

Polyphosphate and omptins: novel bacterial procoagulant agents

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Abstract

Derangement of the blood clotting system contributes strongly to multiple organ failure in severe sepsis. In this review, we examine two microbial modulators of the clotting system: polyphosphates and omptins. Polyphosphates are linear polymers of inorganic phosphate that are abundant in the acidocalcisomes of prokaryotes and unicellular organisms as well as in the dense granules of human platelets. Polyphosphates modulate haemostasis by: (1) triggering clotting *via* the contact pathway; (2) accelerating the activation of coagulation factor V (a key cofactor in blood clotting) and (3) causing fibrin to form clots whose fibrils are thicker and more resistant to fibrinolysis. While polyphosphates are found in all prokaryotes, omptins have a more limited distribution among certain Gram-negative species. Omptins are outer membrane aspartyl proteases which were recently found to proteolytically inactivate tissue factor pathway inhibitor (TFPI), the main inhibitor of the initiation phase of blood clotting. Omptin activity against TFPI requires lipopolysaccharide without O-antigen (rough LPS) such as is found on the surface of *Yersinia pestis*, the etiologic agent of plague. Interestingly, expression of Pla, the *Yersinia pestis* omptin, has a demonstrated virulence role in converting plasminogen into the fibrinolytic enzyme plasmin, which would seemingly antagonize any procoagulant effect of TFPI inactivation. However, since the rate of TFPI inactivation is much higher than the rate of plasminogen activation, we suggest that Pla may have a dual function in supporting the bubonic form of plague which is unique to *Yersinia pestis*.

Keywords: blood clotting • tissue factor pathway inhibitor • plasminogen • sepsis • *Yersinia pestis*

Introduction

The normal response to blood vessel injury involves platelet activation and triggering of the soluble blood clotting system, which together result in the formation of a stabilized haemostatic plug in order to minimize further blood loss. Clotting is typically initiated when the cell-surface protein, tissue factor, is exposed to plasma factor VIIa following vessel injury [1]. The resulting factor VIIa: tissue factor complex initiates a series of proteolytic events that induces a burst of thrombin generation, which in turn converts fibrinogen to fibrin, resulting in the formation of a fibrin clot. Normally, protease inhibitors and other natural anticoagulants in

plasma, as well as the anticoagulant properties of the intact endothelium, function to localize the clotting response to only those areas of vessel damage. During subsequent wound healing, tissue plasminogen activator (tPA) converts plasminogen to plasmin, the fibrinolytic protease that dissolves blood clots.

Thrombotic conditions can arise when the regulatory mechanisms that limit the extent of blood clotting are overwhelmed by a sufficiently intense coagulative stimulus. Such is the case in disseminated intravascular coagulation (DIC) and other coagulopathies in sepsis, in which the host immune response leads to

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overwhelming activation of blood clotting, triggered chiefly by up-regulation of tissue factor expression on monocytes/macrophages [2]. Because DIC contributes to the multi-system organ failure accompanying fatal septicemia [3, 4] several anticoagulant and pro-fibrinolytic agents have been investigated as treatments for severe sepsis. The therapeutic value of such drugs in phase 3 clinical trials proved inconclusive [5–7] until the PROWESS trial in 2001 demonstrated the efficacy of activated protein C (aPC) for this purpose [8]. Perhaps not coincidentally, the therapeutic function of aPC is thought to arise, at least in part, from its anti-inflammatory properties [9] as well as its anticoagulant effects. This raises intriguing questions as to why anticoagulant therapies alone have generally failed to improve mortality in sepsis patients, and underscores our limited understanding of the multiple ways that bacteria interact with the host clotting system. With this in mind, this review focuses on recent findings from our group on two novel microbial mediators of the blood clotting system: the previously unknown procoagulant activities of polyphosphate (polyP), a widespread inorganic molecule that is abundant in microorganisms; and omptins, a family of bacterial proteases with a unique role in the virulence of *Yersinia pestis*.

