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Title: The detrimental impact of extracellular bacterial proteases on wound healing

Running title: Bacterial proteases

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Abstract

In addition to clinical signs of infection (e.g. inflammation, purulence and pain), a microbial count of ≥105 colony-forming units/g has historically been used to define wound infection. However, it is increasingly recognised that, rather than a high bioburden level alone being detrimental to wound healing, it is the virulence of the invading microorganism and the host's immune status that can affect clinical outcomes. Bacteria, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, have developed a range of virulence factors to help them overcome host defences and proliferate within the underlying soft tissue. More specifically, bacterial proteases are one such virulence factor that has been implicated in promoting the invasion and destruction of the host tissue. Because of the complexities of microorganisms, the proteases can negatively impact the wound environment, leading to delayed wound healing. The aim of the present paper is to describe various extracellular bacterial proteases; review the impact they have on the wound environment, the host immune response and biofilms; and discuss potential wound management strategies against them. The evidence discussed suggests that proteases may play a profound role in wound infections, contribute to the development of an inflammatory response and impede wound healing.

Introduction

The wound-healing process consists of four highly integrated and overlapping phases: haemostasis, inflammation, proliferation and tissue remodelling [1]. Multiple factors can lead to impaired wound healing. Some are systemic factors, whereby the overall health or disease state of the individual affects his or her ability to heal [2]. Examples of systemic factors known to impact wound healing are patient age, ischaemia and pre-existing medical conditions such as diabetes [2]. Local factors that directly influence the characteristics of the wound itself may also contribute to delayed healing. Local factors include oxygenation, venous insufficiency and infection [2]. When skin is injured, it allows microorganisms to access the underlying tissues, leading to wound infection. Wound infection has various stages of increasing severity, from contamination to colonisation, local infection/critical colonisation and/or spreading invasive infection [2]. This is known as the continuum of infection [3].

Many of the causative organisms of wound infections are opportunistic pathogens; these microorganisms may be part of the body's normal flora (e.g. *Staphylococcus* spp., *Streptococcus pyogenes*) or be commonly found in the environment (e.g. *Pseudomonas aeruginosa*). These organisms can exploit an ecological advantage, such as an immunocompromised host or a breech in the skin, to cause disease. The ability of such bacteria to cause disease is influenced by a variety of factors, including the number of bacteria present (known as the 'bioburden'), the site of infection and the 'virulence factors' of the microorganism. Virulence factors are produced by microorganisms and contribute to their pathogenicity [4-6].

Occasionally, the physical presence of bacteria may cause disease in the host; for example, high levels of bacteria may obstruct heart valves in endocarditis [7]. More commonly, however, virulence factors, such as enzymes or toxins produced by the microorganism, are the primary cause of detriment to the host [4, 6]. Examples of virulence factors contributing to disease can be found in conditions such as toxic shock syndrome [8] and Clostridium difficile-associated diarrhoea, where the symptoms of pseudomembranous colitis are caused by the effects of bacterial exotoxins [9, 10]. The same trend can be observed in sequelae such as wound infections [4].

Historically, a swab or biopsy sample returning a microbial count of >105/g tissue has been associated with wound infection and delayed wound healing [11, 12]. For some bacteria, such as *S. pyogenes* (β-haemolytic Streptococci; Group A Streptococci), levels far below <105/g tissue have been reported as leading to infection [13, 14]. Conversely, some wounds containing less pathogenic organisms, such as enterococci or diphtheroids, have been reported to heal with bioburden levels above 105/g tissue [4, 15]. Whilst the quantity of pathogenic bacteria in a wound has been shown to influence healing, this quantitative threshold and healing rate is also affected by endogenous host factors, such as the status of the immune system, underlying aetiologies and comorbidities, compounded by the type of

microbial species present and their associated virulence factors [15, 16]. The complexity of the establishment of infection can be expressed as: Infection = microbial bioburden x virulence/host resistance [17].

Overview of bacterial virulence factors

Virulence factors are molecules produced by microorganisms that contribute to the pathogenicity of the organism. There are many types of virulence factors, including adhesins, capsules, endotoxins, exotoxins, flagella, lipases, pilli and proteases. They can have a myriad of functional roles, including the capacity to facilitate microbial attachment, invasion or both as well as the promotion of the growth of a microbe in a host through avoidance of host detection, inhibition of phagocytosis and regulation of the capacity for intracellular survival [18]. Of these, proteases are discussed further in the following sections.

