Introduction

Over the millennia infectious disease has played a major role in determining the outcome of armed conflicts. Disease might be contracted as a consequence of the deployment of troops in areas of the world where it is endemic, as a consequence of traumatic injuries sustained on the battlefield or as a consequence of the use of micro-organisms as biological weapons. Vaccination against these diseases is one important element of a strategy to limit their impact. For some infectious diseases vaccines are commercially available; for others they are not. When vaccines are not available it may be necessary for government agencies to invest in research programmes to develop and licence them. This is the current situation for many vaccines against biological warfare agents. The duration of research and development programmes to devise these vaccines is typically 10–15 years, and they must meet the same regulatory standards as public health vaccines. Although these vaccines are developed to meet the needs of the armed forces, they will often also have uses as niche-market vaccines for the protection of public health.

Clostridium perfringens and disease

Clostridium perfringens is a spore-forming Gram-positive anaerobe which is invariably found where decaying organic matter is present. The bacterium is found in virtually all soil types and in the gut of almost all animal species, and this has led some to suggest that it is the most widely distributed pathogen known (Songer, 2002). The ability of the bacterium to cause disease is ascribed mainly to the production of a range of potent extracellular protein toxins. The so-called major toxins (α-, β-, ε- and t-toxins) are not necessarily produced in large quantities, but the differential production of these toxins is used to assign strains into one of five biotypes. These biotypes are associated with different diseases of humans and animals (Table 1).

Biotype A strains have historically been of interest to the UK Ministry of Defence because they have been associated with the high incidence of gas gangrene on battlefields. For example, during World War I and during the North Africa campaign of World War II, 0·36% and 0·32%, respectively of all casualties developed the disease. Fifty per cent of those who developed gas gangrene during the North Africa campaign died (MacLennan, 1962). Nowadays, battlefield gas gangrene is not a major cause for concern. However, there is evidence that Iraq has considered C. perfringens (or its toxins) to be a possible biological weapon (Bowman, 1998; Cordesman, 1998) and therefore this pathogen, and its toxins, remain of concern.

Gas gangrene is also a disease which occurs in the civilian community, and the elderly, diabetics and accident victims are all especially susceptible to the disease. It is difficult to get good estimates of the incidence of disease, but bearing in mind that the proportion of the elderly and diabetics is increasing, it seems likely that the incidence of gas gangrene will increase in future years.

The pathogenesis of gas gangrene

Gas gangrene is a disease which usually affects muscle, and is often referred to as clostridial myonecrosis. The disease is associated with two events. Firstly, bacteria must be introduced into soft tissues, often following a traumatic injury which involves the ingress of soil or other organic matter containing C. perfringens. Secondly, because the bacterium is an anaerobe, the blood supply to the infected tissues must be impaired in some way. Often, these events occur simultaneously. For example, severe traumatic injuries on the battlefield can result in local damage to the circulatory system as well as allowing the ingress of bacteria. Assuming that these events do occur, then there may be local growth of the bacteria. At this stage the infection is relatively self-limiting, because it is delineated by healthy oxygenated tissues. However, the bacteria can spread into these surrounding tissues if the blood supply into these tissues is reduced and if the ability of the host to mount an inflammatory response involving phagocytes is suppressed. When the infection does develop in this way, then fulminant gas gangrene is the result. Now the infection spreads rapidly from diseased to healthy tissues, and an
entire limb may become gangrenous within the space of a few hours (McNee & Dunn, 1917). At this stage amputation of the infected limb or removal of all of the infected tissues is the only effective treatment. Without this, the toxins produced by the bacteria rapidly kill the patient.