Inorganic polyP in nature

PolyP is a linear polymer of inorganic phosphate linked *via* high energy phosphoanhydride bonds, which can range in size from just a few to over a thousand phosphate units long [10, 11]. PolyP is ubiquitous in nature, being found in all taxonomic kingdoms of life and possibly even predating life itself [10]. PolyP has mainly been studied in unicellular prokaryotes and eukaryotes, with much less known about its roles in higher organisms. For example, the only eukaryote with an identified polyP kinase is the cellular slime mould, *Dictyostelium discoideum* [12]. The development of newer polyP detection assays has spurred recent research into the possible functions of polyP in vertebrates, leading to proposed roles in cancer, cell proliferation [13], angiogenesis [14], osteoblast function [15] and apoptosis [16].

In both prokaryotes and unicellular eukaryotes, polyP is packaged, along with divalent metal ions, in subcellular organelles termed acidocalcisomes (also called volutin or metachromic granules in some organisms). Ruiz *et al.* noted striking morphological and biochemical similarities between acidocalcisomes and platelet dense granules, leading to the assertion that they may be homologous structures [17]. Like acidocalcisomes, platelet dense granules are spherical, acidic [18], electron dense [19] and have high concentrations of divalent cations and pyrophosphate [20]. Ruiz *et al.* reported that platelet dense granules contain abundant polyP, and furthermore that platelets secrete polyP following thrombin stimulation [17]. We recently showed that polyP asserts considerable procoagulant and anti-fibrinolytic effects [21–23]. This raises the possibility that polyP may contribute to consumptive coagulopathies accompanying bacterial sepsis, since polyP can accumulate to abundance in microorganisms.

PolyP modulates haemostasis

PolyP of the size range secreted by activated human platelets enhances blood clotting reactions, acting at three points in the clotting cascade: it triggers blood clotting *via* the contact pathway; it accelerates the rate of factor V activation and it causes fibrin to polymerize into fibrils that are thicker and more resistant to fibrinolysis [21, 23]. Factor V, a procofactor occupying a central place in the blood clotting cascade, is converted to the active cofactor (factor Va) by limited proteolysis by factor Xa or thrombin. PolyP was found to accelerate the activation of factor V by both factor Xa and thrombin [21], although the precise mechanism by which this is achieved is currently unknown. Biochemical studies assessing the relative abilities of factor Xa *versus* thrombin to activate factor V have concluded that factor Xa is not a significant contributor to factor Va generation at low concentrations of tissue factor [24], in the absence of thrombin [25] and at physiological concentrations of clotting factors [26]. However, we speculate that polyP's ability to enhance the factor Xa/factor V interaction might offset this deficiency and allow factor Xa to contribute to factor V activation *in vivo*.

Accelerating the rate of factor V activation by polyP results in an earlier thrombin burst and has interesting consequences for regulatory reactions in the blood clotting system [21]. For example, adding polyP to plasma abrogates the anticoagulant function of tissue factor pathway inhibitor (TFPI), a plasma serine protease inhibitor that is considered to be the major inhibitor of the initiation phase of blood clotting [27]. Studies have demonstrated that factor Xa is protected from inhibition by TFPI in the presence of both its cofactor (factor Va) and its natural substrate, prothrombin [21, 28]. Although the exact mechanism is unknown, prothrombin likely competes with TFPI for a binding site on factor Xa within the prothrombinase complex. In studies by our group, polyP abolished the anticoagulant action of TFPI in clotting reactions in which exogenous TFPI was added to plasma [21]. This effect of polyP was not observed when the same proteins were combined in a plasma-free system, suggesting an additional plasma component such as factor V was needed for TFPI functional inactivation.