Bacterial proteases

Proteases are produced by a variety of microorganisms including both Gram-negative and Gram-positive bacteria, fungi and viruses [19-22]. Many pathogenic bacteria produce a range of proteases [23, 24], of which a number of the bacteria characterised as producing proteases are known wound pathogens and include *Staphyloccocus* spp., *Streptococcus* spp., *Enterococcus* spp. and *P. aeruginosa* [19, 20]. Table 1 lists common organisms and the proteases they produce. It is important to note, however, that despite the importance of bacterial proteases in delayed healing, the majority of proteases in non-healing wounds are endogenous; that is, they are produced by the host themselves as a result of prolonged inflammation [25].

Proteases can be broadly classified according to the location at which they cleave the target protein. Exoproteases cleave at or near the carboxi or amino terminals, whereas endopeptidases can cleave at up to five residues from these terminals [26]. This broad classification is not inclusive of all proteases as some, such as ADP-dependent proteases, do not fit this definition [27]. Proteases can be further categorised according to their catalytic activity and include aspartic proteases, cysteine proteases, glutamic proteases, metalloproteases, serine proteases and threonine proteases [28, 29].

Bacterial proteases can act either extracellularly or intracellularly. Processes such as sporulation and protein maturation within the microbial cell involve/require intracellular proteases [25], whilst extracellular protease are active outside of the microbial cell where they interact with the host environment to aid in the survival and proliferation of the microbial cell. The physiological function of extracellular bacterial proteases is to provide peptidic nutrients for the bacteria by hydrolysing (degrading) proteins in their surrounding environment [20, 28]. However, a fortuitous by-product of protease production for the microorganism is the degradation of host proteins, growth factors and receptors, which can impede the immune response and contribute towards tissue degradation, enabling further microbial dissemination into the underlying soft tissue [19, 23, 30-33]. Arguably, microbial proteases are considered to be among the most important type of microbial virulence factor influencing wound healing [20, 34, 35].

Impact of wound environment on production of bacterial proteases

As with other virulence factors, production and release of bacterial proteases may be mediated by regulatory factors, which govern the transcription of protease genes in response to the local environment of the bacteria [36]. Production may be influenced by a variety of factors, including nutrient availability, quorum sensing (a cell density-dependent signalling mechanism), growth phase, osmolarity, pH and temperature [37-43]. Such factors may be encountered during infection of the soft tissue [36].

Research conducted in vitro on protease production by 95 clinical strains of *Enterococcus faecalis*, specifically looking at Gelatinase (GelE), indicated that production of this protease is influenced by carbon source availability, pH, presence of divalent cations and temperature, suggesting that such conditions could affect the virulence of *E. faecalis* clinically [43]. A notable observation from this study was the effect of pH on GelE production, whereby protease activity peaked at around pH 8 but decreased as the pH of the culture medium was lowered [43]. Additionally, it was also observed that the addition of iron, copper or zinc to the culture media either completely eliminated, or dramatically reduced, GelE activity [43]. Interestingly, iron availability has also been shown to affect protease production in other bacteria, with *P. aeruginosa* protease IV expression found to be enhanced upon iron limitation [42].

Impact of bacterial proteases on the wound environment

The impact of bacterial proteases has been documented in a range of acute and chronic medical conditions, including impairment of lungs in the cystic fibrosis patient [44], eye infections [45-47], gastroenteritis [48] and wound infections [19, 21]. The majority of bacterial proteases research has focussed on the Gram-negative bacterium *P. aeruginosa*, where a strong correlation between the severity of an infection and *P. aeruginosa* protease levels has been reported, with higher levels of the *P. aeruginosa* elastase linked to increased inflammation and tissue damage [49, 50],whilst protease-deficient *P. aeruginosa* strains have been found to be less virulent than their protease-producing counterparts in burn wound mouse models [51, 52].

P. aeruginosa produces a number of proteases, with 155 of 5568 predicted genes of the commonly studied type strain PAO1 strain estimated to encode proteases [53, 54]. Elastase B (pseudolysin; LasB), a major metalloproteinase expressed by *P. aeruginosa*, has been demonstrated to degrade collagen and is thought to play a key role in cystic fibrosis lung infections [55]. This role is supported by several studies that have detected *P. aeruginosa* proteases in the lungs of cystic fibrosis patients [56-58]. Such collagen-degrading activity of *P. aeruginosa* may also occur in wound infections and may contribute to tissue damage [59].

