Review

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Phospholipase A in Gram-negative bacteria and its role in pathogenesis

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Phospholipase A (PLA) is one of the few enzymes present in the outer membrane of Gram-negative bacteria, and is likely to be involved in the membrane disruption processes that occur during host cell invasion. Both secreted and membrane-bound phospholipase A₂ activities have been described in bacteria, fungi and protozoa. Recently there have been increasing reports on the involvement of PLA in bacterial invasion and pathogenesis. This review highlights the latest findings on PLA as a virulence factor in Gram-negative bacteria.

Introduction

Phospholipases constitute a diverse subgroup of lipolytic enzymes that share the ability to hydrolyse one or more ester linkages in phospholipids, with phosphodiesterase as well as acyl hydrolase activity. The amphipathic nature of phospholipids creates obstacles for the enzymes, as the substrates are assembled into bilayers or micelles and are not present in significant amounts as a single soluble substrate. Although all phospholipases target phospholipids as substrates, they vary in the site of action on the phospholipid molecule, their function and mode of action, and their regulation (Waite, 1996). Phospholipases function in various roles, ranging from the digestion of nutrients to the formation of bioactive molecules. This diversity of function suggests that phospholipases are critical to life; the continual remodelling of cell membranes requires the action of one or more phospholipases. The most common phospholipids in mammalian cells are phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE). The plasma membrane of most eukaryotic cells contains predominantly PC and sphingomyelin in the outer leaflet, and PI, PE and PS in the inner leaflet. Therefore, an extracellular phospholipase would not be expected to lyse a target cell unless the outer-leaflet phospholipids were efficiently hydrolysed substrates of the enzyme (Schmiel & Miller, 1999). In contrast to mammalian cells, *Escherichia coli* membranes are composed of PE with some phosphatidylglycerol (PG), cardiolipin and trace amounts of PS.

Invasion of host cells by most pathogens requires penetration and damage of the cell membrane, which is mediated by either physical or enzymic means, or a combination of the two. Phospholipids and proteins represent the major chemical constituents of the host cell envelope. Therefore phospholipases are likely to be involved in the membrane disruption processes that often occur during host cell invasion (Waite, 1996), such as the pH-activated phospholipase A₂ of *Helicobacter pylori* (Berstad *et al.*, 2002).

Classification of phospholipases

The classification of phospholipases is based on their site of attack on the phospholipid. Qualifying letters, such as A, B, C and D, are used to differentiate the phospholipases and to indicate the specific bond targeted in the phospholipid molecule. Phospholipases A are acyl hydrolases classified according to their site of action upon the hydrolysis of the acyl ester bond. Phospholipase A₁ (PLA₁) hydrolyses the fatty acyl ester bond at the *sn*-1 position of the glycerol moiety, while phospholipase A₂ (PLA₂) removes the fatty acid at the *sn*-2 position of this molecule (Fig. 1a). The action of PLA₁ and PLA₂ results in the accumulation of free fatty acids and 2-acyl lysophospholipid or 1-acyl lysophospholipid, respectively. The fatty acid, which is still linked to the lysophospholipid, is, in turn, cleaved by other enzymes termed lysophospholipases (Fig. 1b). Some phospholipases hydrolyse both acyl groups and are termed phospholipases B; they also have high lysophospholipase activity.

Bacterial phospholipase A

Phospholipase A₁ (PLA₁). The phospholipases A₁ in general constitute a large group of 1-acyl hydrolases, some of which also degrade neutral lipids (lipases). PLA₁ has been reported to play an important role as a virulence factor in some bacteria and fungi. Schmiel *et al.* (1998) reported on the *yplA* gene, which encodes secreted PLA₁ activity, linked to lecithin-dependent haemolysis, in the human pathogen *Yersinia enterocolitica*. This *yplA* gene product has 74 % identity and 85 % similarity to the PLA found in *Serratia liquefaciens* (Givskov *et al.*, 1988). It was also reported that this PLA₁ activity plays a role in the pathogenesis of *Y. enterocolitica* and in the following inflammatory process observed in humans and mice, by promoting colonization and increasing the pathological changes resulting from the host inflammatory response (Schmiel *et al.*, 1998).

Fig. 1. Sites of action of various phospholipases. (a) A1 and A2, PLA₁ and PLA₂, respectively; B, PLB; C, PLC; D, PLD. (b) Lyso-PL and Lyso-PL transacylase (Ghannoum, 2000; reproduced with permission from the publisher: <http://www.asm.org/>).

In addition to its physiological roles, PLA₁ is of particular interest for industrial applications as it yields 2-acyl lysophospholipids, which are excellent emulsifiers suitable for use in the food technology, cosmetics and pharmaceutical industries (Song & Rhee, 2000). Secreted (extracellular) PLA₁ activity from *Serratia* sp. strain MK1 isolated from Korean soil was purified and characterized by Kim & Rhee (1996). The cloning and characterization of the *pldA*₁ gene, encoding a phospholipase with a predicted molecular mass of 33.4 kDa, revealed a significant similarity (around 70 %) to PLA₁ from *S. liquefaciens* and *Y. enterocolitica* (Givskov *et al.*, 1988; Schmiel *et al.*, 1998). Furthermore, improving the conditions for a large-scale production of a soluble and active enzyme from *Serratia* sp. MK1 in *E. coli*, which was further improved by evolutionary molecular engineering to increase the thermostability of the produced enzyme, has broadened the possibility of the application of this PLA₁ in various phospholipid-related industries (Song *et al.*, 1999; Song & Rhee, 2000).