PolyP alters fibrin clot structure and stability

We also showed that the presence of polyP in clotting reactions alters the physical structure of fibrin clots, generating thicker fibrin fibrils that are more resistant to fibrinolysis than are clots formed in the absence of polyP [23]. In a purified system containing fibrinogen, Ca²⁺ and thrombin, clots formed in the presence of polyP exhibited higher clot turbidity, contained fibrin fibrils with higher mass/length ratios, and were more resistant to fibrinolysis and elastic stretching than were clots formed in the absence of polyP [23]. This effect required pre-incubation of polyP, fibrinogen and Ca²⁺ together prior to the addition of thrombin, suggesting a direct interaction between fibrinogen and polyP. Toluidine blue staining of clots polymerized in the presence of polyP confirmed that polyP was incorporated into the fibrin clot itself. The exact

mechanism by which polyP alters clot structure is currently unknown. Of note, the anionic polymer heparin has also been shown to increase clot turbidity [29], but unlike clots formed in the presence of polyP, clots formed in the presence of heparin are *more* susceptible to clot lysis [30, 31]. These observations would suggest that polyP affects clot structure in a unique way that is not recapitulated by heparin or other anionic polymers that have been tested [32].

Degradation of polyP in plasma

Uncontrolled coagulation is potentially as deleterious as uncontrolled haemorrhage in terms of generating tissue hypoxia and necrosis. Thus, inhibition of clotting factors *via* protease inhibitors and anticoagulant agents such as aPC limits the extent of coagulation and localizes it to sites of vessel injury and/or platelet activation; subsequent fibrinolytic events dissolve the clot after the damaged vessel is repaired. One would therefore expect some mechanism for controlling the procoagulant effects of polyP, much like there are mechanisms to inactivate and reverse the clotting proteases. One potential mechanism is simply degradation by plasma phosphatases, resulting in a half life of polyP in plasma of about 2 hrs [21, 33]. One can postulate that platelet polyP is released into plasma upon platelet activation, helps promote clot formation and stability and then is degraded to prevent unwanted thrombotic events.

PolyP activates the contact clotting pathway

The so-called contact pathway represents an alternative way to initiate the blood clotting cascade, compared to initiation *via* tissue factor (the main physiological trigger of blood clotting in normal haemostasis). The contact pathway is triggered when specific clotting proteins (factors XI, XII, prekallikrein and high molecular weight kininogen) self-assemble on certain negatively charged polymers or surfaces. Such surfaces or polymers are thought to function as templates for dramatically increasing the local concentrations of the proteins that initiate the contact pathway, thereby facilitating protein-protein interactions. These interactions include the autoactivation of plasma factor XII, which sets off a series of reactions that include activation of prekallikrein and factor XI, and of cleavage of high molecular weight kininogen. Activated factor XI activates factor IX, leading to propagation of blood clotting through the final common pathway (in which factors Xa and Va convert prothrombin to thrombin). The contact pathway is dispensable for haemostasis, since genetic deficiencies of key contact factors are not associated with bleeding diatheses [34]. On the other hand, activation of blood clotting *via* the contact pathway has recently been implicated in thrombotic disease [35, 36]. Furthermore, factor XII gene deletion in mice results in a defective immune response to infection [37] arguing that one of the main physiological roles of the contact system *in vivo* may be in combating infection. In support of this argument, several candidates

for microbial activators of the contact pathway of blood clotting have been identified, including bacterial lipopolysaccharide (LPS) [38], M proteins in *Streptomyces pyogenes* [39] and curli fibres on *E. coli* and *Salmonella* [40]. Contact pathway components are also implicated in complement activation [41], fibrinolysis [42], angiogenesis [43] and kinin formation [44].

We recently showed that polyP is also a potent activator of the contact pathway of blood clotting [21]. Similar to other contact activators, polyP is a highly negatively-charged polymer, a property which likely facilitates its binding to high molecular weight kininogen and factor XII. Furthermore, activation of clotting by polyP exhibits a bell-shaped concentration-dependence, consistent with functioning as a template for the assembly of contact factors (our unpublished results). In addition, triggering blood clotting *via* the contact pathway is optimal with very long polyP polymers, consistent with the sizes of polyP typically found in bacteria. PolyP chains consisting of 200 phosphate units or more are the predominant form found in bacteria, although bacteria are able to alter the size and abundance of polyP based on environmental cues [45]. In contrast, polyP polymers of the size released from human platelets (approximately 75-mers [17]) exhibit far less ability to activate the contact pathway (manuscript in preparation).