Impact of bacterial proteases on the host immune response

If the protective barrier of the epidermis is breached due to a cut, abrasion or bite for example, it allows bacteria access to the underlying tissue where they may colonise, migrate and proliferate, leading to localised infection. During these initial phases, it is of benefit to the organisms to impede the immune response and so ensure the best possibility of its survival. Bacterial proteases play a significant role in the inhibition of the hosts' immune response through a range of mechanisms including induction of an inflammatory reaction, reduction in

phagocytosis, inactivation of the complement system, cytokine degradation, immunoglobulin degradation and inactivation of antimicrobial peptides (AMPs).

Induction of inflammatory reaction

Wound healing is a complex series of overlapping phases (inflammation, proliferation and tissue remodelling) that involves a myriad cells and mediators [60]. An inflammatory response is a typical and necessary part of normal wound healing and occurs as blood vessels dilate, which allows antibodies, white blood cells, enzymes and other beneficial elements into the affected area [61]. In some instances, bacterial proteases can also induce a host inflammatory response. For example, *P. aeruginosa* elastase A (LasA) protease enhances activity of several host elastolytic proteases, including human leukocyte elastase and human neutrophil elastase [62]. Whilst this may appear counterintuitive for the survival of the organism as it aids the removal of bacterial organisms from the site, if this inflammatory phase is prolonged, this can result in a prolonged elevation of the host's immune response, including host proteases, leading to wound chronicity [19, 63]. In these cases, the host's own immune components actively degrade the surrounding tissue without resolving the infection, facilitating the further dissemination of the infection into the surrounding and deeper-seated tissues.

One of the most notorious examples of a host immune component providing a dual role in wound healing are the matrix metalloproteinases (MMPs), which function in the extracellular environment of cells and degrade both matrix and non-matrix proteins. They play central roles in morphogenesis, wound healing, tissue repair and remodelling in response to injury, with several studies indicating that bacterial proteases may up-regulate host MMP production [64, 65]. MMPs play an important role in wound healing, facilitating several important processes including angiogenesis; removal of damaged extracellular matrix (ECM); transition of epithelial cells, fibroblasts and vascular endothelial cells across the ECM; contraction of scar ECM; and scar remodelling [66-71]. However, some chronic wounds become ‘stalled’ in the inflammatory phase of wound healing. In these instances, components pivotal in wound healing, such as growth factors, are degraded, and host proteases are abnormally elevated [72]. A direct consequence of abnormally elevated MMP activity includes a reduction in wound closure rates [73-75].

A further example of bacterial proteases contributing to induction of an inflammatory reaction in the host is through the proteases of *S. pyogenes* and *Staphylococcus aureus*. Proteases produced by these bacteria have been found to activate the kinin system and degrade kininogens, which subsequently induce an inflammatory reaction of oedema, redness and pain [34]. In addition, release of bacteria into the circulation may be promoted by kinin-enhanced vascular leakage, which will potentially allow for the spread of infection and may further perpetuate the pathophysiology of infectious diseases [34].

Reduction in phagocytosis

Similar to other immunological factors, phagocytosis can also be hindered by bacterial proteases [76]. The *P. aeruginosa* proteases alkaline protease (aeruginolysin; AprA) and LasB have been found to reduce leucocyte activity [77], inhibit the function of neutrophils and interfere with their chemotaxis [78]. The *S. aureus* cysteine protease staphopain B (SspB) can inhibit neutrophil phagocytosis and can also reduce neutrophil chemotactic activity [79, 80]. The intracellular survival of *S. pyogenes* in macrophages has been shown to be enhanced by

the streptopain (SpeB) cysteine protease in vivo [81], while Chiang-Hi and colleagues reported that SpeB can also prevent immune clearance of *S. pyogenes* by causing mitochondrial damage in polymorphonuclear neutrophils (PMN) [82].