Two phospholipases with PLA₁ activity have been purified from *E. coli* based on their differential sensitivity to treatment with detergents. The first is a detergent-resistant enzyme, of the outer membrane (OMPLA), with broad substrate specificity, hydrolysing all phospholipids and many neutral glycerides (Rock *et al.*, 1996). This enzyme is unusually resistant to inactivation by heat and ionic detergents and requires calcium for maximal activity. The second enzyme is a detergent-sensitive PLA₁ found as a soluble fraction on the cytoplasmic membrane. This cytoplasmic PLA₁, with a high degree of specificity for phosphatidylglycerol, can be inactivated by heat and by ionic detergents. This enzyme also acts as a transacylase and requires calcium for activity (Rock *et al.*, 1996).

Phospholipase A₂. The eukaryotic phospholipases A₂ (PLA₂) were the first of the phospholipases to be recognized. The pancreatic phospholipase has been known to degrade phosphatidylcholine since 1878, and at the turn of the century, cobra venom was shown by Keyes in 1902 to have haemolytic activity directed toward the membranes of erythrocytes (Waite, 1996). The family of eukaryotic

PLA₂ enzymes is diverse and has been shown to function in digestion of lipids, microbial degradation, inflammation, cell signalling and membrane remodelling. PLA₂ proteins are of great interest to the pharmaceutical industry since they are responsible for the release of arachidonic acid from membranes, and the subsequent conversion of this fatty acid to leukotrienes and prostaglandins is part of the inflammatory response (Farber *et al.*, 1999).

Secreted and membrane-bound PLA₂ activity has been described in bacteria, fungi and protozoa (Waite, 1996; Ghannoum, 2000; Matoba *et al.*, 2002). This activity is reported to be calcium-dependent and related to pathogenesis in most micro-organisms. The crystal structure and the tertiary structure of the secreted PLA₂ (EC 3.1.1.4) from *Streptomyces violaceoruber* A-2688 have been determined by NMR and X-ray analyses. Interestingly, the X-ray diffraction data of this PLA₂ indicated that although the tertiary structure of the microbial PLA₂ is strikingly different from that of the bovine pancreatic phospholipase, the internal motion, which is associated with the calcium binding, phospholipid binding and allosteric interfacial activation, is common to both (Matoba *et al.*, 2002; Matoba & Sugiyama, 2003).

Outer-membrane PLA in Gram-negative bacteria

Molecular and biochemical characteristics of the enzyme. Outer-membrane PLA (OMPLA; EC 3.1.1.32) is one of the few enzymes present in the outer membrane of Gram-negative bacteria. PLA and lysophospholipase activities were first reported in *E. coli* in 1971 and the membrane-bound PLA activity was isolated and purified later in the same year (Scandella & Kornberg, 1971; Doi *et al.*, 1972). OMPLA of *E. coli*, the most-studied protein among this group of phospholipases, is a 31 kDa protein encoded by the structural gene *pldA*; the amino acid sequence is composed of 269 amino acids residues preceded by a 20 amino acid signal sequence which targets the protein across the inner membrane. The gene is under the control of a constitutive promoter, resulting in the presence of 500 copies of the enzyme in the outer membrane of

normally growing cells (de Geus *et al.*, 1983; Homma *et al.*, 1984). This enzyme is strictly calcium dependent and displays broad substrate specificity. It hydrolyses the acyl ester bonds in phospholipids with PLA₁ and A₂ activities, and also has 1-acyl and 2-acyl lysophospholipase activity, as well as mono- and diacylglyceride lipase activities (Horrevoets *et al.*, 1989).

Brok *et al.* (1994) suggested that OMPLA is not required for the bacterial cell under laboratory conditions but it might be essential for growth in the natural environment. Kingma & Egmond (2002a) suggested a function for OMPLA as a housekeeping enzyme, indicating that the inhibition of OMPLA by its substrate provides the means to prevent this enzymic activity from being lethal to the cell once activated. Another speculative function of OMPLA is related to organic solvent tolerance in the bacterium; however, the role of OMPLA in this process needs to be clarified (Snijder & Dijkstra, 2000).

Merck *et al.* (1997) used genetic approaches to propose a model for the topology of OMPLA in *Salmonella typhimurium* based on the prediction of turns and loops and calculations of hydrophobicity and hydrophobic moment. In this model, OMPLA traverses the outer membrane 14 times as antiparallel amphipathic β -strands, thereby exposing hydrophilic loops to the cell surface, while the β -strands are connected by short turns on the periplasmic side. A similar β -barrel structure, but only involving 12 strands, was reported for OMPLA in *E. coli* by Brok *et al.* (1994).

Activation and functions of OMPLA. Although OMPLA of Gram-negative bacteria is an integral membrane protein that is embedded in its own substrate in the cell envelope, no enzymic activity can be detected in normally growing cells. Genetic regulation cannot explain the absence of enzymic activity because OMPLA is constitutively expressed, and the expressed protein is correctly transported to and inserted into the outer membrane, which is composed of phospholipids in the inner leaflet and of lipopolysaccharide (LPS) in the outer leaflet. As uncontrolled breakdown of the outer membrane would have lethal consequences for the cell, *in vivo*, OMPLA is present in the outer membrane as a monomeric protein in a dormant state. Hence, enzymic activity can only be induced after severe perturbation of the cell envelope integrity, which occurs during various processes such as phage-induced lysis, temperature shock and colicin secretion. Membrane-perturbing peptides, such as polymyxin B, melittin or cardiotoxin, can also activate the enzyme (Dekker, 2000; Snijder & Dijkstra, 2000).

