Non-proteolytic functions of microbial proteases increase pathological complexity

Veronica M. Jarocki¹, Jessica L. Tacchi¹ and Steven P. Djordjevic¹,²

¹ The ithree institute, Proteomics Core Facility, University of Technology, Sydney, NSW, Australia
² Proteomics Core Facility, University of Technology, Sydney, NSW, Australia

Proteases are enzymes that catalyse hydrolysis of peptide bonds thereby controlling the shape, size, function, composition, turnover and degradation of other proteins. In microbes, proteases are often identified as important virulence factors and as such have been targets for novel drug design. It is emerging that some proteases possess additional non-proteolytic functions that play important roles in host epithelia adhesion, tissue invasion and in modulating immune responses. These additional “moonlighting” functions have the potential to obfuscate data interpretation and have implications for therapeutic design. Moonlighting enzymes comprise a subcategory of multifunctional proteins that possess at least two distinct biological functions on a single polypeptide chain. Presently, identifying moonlighting proteins relies heavily on serendipitous empirical data with clues arising from proteins lacking signal peptides that are localised to the cell surface. Here, we describe examples of microbial proteases with additional non-proteolytic functions, including streptococcal pyrogenic exotoxin B, PepO and C5a peptidases, mycoplasmal aminopeptidases, mycobacterial chaperones and viral papain-like proteases. We explore how these non-proteolytic functions contribute to host cell adhesion, modulate the coagulation pathway, assist in non-covalent folding of proteins, participate in cell signalling, and increase substrate repertoire. We conclude by describing how proteomics has aided in moonlighting protein discovery, focusing attention on potential moonlighters in microbial exoproteomes.

Keywords: Microbiology / Moonlighting / Multifunctional proteins / Pathogenesis / Protease

1 Introduction

Proteases (also known as peptidases and proteinases) are ubiquitous enzymes that cleave peptide bonds in polypeptide chains and profoundly influence protein shape, size, composition, localisation, turnover and degradation. The effects of such control include post-cleavage gain of function, loss of function or switching between functions. Proteases are thereby responsible for a multitude of physiological processes in all organisms [1]. Classification of proteases is based on the amino acid at the catalytic site for substrate hydrolysis, which can either be serine, cysteine, threonine, aspartic or glutamic acid. For some proteases, the mode of action is mediated through coordination with a metal ion, so they are called metallopeptidases. Further classification is determined by the location of the protease cleavage site: proteases cutting at the amino terminus are called aminopeptidases, at the carboxyl terminus are called carboxypeptidases and towards the centre are called endopeptidases. Though uncommon, some proteases possess both endo- and exopeptidase function [2].

Microbial proteases have been implicated in prominent pathological roles and are recognised as important virulence factors. This has driven research aimed at developing...
inhibitors that target their catalytic sites [3] and proteomics has been used to identify promising vaccine candidates for significant human pathogens, including *Streptococcus pyogenes* [4] and *Plasmodium falciparum* [5], and animal pathogens, such as *Vibrio harveyi* [6]. The virulence modulating functions of proteases are to be expected, as host immune effector and signalling molecules are often proteins or peptides. However, as proteases often function within intricate proteolytic networks, their exact targets and biological actions are often difficult to ascertain or even predict.

Some proteases perform additional non-proteolytic actions, due to either the presence of extra functional domains or alternative conformations. Proteases are macromolecules with a limited number of residues required for catalysis. The remaining structure is generally made up of non-catalytic domains, including sorting signals that direct correct cellular localisation, autoinhibitory prodomains to prevent premature activation and ancillary domains that facilitate protein–protein interactions [7]. Alternative protease conformations may involve ligand binding, post-translational modifications, changes in molecular environment or oligomerisation [8]. The changes to conformational structure may also be facilitated by intrinsically disordered regions, which provide structural malleability, permitting various and often opposing functions to reside within the same region [9].

Enzymes that have multiple activities, with each activity dependent on cell localisation, substrate availability or interactions with other proteins, are said to be “moonlighting”. A moonlighting protein, by simplest definition, is a protein capable of performing two or more distinct biological functions within a single polypeptide chain [9]. It has been hypothesised that moonlighting functions arise from selective pressure on an advantageous novel function [10], and may benefit an organism by increasing complexity without resorting to genome expansion [11]. To differentiate moonlighters from other multifunctional proteins, *bone fide* members do not encompass products of gene fusions, protein fragments, or splice variants [8]. That is, whilst a proprotein can be cleaved to produce a protease and another functional unit, a moonlighting protease can exert both functions without prior processing. Additionally, moonlighting functions are not always conserved among the same protein across different organisms [12]. Terminology used to describe the presence of more than one catalytic function in a protein molecule is not applied consistently in the literature [11, 13, 14]. To clarify, bifunctional enzymes have two or more catalytic sites that function independently of each other; moonlighting proteins possess catalytic activity and an additional structural or regulatory function, while promiscuous enzymes have a single catalytic domain capable of catalysing more than one chemical reaction. These enzyme classifications are not exclusive — as some moonlighting proteases also have promiscuous and/or bifunctional activities.