Although all strains of *C. perfringens* produce α-toxin (it is actually diagnostic for *C. perfringens*), type A strains produce this protein in especially large amounts. Since the 1940s it had been suspected that α-toxin was a major virulence determinant of gas gangrene caused by *C. perfringens* (MacFarlane, 1955; MacLennan & MacFarlane, 1944). However, many of the studies to investigate the properties of the toxin used protein which was purified from *C. perfringens* culture supernatant fluid. This approach is not without its limitations. Firstly, the purification of the toxin is not a simple task, and secondly, it is extremely difficult to remove all traces of the other (minor) toxins. This is of especial concern because the minor toxins are not necessarily produced in small quantities, nor do they necessarily have low toxicities. Rather, they are termed minor toxins because they are not used to type *C. perfringens* strains. Therefore, it is difficult to be certain that the properties previously ascribed to α-toxin are not really due to any one of the minor toxins.

A major step forwards in resolving the question of the role of α-toxin in disease came from Julian Rood’s laboratory in the mid 1990s. An allelic replacement mutant in a virulent type A strain was constructed and tested in the murine model of gas gangrene (Awad et al., 1995). The mutant showed almost complete loss of virulence. In addition, the characteristic signs of gas gangrene in the mouse (foot swelling, foot blackening and muscle necrosis) were almost completely absent in mice challenged with the α-toxin mutant (Awad et al., 1995). The reintroduction of the gene encoding α-toxin into the mutant restored the virulence properties of the wild-type, fulfilling molecular Koch’s postulates. Overall this work confirmed for the first time that α-toxin is the major virulence determinant in gas gangrene.

**The effect of α-toxin on host cells**

To resolve the problems associated with the isolation of pure α-toxin from *C. perfringens*, the encoding gene has been cloned and overexpressed in *Escherichia coli* (Basak et al., 1994; Titball et al., 1989). Although α-toxin accumulates in the periplasmic space, this does not appear to be detrimental to the bacteria. The protein is relatively easily isolated and purified using column chromatography (Basak et al., 1994). Purified recombinant α-toxin has been used for many of the studies on the mode of action of the toxin, and for structural biology studies, over the past decade.

The α-toxin is a phospholipase C and is active towards phospholipids which are in micellar or monodispersed forms. This activity can be measured relatively simply in the laboratory. However, most phospholipid in the host is present in cell membranes, rather than in micellar or monodispersed forms. Incubation of cells with suitably high concentrations of α-toxin does result in cell lysis as a consequence of extensive damage to the membrane, and this can easily be measured. One simple assay involves measuring the lysis of erythrocytes (Titball et al., 1989). However, such crude indicators of the interaction with host cells may not fully indicate the nature of the interaction with α-toxin. For example, sublytic quantities of the toxin have been shown to activate the arachidonic acid cascade in a range of cell types (Fuji & Sakurai, 1989; Gustafson & Tagesson, 1990). The end-products of this cascade include prostaglandins, thromboxanes and leukotrienes (Samuelsson, 1983). These compounds play important roles in regulating inflammatory processes. However, the production of thromboxanes is known to be associated with platelet aggregation. The aggregation of platelets does occur after the administration of α-toxin (Fuji et al., 1986; Ohsaka et al., 1978; Sugahara et al., 1977), and this may well be due to the activation of the arachidonic acid cascade in these cells. The formation of platelet aggregates occludes blood vessels (Bryant et al., 2000b) and appears to be responsible for the steep decline in blood flow to tissues (Bryant et al., 2000a). It is possible to envisage the situation where α-toxin diffuses away from the initial site of infection into adjacent healthy tissues, and the resultant reduction in blood supply to these tissues then provides the appropriate conditions for the spread of the infection into these tissues.

The spread of the infection in this way is also dependent on the suppression of the host inflammatory response. Remarkably, as long ago as 1917 two military surgeons who had dealt with cases of gas gangrene on the battlefield noted that ‘leucocytes are generally conspicuous by their absence in the muscular tissue involved’ (McNee & Dunn, 1917). At this stage amputation of the infected limb or removal of all of the infected tissues is the only effective treatment. Without this, the toxins produced by the bacteria rapidly kill the patient.