Inactivation of the complement system

Complement involves a group of proteins that provide enzymatic activity and produce effector molecules, facilitating a range of immunological functions such as cell lysis (C5b-9), inflammation (C3a, C5a) and phagocytosis (C3b) [83]. Proteins C3 and C5 are involved in the initiation of an immune response and, as such, present as targets for bacterial proteases [84]. *P. aeruginosa* protease IV (lysyl endopeptidase; iron-regulated protein PrpL) can degrade a range of biologically important host proteins, such as the complement components C3 and C1q [85], whereas the *S. pyogenes* protease SpeB can prevent formation of C5 by degrading C3 [86, 87]. Consequently, as coating of bacteria with C3 is prevented, opsonisation and neutrophil phagocytosis is hindered or even prevented [84]. A further role of SpeB with respect to disarming the complement system is to cleave properdin. Properdin stabilises the formation of the C5 [88]. As such, cleavage of properdin can make the bacteria less susceptible to opsonophagocytosis by neutrophils [84]. Other bacterial species, such as the Gram-positive enteric bacterium E. faecalis, are also capable of inactivating complement. The protease gelatinase (coccolysin; GelE) of this microorganism is able to inactivate the host complement system by degrading C3 [89].

Cytokine degradation

Cytokines are small proteins (8–15 kDa) that include chemokines, colony-stimulating factors (CSF), interferons (IFN), interleukins (IL) and tumour necrosis factors (TNF) and are released in response to tissue damage. The many functions performed by cytokines include activation of phagocytic cells, antiviral and anti-parasitic activity, chemotaxis of neutrophils and T-cells, growth of macrophage colonies and proliferation of B- and T-cells. As such, cytokines represent an ideal target for bacteria in overcoming the host immune system, and a range of bacterial proteases have been found to be able to degrade cytokines and their receptors [84]. *P. aeruginosa* proteases hinder a range of cytokine activities and are also able to induce degradation of cytokines [59]. Examples include AprA degradation and inactivation of human interferon γ (INF-γ) [90], and inactivation of human tumour necrosis factor-α (TNF- α) by LasB [90, 91]. Both INF-γ and TNF-α play an important role in the host immune response, with a lack of INF-γ resulting in auto-inflammatory diseases [92, 93] and TNF-α involved in systemic inflammation and apoptosis [77]. The *P. aeruginosa* large extracellular protease (LepA) also increases IL-8 production and secretion [50, 94], which may have a detrimental effect on the host by elevating and prolonging an inflammatory response [95]. Another putative serine protease of *P. aeruginosa* (PA0328, also designated AaaA) has been shown to provide the bacterium with a selective advantage at establishing infection and long-term survival in a chronic mouse wound model. The authors also noted that higher levels of TNF-α and IL-1α expression was detected in response to the wild-type *P. aeruginosa* strain compared with an AaaA deletion mutant [96]. Bacterial proteases from other organisms such as L. monocytes, Serratia marcescens and *S. aureus* have also been shown to elevate interleukin levels [22].

Proteases of the Gram-positive skin pathogen *S. pyogenes* can also affect cytokine activity. The *S. pyogenes* protease SpeB can cleave the IL-1 precursor to produce biologically active IL-1, a principle mediator of inflammation [97]. An additional protease of *S. pyogenes*,

Streptococcal chemokine protease (ScpC), has been found to degrade IL-8 [34]. Given that IL-8 mediates neutrophil migration and activation, expression of ScpC can be detrimental to the host immune response. Proteases produced by other bacteria – for example, the Gram-positive skin pathogen *S. aureus* – can also interfere with IL-8 function. The serine proteases of this bacterium can modulate IL-8 synthesis [98].

Degradation of immunoglobulins

A further function of bacterial proteases in overcoming the host immune system is in the degradation of host immunoglobulin [59]. This can be particularly detrimental to the host given the role of immunoglobulins in recognising and contributing to the neutralisation of invading microorganisms. Various groups have reported the impact of *P. aeruginosa* proteases on the degradation of immunoglobulins and include the degradation of immunoglobulin A (IgA) and immunoglobulin G (IgG) by *P. aeruginosa* protease LasB and protease IV [47], respectively [99]. The *Proteus mirabilis* metalloprotease ZapA has also been implicated in degrading IgA [100, 101].

Inactivation of antimicrobial peptides

AMPs are antimicrobial agents produced by eukaryotic organisms to prevent microbial invasion. In humans, specific roles of antimicrobial peptides include killing invading bacteria primarily by disrupting the membrane integrity of the bacterial cell wall [84]. In general, AMPs are relatively resistant to proteolytic degradation, although there are some bacteria that are capable of producing proteases effective at cleaving and inactivating AMPs [84].

