during *in vivo* passage of isolates [62]. It was shown that high levels of OmpW appear to sensitise EHEC to oxidative stress and the change in OmpW stress response is a hallmark in the change in stress sensitivity that occurs during *in vivo* colonisation. In *V. cholerae*, OmpW is one of a number of genes that is modulated by a cyclic AMP receptor protein, including QS genes and other genes that are involved in intestinal colonisation [63].

OmpW also functions as a porin which is involved in the efflux of a diverse range of molecules from paraquat (*S. typhimurium*) [64], to naphthalene (*P. fluorescens*) [65]. It is involved in resistance to a range of antibiotics in addition to antimicrobial peptides. OmpW was down-regulated when *E. coli* was exposed to chlortetracycline, and deletion of OmpW from *E. coli* decreased MICs for a range of antibiotics [66]. In *S. typhimurium*, the expression of the *ompW* gene was increased two-fold in the presence of paraquat and contributes to paraquat resistance by a different pathway than the porin, OmpD [64]. Furthermore, it was demonstrated that paraquat may be effluxed through OmpW in this species [64]. The *P. aeruginosa* homolog, OprG, is induced at high iron concentrations (100uM) but does not appear to function either as an iron-uptake system nor in antibiotic resistance [53]. It has been demonstrated to function as a porin, like its homolog OmpW, however the majority of the dissimilarity between these two porins are associated with residues involved in substrate binding [58]. Structural studies have shown that the majority of residues (approximately 89%) that face the lumen of the barrel on the extracellular side of the molecule are hydrophobic, resulting in a hydrophobic funnel, which does not continue all the way down the channel [59]. Polar residues close off the channel on the periplasmic side.

Role of OmpW family proteins in pathogenesis: It has been associated with attachment of, and immune response to, a number of intestinal pathogens. Mutant strains of the intestinal pathogen, *Vibrio cholerae*, which expressed no OmpW were shown to have reduced colonisation of mice by 10 fold relative to those that expressed OmpW [54]. This dramatic effect was limited to mutants of serogroup 139 and were not observed in parallel studies on serogroup 1, *ompW* mutants of which exhibited only a 1.8-fold reduction in colonisation [54], suggesting that OmpW is not the only protein

involved in colonisation of this species. McPhee *et al.* [53], have shown that in the absence of the OmpW homolog of *P. aeruginosa*, OprG, mutants were over three-fold less cytotoxic towards human bronchial epithelial cells, 16HBE14o-, relative to WT within 4 hours of co-culture. The *oprG* gene was also shown to be down-regulated (9-fold) in *P. aeruginosa* cells which were adherent to HBE cells compared cells which did not adhere in the same experiment after 4 hours of interaction, indicating that OprG promotes host cell interactions in the parental strain leading to cytotoxicity and it is subsequently down-regulated after initial interaction.

Role of OmpW in host response. Like OmpA, OmpW is immunogenic in *Salmonella* induced reactive arthritis; all fractions producing immunoproliferative responses contained OmpA and OmpW [46]. Sixty percent of Celiac disease patients were seropositive for *Bacterioides* OmpW antibodies [67], and serum levels decreased significantly when patients were on a gluten free diet [68]. It was suggested that OmpW may contribute to the pathogenicity of inflammation in celiac disease [67]. Serum antibodies to OmpW also are elevated in pediatric Crohn's disease patients [69]. This protein has also been identified as being related to the immune response in inflammatory bowel disease associated with an oral pathogen: *Porphyromonas gingivalis* [70].

Immunoprophylactic potential of OmpW: Antiserum raised against *V. cholerae* OmpW was moderately protective to suckling mice against *in vivo* challenge with a *V. cholerae* O1 classical strain (66.6% protection at 1:50 dilution) and an O1 El Tor strain (50% protective) suggesting the potential for OmpW as a vaccine candidate [54]. *Vibrio alginolyticus*, is a marine bacterium that is one of the major human *Vibrio* pathogens, causing wound infections, otitis media gastroenteritis and septicemia [71]. Immunisation of fish with recombinant *V. alginolyticus* OmpW, demonstrated that OmpW was an effective vaccine candidate and was protective to challenged fish with a relative percent survival of 78% [72]. The effectiveness of OmpW as a vaccine against this marine pathogen further highlights the potential of the conserved OmpW as a vaccine candidate for other pathogenic *Vibrios*.

OmpX family.