**Table 1. Association of *C. perfringens* biotypes with diseases of humans and animals**

<table>
<thead>
<tr>
<th>Biotype</th>
<th>Major toxins produced</th>
<th>Disease association</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>α</td>
<td>Gas gangrene of humans and animals, fowl and porcine necrotic enteritis, bovine and ovine enterotoxaemia, food poisoning in man, colitis in horses, canine haemorrhagic gastroenteritis</td>
</tr>
<tr>
<td>B</td>
<td>α, β, ε</td>
<td>Lamb dysentery, enterotoxaemia of foals, sheep, goats</td>
</tr>
<tr>
<td>C</td>
<td>α, β</td>
<td>Pig-bel (necrotic enteritis) in man, enterotoxaemia of sheep (struck), calves, lambs, piglets</td>
</tr>
<tr>
<td>D</td>
<td>α, ε</td>
<td>Enterotoxaemia of lambs and sheep (pulpy kidney), goats, cattle (human)</td>
</tr>
<tr>
<td>E</td>
<td>α, i</td>
<td>Rabbit enteritis, enterotoxaemia of calves and lambs</td>
</tr>
</tbody>
</table>
More recently, in mice, it has been shown that tissues surrounding the site of challenge with wild-type *C. perfringens* are devoid of neutrophils whereas after challenge with an χ-toxin mutant the expected influx of phagocytes occurs (Awad et al., 1995). Additional studies have shown that after the administration of χ-toxin, neutrophils accumulate along the walls of blood vessels rather than migrating into tissue spaces (Bryant et al., 2000a). Collectively, these observations clearly point towards a role for χ-toxin in modulating the host immune response.

Damage to the circulatory system and the consequential reduction in blood supply to tissues might also enhance the susceptibility of host cells to χ-toxin in a completely unexpected way. Mammalian cells grown under low-oxygen or low-glucose conditions become deficient in UDP-glucose, and UDP-glucose deficiency has been reported in the skeletal muscle of diabetic animals (Flores-Diaz et al., 1998). A mutant fibroblast cell line, deficient in UDP-glucose, has been shown to be 10-fold more sensitive to χ-toxin (Flores-Diaz et al., 1998). However, it is not clear at this stage why UDP-glucose-depleted cells show this enhanced susceptibility to the toxin. Whatever the molecular mechanism, the conditions found in the muscle tissues of some diabetics or in muscle tissues following a traumatic injury might enhance the effect of the toxin on host cells.

**The molecular basis of toxicity**

Although the χ-toxin is a phospholipase C, there are many non-toxic phospholipase enzymes produced by other bacteria. An emerging theme is that toxicity can be ascribed to the ability of the enzyme to interact with membrane phospholipids, thereby perturbing host cell metabolism and promoting the effects outlined above which allow the spread of the bacteria into otherwise healthy tissues. To fully understand the molecular basis of toxicity the crystal structure of χ-toxin has been determined (Naylor et al., 1998). This reveals a two-domain protein (Fig. 1). The χ-helical amino-terminal domain contains three zinc ions, located within a cleft. These zinc ions appear to play roles both in stabilizing the structure of the protein and in the phospholipase C catalytic activity (Krug & Kent, 1984; Titball & Rubidge, 1990). There are several pieces of evidence indicating that this cleft is the phospholipase C active site. Firstly, when the surface of the protein is modelled, a phospholipid molecule can be accommodated within the charged cleft (Naylor et al., 1998). More importantly, when recombinant amino-terminal domain is produced in *E. coli*, the purified polypeptide retains phospholipase C activity (Titball et al., 1991). However, the amino-terminal domain lacks the toxicity and the cytolytic activity of the holotoxin (Titball et al., 1991).

Although the amino-terminal domain alone is non-toxic, it is clear that its enzymic activity is required for toxicity. The site-directed mutagenesis of zinc-binding ligands in the amino-terminal domain (Table 2) simultaneously abolishes phospholipase C, toxic and cytolytic activities (Guillouard et al., 1996; Nagahama et al., 1995a, 1997). This finding suggests that the carboxy-terminal domain confers cytolytic and toxic activities on the enzyme (Titball et al., 1993). The isolated carboxy-terminal domain has no detectable toxic or cytolytic activity, but mixing the purified amino- and carboxy-terminal domains together in solution restores haemolytic activity, presumably because these domains can associate via the hydrophobic face between these domains in the holotoxin (Titball et al., 1993).