The strict anaerobe and opportunistic bacterium *Finegoldia magna* associated with skin infections produces a subtilisin-like serine protease SufA, which targets the human cathelicidin AMP LL-37 [102]. AMP LL-37 is also targeted by other bacterial proteases including SpeB of *S. pyogenes*, elastases of *P. aeruginosa*, GelE of E. faecalis and ZapA of P. mirabilis [102]. Proteolytic degradation of AMP LL-37 prevents binding of this antimicrobial peptide to the invading bacteria and, as such, destroys the bactericidal activity of the peptide [84]. Interestingly, recent data indicate that inactivation of LL-37 by the *S. pyogenes* protease SpeB can be found in patients with severe *S. pyogenes* soft tissue infections [103].

Bacterial proteases contributing to invasion

Once the innate barrier of the skin has been compromised and bacteria have gained entry to the underlying soft tissue, bacterial proteases can help the microorganism spread from the initial site of infection and invade the surrounding tissue [19, 20, 77, 104]. The presence of bacterial proteases and additional disruption of the epithelial barrier by these enzymes further compromises the protective barrier of the skin, which may allow other microbial species access to the location [34]. Specific examples of potential wound pathogens using proteases to contribute to invasion are discussed below.

Pseudomonas aeruginosa

P. aeruginosa proteases, including AprA, LasA, LasB and protease IV, can cause tissue damage during *P. aeruginosa* infections [59]. These proteases cause the proteolytic inactivation of the pathogen's adhesive molecules, which aids in the dissemination of bacteria from the initial site of infection [34]. Components of connective tissue, including collagen and elastin, have been demonstrated as being degraded by *P. aeruginosa* proteases in vitro [105, 106]. This

may have a detrimental effect on wound healing because collagen controls cellular functions (e.g. cell differentiation and cell migration) that are important during the phases of wound healing [107]. *P. aeruginosa* elastase B and alkaline proteases have also been found to degrade laminin α3 LG4-5, a component of the basement membrane in human skin [108]. Additionally, *P. aeruginosa* proteases may have a role in invasion and haemorrhagic tissue necrosis in infections [77], whilst protease IV can degrade fibrinogen [109].

LasA and LasB are among the most researched *P. aeruginosa* proteases and are thought to play a role in the pathogenesis of some *P. aeruginosa* strains [77, 110-113]. *P. aeruginosa* elastases have been found in clinical wound fluid samples [59] and are capable of degrading proteins on the surface of fibroblasts and inhibiting fibroblast growth [34]. Moreover, the *P. aeruginosa* protease LasA is involved in host ectodomain shedding whereby cell surface proteins are cleaved [114, 115], leading to epithelial disruption, tissue penetration and endothelial damage [116, 117]. *P. aeruginosa* strains producing LasB have also been found to inhibit fibroblast growth and degrade proteins from human wound fluid and skin biopsies [21, 59]. These observations suggest that *P. aeruginosa* proteases may be detrimental to wound healing [59].

Quorum sensing has been shown to contribute to the virulence of *P. aeruginosa*. For example, quorum sensing can regulate the expression of various virulence factors in *P. aeruginosa*, including pyocyanin, rhamnolipids and proteases such as the elastases LasA and LasB [77, 118]. The role of quorum sensing in infection has been demonstrated using quorum sensing-deficient *P. aeruginosa* strains in a range of in vivo models designed to mimic various conditions, including acute and chronic lung infections, burn wound infection and microbial keratitis. In these studies, the inability of quorum sensing-deficient strains to induce infection was thought to be due to decreased production of proteases and rhamnolipid [119-122]. These observations would appear to suggest that protease production in wound infections with *P. aeruginosa* increases as the density of the *P. aeruginosa* reaches a critical threshold.

Staphylococcus aureus

S. aureus proteases, such as Ssp (V8, a serine protease), can mediate a phenotypic change in the bacterium from adhesive to invasive by degrading its surface-associated adhesins [34]. The proteolysis of fibronectin-binding proteins by V8 decreases the adhesive phenotype of *S. aureus*, allowing for the diffusion of the pathogen. Such proteases (e.g. staphopain A) can also degrade host tissue, including collagen and elastin [34]. For example, the Staphopain A (ScpA) protease of *S. aureus* has comparable elastinolytic activity to host neutrophil elastase. This may contribute to the degradation of connective tissue in staphylococcal infections [123]. Additionally, similar to *P. aeruginosa* proteases, metalloprotease aureolysin and the serine proteinase V8 of *S. aureus* can also cleave laminin α3 LG4-5 [108].