The third family of eight-stranded β -barrel proteins that has been associated with cellular attachment and neutralization of host defence mechanisms includes OmpX. It was first described in *Enterobacter cloacae* and has a number of homologues, e.g. Ail in *Yersinia enterocolitica*; PagC and Rck in *S. typhimurium* [4], which share between 85 and 32% identity [73]. Although its architecture is similar to OmpA, OmpX has a lower shear number (of eight, as opposed to ten), a measure of the stagger of the strands in the β -sheet [4]. It also protrudes further outside the outer membrane than OmpA, as a result of having four β -strands which are significantly longer than those in OmpA [74] (Figure 1). These protruding extracellular loops alone suggested that this protein promoted cell adhesion, invasion and defence against host complement [74]. It is the smallest of the eight-stranded barrel proteins, being only 18kDa in size. OmpX, like OmpA and OmpW is environmentally regulated, and its expression is increased as an early response to drug exposure or to environmental stress, such as higher osmolarity and temperature [75]. OmpX is QS regulated in *Serratia marcescens* [76].

Role of OmpX in Pathogenesis: Prior to its discovery in *E. coli*, it was identified in *Y. enterocolitica* and in *Y. pseudo-tuberculosis* as Ail (adhesion and invasion locus) and its role in serum resistance and host cell invasion was demonstrated [77, 78]. Another homologue is a 21.5kDa protein, referred to both as OmpX and as Ail, has been identified in *Y. pestis*, the causative agent of plague. This pathogen invades epithelial cells as one of its mechanisms to avoid the host immune system; however it does not express either invasins or YadA, two well-studied adhesins that are essential to the host cell interactions of *Y. enterocolitica* and *Y. pseudotuberculosis*. OmpX is one of the most abundant proteins found in the outer membrane of this organism and is an essential virulence factor of *Y. pestis*. Ail mutants required over 3000-fold more bacteria to attain the 50% lethal dose in mice [79]. Clear demonstration of the role that OmpX plays in *Y. pestis* was demonstrated by its inactivation which reduced the interaction of *Y. pestis* with epithelial cells association and its internalization by 90 and 98 %, respectively [80]. Furthermore, expression of OmpX from *Y. pestis* into *E. coli* was associated with an increase in adhesion to epithelial cells by up to 7-fold and an increase in internalized bacteria

by up to 30-fold. OmpX has recently been shown to bind to fibronectin on host cells and facilitates the delivery of cytotoxic Yops to the host cell [81]. Anti-fibronectin antibodies blocked delivery of Yop and Ail-mediated cytotoxicity. It was recently shown that the invasion activity of OmpX in *Y. pestis* and its ability to confer serum resistance depends on the length of the LPS core in the OmpX expressing strains [82].

Although OmpX in *E. coli* is 40% identical to Ail of *Y. enterocolitica*, it is not involved in adherence to mammalian cells. Furthermore unlike Ail and Rck in *S. typhimurium*, it does not contribute to serum resistance. The largest divergence between OmpX in *E. coli* and the other proteins is in the surface exposed residues, confirming the role for these regions in both complement and host cell interactions [73].

OmpX is also a potent immunogen. Together with OmpA, OmpW and PAL, it has shown considerable immunogenicity and T-cell activation in salmonella-induced arthritis [46]. OmpX from *E. coli* binds to both immature dendritic cells and macrophages and induced both humoral and cellular immune responses [83]. However, it did not activate these antigen presenting cells, suggesting that OmpX in *E. coli* does not provide a danger signal to antigen presenting cells.

OmpA-like proteins and lipoproteins:

There are a diverse and expanding group of proteins which are described as “OmpA-like”, predominantly because they share sequence homology with the carboxy terminal of OmpA, and are therefore peptidoglycan-binding proteins and lipoproteins. These proteins also interact with host cells and have been shown to play roles in cellular attachment and virulence.

Peptidoglycan associated lipoprotein

A large group of PAMPs which are related to OmpA include peptidoglycan-associated lipoproteins (PAL). These are ubiquitous in Gram negative organisms and sequence homologs have been

identified in over 100 species [84], and associated with the pathogenesis of several organisms [85-87]. PAL has been identified in *B. multivorans*, *Pseudomonas putida*, *Campylobacter jejuni*, *H. influenzae* and *E. coli* among other species [84, 85, 88-90]. The C-terminus of PAL has homology with both OmpA and OprF and also with other lipoproteins that are tightly but non-covalently bound to peptidoglycan [91]. It also has homology with inner membrane proteins Mot B of *E. coli* and with proteins in Gram positive bacteria, e.g. *Bacillus subtilis* OrfB. This homology most likely is due to the fact that these proteins share a common function, i.e. binding to peptidoglycan. PAL (also identified as OpcL) has been shown to be conserved among all *Burkholderia cepacia* complex species, an opportunistic pathogen in CF patients, and contributes to membrane integrity and to both detergent and antibiotic resistance [92].