A comparison of the overall topology of the carboxy-terminal domain reveals that it has a fold very similar to that of the phospholipid-binding domains of eukaryotic proteins such as human pancreatic lipase, rat phosphatidylinositol phospholipase C and synaptotagmin (Naylor et al., 1998). Some of these domains have been found complexed with calcium ions, which appear to play a key role in phospholipid binding. Under some conditions the carboxy-terminal domain of χ-toxin is also able to bind calcium ions along one face (Naylor et al., 1999). These calcium ions appear to be associated with changes in the organization of the active site such that the flanking loop regions adopt a conformation which allows substrate entry (Eaton et al., 2002). The site-directed mutagenesis of calcium-binding ligands (Table 2) results in almost complete loss of cytolytic activity of χ-toxin, although phosphorylase C activity towards egg yolk phospholipid is relatively unaffected (Alape-Giron et al., 2000; Walker et al., 2000). All of these findings are in accordance with observations from several decades ago which indicated the requirement for calcium ions in buffers used for *in vitro* measurements of cytolytic activity (Krug & Kent, 1984).

Therefore the available evidence indicates that the amino-terminal domain of χ-toxin contains the phospholipase
C active site and the carboxy-terminal domain allows binding of the toxin to membrane phospholipids. Calcium ions play a key role in binding of the toxin to membranes, and also appear to be associated with structural changes which open the active site and allow the side chains of Trp214 and Phe334 to become inserted into the hydrophobic core of the membrane.

The development of a vaccine

One aim of the work to understand structure–function relationships of α-toxin was to devise a vaccine which could protect against gas gangrene. Formaldehyde toxoids have previously been used as immunogens (Evans, 1945; Kameyama et al., 1975) and have even been used in experimental vaccines used in humans (Boyd et al., 1972). However, the efficacy of these vaccines appeared to be quite variable (Evans, 1945; MacLennan, 1962), possibly reflecting the reported difficulties of reproducibly obtaining immunologically active toxoids (MacLennan, 1962).

Two approaches to the development of improved toxoids have been proposed. It might be possible to use site-directed variants with reduced toxic activity but an alternative strategy would be to use immunologically active fragments of the toxin. Immunization with either the amino- or carboxy-terminal domains of α-toxin induces high titres of antibody which reacts with the holotoxin (Table 3). However, in mice, antibody capable of neutralizing the cytotoxic activity develops only after immunization with the carboxy-terminal domain (Williamson & Titball, 1993). Mice immunized with recombinant carboxy-terminal domain are protected against at least 50 median lethal doses (MLDs) of α-toxin (Williamson & Titball, 1993). In the murine model of gas gangrene, immunized mice are protected against at least 10 MLDs of C. perfringens type A (Williamson & Titball, 1993). More detailed studies have shown that foot blackening was barely detectable after the challenge of immunized mice, and although limb swelling occurred it was minimal by 18 h post challenge (Stevens et al., 2004). This vaccine might well have a utility for the protection of at-risk groups of humans.

An additional use for this vaccine might be to protect domesticated livestock from diseases where α-toxin plays a pivotal role (Heier et al., 2001; Van Immerseel et al., 2004). Recently, the incidence of necrotic enteritis in fowl has increased in some European countries and has been associated with the removal of antibiotic growth promoters from feedstuff (Grave et al., 2004). The incidence of necrotic enteritis does vary from flock to flock, and there is evidence that the incidence of disease is related to the levels of

### Table 2. Properties of reported site-directed mutants of C. perfringens α-toxin with altered metal-binding ligands

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Proposed function of target amino acid</th>
<th>Phospholipase activity</th>
<th>Haemolytic or cytotoxic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>W15*</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H115*</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H68S*</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H68G†</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H126S*</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H126G†</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H136S*</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H136G†</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H136A†</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H148S*</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H148G†</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H148L†</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E152D‡</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E152Q‡</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E152G‡</td>
<td>Zn2+ binding</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D269N§</td>
<td>Ca2+ binding</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>D293S</td>
<td></td>
<td></td>
<td>Ca2+ binding</td>
</tr>
<tr>
<td>D336N§</td>
<td>Ca2+ binding</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

*Guillouard et al. (1996); †Nagahama et al. (1995b); §Nagahama et al. (1997); ‡Alape-Giron et al. (2000); ||Walker et al. (2000).