Structural analysis of the OMPLA molecule. The 12- β -stranded architecture of *E. coli* OMPLA resembles the folds of other β -barrel outer-membrane proteins (OMPs) that have 8–22 strands (Brok *et al.*, 1994). The crystal structures of the monomeric and the inhibited dimeric enzymes were determined by Snijder *et al.* (1999); this

revealed that OMPLA monomers are folded into a 12-stranded antiparallel β -barrel with a convex side and a flat side with approximate dimensions of $20 \times 30 \times 45$ Å (Fig. 2). As reviewed by Dekker (2000), the β -strands are connected by long extracellular loops and short periplasmic turns. The interior of the β -barrel is hydrophilic, with extensive hydrogen-bonding networks and electrostatic interactions, resulting in a very stable structure. The β -barrel has a hydrophobic outer surface with which the protein is embedded in the outer membrane. The active site of the enzyme is located at the hydrophilic–hydrophobic boundary (apolar–polar boundary) of the outer leaflet of the outer membrane (Dekker, 2000).

Ser-144 and His-142 have been identified by chemical modification and by site-directed mutagenesis as essential residues in the active site, in an arrangement resembling the corresponding residues of a serine hydrolase catalytic triad. However, instead of the Asp or Glu that is normally present in the triad of serine hydrolases, a neutral asparagine (Asn-156) was found in OMPLA (Kingma *et al.*, 2000).

As reviewed by Snijder & Dijkstra (2000), dimeric and monomeric OMPLA hardly exhibit structural differences, as OMPLA dimers are formed by the association of two enzyme monomers interacting via the flat barrel side (Fig. 2). Dimerization creates two extended clefts along the subunit interface, which run down from the two active sites. Thus, although the monomer contains a catalytic triad, the monomeric enzyme form lacks catalytic activity due to

Fig. 2. 3D structure for the OMPLA molecule of *E. coli* in the active dimeric state (Snijder *et al.*, 1999; reproduced with permission from the publisher: <http://www.nature.com/>).

absence of substrate-binding pockets, which are only formed in the dimeric complex. The architecture of the catalytic site and substrate-binding clefts allows a broad range of substrates to bind; therefore, substrates with either one or two acyl chains of various lengths can be productively bound (Snijder & Dijkstra, 2000).

Dimerization as an essential step for OMPLA activity.

Snijder *et al.* (1999) proposed that bacterial phospholipids are only found in the inner leaflet of the outer membrane, where OMPLA is present in the inactive monomeric form, but that the enzyme will be activated when the bacterial membrane structure is disturbed. They suggested that the relief of lipid asymmetry and the presentation of substrate to the outer leaflet induces activation by promoting dimerization and calcium binding. Kingma & Egmond (2002b) constructed a well-defined covalent OMPLA dimer to study the importance of dimerization for activity regulation, both in a detergent system and after reconstitution in a phospholipid bilayer. This study revealed that a proper OMPLA dimer displays high affinity for calcium as OMPLA variants with an impaired dimeric interface also lacked high-affinity calcium binding. The structure of the detergent in crystals of OMPLA was determined by Matoba & Sugiyama (2003). Further research indicated that the enzyme's active site is positioned just outside the hydrophobic detergent zone; therefore, it is in a proper location to catalyse the hydrolysis of phospholipids in a natural membrane (Snijder *et al.*, 2003). Baaden *et al.* (2003) studied the possible differences in conformational dynamics that may be related to enzyme activation. They indicated that dimeric OMPLA is less flexible than monomeric OMPLA, especially around the active site, and also proposed that the increased stability of the active site in dimeric OMPLA is a consequence of the local ordering of water around the nearby calcium ion.

Role of calcium in activation of OMPLA. OMPLA has two calcium-binding sites per monomer: one high-affinity site (K_d 36 μ M) and a binding site with a 10-fold lower affinity (K_d 358 μ M) (Ubarretxena-Belandia *et al.*, 1998). As dimerization occurs at low calcium concentration, Snijder & Dijkstra (2000) suggested that the first calcium ion plays a role in dimer stabilization. However, in the colon, the normal habitat of *E. coli*, the calcium level exceeds the determined binding affinities for calcium to OMPLA. Therefore, it is unlikely that the requirement for the cofactor calcium is involved in the regulation of OMPLA activity *in vivo* (Dekker, 2000). One calcium-binding site of the OMPLA monomer is located approximately 10 Å away from the active site and is bound between loops L3 and L4 (L3L4 site). After dimerization a second calcium-binding site (catalytic site or interfacial site) at the dimer interface is present (Fig. 3). The calcium ion is ligated by the side chain of Ser-152 and by one main-chain carbonyl oxygen atom from each monomer (Snijder & Dijkstra, 2000; Dekker, 2000).