PAL Structure: In *E. coli*, PAL has a MW of 16kDa without acylation and it migrates at 20kDa due to acylation at the N-terminus. Its homolog in *H. influenzae* is 16kDa, while PAL in *C. jejuni* is 18kDa [84, 85]. It is anchored to the outer membrane through its N-terminal lipid attachment (figure 2). It forms a tight but non-covalent link with peptidoglycan. *E. coli* PAL was shown to have 32% sequence identity with *E. coli* OmpA, while the PAL in *H. influenzae* shared 68% sequence identity over residues 29 to 134 with *E. coli* PAL [84]. Indeed, 30% sequence identity was observed between this region of *H. influenzae* PAL and RmpM from *N. meningitidis*. Using NMR spectroscopy, Parsons et al., [84] demonstrated that there are six residues that are invariant in the PAL family. Three of these are at, or near, the peptidoglycan binding site: the carboxylate group of D71 forms a hydrogen bond with PGN, while hydrophobic interactions between L82 and PGN were observed. In addition to these six residues, a number of surface residues near the Pgn binding site are conserved in PAL sequences, but not in sequences of the broader PAL-related family. This was suggested to be due to an additional, non-PG binding function of PAL [84].

PAL Function: The function of PAL is not fully understood. As recently reviewed by Godlewska et al., [86], PAL interacts with Tol B, a periplasmic protein as well as with outer membrane proteins,

OmpA and lipoprotein precursor, Lpp. PAL forms dimers with OmpA (54kDa) and with lipoprotein precursor (30kDa)[89, 91]. All these interactions are independent of each other. Ray et al.,[93] have shown that the region between residue 94 and 121 of PAL is responsible for interactions with both TolB and with peptidoglycan, but PAL cannot bind both molecules at the same time. The interaction with Tol B is via an induced-fit mechanism which involves conformational changes in both proteins. PAL interacts with OmpA at a site distinct from its TolB/PGN binding site: Residues 44 to 61.

Role of PAL in pathogenesis: PALs have been shown to be highly immunogenic lipoproteins in Gram negative pathogens including *H. influenzae*, *S. typhimurium*, *Legionella pneumophila*, *C. jejuni* and *P. multocida* [46, 85, 94-97] (Table 2). Like OmpA and OmpW, PAL was found to be present in all fractions of OMPs from *Salmonella*-induced arthritis that elicited an immunoproliferative response [46]. PAL is among the lipoproteins which are secreted during Gram negative infections resulting in septic shock [87]. *E. coli* PAL is a potent activator of TLR-2 in macrophage, acting synergistically with LPS to stimulation inflammation in sepsis [98]. More recently, it has been shown that PAL binds to the myocardium and contributes to cardiac dysfunction during sepsis mediated by TLR2/MyD88 signalling [99]. PAL of *L. pneumophila* also activated TLR-2 mediated signalling in murine macrophages, inducing PAL-specific B-cell and T-cell responses in mice [100].

PAL is crucial for virulence in *Haemophilus ducreyi* as was demonstrated in human trials using PAL mutants [101]. It has been associated with pathogenesis of a number of oral pathogens, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. PAL (AaPAL) was released during *A. actinomycetemcomitans* periodontosis infections and has been shown to stimulate pro-inflammatory cytokine (Il-6, Il-8 and macrophage inflammatory protein, MIP-1b) responses in an *ex-vivo* model [102].

Immunoprophylactic potential of PAL: The potential for the application of PAL in vaccines among several Gram negative bacteria including *Legionella*, *Haemophilus influenzae* and *C. jejuni* was highlighted in a recent review [86]. A protein with significant sequence homology to PAL was

identified in *Bordetella pertussis* as eliciting strong antibody responses [103]. However, the serum antibody response to GST-PAL conjugate alone was not protective against *B. pertussis* challenge, when administered as a vaccine to mice [103] suggesting that PAL was not a strongly protective antigen for *B. pertussis*. In contrast, the *H. influenzae* PAL protein, P6, protected mice against infection and induced strong specific mucosal antibody responses [84] further demonstrating the potential of PAL as a vaccine candidate against certain pathogens.