In bacteria, the majority of moonlighting proteins identified have been cytosolic “housekeeping” proteins, including those involved in the glycolytic pathway and tricarboxylic acid cycle all of which have been reviewed extensively [15–18]. These intracellular proteins demonstrate additional functions when secreted or localised to the cell surface, and were originally termed anchorless or non-classically secreted proteins as translocation occurred in the absence of any known signal peptide. The debate remains whether lysis or a novel secretion mechanism is responsible for the presence of cytosolic proteins on the cell surface [19]. Regardless, these proteins have frequently been shown to act as adhesins, invasins, and modulators of host immune responses, thereby playing important roles in colonisation, dissemination, and avoiding immune clearance [16].

Identifying which proteins have moonlighting functions has relied heavily on serendipitous empirical data [20]. Identification of additional functions of proteases and determination of their role in virulence remains a challenge. Such knowledge has implications for host–pathogen interactions and drug design strategies. Global methods for identifying moonlighting proteins include bioinformatic analysis and mass spectrometry. Bioinformatic analysis is better suited to identifying proteins that have evolved new function via the acquisition of additional domains rather than those that have acquired it via conformational changes. This is because there are currently no reliable prediction algorithms [21], no apparent moonlighting motifs [22] and no obvious links to sequence homology between moonlighting and non-moonlighting proteins of the same class and between species [23]. Furthermore, a moonlighting function can be introduced by as few as four amino acid substitutions [20]. Detailed genomic comparisons of high and low virulence isogenic bacteria describe the accumulation of point mutations in surface accessible proteins [16], suggesting that minor sequence variation may have profound implications for protein function, including many moonlighting functions that are yet to be described. Mass spectrometric and proteomic analyses have been previously identified as key techniques in the search for moonlighting proteins due to their ability to identify the presence of a protein in varying cell types, locations and multiprotein complexes [24].

Bacterial moonlighting proteins with functions important in virulence would be expected to be found within microbial exoproteomes. There have been hundreds of microbial surfaceome and secretome studies published to date. Many report the presence of cytosolic proteins in extracellular fractions, providing tantalising suggestions of possible moonlighting function. Microbial proteases have established roles as virulence factors, yet the presence of traditionally cytosolic proteases within exoproteomes has generally been overlooked. Microbial proteases that have more than one function additional to proteolytic activity will form the focus of this review. Examples will be grouped into five main types of functions that the proteases have as additional to their proteolytic activity: adhesin, plasminogen-binding, chaperone (Cpn), receptor, and non-proteolytic catalysis. We will discuss how these additional functions may contribute to pathogenesis...
and point to future research directions by highlighting other potential moonlighters within microbial exoproteomes.

2 Proteases as virulence factors

Although their role was once thought to be limited to degradation of unneeded proteins, proteases are now known to be mediators of many pathological processes. In these roles, their proteolytic function ranges from less specific cleavage events, such as degradation of extracellular matrix (ECM) components for nutrient acquisition [25], to precise cleavage events. Through peptide bond cleavage many pathogens are capable of modulating host innate immune responses, including disruption of cascade systems, such as complement, coagulation and fibrinolysis, and inactivation of immunoglobulins, cytokines, chemokines and antimicrobial peptides [26]. Additionally, some proteases can affect virulence directly by acting as toxins [27] or indirectly through the processing of other virulence factors [28].

Pathogens indisputably utilise proteolysis to establish successful infections and this has driven research into developing protease inhibitors as therapeutic agents. Efficacious examples include human immunodeficiency virus aspartyl protease inhibitors, which are used to prevent the progression of acquired immunodeficiency syndrome [29], and two serine protease inhibitors used for the treatment of chronic hepatitis C infections [30]. Like all surface accessible proteins, extracellular proteases will be under selective pressure from the host immune response and thus are likely to evolve faster that proteins strictly residing within the cytosol. The virulence attributes of proteases are not solely reliant on their enzymatic capabilities and their role in pathogenesis may be significantly underestimated. Moonlighting functions may increase pathogenic complexity and impede current drug design strategies. The literature contains many examples of microbial proteases that demonstrate additional functions independent of proteolysis, several of which have been described specifically as “moonlighting” activities (Table 1).

2.1 Proteases with a role in adhesion

Adherence to the mucosal epithelium frequently marks the first essential stage of infection and successful colonisation. This adherence is often facilitated by adhesive surface-bound molecules called adhesins that bind to the ECM covering mucosal cells. ECM typically comprises a heterogeneous mixture of glycoproteins, such as collagen, elastin, fibronectin laminin, and glycosaminoglycans (GAGs) [31]. These molecules are targeted by microbial adhesins to aid colonisation and invasion. There is also increasing evidence suggesting that microbial binding of ECM molecules elicits more than just adhesion. For example, fibronectin not only provides a bridge between the intracellular cytoskeleton and other ECM components, but is also involved in inflammation by augmenting chemotaxis and leucocyte function, cell signalling [32] and cell invasion [33]. Fibronectin-binding proteins are known virulence factors in important human pathogens, such as *Staphylococcus aureus* and group A *Streptococcus* [32], and *Campylobacter jejuni* fibronectin-binding proteins have been implicated in membrane ruffling stimulation and consequent host cell invasion by activating the epidermal growth factor pathway [34]. The co-localisation of fibronectin and veterinary pathogen *Mycoplasma hyopneumoniae* on the ciliated lining of the porcine respiratory tract has also been demonstrated [35]. GAG binding is also associated with more than adhesion, including facilitating intracellular survival in macrophages [36], and central nervous system entry [37]. The importance of identifying microbial adhesins is highlighted by the drive to implement anti-adhesion methods as novel therapeutics against infections, particularly within the context of ever increasing antibiotic resistance [38].