Crystallographic studies by Snijder *et al.* (2001) revealed that the L3L4 site was found only in the structure of monomeric OMPLA, whereas the interfacial site (catalytic site) was only found in the dimeric enzyme, with no calcium binding observed at the L3L4 site. This indicates that calcium binding occurs in the L3L4 site of OMPLA monomers. However, the calcium ion is displaced from this site when the dimers are formed, which indicates that calcium plays an important role in the formation of the active dimer from two inactive monomers. Furthermore, Snijder *et al.* (2001) suggested that the L3L4 site could serve as a sink for calcium ions, and that it might complex with the LPS matrix, thus maintaining the barrier function of the outer membrane. They also indicated that the key elements of this model (calcium binding and activation) are conserved in Gram-negative bacteria, suggesting that OMPLA architecture in this group is most likely the same, and that its activity is calcium- and dimerization-dependent. Several of the conserved residues are close in sequence, and form a complete and highly specific consensus sequence motif (YTQ-X_n-G-X₂-H-X-SNG), which proves to be much more specific than a motif based on the active-site residues alone as indicated in Fig. 4 (Snijder *et al.*, 2001).

Molecular analysis of OMPLA sequences encoded by the *pldA* gene in Gram-negative bacteria

Brok *et al.* (1994) compared the predicted primary structures of the *pldA* gene in *S. typhimurium*, *Klebsiella pneumoniae* and *Proteus vulgaris* with that of the *E. coli* *pldA* gene. This sequence analysis revealed a high degree of homology, with 79% of the amino acid residues being identical in all four proteins. Furthermore, enzymically active gene products were expressed when these *pldA* genes were cloned in *E. coli*. In a subsequent publication Brok *et al.* (1998) screened databases of whole-genome sequencing projects, which revealed the presence of proteins with homology to OMPLA in *H. pylori*, *Campylobacter jejuni*, *Yersinia pestis*, *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Comparison of OMPLA amino acid sequences in these bacteria also revealed that 30 amino acid residues are completely conserved, indicating an important physiological role of this enzyme in Gram-negative bacteria (Brok *et al.*, 1998). However, in a following study which involved sequence alignment of complete OMPLA sequences in 16 different species of Gram-negative bacteria, Snijder *et al.* (2001) indicated that only 20 amino acid residues are absolutely conserved and another 17 are similar in the sequences of *E. coli*, *Bordetella pertussis*, *Campylobacter coli*, *C. jejuni*, *Enterobacter agglomerans*, *H. pylori* 2669551, *H. pylori* J99, *Salmonella typhi*, *S. typhimurium*, *K. pneumoniae*, *N. gonorrhoeae*, *N. meningitidis*, *Pasteurella multocida*, *Proteus vulgaris*, *Y. pestis* and *Y. pseudotuberculosis*, therefore yielding a homology of only 13.5% (Fig. 4). The study indicated that the best-conserved residues are the active-site residues His-142 and Ser-144 and also the residues Asn-145 and Gly-146 which participate in the oxyanion-hole

Fig. 3. Diagrams for calcium-binding sites in monomeric and dimeric molecules of *E. coli* OMPLA by Kingma *et al.* (2002) (reproduced with permission from the publisher: <http://www.elsevier.com/>). (a) The structure of monomeric OMPLA (I), with a calcium ion bound between a carboxylate oxygen atom of Asp-149 in loop 3 and a carboxylate oxygen atom of Asp-184 in loop 4 (II). (b) Crystal structure of inhibited, dimeric OMPLA with one calcium ion per monomer bound at the dimer interface (I). The calcium ion is coordinated by the hydroxyl group of Ser-152 and the Ser-106 carbonyl oxygen atom of one monomer, the Arg-147 carbonyl oxygen atom of the other monomer, and three water molecules (II).

formation. Furthermore, the catalytic calcium site is conserved among all OMPLA sequences, as Ser-152, which ligates the calcium ion with its hydroxyl group, is absolutely conserved in all Gram-negative bacteria investigated. In contrast, the L3L4 calcium site is not absolutely conserved, as Asp-149 is replaced by an arginine residue in *Neisseria* species, and by a glycine residue in the *Helicobacter* strains. The second calcium ligand, Asp-184, is conserved in all except in *Helicobacter* strains, where it is replaced by a serine residue (Snijder *et al.*, 2001).

The biochemical relevance of OMPLA's calcium-binding sites was investigated in *E. coli* using site-directed mutagenesis (Kingma *et al.*, 2002). The study concluded that the interfacial calcium site is essential for both catalysis and calcium-dependent dimerization, whereas the L3L4 site is redundant *in vitro*. These results are in accordance with sequence homology noted for several OMPLAs by Snijder *et al.* (2001), which revealed absolute conservation of Ser-152, whereas Asp-149 is not conserved in *Neisseria* species, and neither Asp-149 nor Asp-184 is conserved in *H. pylori*.

and *B. pertussis*. However, although OMPLA in *H. pylori* lacks the L3L4 calcium site, its activity still depends on the cofactor calcium (Kingma *et al.*, 2002). The lack of the L3L4 calcium site may be related to differences in outer-membrane composition compared to that of *E. coli*. In *Neisseria* spp., phospholipid molecules are present in the outer leaflet of the membrane, while the outer membrane of *H. pylori* may contain substantial amounts of lysophospholipids. Alternatively, those OMPLAs, which are least similar to the *E. coli* OMPLA, may still have an L3L4 site that is obscured by sequence differences and insertions (Snijder *et al.*, 2001).