NspA: Another eight stranded β -barrel OMP with a hydrophobic pocket is Neisserial cell adhesion protein, NspA (figure 1, Table 1). This protein is expressed by *Neisseria meningitides*, a major cause of bacterial meningitis and sepsis globally. Expression of NspA confers serum resistance to *Neisseria* [104] and the shallow hydrophobic pocket is considered to be involved in attachment to host lipids, rather than lipid transport [105]. Based on the sequence similarity of NspA to Opa proteins, which are adhesins facilitating colonization of the human naso-pharynx, it was suggested that NspA was also an adhesin. When compared to the structures of other β -barrel OMPs in *E. coli*, OmpA, OmpX and PagP, it was found that the crystal structure of NspA most closely resembled that of OmpA with 144 C α atoms overlaying on OmpA C α atoms [105]. OmpX was also structurally homologous to NspA, albeit to a lesser degree (139 C α atoms overlay on OmpX). Recent data have indicated that NspA also evades the host immune system by binding to complement pathway inhibitor factor H and subsequently evading complement-associated bacterial killing [104].

OmpATb: OmpATb in *Mycobacterium tuberculosis* shares high sequence identity of its carboxy-terminal part with the carboxy-terminal domain of OmpA from *E. coli* [106]. It is expected to be located inside the outer membrane of virulent strains. However, unlike OmpA, the N-terminal domain of OmpATb does not fold as a β -barrel (figure 3), although it is capable of interacting intimately with the membrane to form channels [106]. While the function of OmpATb is not known, it was proposed that it could interact with arabinogalactan-peptidoglycan layer of the mycobacterial envelope. It was also observed that OmpATb channels exhibit more frequent and more prolonged

closure events at acidic pH. This behavior of OmpATb at low pH conditions is beneficial to *M. tuberculosis* survival in the mildly acidic environment encountered in the phagocytotic vacuole of host macrophages. Moreover, it was shown that OmpATb appears to be expressed only in pathogenic species (i.e., the members of the *M. tuberculosis* complex) underscoring its role in the virulence of these mycobacterial strains [107].

Other OmpA-like Lipoproteins:

Lipotoxin F (LptF): When *P. aeruginosa* chronically colonises the cystic fibrosis lung it changes phenotype from a non-mucoid to a mucoid form. Lipotoxin F (LptF, PA3692), is a major OmpA-like outer membrane lipoprotein of 27kDa that exhibited increased expression in a mucoid PAO1 mutant with dramatically increased alginate production [108]. Deletion of LptF resulted in increased susceptibility of *P. aeruginosa* to hypochlorite and also reduced adhesion to lung epithelial cells to 70% of controls. The potentially protective effect of LptF against hypochlorite suggests that it protects the organism in the niche of the CF lung. Lipotoxins such as LptF stimulate inflammatory responses via TLR-2 [109]. The anti-inflammatory macrolide azithromycin has been shown to down-regulate lipotoxin LptF amongst other *P. aeruginosa* lipotoxins [110] and may provide a mechanism for the one of the observed anti-inflammatory effects of Azithromycin in CF patients.

Loa22: Another lipoprotein that is reported to be OmpA-like is Loa22, a surface exposed protein in *Leptospira interrogans*, the causative agent of leptospirosis [111]. It is a 22 kDa protein, which like PAL, has a large OmpA-like domain at its C terminus, sharing significant sequence similarity with the OmpA family and has a predicted peptidoglycan-associating motif [112]. Its C-terminal 110 amino acid residues are 49% identical to OprF. Its N-terminal domain (residue 1 to 77) is not related to any other identified protein. Loa22 has been shown to play an important role in leptospirosis infection and was the only antigen to be significantly up-regulated during acute host infection [113], suggesting a prominent role in the infection process. While its role in pathogenesis has yet to be

determined, it has recently been identified that Loa22 is required for virulence of *Leptospira* within animal models. Strains of *L. interrogans* which were defective for Loa22 were attenuated in their ability to cause disease and death in guinea pig and hamster models, demonstrating that it is a virulence factor in leptosporosis [111]. In the human host, this protein is immunogenic as it is strongly recognised by sera from leptosporosis patients [114]. It has recently been shown to be involved in nephropathy via direct cytotoxicity on nephronal cells and enhanced inflammatory responses [115]. This lipoprotein also up-regulated TLR2, nitric oxide and the macrophage recruitment chemokine MCP-1 within 48 hours of treatment of nephronal cells. Loa22 is up-regulated during the acute phase of infection [114] and is considered to play an important role in inducing inflammatory responses through TLR2 which may underlie the pathogenesis of leptospiral nephritis[115].