PolyP and/or the enzymes of its metabolism have been shown to play roles in the responses of bacteria to a variety environmental and chemical signals and stresses. These include a role in the SOS response induced by DNA damage [46], the stringent response system which reacts to low levels of phosphate, carbon, or amino acids [47], the general stress response where polyP increases levels of RpoS [48] promoting a shift away from bacterial growth and towards dormancy [11, 49, 50]. Whatever their endogenous functions in bacteria, polyP accumulating in bacterial cells can potentially activate the blood clotting cascade in cases of sepsis. While this has yet to be examined *in vivo*, administration of recombinant TFPI failed to ameliorate sepsis in a large clinical trial [5]. It is tempting to speculate that the ability of polyP to bypass the TFPI control on coagulation may have played a role.

Bacterial omptins and coagulation

Omptins are a family of aspartyl proteases expressed in Gram-negative bacteria, which to date include 13 orthologues in nine genera [51]. Functionally, the best characterized omptin is a protein known as plasminogen activator or Pla, which is expressed by *Y. pestis*, the causative agent of plague. Numerous reports have delineated a specific fibrinolytic role for the Pla protein in *Y. pestis* virulence [52–55]. Eliminating Pla expression in a fully virulent *Y. pestis* strain decreased its LD₅₀ by 6 orders of magnitude in a mouse model of bubonic plague [52]. This finding led to the hypothesis that Pla, by converting plasminogen into plasmin, allowed *Y. pestis* to disseminate throughout the host *via* plasmin-mediated degradation of fibrin, basement membranes and extracellular matrix barriers [52].

Recently, we showed that the interaction between Pla and the host clotting/fibrinolysis system is more complex than previously thought, as we found that Pla as well as other bacterial omptins can proteolytically degrade TFPI, completely abrogating its anticoagulant function [56]. This has the net effect of accelerating blood clotting. *In vitro*, TFPI was found to be a much better substrate for Pla than was plasminogen [56], raising questions as to how this newfound activity alters our view of the virulence role of Pla. In the sections that follow, we review what is known about Pla, and speculate on how *Y. pestis* may modulate the host haemostatic system to its advantage.

Omptin structure

Omptins are a family of outer membrane proteases expressed in Gram-negative bacteria that share about 40 to 50% sequence homology. They are composed of 10-antiparallel strands which form a vase-shaped barrel embedded in the outer membrane [57]. Comprising the rim of this vase are five surface exposed loops which confer much of the substrate specificity of the various omptins [58]. The active site cleft is formed just beneath the rim of this 'vase' and is composed of two opposing pairs of catalytic residues, a His-Asp dyad resembling the serine protease His-Asp-Ser triad and an Asp-Asp dyad resembling the active site of aspartyl proteases. Mechanistically, it is thought that the His-Asp dyad activates a water molecule for nucleophilic attack on the substrate's scissile bond [59], while the Asp-Asp dyad has been proposed to participate in catalysis by proton translocation or stabilization of the oxyanion intermediate (reviewed by Haiko *et al.* [60]). OmpT in *E. coli* exhibits a preference for cleaving substrates between consecutive basic residues [61, 62]; since the aforementioned catalytic residues are conserved in all omptins, it is assumed that Pla displays a similar substrate preference. LPS is required for the enzymatic activity of omptins, and in particular, LPS with short O-antigen side chains (rough LPS) is required for omptin activity toward many exogenous macromolecular substrates [63, 64]. It is thought that an extended O-antigen side chain (smooth LPS) sterically interferes with substrate binding [65], a significant observation since *Y. pestis* is one of the few omptin-expressing bacteria that naturally express rough LPS. Indeed, the evolution of Pla's activity toward foreign substrates may have been a major determinant in eliminating O-antigen expression in *Y. pestis* [60, 63].

Evolution of *Y. pestis* and its life cycle

Genomic comparisons indicate that *Y. pestis* recently evolved from the closely related *Yersinia pseudotuberculosis*, emerging within the last 1500 to 20,000 years [66]. Both species are remarkably similar genetically, displaying >90% sequence homology. The *pla* gene of *Y. pestis* is notably absent from *Y. pseudotuberculosis* and *Yersinia enterocolitica*, however [60].