In addition to proteins with primary adhesin functions, many examples of surface-associated moonlighting proteins have also demonstrated adhesive interactions with ECM components. It has been proposed that this redundant adhesive activity of moonlighters is significant in host–microbe interactions [8, 16]. The literature contains many examples of cytosolic proteins, particularly metabolic enzymes that moonlight on the cell surface, which may skew the perception of moonlighting to consideration of only multifunctional proteins found in atypical cellular compartments. Moreover, as proteases typically feature on microbial cell surfaces, those that are characteristically cytosolic may have been overlooked. Here, we examine several proteases that exhibit adhesin functions secondary to their proteolytic function.

*Streptococcal* pyrogenic exotoxin B (SpeB), also known as streptopain, of *S. pyogenes* is a cysteine protease with a role as a toxin that induces apoptosis and reduces phagocytic function in human monocytes. This important virulence factor is also responsible for ‘strepadhesin’ activity, with capacity to bind to thyroglobulin, asialofetuin and submaxillary mucin [39]. SpeB also binds laminin, a major glycoprotein found within mammalian basement membranes (BMs). Laminin is targeted by many pathogens during the initial stages of adherence and during tissue invasion [40]. Notably, experiments using cysteine protease inhibitors and inactive forms of SpeB have demonstrated that adhesion to glycoproteins was independent of proteolytic function. These data suggested that the adhesive motif and the substrate-binding site are distinct entities [39]. SpeB adhesive properties are essential during the initial stages of infection by group A *Streptococcus*; however, after systemic infection they become problematic due to their affinity for α1 antitrypsin. SpeB binding of α1 antitrypsin mediates contact-system mediated bacterial killing, so the protease is downregulated after dissemination into the blood stream [41].

Plasminogen activator (Pla) of *Yersinia pestis* is another multifunctional protease that binds ECM molecules. As its name suggests, the protease can directly cleave plasminogen at the Arg<sup>561</sup>-Val<sup>562</sup> bond, releasing the active serine protease
**Table 1.** Examples of proteases with additional non-proteolytic functions

| Name (Synonyms) | Protease classification (Family) | Other function(s) | Organism | Predicted localisation | Found to be surface exposed |
|-----------------|---------------------------------|-------------------|----------|------------------------|-----------------------------|
| Aminopeptidase S* (SgAP) | Metalloaminopeptidase (M28) | Hydrolyses ester bonds | *Streptomyces griseus* | Unknown | Unknown |
| C5a peptidase (scpA, scpB) | Serine endopeptidase (S8) | Adhesin | *Streptococcus agalactiae* | Cell wall | Yes |
| Cpn60.2* (chaperone 60, HSP60/5) | Trypsin-like endopeptidase | Chaperone | *Myco bacterium leprae* | Cytoplasmic | Yes |
| DPP IV (dipeptidyl peptidase 4, Xaa-Pro dipeptidyl peptidase, PepX, X-prolyl dipeptidyl aminopeptidase) | Serine oligopeptidase (S9/S15) | Adhesin | *Porphyromonas gingivalis* | Extracellular | Yes |
| Endopeptidase O (PepO, oligopeptidase 01, endopeptidase 2) | Metalloendopeptidase (M13) | Adhesin; accelerates plasminogen activation | *Streptococcus pneumonia* | Cytoplasmic | Yes |
| FtsH* (high-frequency lysogenisation by phage λ, hfIB) | Metalloendopeptidase | Chaperone; AAA+ ATPase | *Multiple organisms* | Outer membrane | Yes |
| Gingipain (HRgpA/RgpB/Kgp, Arg/Lys-gingipain) | Cysteine endopeptidase (C25) | Adhesin; Exopeptidase | *Porphyromonas gingivalis* | Outer membrane and periplasmic space | Yes |
| Glutamyl aminopeptidase* (aminopeptidase A) | Metalloaminopeptidase (M42) | Adhesin; Accelerates plasminogen activation | *Mycoplasma hyopneumoniae* | Cytoplasmic | Yes |
| HtrA* (high-temperature requirement A, DegP, protease Do) | Serine endopeptidase (S1) | Chaperone | Multiple organisms | Cytoplasmic/periplasmic | Yes |
| Leucine aminopeptidase* (cytosol aminopeptidase, LAP3, leucyl peptidase) | Metalloaminopeptidase (M17) | Adhesin; Accelerates plasminogen activation; DNA-binding protein | *M. hyopneumoniae* | Cytoplasmic | Yes |
| Lon* ATP-dependent serine (proteinase, endopeptidase L, Lon, Lon type 1) | Serine endopeptidase (S16) | Chaperone; AAA+ ATPase; DNA-binding protein | *Multiple organisms* | Cytoplasmic | No |
| NS3/4A (hepacivirin, Cpro-2) | Serine endopeptidase (S29) | ssrA tagging | *Hepatitis C virus* | Viral capsid, host endoplasmic reticulum | Unknown |
| Papain-like protease (PLP) | Cysteine endopeptidase (C16) | Helicase; nucleotide-binding protein | *Coronaviridae* and *Arteriviridae* viral families | Host cytoplasm | Unknown |
| PgtE (protein E) | Aspartic endopeptidase (A26) | Adhesin | *Salmonella enterica* senovar Typhimurium | Outer membrane | Yes |
| Prolidase (Xaa-Pro dipeptidase, imido/dipeptidase, gamma peptidase, pepO, proline dipeptidase) | Metalloaminopeptidase (M24) | Hydrolyses ester bonds | *Alteromonas haloplanktis* | Cytoplasmic | Unknown |
| Pla* (plasminogen activator) | Aspartic endopeptidase (A26) | Adhesin; coagulase | *Yersinia pestis* | Outer membrane | Yes |
| OmpT (protease 7, protease A) | Aspartic endopeptidase (A26) | Adhesin | *Escherichia coli* | Outer membrane | Yes |
| SpeB (streptopain, erythrotoxin B, histase, In1Is convertase, *Streptococcus* peptidase A) | Cysteine endopeptidase (C10) | Adhesin | *Streptococcus pyogenes* | Extracellular | Yes |
| TAP (thioesterase I) | Serine endopeptidase | Thioesterase, alyesterase, esterase, lysophospholipase | *Escherichia coli* | Unknown | Unknown |
plasmin. Plasmin is a proinflammatory agonist that dissolves fibrin clots, activates collagenase and gelatinase precursors, and degrades laminin [42]. Whilst laminin is not degraded by Pla, the protease binds strongly to this BM protein [43]. Thus, Pla-mediated laminin adhesion and plasminogen activation work together to produce localised proteolysis at the mammalian BM, facilitating Y. pestis invasion. Pla also binds the GAG heparan sulphate, and collagen [44], though the latter has been contested [45]. Pla further mediates invasiveness by utilising the lectin receptor DEC-205 to promote macrophage uptake, thus leading to dissemination [46]. Pla also possess some weak coagulase activity but whether this function plays a role in Y. pestis pathogenesis has not been assessed [47].