**Fig. 2.** Model depicting the interaction of C. perfringens α-toxin with the outer leaflet of a eukaryotic cell membrane. Calcium ions which may form bridges to the phospholipid head group and surface-exposed hydrophobic amino acids which may become inserted into the membrane are highlighted. Reproduced from Naylor et al. (1998) with the permission of Nature Publishing Group (http://www.nature.com/namb/index.html).
maternal antibody to z-toxin, which is transferred to chicks (Heier et al., 2001). Therefore, one suggested approach to the control of necrotic enteritis in fowl is to vaccinate flocks with z-toxoid (Lovland et al., 2004). To exploit any of the vaccines which have been devised for the control of gas gangrene it is essential that avian isolates of C. perfringens produce z-toxin which is immunologically cross-reactive. There has been some work to investigate this possibility. In one study avian isolates were found to show extensive sequence homology with mammalian disease isolates (Sheedy et al., 2004). However, another study showed that an isolate of C. perfringens from a dead swan produced a sequence-divergent form of z-toxin (Justin et al., 2002). The extent of immunological cross-reactivity between these toxins is not known.

It is possible that the z-toxoid will be valuable for the prevention of other diseases in domesticated livestock. For example, there is evidence that z-toxin plays a key role in the pathogenesis of some enteric diseases of calves and piglets (Cygan, 1997; Ginter et al., 1996; Wierup, 2001). There is some evidence that these isolates produce a form of z-toxin with increased resistance to proteolysis (Ginter et al., 1996). This might be consistent with the site of production of the toxin in the gut. Again, the extent of immunological cross-reactivity of z-toxin from these strains with z-toxin from gas gangrene isolates is not known.

Other bacterial phospholipases and virulence

Phospholipases A, C or D are produced by a diverse range of Gram-positive and Gram-negative pathogens (Titball, 1999; Titball & Rood, 1999). Therefore, what of the role of other bacterial phospholipases in the pathogenesis of disease? It is known that other clostridial species which are occasionally associated with gas gangrene, such as Clostridium novyi and Clostridium absomum, produce phospholipase Cs which share amino acid sequence and structural homology with z-toxin (Clark et al., 2003; Tsutsui et al., 1995). It seems likely that these enzymes play similar roles to the C. perfringens z-toxin in the pathogenesis of disease. For other pathogens, phospholipases can play very different roles in different diseases. For example, mucosal surfaces are generally coated with a phospholipid-rich surfactant, and there is an obvious possible role for these enzymes in the degradation of the surfactant layer, allowing access to the underlying tissues. Surfactant degradation has been demonstrated in vitro (Holm et al., 1991) but has yet to be demonstrated in vivo during disease. Nevertheless, a role for Helicobacter pylori and Pseudomonas aeruginosa phospholipases in the colonization of the stomach and respiratory tract, respectively, has been proposed (Langton & Cesareo, 1992; Saiman et al., 1992; Titball, 1999). In the former case it may be significant that bismuth salts, which were in the past often used to treat stomach ulcers, are potent inhibitors of the H. pylori phospholipase (Ottlecz et al., 1993).

In addition to the C. perfringens z-toxin, there are a number of bacterial phospholipases which have been directly implicated in promoting the growth of pathogens in vivo. For example, the phospholipase D of Corynebacterium pseudotuberculosis increases vascular permeability and is known to be a major virulence determinant in cases of lymphadenitis/lymphangitis in ruminants (Hodgson et al., 1992; McNamara et al., 1994). Listeria monocytogenes produces two phospholipases C, PLC-A, which is active against phosphatidylinositol, and PLC-B, which preferentially hydrolyses phosphatidylcholine. These enzymes have overlapping functions, and a ΔplcA ΔplcB double mutant was 500-fold attenuated in a mouse model of disease (Smith et al., 1995). These phospholipases appear to promote escape of the bacteria from the phagosome and allow cell-to-cell spread (Smith et al., 1995).