Y. pseudotuberculosis is motile, free-living and infects mammalian hosts perorally, inducing relatively mild gastrointestinal infections that may or may not persist in the host. In contrast, *Y. pestis* utilizes a flea vector to infect susceptible hosts *via* the intradermal or subcutaneous route, often causing fulminate septicemic infections with high mortality rates. The natural reservoir for *Y. pestis* is thought to be resistant rodent populations which become the source for periodic epizootics among more susceptible rodent populations and occasionally human populations [67]. The intradermal route of transmission *via* fleabite is the most common mode of *Y. pestis* host entry [68] and leads most often to the bubonic form of plague [55]. This type of *Y. pestis* infection is thought to progress by migration of the infectant from the dermis to a regional draining lymph node, possibly inside a macrophage [69]. *Y. pestis* then multiplies extracellularly in the infected lymph node, also called a bubo, and subsequently spreads to other organs and tissues throughout the body *via* blood and lymphatic routes [70]. Primary pneumonic plague is caused by aerosol transmission into pulmonary tissue, while primary septicemic plague reflects direct entry of *Y. pestis* into the vascular system of the host. All three forms often result in fatal septicaemia in rodent as well as human populations.

Expression of Pla increases *Y. pestis* virulence

The virulence role of Pla is intimately tied to the bubonic (and pneumonic) forms of the disease, since expression of Pla has no significance in infectivity or lethality when the pathogen is introduced intravascularly [52]. Pla expression increases virulence in animal models of pneumonic plague although not to the extent seen in bubonic plague models [71]. These two experimental findings give our first clue to the functional role of Pla in *Y. pestis* virulence, namely that Pla must act in an extravascular setting. The exact mechanism(s) by which Pla exerts its effects is not completely understood, as most studies using bubonic plague models have focused on morphological alterations in the host or differences in bacterial or inflammatory cell counts as a result of Pla expression [52–55]. These findings can be generalized as follows: (1) Pla expression results in attenuated inflammatory cell recruitment (particularly neutrophils) to infected lesions; (2) Pla expression causes a structural derangement of infected lymph tissue characterized by lymphadenitis, necrosis, haemorrhage, thrombosis and disorganized masses of infiltrating bacteria and (3) Pla expression promotes the systemic dissemination of the infection.

Animal studies also show that the number of *Y. pestis* bacteria localized to subcutaneous injection sites is not altered by Pla expression [52, 54], indicating that Pla exerts its effects after initial colonization in the dermis but before systemic infiltration of the vasculature. What is altered, however, is the extent of the host inflammatory response in infected draining lymph nodes as the number of inflammatory cells is greatly reduced upon expression of Pla [52, 54]. In addition, these lymph nodes exhibit major alterations in gross architecture and sustain greater bacterial growth

with expression of Pla. Wild-type *Y. pestis*-infected rat lymph nodes exhibited 'necrotizing fibrinous and septic lymphadenitis and periadenitis' [72] with normal node structure eliminated and replaced by large masses of bacteria interspersed among necrotized disordered tissue and haemorrhagic and thrombotic lesions. Thus, although a role in *Y. pestis*' migration from the dermis to the lymph node cannot be eliminated, it seems more probable that Pla activity is necessary once the pathogen has reached a regional draining lymph node, where *Y. pestis* undergoes rapid extracellular multiplication and the lymph node then becomes the nidus for subsequent systemic dissemination. This idea is supported by other studies which show that Pla-deficient *Y. pestis* disseminate to regional lymph nodes after subcutaneous inoculation but do not cause the lymphadenopathy observed with wild-type *Y. pestis* infections [54], even though they may subsequently infect other organs such as the liver and spleen and result in mortality to the host [55]. Guinet *et al.* [73] also noted that rat lymph nodes infected by Pla-deficient *Y. pseudotuberculosis* retained their general architecture, along with abscess-type polymorphonuclear infiltrates which sequestered the infection. This happened even though the bacterial count in the lymph node 24 hrs after infection was similar to the count in *Y. pestis* infections.