The serine protease C5a peptidase of *Streptococcus agalactiae* (group B streptococcus) is a cell wall anchored protease with a highly specific catalytic function of degrading neutrophil chemotaxin C5a [48]. Whilst its proteolytic function has been shown to lie within the N terminus, the protease has two Arg-Gly-Asp motifs implicated in integrin binding and the C-terminus possess three fibronectin type III domains [49]. Group B streptococcus C5a peptidase has a high affinity for fibronectin, an interaction shown to be biologically significant as evidenced by streptococcal strains defective in the expression of C5a peptidase having 50% less fibronectin-binding capacity than wild type strain [50]. Furthermore, mice immunised with C5a peptidase poorly colonise the lungs [51].

Dipeptidyl peptidase IV (DPPIV, also known as cluster differentiation 26) is a serine exopeptidase widely studied in eukaryotes largely due to its role in tumour biology [52] and in regulating blood glucose levels [53]. It is considered a moonlighting protease as it exerts numerous additional functions to proteolysis that are dependent on cell localisation. These functions include acting as an adhesin, receptor, co-stimulatory protein and a role in apoptosis [54]. The functions of DPPIV in microbes, moonlighting or otherwise, are less well established. However, DPPIV in *P. gingivalis* and *Streptococcus suis* is known to function as an adhesin. In *P. gingivalis*, DPPIV binds fibronectin and this interaction is required to establish colonisation [55]. Fibronectin was not degraded by DPPIV and its binding was evident even in proteolytically inactive DPPIV mutants. Furthermore, binding fibronectin inhibited fibroblast recruitment and it was suggested that this could affect recovery from damage caused by inflammation [55]. Similarly, DPPIV in *S. suis* bound fibronectin and the inactivation of the DPPIV gene caused a significant decrease in the pathogens cellular adhesion ability and impaired virulence [56]. In the same study, mice that were vaccinated with purified DPPIV and challenged with a virulent strain of *S. suis* had a 100% survival rate after a week compared to a 100% mortality in the unvaccinated control group.

The gingipains of *P. gingivalis* are a group of trypsin-like cysteine proteases that contribute to periodontitis. The group consists of a lysine-specific gingipain (Kgp) and two arginine-specific gingipains, HRgpA and RgpB. Through their catalytic activity all three proteases are responsible for major tissue destruction and deregulation of immune cascade pathways, inducing inflammation and periodontal bone loss [57]. Incidentally, the arginine gingipains exhibit both endo- and exopeptidase functions [58]. HRgpA and Kgp also possess four non-catalytic haemagglutinin/adhesin (HA) regions. These structural domains together with the proteolytic active sites work in concert to bind and degrade haemoglobin, which is fundamental for both the growth and virulence of *P. gingivalis* [59]. The HA domain also has a high affinity for phospholipids, fibronogen, laminin, fibronectin and fibrin, and is thought to contribute to the greater tissue destruction capacity of HRgpA than RgpA [57]. As the HA regions enhance proteolytic activity, the gingipains differ from the other examples provided above, which appear to bind substrates that are not degraded by their protease function.