Conclusion:

We are entering the post-antibiotic era with limited treatment options for many Gram negative bacterial infections. An anti-virulence strategy has advantages over antibiotic strategies because in the absence of biocidal activity, the evolutionary pressure is significantly reduced, thereby reducing the potential for resistance [116]. Many Gram negative pathogens are becoming difficult to eradicate due to multidrug-resistance and their intrinsic resistance to antimicrobial peptides. A greater understanding of the mechanisms of virulence may lead to the development of new anti-virulence drug targets. In addition, we need to understand the interactions between bacterial OMPs and both the host immune system and tissues that are colonised which are crucial to the pathways either controlling host inflammation or virulence, with a view to developing new drug targets which will prevent that interaction. A common theme throughout the OMPs and OM lipoproteins discussed in this review is their immunogenicity and involvement in host cell interactions. Therefore, continued investigation of these PAMPs offers the potential to develop new immunoprophylactic agents to prevent infection of many Gram negative bacteria.

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Figure Legends:

Figure 1: Ribbon structures of five eight-stranded β -barrel outer membrane proteins. OmpA and structurally homologous protein NspA, OprG and related protein, OmpW and OmpX. Images were made in PyMOL with the following files from the Protein data bank: 1G90 (OmpA), 1P4T (NspA), 2X27 (OprG), 2FLT (OmpW), 1QJ8 (OmpX). Protein domains of unknown structure, C terminus of OmpA represented as an oval shape.

Figure 2: Ribbon structure of peptidoglycan associated lipoprotein from *H. influenzae*. Image prepared in PyMOL using the 2aiz file from the Protein Data Bank. Lipid component shown as spheres.

Figure 3: Ribbon structure of N- terminus of OmpATb from *M. tuberculosis*. Image was made in PyMOL with using the 2KGS file from the Protein data bank.

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Figure 1

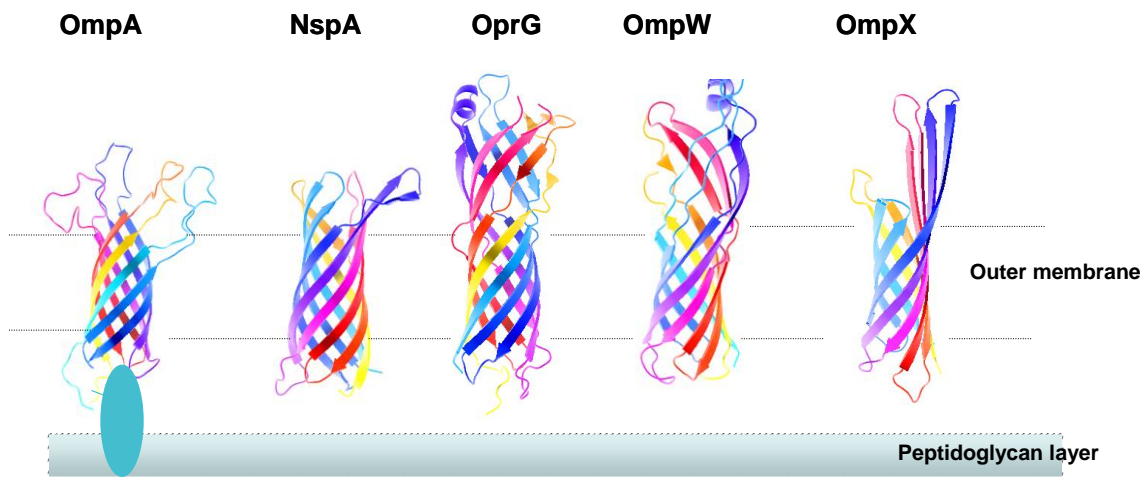


Figure 2:



Figure 3:

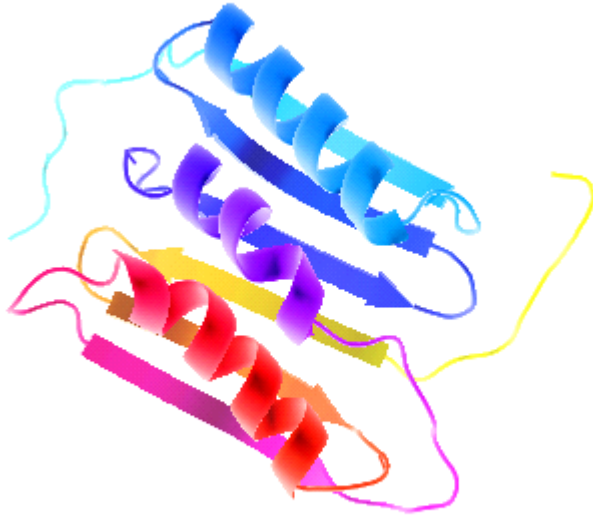


Table 1: Examples of eight-stranded β -barrel outer membrane proteins involved in host cell interactions.

<i>Family / name</i>	<i>Species/ strain</i>	<i>Host cell interaction</i>	<i>ref</i>
OmpA family			
OmpA	<i>E. coli (K-1)</i>	Attachment and invasion of human brain endothelial cells. Allows survival in macrophages and prevents maturation of dendritic cells.	[14, 27]
OmpA	<i>EHEC</i>	Attachment to Hela & Caco-2 cells	[18]
OmpA	<i>Uropathogenic E. coli</i>	Post invasion pathogenesis of cystitis, development of persistence, but not invasion.	[30]
OmpA	<i>C. sakazakii</i>	Invasion of human Caco-2 cells, microtubule condensation	[31, 32]
OmpA	<i>A. baumannii</i>	Induces cell death in epithelial cells and dendritic cells	[44]
PmOmpA	<i>P. multocida</i>	Attach to extracellular matrix components, bridging to host cells.	[6]
OmpA	<i>S. typhimurium</i>	Activates dendritic cells, drives polarised Th1 response	[50]
OmpA	<i>K. pneumoniae</i>	Binds to dendritic cells and macrophages	[34]
Oprf	<i>P. aeruginosa</i>	Interferon gamma, attachment to Caco-2 and glial cells	[25, 43]
Omp5	Non-typeable <i>H. influenzae</i>	Adheres to and upregulates ICAM	[17]
OmpW family			
OmpW	<i>V cholerae</i>	Intestinal colonisation	[54]
OmpW	<i>E. coli</i>	Antibiotic resistance	[66]
OmpW	<i>S. typhimurium</i>	Paraquat resistance	[60]
OprG	<i>P. aeruginosa</i>	OprG mutants less cytotoxic to lung cells	[53]
OmpX Family			
OmpX	<i>E. coli</i>	Not involved in adherence	[73]
OmpX	<i>C. sakazakii</i>	Invasion into gastrointestinal epithelial cells	[31]
Ail	<i>Y enterocolitica</i>	Host epithelial cell invasion	[78]
Ail	<i>Y. pseudotuberculosis</i>	Attachment to Caco-2 cells	[117]
Ail /OmpX	<i>Y. pestis</i>	Attachment to and invasion of epithelial cells, binding to fibronectin, facilitation of Yops delivery	[80, 81]
Pag C	<i>S. typhimurium</i>	Essential for virulence and survival in macrophages	[118]

Other OmpA like proteins

NspA	<i>N. meningitides</i>	Attachment to host lipids; Evasion of host immune system	[104, 105]
OmpATB	<i>M. tuberculosis</i>	Expressed only in pathogenic strains, enables survival in phagocytic vacuole.	[107]

Table 2: OmpA-like lipoproteins involved in pathogenesis.

<i>Lipoprotein</i>	<i>Species/ strain</i>	<i>Host cell interaction</i>	<i>reference</i>
PAL	<i>E. coli,</i>	Released in septic shock; activator of TLR-2 in macrophages, binds to myocardium	[87, 98]
PAL	<i>L. pneumophila</i>	Interacts with murine macrophage; activated TLR-2 mediated signalling	[100]
PAL	<i>H. ducreyi</i>	Essential for virulence	[101]
PAL	<i>A. actinomycetemcomitans</i>	Released during infection and stimulates cytokine production	[102]
Lipotoxin F	<i>P. aeruginosa</i>	Resistance to oxidative stress; adhesion to lung epithelial A459 cells	[108]
Loa22	<i>Leptospira interrogans</i>	Upregulated during host infection; virulence factor	[113, 115]