These results argue that the evolution of Pla facilitates the bubonic form of *Y. pestis* infection, exerting its virulence function mainly within the context of a regional draining lymph node. This is not surprising, as infection of the bubo can be considered a bottleneck in the systemic spread of the disease. It is at this stage of the infection where *Y. pestis* first multiply in great numbers, where the first signs of gross morphological changes are observed, and where subsequent Pla expression has no demonstrated impact on LD₅₀.

Plasminogen activation by Pla

The ability of Pla to promote systemic dissemination has been attributed to its ability to generate plasmin, an enzyme that can promote general proteolysis, and also to the ability of Pla to inactivate the main physiological inhibitor of plasmin, α_2 -antiplasmin [58]. Pla has been shown to proteolytically activate human Glu-plasminogen to plasmin with a k_{cat} of 0.21/min. [52]. For comparison, urokinase-type plasminogen activator converts plasminogen to plasmin with a k_{cat} of 89/min. [74]. Plasmin is rather promiscuous in its substrate recognition, and has been shown to degrade extracellular matrix components, activate procollagenases, inactivate collagenase inhibitors and promote cell migration [75–77]. Lathem *et al.* observed that fibrin(ogen) deposition was greatly decreased in lung tissues upon inducible expression of Pla in *Y. pestis*, which also correlated with an increase in mortality in infected mice [71, 78]. Because a pneumonic plague model was used, these results may not represent the level of plasminogen activation and/or fibrinolysis occurring in infected lymph nodes. Using a bubonic plague mouse model, Degen *et al.* noted that survival of mice infected with Pla-deficient *Y. pestis* was greater than cohorts infected with wild-type *Y. pestis* [79]. This survival

advantage was negated, however, if fibrinogen knockout mice were infected instead, directly implicating the host coagulation system as a target for Pla's role in virulence. Interestingly, these authors also observed a survival advantage of infecting plasminogen knockout mice with wild-type *Y. pestis* compared to infection of wild-type mice; however, this advantage was not as great as the survival advantage of Pla deletion in infective *Y. pestis*. This suggests that activation of plasminogen is only one of the ways that Pla increases *Y. pestis* virulence.

TFPI inactivation and factor VII activation by Pla

We recently observed that adding live *E. coli* cells to human plasma resulted in rapid loss of TFPI anticoagulant activity, prompting us to investigate the mechanism [56]. Cell fractionation experiments showed that lysed bacteria could also abrogate TFPI anticoagulant function, with the activity being localized to the cell envelope fraction, implicating an outer membrane component. Targeted gene deletion experiments subsequently showed that OmpT was responsible for the ability of these *E. coli* cells to antagonize TFPI function. We found that two other Gram-negative bacteria, *Y. pestis* and *Salmonella enterica* serovar Typhimurium, also efficiently inactivated TFPI anticoagulant activity in human plasma, and targeted gene deletions confirmed that these bacteria also abrogated TFPI function *via* oмпtins: Pla in the case of *Y. pestis* and PgtE in the case of *S. Typhimurium*. Further experiments established that the bacteria had to express both the oмпtin and short O-antigens (rough LPS) in order to abrogate TFPI function, consistent with many previous studies that have shown that long O-antigens decrease the ability of oмпtins to proteolyse exogenous substrates.