### 2.2 Proteases that bind plasminogen

The contact activation pathway of the coagulation cascade is an important component of innate immunity. Following contact activation by antigenic stimuli, a series of events occurs that ultimately results in the deposition of a fibrin mesh that captures invading pathogens and exposes them to bacterial peptides released from platelets and kinins [60, 61]. Once the infection has been cleared by leukocytes, the proenzyms plasminogen is cleaved by two serine proteases, tissue plasminogen activator (tPA) and urokinase plasminogen activator, to release serine protease plasmin, which degrades the fibrin clot [62]. Despite this formidable defence system, numerous bacteria have developed strategies to avoid fibrin clot confinement and subsequent immune clearance. One common mechanism involves the expression of surface proteins that bind plasminogen. These recruit host plasminogen to the cell surface and utilise host tPA and urokinase plasminogen activator to induce localised plasmin activity, thus facilitating fibrinolysis [60, 63]. Interestingly, some of the most extensively characterised plasminogen-binding proteins have been cytosolic or glycolytic pathway proteins localised to the cell surface by a yet unknown secretion mechanism [63]. Several extracellular and intracellular proteases have been shown to bind plasminogen, including endopeptidase O (PepO) from *Streptococcus pneumoniae*, two aminopeptidases from *M. hypneumoniae* and DPPIV from *Pseudomonas aeruginosa* [64–66]. PepO from *S. pneumoniae* [65] and glutamyl aminopeptidase [66] and leucine aminopeptidase (LAP) [67] from *M. hypneumoniae* were shown to bind plasminogen in a dose-dependent manner and that bound plasminogen was readily converted to plasmin in the presence of tPA. Notably, in the absence of tPA, these proteases did not directly cleave plasminogen to release plasmin. Adhesion was inhibited in the presence of a lysine analogue e-aminocaproic acid indicating a reliance on surface-exposed lysine residues for plasminogen binding [68]. Using a variety of complementing methodologies, including immunofluorescence, enzymatic shaving and surface biotinylation, all three proteases were found to be surface exposed despite the absence of any...
transmembrane domains, secretion motifs or signal peptides. Additionally, both *M. hyopneumoniae* aminopeptidases bound heparin, and the LAP also bound DNA, a moonlighting function shared with LAP from *E. coli* in which it has been linked to site-specific recombination and transcriptional control of the *carAB* operon [69]. PepO is a multifunctional adhesion by additionally binding fibronectin, and the inactivation of the pepO gene in *S. pneumoniae* was correlated with a significant decrease in epithelial invasiveness [65].

### 2.3 Proteases with Cpn functions

Molecular Cpons are proteins that assist in non-covalent folding or unfolding of other macromolecules. They often work in conjunction with proteases to degrade malfunctioning proteins by enabling greater substrate access by the protease. In all organisms, collaborative protease activity and Cpn protein quality control is driven by ATPases associated with diverse cellular activities (AAA⁺). AAA⁺ proteins are a class of Cpn-like ATPases involved in many vital cellular processes. Through ATP hydrolysis AAA⁺ proteins are able to generate a mechanical force that enables the remodelling of bound substrates [70]. In bacteria, AAA⁺-mediated protein degradation is usually performed by an AAA⁺ protein (such as ClpA or ClpX) that binds and unfolds a target substrate and then delivers it to a protease such as ClpP for proteolysis. There are currently five known AAA⁺-containing degradation pathways, comprising ClpXP, ClpAP, HslUV, FtsH and Lon. For most systems, the AAA⁺ domain and protease are encoded on separate polypeptides; however, both are present within a single polypeptide in FtsH and Lon [71]. Lon also possesses a non-catalytic DNA-binding domain [72]. As many aspects of both Lon and FtsH have been studied extensively, including the mechanics behind their functions [70], only a summary will be provided here.

Lon proteins are generally intracellular and are classified, with some contention, as belonging to the S16 family of serine proteases. Contention arises because the catalytic site is composed of a Ser–Lys dyad as opposed to the classical Ser–His–Asp triad typical of other serine proteases, and also because traditional inhibitors of serine proteases poorly impede Lon activity. Nevertheless, Lon plays multiple important roles in cells. Firstly, Lon degrades aged, misfolded or aggregated proteins [73]. In the genome-reduced mycoplasmas, Lon mimics the function of the ClpXP proteolysis system. In most other bacteria, ClpXP is responsible for the tmRNA-mediated aging and ribosome rescue system that appends an ssrA tag to the C-terminus of proteins targeted for proteolysis. Having lost genes encoding ClpX and ClpP, the mycoplasmal Lon protease not only executes ssrA tagging, but does so in a more efficient manner than typically seen for ClpXP [74].

Secondly, Lon plays an important role in various regulatory circuits by processing substrates involved in cell division, capsule synthesis and DNA transcription [73]. Thirdly, Lon acts in regulating virulence factors in pathogenic bacteria. For example, in *Pseudomonas aeruginosa* mutational analysis has shown Lon to have roles in swimming motility, twitching motility, biofilm formation, antibiotic susceptibility, lung colonisation and quorum sensing [75]. Regulation of virulence gene expression relies on the site-specific DNA-binding capability of Lon [76]. Virulence genes regulated by Lon include those carried on Salmonella pathogenicity island 1 [77] and the hpmBA and zapa genes of *Proteus mirabilis* [78]. This highlights the need to have a comprehensive understanding of the multifunctional nature of some proteases.

FtsH (also known as high-frequency lysogenisation by phage Λ or hlfB) is the only membrane-bound protein classified as both an AAA⁺ and zinc-dependent metalloprotease [79]. Due to this localisation, identified FtsH substrates are both cytosolic and membrane bound. FtsH substrates include components involved in the transcription of the heat shock operon, protein translocation and membrane biosynthesis [80]. In *Streptomyces peucetius*, FtsH exerts both Cpn and protease activities upon the DrrA and DrrB proteins, which form an ABC-type permease involved in the export of antibiotics [81].

The effects of *ftsH* gene knockouts appear to differ drastically between bacterial species. In *E. coli*, *Bradyrhizobium japonicum* and *Helicobacter pylori* *ftsH* mutations are lethal, yet in some bacteria they cause only mild growth impairments. However, all mutations in *ftsH* examined thus far have been associated with increased susceptibility to heat shock and osmotic stress [82].