Relationship between bacterial phospholipases and pathogenesis

Phospholipase activity has been linked to pathogenesis in bacterial species which cause different disease syndromes such as the massive tissue destruction related to gas gangrene, skin and lung infections caused by *Pseudomonas aeruginosa*, and food-borne listeriosis (Schmiel & Miller, 1999). However, although the importance of some bacterial phospholipases in pathogenesis is certain, the exact mechanism of the phospholipase action *in vivo* has not been definitively determined. Phospholipase toxicity has been linked to cytolytic activity resulting from the accumulation of membrane-destabilizing products or by the extensive destruction of membrane phospholipids. Cytolysis can be caused directly by a very active bacterial phospholipase with broad specificity or in concert with host degradative enzymes induced by the bacterial phospholipase. The cytolytic properties of the phospholipase are dependent on its ability to interact directly with and hydrolyse phospholipids in the membrane. Frequently, for convenience the lytic activity is measured as haemolysis, regardless of the likelihood of a particular pathogen causing systemic infection or a bacterial phospholipase entering the bloodstream (Schmiel & Miller, 1999). Although phospholipases C (PLCs) are the best-studied bacterial phospholipases as virulence factors, various studies have indicated that many bacterial outer-membrane or secreted phospholipases are cytolytic and therefore responsible for tissue damage during infection. Examples include PLA₁, A₂ and C activities in *Helicobacter pylori*, which were linked to the degradation of the phospholipid components of the mucosal barrier, and the secreted PLA₁ activity of the human pathogen *Y. enterocolitica* (Nilius & Malfertheiner, 1996; Songer, 1997; Schmiel *et al.*, 1998).

Phospholipase A as a virulence determinant in Gram-negative bacteria

***Helicobacter pylori*.** *H. pylori* possesses several different phospholipase activities, such as PLA₁, PLA₂ and PLC, which are linked to the degradation of the phospholipid components of the mucosal barrier. In addition to direct disruption of the mucosal barrier, phospholipases are also able to initiate production of leukotrienes and other eicosanoids, converted from arachidonic acid, which could also affect membrane permeability and mucous discharge (Nilius & Malfertheiner, 1996). The *H. pylori* 26695 and J99 genome sequences revealed the presence of an open reading frame (HP0499) encoding a putative protein of around 42.5 kDa with homology (54.1 % similarity) to *E. coli* OMPLA; the gene was confirmed to encode an active phospholipase with lecithinase, PLA₂, and haemolytic activities (Dorrell *et al.*, 1999). The researchers constructed an isogenic *pldA* mutant in *H. pylori*, which showed a marked reduction in PLA₂ and haemolytic activities and, unlike the wild-type, was unable to colonize mice at 2 and 8 weeks. Although the ability of the mutant to adhere to human gastric adenocarcinoma cells was unaffected, it did induce a significant immune response, indicating a role for PLA in colonization of the gastric mucosa and possible tissue damage after colonization (Dorrell *et al.*, 1999). These findings were confirmed by Zhang *et al.* (2002); they indicated that mutations in genes encoding the urease (*ureB*) and phospholipase (*pldA*) enzymes did not affect adherence of the bacteria to gastric epithelial cell lines. However, adherence to gastric epithelial cells *in vitro* does not imply that the mutant can successfully colonize *in vivo*.

Xerry & Owen (2001), in a study to determine the degree of variation in the PLA gene in *H. pylori* isolates, found that the *pldA* gene is a highly conserved feature of the *H. pylori* genome irrespective of the geographical origin of the isolate. The study also investigated links between *pldA* genotype and clinical disease severity, as well as with variation in *cagA* status and *vacA* genotypes. It was concluded that *pldA* is a conserved feature of the *H. pylori* genome, with isolates of the same PCR-RFLP genotype having a widespread geographical distribution. The study also supported the theory that the role for PLA activity in *H. pylori* is in colonization and persistence rather than in chronicity of infection (Xerry & Owen, 2001).

H. pylori can spontaneously and reversibly change its membrane lipid composition, under slightly acidic conditions,

Fig. 4. Sequence alignment of *E. coli* OMPLA and 15 homologous enzymes from other Gram-negative bacteria highlighting the conserved residues in the active site, with the highly specific consensus sequence motif (YTQ-X_n-G-X₂-H-X-SNG) by Snijder *et al.* (2001) (reproduced with permission from the publisher: <http://www.elsevier.com/>). Residues involved in calcium binding are indicated above the alignment by open circles for the L3L4 site and black circles for the catalytic site. The active-site residues are indicated by the black arrowheads below the sequences. Strictly conserved residues are shown in black boxes, homologous residues in grey boxes. Residues involved in substrate binding and dimerization are indicated by the lower-case letters below the alignment: substrate binding (s), dimerization (d), and both substrate binding and dimerization (b).