It is studies with the P. aeruginosa phospholipases which have revealed some of the most unexpected roles of these enzymes in the pathogenesis of disease. The P. aeruginosa PLC-H phospholipase C appears to play a role in disease of the respiratory tract (Granström et al., 1984; Saiman et al., 1992). Like C. perfringens z-toxin, PLC-H appears to be able to modulate the metabolism of mammalian cells by activating the arachidonic acid cascade (König et al., 1997). Additionally PLC-H is able to convert phosphatidylcholine

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**Table 3. Properties of antisera against the amino- or carboxy-terminal domains of z-toxin, in mice**

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Antibody titre*</th>
<th>Neutralization of phospholipase C activity†</th>
<th>Neutralization of haemolytic activity‡</th>
<th>Protection against toxin§</th>
<th>Protection against gas gangrene$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;1000</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Formol-toxoid</td>
<td>51 200</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Amino domain</td>
<td>51 200</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Carboxy domain</td>
<td>12 800</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

ND, Not determined.

*Against holotoxin after immunization of mice with 0-36 pM of purified protein.
†In vitro neutralization assay.
‡Immunized mice challenged with 50 MLD of toxin.
§Immunized mice challenged with 10 MLD of C. perfringens.
into choline-betaine, which accumulates in the bacterial cell and acts as an osmoprotectant (Shortridge et al., 1992). The bacterium might rely on this pathway for protection against the high osmotic strength environment in the lung.

A different role in disease has been proposed for the \textit{P. aeruginosa} PLC-B phospholipase, which appears to play a role in the generation of diacylglycerol-based compounds which act as a chemoattractant (Barker et al., 2004). It is possible that this compound attracts bacteria to ‘preferential’ sites in the respiratory tract. Yet another \textit{P. aeruginosa} phospholipase, ExoU, is one of the effector proteins of the type III secretion system. After delivery into the host cell, the enzyme appears to cause damage to the membranes of different organelles and leads to necrotic cell death (Sato & Frank, 2004).

Other bacterial phospholipases have either been shown to play a role in virulence or are strongly implicated in disease, but their precise function is yet to be determined. For example, inactivation of the cell-surface phospholipases of \textit{Yersinia pseudotuberculosis} and \textit{Yersinia enterocolitica} results in partial attenuation (Darwin & Miller, 1999; Karlyshev et al., 2001). There appears to be multiple redundancy of the \textit{Mycobacterium tuberculosis} phospholipases C, and triple or quadruple mutants show a 10-fold reduction in the colonization of mouse lungs in the later stages of disease (Raynaud et al., 2002). It is possible that these enzymes play a role in the establishment of chronic disease.

Some phospholipases actually appear to play a role in reducing the virulence of pathogens – a phospholipase (β-toxin) mutant of \textit{Staphylococcus aureus} colonized tissues at a lower level than the wild-type in the murine model of mastitis (Bramley et al., 1989). Others appear to play roles in the colonization of non-mammalian hosts – \textit{P. aeruginosa} PLC-H plays a role in disease of plants (Rahme et al., 1995) whilst the \textit{Yersinia pestis} phospholipase D is essential for the colonization of flea vectors (Hinnebusch et al., 2002).

Phospholipases are true multifunctional virulence determinants, playing a range of roles in the pathogenesis of disease. Additional enzymes which play a role in virulence will probably be identified in the future. It seems likely that some of these enzymes will interact with host cells using mechanisms similar to those identified for \textit{C. perfringens} α-toxin. For others, the molecular basis of activity is yet to be determined.

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**References**


Justin, N., Walker, N., Bullifent, H. L. & 8 other authors (2002). The first strain of Clostridium perfringens isolated from an avian source has an alpha-toxin with divergent structural and kinetic properties. Biochemistry 41, 6253–6262.