Dual protease and Cpn function is not limited to members of the AAA⁺ superfamily. High-temperature requirement A (HtrA), also known as DegP, is a serine protease that is crucial for protein homeostasis in bacteria. Like Lon and FtsH, HtrA combines proteolytic and remodelling activities on a single polypeptide chain. The switch between the two functions is mediated by a structural loop covering the active sites and the movement of this loop appears to be temperature dependent [83]. HtrA is an important virulence factor in a number of Gram positive and Gram negative bacteria, an effect that has mainly been attributed to the protein’s role in protein quality control and the heat shock response [84]. However, despite traditionally being thought to be strictly periplasmic in Gram negative bacteria, HtrA is in the growing list of proteins found to be surface exposed or secreted via an unknown pathway. On the surface, HtrA protease function has been linked to dissemination in a number of pathogens by degrading the cell junctional protein E-cadherin [85] and in the pathogenesis of Lyme disease by binding and degrading aggrecan, a proteoglycan required for joint function [86].

One reason that molecular Cpons have been a focus of moonlighting research is that despite traditionally thought to be intracellular proteins, many are expressed extracellularly and act as cell signalling proteins or in bacterial adherence to host cells [87]. There is abundant evidence of Cpons functioning as chemoattractants and cytokine modulators [88]. In prokaryotes, only four Cpons have thus far been shown to communicate with host cells – Cpn 60 (also known as
HSP60 and GroEL), Cpn10 (HSP10), DnaK (HSP70) and peptidylprolyl isomerase [15]. Of particular interest is the Cpn60.2 mycobacterial molecular Cpn, the first identified Cpn with signalling properties [12]. Cpn60.2 moonlights as an adhesin that facilitates alveolar macrophage entry by binding the transmembrane protein CD43 found on the surface of several immune effector cells [89]. However, it has also become apparent that not all Cpn60.2 proteins possess the same moonlighting functions. A study using a murine asthma model found that out of the five Cpn60 proteins from various bacteria, only Cpn60.2 from Mycobacterium leprae had an anti-inflammatory effect, despite 95% sequence identity with the M. tuberculosis Cpn60.2, which was also tested [12]. Inhibition of inflammation and hyper-responsiveness induced by M. leprae Cpn60.2 may be linked to the fact that this Cpn is the only Cpn60 protein with demonstrable proteolytic activity [90], since proteases are established modulators of innate immune responses. The proteolytic activity is catalytically related to HsIV, which is the protease counterpart in the HsIVU AAA+ protein degradation system mentioned above.

Dual functioning protease/Cpns may be more common than currently appreciated. One study illustrated that both eukaryotic and prokaryotic aspartic proteases possess structural similarities to AAA+ by way of a double-β barrel domain at the N-terminus. This study also demonstrated that both pepsin and human immunodeficiency virus 1 protease exhibited inherent Cpn functions when their proteolytic action was blocked [91].

2.4 Proteases that act as receptors

Proteolytic cleavage events are well known to activate or inactivate various host receptor molecules and modulate host immune responses to microbial invasion. Several proteases are known to cleave protease-activated receptors, Toll-like receptors, receptors within the complement cascade and various cytokine receptors, thereby disrupting communication within the innate immune system and its signalling pathways [60]. In contrast, there are considerably fewer published examples of a protease functioning as a receptor.

However, some proteases are known to function as receptors for viruses on host cells. The coronaviridae are a large family of viruses that can cause disease in both humans and other animals ranging from the common cold, the recently discovered Middle East respiratory syndrome and severe acute respiratory syndrome. Exopeptidases are the sole identified class of receptors required for the process of attachment and membrane fusion of coronaviruses within host tissues [92]. The host cell receptor for severe acute respiratory syndrome is the metallocoxypeptidase angiotensin-converting enzyme 2. This receptor also binds the Human (H) CoV-NL63 virus. Fibronectin-binding DPP4 is a receptor for Middle East respiratory syndrome [92]. Perhaps the most well-known example of a protease viral receptor is aminopeptidase N (also known as CD13), as it is targeted by a number of human, porcine, feline and canine CoVs, as well as porcine transmissible gastroenteritis virus and feline infectious peritonitis virus [93].

It is not known whether microbial exopeptidases function as receptors for viruses. However, exopeptidase counterparts are found in the exoproteomes of a number of bacteria including S. pneumoniae, Burkholderia pseudomallei and Moraxella catarrhalis (Table 2). Furthermore, synergism occurs between viral and bacterial coinfections, particularly in the respiratory tract [94]. Over the last ten years, it has been established that viral infections aid bacteria colonisation and dissemination through various means, including disrupting mucociliary function [95], impairing immune effector cell function [96] and providing easy access to adhesin targets and nutrients via epithelial damage [97]. As the respiratory tract is a reservoir to a number of pathobionts, one hypothesis is that these microbes secrete potential viral protease receptors or chemoattractants to enhance their own colonisation.

2.5 Proteases with additional catalytic functions

The potential for proteases to possess additional, unexpected activities adds layers of complexity to our understanding of physiological processes. Although there are several examples of microbial proteases that catalyse reactions unrelated to proteolysis, information about their biological significance is scarce due to the intrinsic challenges faced when ascertaining specificity, redundancies and natural substrates in complex biological samples.