from the 'normal' L-variant, with less than 2 % lysophospholipids, to the 'lyso' S-variant, with over 50 % lysophospholipids in the membrane (Bukholm *et al.*, 1997). The 'lyso' variant is more haemolytic, with enhanced affinity for epithelial cells and increased release of urease and VacA toxin. The deletion of the *pldA* gene in the S-variant restored the normal phenotype, suggesting that the L- to S- transition is due to the activation of OMPLA (Bukholm *et al.*, 1997). In an extension of this study Tannaes *et al.* (2001) indicated that the change in membrane lipid composition is actually due to phase variation in the *pldA* gene. They showed that a change in the C-tract length of this gene results in reversible frame-shifts, translation of a full-length or truncated *pldA*, and the production of active or inactive OMPLA. Furthermore, growth at low pH resulted in a selection of the best-adapted (S) phenotype, with an almost complete conversion of the normal L-variant (C7, OMPLA 'off') to the lyso S-variant (C8, OMPLA 'on'). The observed difference in colony morphology could not be explained by an LPS variation; therefore it was concluded that the *pldA* gene might contribute to survival of *H. pylori* at low pH (Tannaes *et al.*, 2001). More recently, Tannaes *et al.* (2005) investigated the significance of phase variation in the *pldA* gene of *H. pylori* and its association with peptic ulcer disease. The study suggested that OMPLA activity is a virulence factor in *H. pylori*, as isolates with high OMPLA activity are significantly associated with patients suffering from peptic ulcer. The study also suggested a two-step regulation of OMPLA in *H. pylori*: (i) phase variation in the homopolymorphic tract of the *pldA* gene from 'off' to 'on' and (ii) protein level regulation among the *pldA* 'on' bacterial cells (Tannaes *et al.*, 2005). Salaun *et al.* (2005) investigated 31 phase-variable genes in *H. pylori* following murine gastric colonization for up to one year in three unrelated *H. pylori* strains. They concluded that genes which are most likely to be associated with immediate niche fitting, including the *pldA* gene and LPS biosynthesis genes, changed more rapidly than other surface-protein genes which may be under control of the adaptive immune system. These findings suggest a role for these genes in determining initial fitness for colonization of *H. pylori* as well as in subsequent niche adaptation (Salaun *et al.*, 2005). Moreover, Berstad *et al.* (2002) suggested that the pH-activated phospholipase A₂ in *H. pylori* acts as an initial mucosal barrier breaker and is considered to have a pivotal role in the pathogenesis of peptic ulcer. The research also indicated that the gastric juice of *H. pylori*-infected individuals contains significantly higher PLA₂ and lyso-PC concentrations than that of healthy individuals (Berstad *et al.*, 2002).

***Neisseria* spp.** The presence of outer-membrane-associated phospholipase activity in *N. gonorrhoeae* was first reported by Wolf-Watz *et al.* (1975). Later Senff *et al.* (1976) reported that this activity was calcium dependent, with a pH optimum of 8.0 to 8.5, and was sensitive to detergents, such as 0.1 % Triton X-100. The presence of a putative *pldA* gene was also demonstrated in the genome sequences of the human pathogens *N. meningitidis* and *N.*

gonorrhoeae, indicating that the deduced meningococcal and gonococcal OMPLA proteins differ in only a few amino acid residues (Brok *et al.*, 1998). Recently, Bos *et al.* (2005) studied the presence and conservation of OMPLA among meningococcal strains and tested its immunogenicity in an animal model. The vaccine potential of OMPLA in this human pathogen was also investigated, as a high level of conservation of OMPLA is maintained among meningococcal strains. However, the study showed that OMPLA is expressed *in vitro* by both pathogenic and non-pathogenic neisserial strains. The investigators demonstrated that OMPLA functions as an autolysin that acts under specific conditions, such as prolonged growth of the bacteria. The vaccine potential of the protein was investigated by immunizing mice with *in vitro* refolded, recombinant OMPLA. Although high levels of antibody to the OMPLA were obtained, the murine sera were neither bactericidal nor protective. Also, convalescent patients and vaccine sera did not contain detectable levels of anti-OMPLA antibodies, indicating that OMPLA may not be sufficiently immunogenic to be included in a meningococcal vaccine (Bos *et al.*, 2005).

***Yersinia* spp.** *Y. enterocolitica* produces a virulence-associated PLA₂ (YpLA) that is secreted via its flagellar type III secretion apparatus (Young *et al.*, 1999). YpLA appears to be closely related to PhLA of *S. liquefaciens*. However, initial reports suggested that the native form of YpLA was 59 amino acids shorter than PhLA (Schmiel *et al.*, 2000). The role of YpLA in *Y. enterocolitica* pathogenesis has not been fully described, but this enzyme is proposed to contribute to increased inflammation in mesenteric lymph nodes during *Yersinia* infection (Schmiel *et al.*, 1998). The *pldA* gene in *Y. pseudotuberculosis* encodes a phospholipase that is active towards both phosphatidylcholine and sphingomyelin, which are the major phospholipid components of the outer leaflet of eukaryotic cell membranes. The determined 1468 bp sequence, including the *pldA* gene with flanking regions, was found to be 100 % identical to the corresponding sequence of *Y. pestis*, the causative agent of human plague (Karlyshev *et al.*, 2001). Moreover, the researchers indicated that a PldA mutant strain of *Y. pseudotuberculosis* exhibited reduced phospholipase activity compared to the wild-type strain and confirmed *in vivo* attenuation of the mutant, therefore suggesting that PldA is an essential virulence factor in the murine yersiniosis model of infection (Karlyshev *et al.*, 2001).