We confirmed that oмпtin inactivation of TFPI was due to rapid proteolysis of TFPI, mapping the initial proteolytic cleavage event to within the highly basic, C-terminal domain of TFPI that is required for both anticoagulant function and binding to a variety of cell surface receptors [56]. Longer exposure of TFPI to oмпtin-bearing bacteria resulted in further proteolysis, yielding even smaller TFPI fragments. Bacteria expressing Pla, OmpT or PgtE proteolysed TFPI efficiently even in whole plasma, which contains a very high concentration of potential competing substrates, indicating a high degree of specificity in the recognition of TFPI as a substrate. In addition, bacteria expressing these oмпtins did not detectably alter the activities of several other blood clotting proteins tested, with one exception: Pla protein (but not the other two oмпtins) proteolytically converted zymogen factor VII to the active form, factor VIIa. Factor VIIa (when bound to tissue factor) is the enzyme responsible for initiating the clotting cascade in normal haemostasis, and it is also a major target of the anticoagulant action of TFPI. Taken together, these results indicate that Pla works to accelerate the initiation phase of blood clotting by activating the first enzyme in blood clotting (factor VIIa) and inactivating its most important protease inhibitor in plasma (TFPI). This is highly reminiscent of the ability of Pla to activate plasminogen to plasmin and to inactivate its plasma inhibitor, α_2 -antiplasmin.

We compared the catalytic efficiencies of omptins toward TFPI and found that Pla (*Y. pestis*) and OmpT (*E. coli*) proteolytically inactivated TFPI at comparable rates, while PgtE (*S. Typhimurium*) was some 100-fold less active toward TFPI [56]. Interestingly, TFPI appears to be a far better substrate for Pla than is plasminogen: Pla proteolytically inactivated TFPI at a rate that was more than 100-fold faster than the rate at which it activated plasminogen. Furthermore, Pla activated factor VII about twice as fast as it activated plasminogen.

If Pla does indeed activate factor VII and degrade TFPI *in vivo*, this would seem to counteract the pro-fibrinolytic effects of plasminogen activation by Pla. How can we reconcile these two seemingly opposing functions? For one, a distinction needs to be made between coagulant and fibrinolytic processes. Fibrinolysis entails the degradation of clots but not necessarily the prevention of their formation. Physiologically, fibrin enhances plasminogen activation and plasmin activity, allowing orderly formation and dissolution of fibrin clots. Perhaps *Y. pestis* utilizes a strategy whereby it initially promotes activation of the clotting system to form a protective fibrin barrier around the infected area, decreasing the chance of elimination by host inflammatory cells at the regional lymph node (and possibly preventing premature dissemination of the bacteria into the bloodstream). A similar argument could be made for the pneumonic form of plague, in which fibrin deposition *via* local activation of the blood clotting system in the infected lung tissue might initially be protective for the bacteria. This period of safety from immune attack could then be used by *Y. pestis* to allow for multiplication of bacterial numbers and up-regulation of genes to overcome subsequent immune attack, followed by generation of a sufficiently high local Pla concentration to compensate for its relatively low catalytic efficiency towards plasminogen. This scenario is supported by a recent study suggesting that spatial clustering of *Bacillus cereus* and *Bacillus anthracis* could trigger blood coagulation by increasing the local concentration of the bacterial metalloprotease InhA1 (a direct activator of prothrombin and factor X) above a threshold

concentration needed to sustain the coagulation cascade, in a mechanism that has been termed, 'quorum acting' [80]. Also, microarray analyses of *Y. pestis* isolates from buboes have indicated that *pla* expression is substantially up-regulated in this setting, as well as several genes involved in defense against host immune effectors including type III secretion system and F1 capsular antigen genes [81].

Other functions of Pla

Although plasminogen is the best known substrate of Pla, various other proteins and peptides are also known to be proteolysed by Pla. As mentioned above, this includes the circulating plasmin inhibitor, α_2 -antiplasmin, cleavage of which negates its ability to inhibit plasmin, thus strengthening the argument for a pro-fibrinolytic role for Pla in *Y. pestis* virulence. Pla is also known to proteolyse complement C3 [52] which may possibly ameliorate the host inflammatory response by abolishing its chemoattractant properties. Recently, Pla has also been shown to confer protection against the bactericidal activity of the antimicrobial peptides and cathelicidins, LL-37 and rCRAMP, through proteolytically degradation [82]. Pla is also thought to act as an adhesin to mammalian basement membranes by binding laminin [83] and expression of Pla was also found to be critical for the invasion of HeLa cells by *Y. pestis* [84]. How these other potential functions of Pla aid *Y. pestis* in its ability to generate bubonic infections will undoubtedly be a focus of much future research.

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