Site-directed mutagenesis, gene deletion studies and molecular modelling have been key in identifying unusual modes of action for proteases. For example, the papain-like proteases (PLPs) from the viral families Coronaviridae and Arteriviridae are responsible for processing the critical replicase polyprotein into its numerous functional units [98]. Structural similarities of PLPs to deubiquitinating enzymes showed that PLPs not only function as deubiquitinating enzymes, but are also able to block induction of several endogenous proinflammatory cytokines, including IFNβ, CCL5 and CXCL10 [98]. Another essential multifunctional viral protein is the hepatitis C virus NS3/4A, which possesses both chymotrypsin-like serine protease and helicase activities in two individual domains. As this protein is vital for viral propagation, it has been the target for the small-molecule inhibitors that have been commercially available for hepatitis C virus treatment since 2011 [99].

For other multi-catalytic proteases, biological relevance is less certain and studies often infer evolutionary and environmental advantages to an affinity for several different types of substrates. For example, there are enzymes that are able to hydrolyse different types of bonds. An aminopeptidase from the antibiotic-producing bacterium Streptomyces griseus [100] and a prolidase from the marine bacterium Alteromonas haloplanktis [101] are capable of cleaving amide, phosphoester
Table 2. Examples of cytosolic/periplasmic proteases found in microbial exoproteomes

| Protease | NCBI locus | Organism | Reference | Additional conversed domains |
|----------|------------|----------|-----------|-------------------------------|
| Aminopeptidase I | BB0366 | Borrelia burgdorferi | [126] | |
| Aminopeptidase M | XP_001682593 | Leishmania major | [127] | |
| Aminopeptidase N | AAK9510 | Streptococcus pneumoniae | [123] | |
| Aminopeptidase N | ZP_01769994 | Burkholderia pseudomallei | [128] | |
| Aminopeptidase N | ADG61634 | Moraxella catarrhalis | [129] | |
| Aminopeptidase T | NP_830199 | Bacillus cereus | [130] | |
| Carboxypeptidase | Q18AU1_CLOD6 | Clostridium difficile | [131] | Aminoacylase 1 |
| C-terminal processing peptidase | BP0609 | Bordetella pertussis | [132] | |
| ClpB | BP1196 | Bordetella pertussis | [132] | AAA+ ATPase |
| ClpC | NP_371049 | Staphylococcus aureus | [133] | |
| ClpP | ZP_21019400 | Escherichia coli | [133] | |
| Collagenase | XP_001682593 | Leishmania major | [127] | |
| Deblocking aminopeptidase | NP_834277 | Bacillus cereus | [130] | |
| DPPIII | XP_001687573 | Leishmania major | [132] | |
| DPPIV | AAL00295 | Streptococcus pneumoniae | [123] | |
| Endopeptidase O | AAL00485 | Streptococcus pneumoniae | [123] | |
| HtrA | E07_04918 | Haemophilus parasuis | [139] | |
| | BP2434 | Bordetella pertussis | [132] | |
| | POC0V | Escherichia coli | [140] | |
| | CAB13147 | Bacillus subtilis | [141] | |
| | CAD00819 | Listeria monocytogenes | [142] | |
| | ? | Brucella abortus | [143] | |
| L-Aminopeptidase d-amidase/o-esterase | YP_00505390 | Filifactor alocis | [136] | Endo-type 6 aminohexanoate-oligomer hydrolase |
| Leucine aminopeptidase | XP_00168531 | Leishmania major | [127] | |
| Lon protease | YP_00504101 | Filifactor alocis | [136] | AAA+ ATPase |
| Oligopeptidase F | AAT30295/AAT30449 | Bacillus anthracis | [134] | |
| Peptidase T | YP_00504583 | Filifactor alocis | [136] | Molecular chaperone |
| Peptidase T | NP_833858 | Bacillus cereus | [130] | |
| Peptidase T | AAT33487 | Bacillus anthracis | [134] | |
| Protease HsIV | PPF1_PYRFU | Pyrococcus furiosus | [137] | Glutamine aminotransferase |
| Protease RsIV | YP_513676 | Francisella tularensis | [144] | |
| Pyroglutamyl peptidase | YP_513002 | Francisella tularensis | [144] | |
| Signal peptide peptidase A | YP_514244 | Francisella tularensis | [144] | |
| TAP | PADA1 | Escherichia coli | [139] | |
| Thermolabile carboxypeptidase | NP_831344 | Bacillus cereus | [130] | |
| | CBP1_PYRFU | Pyrococcus furiosus | [137] | |
| | XP_001686015 | Leishmania major | [127] | |
| Thimet oligopeptidase | XP_00168416 | Leishmania major | [127] | |
| Transpeptidase | ZP_21022097 | Escherichia coli | [136] | Transglycosylase |
| Xaa-His dipeptidase | NP_372275 | Staphylococcus aureus | [133] | |
| Xaa-Pro aminopeptidase | CBA6449/ CBA64694 | Clostridium difficile | [131] | Creatinase |
| Zinc metalloproteases | XP_003722629 | Leishmania major | [127] | Prolidase |
| Zinc metalloproteases | XP_00364239 | Leishmania major | [127] | LysM |
| Zinc metalloproteases | BP1721 | Clostridium difficile | [146] | Acetylornithine deacetylase |

and phosphofluorinate bonds. This is interesting as the mechanics behind peptide and ester hydrolysis are dissimilar [100]. Another enzyme capable of mechanistically diverse activities is the thioesterase I (TAP) of *E. coli*, which exhibits thioesterase, arylerase, esterase, lysophospholipase and serine protease activities [102]. However, despite an ability to interact with a great number of different substrates, the role of TAP in vivo is not yet well established. Finally, the extremophilic bacterial species *Deinococcus radiodurans*, *Pyrococcus horikoshii* and *Thermoanaerobacter tengcongensis*...
all possess an enzyme that is both an aminoacylase (hydrolysing N-acetylated amino acids) and a carboxypeptidase. It has been suggested that the role of these multifunctional enzymes is in enabling peptidolytic growth in these organisms [103].