***Legionella* spp.** Two PLA activities secreted by several *Legionella* spp. were considered as possible powerful agents of legionellae in causing lung disease due to destruction of lung surfactant and epithelial cells (Flieger *et al.*, 2000). *L. pneumophila* secretes, via its type II secretion system, PLA activities that are distinguished by their specificity for certain phospholipids. However, the timing of PLA and lysophospholipase A secretion in *L. pneumophila* differed as compared to other bacterial species. PLA activity

was secreted prior to lysophospholipase A activity in *L. pneumophila*, which may lead to an accumulation of the cytotoxic agent (Flieger *et al.*, 2001). The *L. pneumophila* *plaA* gene, encoding a 309 amino acid protein (PlaA) that cleaves fatty acids from lysophospholipids, was identified and characterized by Flieger *et al.* (2002). Overexpression of *plaA* completely protected *L. pneumophila* from the toxic effects of lysophosphatidylcholine, suggesting a role for PlaA in bacterial detoxification of lysophospholipids. By screening a genomic library of *L. pneumophila*, Flieger *et al.* (2004) found an additional *L. pneumophila* gene, *plaB*, which encoded a cell-associated haemolytic activity and contained a lipase consensus motif in its deduced protein sequence. *L. pneumophila* PlaB shares several properties with the OMPLA of various enteropathogenic bacteria, including *E. coli* and *S. typhimurium*, and other Gram-negative bacteria, including *H. pylori* or *N. meningitidis*. Flieger *et al.* (2004) generated isogenic *L. pneumophila* *plaB* mutants and tested them for haemolysis, lipolytic activities and intracellular survival in amoebae and macrophages. Compared to the wild-type, the *plaB* mutant showed reduced haemolysis of human red blood cells and almost completely lost its cell-associated lipolytic activity. Therefore it was concluded that *L. pneumophila* *plaB* is the gene encoding the major cell-associated PLA, possibly contributing to bacterial cytotoxicity due to its haemolytic activity. On the other hand, in view of the fact that the *plaB* mutant multiplied like the wild-type both in U937 macrophages and in *Acanthamoeba castellanii* amoebae, *plaB* is not essential for intracellular survival of the pathogen (Flieger *et al.*, 2004).

Campylobacter spp. OMPLA is considered to be a major haemolytic factor in *C. coli*. Grant *et al.* (1997) identified a *pldA* gene upstream of an operon encoding an enterochelin transport system in *C. coli*. The *pldA* gene product

is a 35 kDa protein with significant similarity to the *E. coli* OMPLA. The recombinant protein displayed calcium-dependent lysophospholipase and phospholipase activities *in vitro*, indicating similar expression and recognition of this gene product by *E. coli*. Furthermore, a constructed *pldA* mutant in *C. coli* showed reduced haemolytic activity compared to the wild-type strain, indicating a role for PLA in the lysis of erythrocytes and suggesting a possible role for PLA in virulence of *Campylobacter* species. A *pldA* gene sequence was also reported for *C. jejuni*, encoding a putative PLA protein with significant similarity to PLA in *C. coli* (Brok *et al.*, 1998). Istivan *et al.* (2004) reported a membrane-bound haemolytic PLA activity in *Campylobacter concisus* strains isolated from gastroenteritis cases in children and also in two *C. concisus* reference strains, ATCC 51561 and ATCC 51562. The cell-associated PLA was extracted and partially purified and was found to be a heat-stable, calcium-dependent PLA with both haemolytic and cytolytic effects on different types of erythrocytes and on tissue culture cell lines respectively (Istivan *et al.*, 2004). The sequence of the *pldA* gene in *C. concisus* strain RCH3 (GenBank accession no. AJ786391) was analysed, and the translated 297 amino acid sequence for this gene product (Q6A175) has a predicted molecular mass of 35.076 kDa (www.ebi.uniprot.org/uniprot-srv). A BLAST search performed on Q6A175 showed that it has 97% identity to the amino acid sequence for PLA in *C. coli* (O32349) and 76% identity to PLA of *C. jejuni* (Q9PMU8), while it has only 34% identity to the OMPLA sequence of *E. coli* (Q8CVJ6) (Fig. 5). A three-dimensional structure for the monomeric form of OMPLA in *C. concisus* and *C. coli* showing the high similarity between the beta-barrel molecules in these two species is illustrated in Fig. 6. The β -barrel structure of OMPLA in *Campylobacter* spp. has a significant resemblance to the structure of OMPLA protein molecules of other enteric Gram-negative bacteria.

<i>C. concisus</i>	1	MQIYKKLALQNSPKQDLKENNASLLSSIKHETQNTQKTPSTKEDFSRIALANYLGENSESF
<i>C. coli</i>	1	MQIYKKLALQNSPKQDLKENNASLLSSRIKHETQNTQKTPSTKEDFSRIALANYLGENSESF
<i>C. jejuni</i>	1	MEIYKKALKNSSVLISQEQNNSSQATQTONSITIKKE--EKQDFSRLALANYLGENSESF
<i>E. coli</i>	1	--MRTLQGWLLFVFMFMAVYAQEAIVKEVHDAPAVR-----G--SIANMLQEHDD--
<i>C. concisus</i>	61	NPLGISSYKMNYPFLPFAYSFG-----SLGGENRKTEMKFQLSIKKRLFEDLLGLGEK
<i>C. coli</i>	61	NPLGISSYKMNYPFLPFAYSFG-----SLGGENRKTEMKFQLSIKKRLFEDLLGLGEK
<i>C. jejuni</i>	59	NPLGISSYKMNYPFLPFAYSFN-----SLGVNNKSEAKFQLSVKKRLFENLLGLDEK
<i>E. coli</i>	48	NPFTLYSYDNTNLIYTTQTSDLNKEAIASYDWAENARKDEVKQFQLSIAFLPWRGILGPNSV
<i>C. concisus</i>	113	YYVGYYTQTSWQNN--YKHSSPFRETNYQPEFFVDIPLHFEDYKFLN-NLRVGILHESNGK
<i>C. coli</i>	113	YYVGYYTQTSWQNN--YKHSSPFRETNYQPEFFVDIPLHFEDYKFLN-NLRVGILHESNGK
<i>C. jejuni</i>	111	YYIAYTQTSWQNI--YEHSSPFRETNYQPEFFIDLPYLKDYEFFN-NLRVGILHESNGK
<i>E. coli</i>	108	LGASVYTSKSWQLNSNEESSPFRETNYEQPLFLGFATDYRFAGWTILRDVEMGYNHDSNGR
<i>C. concisus</i>	170	GDENLESRSWNRIYASSVFLYQRFIFPRIWYRIPENSKDDDNPEITHYMGNFNDINIGYL
<i>C. coli</i>	170	GDENLESRSWNRIYASSVFLYQRFIFPRIWYRIPENSEDDDNPEITHYMGNFNDINIGSL
<i>C. jejuni</i>	168	GDENLQSRWNRIYVSTAILYNKFLFVPRWYRIPENKDDDNPAILHYMGNFNDVNLAYL
<i>E. coli</i>	168	SDP--TSRSWNRIYTRIMAEANGNWLVEVKEFWYVVG---NTDDNPDITKMGYQLKIGYH
<i>C. concisus</i>	230	GNDYFINLMLRNLDLFDHDKGAVQVDIGYDIFDNGIYWYLQYFNGYGESLIDYNKRLQRL
<i>C. coli</i>	230	GNDYFINLMLRNLDLFDHDKGAVQVDIGYDIFDNGIYWYLQYFNGYGESLIDYNKRLQRL
<i>C. jejuni</i>	228	GDDYFINLMLRNLDLFDHDKGAVQVDIGYDIFDNGIYWYLQYFNGYGESLIDYNKHLQRL
<i>E. coli</i>	223	LGDAVLAKG--QYNWNTGYGGAELGLSYPIETH-VRLYTQVSYSGYGESLIDYNFNQTRV
<i>C. concisus</i>	290	STGFLISY--
<i>C. coli</i>	290	STAFILISY--
<i>C. jejuni</i>	288	STGFLISY--
<i>E. coli</i>	280	GVGVMLNDLF

Fig. 5. Alignment and analysis of OMPLA sequences in *C. concisus*, *C. coli*, *C. jejuni* and *E. coli*. The green colour indicates the strictly conserved residues among *pldA* gene sequences of Gram-negative bacteria. The black arrowheads indicate the active sites in OMPLA sequences. The grey highlighted region indicates the consensus sequence motif for this family of phospholipases (YTQ-X_n-G-X₂-H-X-SNG).

Fig. 6. The β -barrel protein molecule structure for a PLA monomer of *C. concisus* (left), and *C. coli* (right). The 3D protein structure for the PLA molecule was constructed using the 3-D JIGSAW Protein comparative modelling server. RasTop 1.3 by Philippe Valadon (2000) and RasMol molecular graphics programs were used for visualization and to create the 3D representation of the PLA molecule.

The genome sequence of *C. jejuni* NCTC 11168 (Parkhill *et al.*, 2000) revealed the presence of a *pldA* gene sequence (Cj1351) upstream of the *ceuB* and *ceuC* genes, similar to the position of the *pldA* gene in the *C. coli* genome (Grant *et al.*, 1997) and in the *C. concisus* genome (T. S. Istivan, unpublished data). The ability of *pldA* mutant strains in *C. jejuni* to serve as viable bacterial vaccines against caecal colonization was investigated by Ziprin *et al.* (2001). They found that *C. jejuni* strains containing mutations in the *ciaB* and *pldA* genes were extremely poor colonizers, and were avirulent as a result of their impaired ability to colonize the chicken caecum. However, although the mutants proved avirulent, inoculation with these strains did not induce biologically significant resistance against subsequent challenge with the parental strain; this was in contrast to the *H. pylori* *pldA* mutant, which was unable to colonize mice but did induce a significant immune response (Dorrell *et al.*, 1999; Ziprin *et al.*, 2001).

Conclusion

Bacterial PLA is a key factor in bacterial-induced haemolysis. It is likely that this activity provides a favourable source of nutrients for bacterial growth in addition to providing a suitable environment for replication and survival. The *pldA* gene encoding OMPLA is highly conserved in Gram-negative bacteria, suggesting that it has a significant role in bacterial survival. However, the role of PLA in bacterial virulence is yet to be fully elucidated. In some species, such as *C. jejuni*, *pldA* gene mutants with no haemolytic activity are poor colonizers but do not provide protection against colonization with wild-type bacteria, suggesting that OMPLA may play a role in colonization but not necessarily in virulence. Conversely for *H. pylori*, a *PldA* mutant did not colonize mice but did offer some protection against challenge, suggesting that for this highly specialized pathogen, in a unique niche, OMPLA is important in inducing pathogenesis.

Many Gram-negative bacteria possess OMPLA activity, including most of the species that are effective colonizers of mammals and birds. The presence of OMPLA enables the

bacteria to modify the environment, often the intestine or respiratory tract, to enhance their survival *in vivo*. However, further research is required to identify the role of PLA in the pathogenesis and survival of Gram-negative bacteria.

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