3 Identification of moonlighting proteases

Proteomic technologies have been at the forefront of efforts to characterise the identities of proteins that reside on the cell surface (surfaceome) or are secreted into the extracellular milieu (secretome) for a wide range of pathogenic Gram positive and Gram negative bacteria [18, 104]. These hypothesis-free methods have identified the presence of a diverse range of proteins that are localised on the cell surface or excreted into the extracellular milieu, regardless of transport mechanisms (exoproteome). The presence of proteins with well-defined enzymatic roles in the cytosol and their detection in the exoproteome continue to challenge our understanding of the mechanisms that enable these proteins to reach these extracellular sites and the role played by cell lysis [15, 19].

Generally, proteases need to be secreted or surface exposed to function as virulence factors. Notably, several of the known multifunctional proteases discussed in this review were initially predicted to be cytosolic but were then found to be surface exposed, a location typical of the largest group of known moonlighting proteins [13]. Table 2 provides examples of typically cytosolic proteases found in recent microbial exoproteomes.

One approach to identifying moonlighting proteases is to evaluate extracellular proteolytic activity in the exoproteome or via whole cell assays. Proteomic techniques including terminal amine isotopic labelling of substrates [105] have recently been developed [106] for the identification of proteolytic cleavage products and substrate, as well as proteomic identification of cleavage sites [107], providing information about protease cleavage site specificity.

Cell surface shaving with trypsin and other proteolytic enzymes and surface labelling of proteins with biotin are two powerful methodologies for identifying surface-accessible proteins. These approaches also detect potential moonlighting proteins. Regardless of the many proteomic methodologies available for identification of surface-exposed and secreted proteins (reviewed in [108, 109]), careful controls are necessary to estimate the role cell lysis has on the interpretation of these datasets. Despite extensive efforts to develop strategies to estimate cell lysis, it is difficult to determine if a protein has a bona fide role on the cell surface. Ultimately, alternative methods are required to confirm the surface location of putative moonlighting proteins identified using the high-throughput approaches described above.

The degree to which cytosolic proteins are dismissed seems to correspond to the type of analysis. For example, a surfaceome analysis implies proteins expressed at or specifically bound to the cell surface, and this results in proteins with annotated cytosolic functions being viewed as contaminants. The reporting of cytosolic proteins in surfaceome analyses varies from none in a study where 28 proteins were identified [110], to 17% cytosolic proteins reported using a false-positive control (50 proteins identified) [111], to >50% cytosolic proteins reported using a combination of methodologies [112]. Experiments cataloguing the exoproteome more often acknowledge the presence of cytosolic proteins, with the caveat that cell lysis will occur in a population of cells during the analysis and this constitutes a part of the exoproteome. Furthermore, the exoproteome can be interrogated using sera from infected animals or patients to identify the immunoproteome, providing valuable biological insights.

Recently, Solis et al. [113] addressed the issue of proteins that are secreted into the extracellular milieu that have the capacity to bind back to the cell surface as part of the surfaceome in S. aureus. Using cell surface shaving techniques with false-positive controls coupled with iTRAQ labelling, a scoring approach was proposed to calculate the probability of a protein being surface-exposed by taking into account the relative abundance of peptides identified in the shaved and control datasets. This kind of analysis is a significant move forward in the ability to identify geographical moonlighting proteins, although again there are caveats. Where proteases are present at the cell surface (whether moonlighting or not), proteolytic processing of surface proteins may occur, resulting in cleavage products, rather than intact proteins, being variably present on the cell surface or released into the extracellular milieu, and this may skew attempts at quantitative normalisation analyses. This “exo-processing” of proteins is akin to ectodomain shedding, a common phenomenon in eukaryotes [114], and can be seen in many pathogenic bacteria including Clostridium difficile with surface layer protein A [115], and in M. hyopneumoniae with the P97 and P102 paralogous families of adhesins [116–122].

An ideal workflow for characterising suspected moonlighting proteins would include a series of orthogonal methods to interrogate the identity of proteins on the cell surface, such as trypsin shaving and surface biotinylation coupled with immunofluorescence microscopy. Functions could be inferred by characterising the immunoproteome and applying affinity chromatography using known host components (e.g. fibronectin) as bait. Functions inferred by these methods would require cloning and expression of the putative moonlighting protein followed by detailed binding studies [123]. Whilst it is not always feasible or practical to pursue these follow-up investigations for each cytosolic protein identified in large-scale proteomic analyses, there is good reason to focus research efforts on microbial proteases in unusual locations: proteolysis is central to a multitude of pathological processes and several microbial proteases have now been shown to have additional functions that are frequently linked to virulence. A
full understanding of the pathogenic complexity of microbial proteases can contribute to combating microbial-associated disease.

The authors have declared no conflict of interest.

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