Staphylococcus epidermidis

Staphylococcus epidermidis, a Gram-positive bacterium associated with the normal flora of healthy skin, may be pathogenic in immunocompromised patients and has been found to be responsible for surgical wound infections. Research indicates that the *S. epidermidis* cysteine protease (Ecp) has a similar sequence to ScpA and SspB proteases of *S. aureus* [124]. Moreover, Ecp mode of action is similar to ScpA and SspB in that it has elastinolytic activity.

Consequently, this may contribute to the invasiveness and pathogenicity of *S. epidermidis* in wounds [124].

Streptococcus pyogenes

Proteases play a pivotal role in the invasiveness of *S. pyogenes*, as indicated by *S. pyogenes* protease deletion mutants that were found to be two- to threefold less invasive than the wild-type strains when assessed in vitro on epithelial cells [125]. Additionally, numerous authors report that SpeB (streptopain) may affect the severity and migration of *S. pyogenes* infections [126-131]. SpeB has also been shown to be produced in vivo during infection in mouse and primate models [132-134] and can degrade fibronectin (1993) [135]. Other *S. pyogenes* proteases include Streptolysin S, which is involved in skin penetration [34], and IdeS (immunoglobulin G-degrading enzyme), which inhibits opsonophagocytosis [136].

Finegoldia magna

Finegoldia magna is a Gram-positive anaerobic bacterium associated with the normal microbiota of the skin. In immunocompromised hosts or when the normal microflora of the skin is disrupted, however, *F. magna* may act as an opportunistic pathogen [137]. In such circumstances, *F. magna* has been commonly isolated from chronic wounds including diabetic and pressure ulcers [138-143].

Contributing to tissue invasion by *F. magna* is the serine protease SufA [102, 137, 144]. Using *F. magna* SufA deletion mutants and electron microscopy, Murphy and colleagues eloquently demonstrated that SufA can degrade collagen IV and collagen V, potentially enabling this opportunistic pathogen to establish a deep-seated infection [137].

A further example of the influence of environmental conditions on the production of proteases can be found with *S. pyogenes* [36, 145]. Using a mouse soft tissue model, Loughman and Caparon identified a number of environmental factors, including growth phase, pH and NaCl concentration, which altered the activity of the SpeB protease [36]. Consistent with other publications, the authors also found that SpeB protease activity was associated with low pH [109, 146, 147]. The authors noted that as *S. pyogenes* entered stationary phase, the culture medium fell from an initial pH 7·5 to pH 6, with SpeB activity peaking in stationary phase. When a culture medium was buffered to maintain a constant pH of around pH 6, SpeB activity was independent of growth phase, meaning that protease activity could be induced in exponential phase. NaCl concentration was also shown to affect the activity of SpeB, with limited protease expression detected at physiological levels of NaCl (150 mM) and increasing protease activity detected as the NaCl concentration was increased [36]. Such conditions may be encountered in a clinical setting, and variations in the wound environment could impact bacterial protease production.

Protease activity in biofilms

It is increasingly acknowledged that many microorganisms have a predisposition to attach to surfaces, aggregate and form biofilms [148]. Biofilms are complex microbial communities containing bacteria and fungi. The microorganisms synthesise and secrete a protective matrix that attaches the biofilm firmly to a living or non-living surface [149].

Given the frequent isolation of biofilms from a wide range of environments, it is perhaps unsurprising that they have been detected in chronic wounds, which provide ideal conditions for bacterial attachment and proliferation [150]. The wound bed often contains necrotic tissue and debris, aiding bacterial adherence, while exudate provides nutrients to support bacterial growth [151, 152]. Additionally, chronic wounds are often associated with an impaired host immune response, increasing susceptibility to infection [151-153].

A study by James *et al.* using microscopy techniques reported that 60% of chronic wound specimens contained a biofilm, compared with only 6% of acute wound samples examined [150]. Other research groups reported biofilms in 47–59% of chronic wounds tested, correlating well with James' data [154, 155]. A further study suggests the figure could even be as high as 90% [156].

Upon the transition from planktonic or 'free-floating' bacteria to the establishment of a biofilm, bacteria undergo a general reduction in growth rates and metabolic activity, possibly contributing to a reduced susceptibility to antimicrobials [157]. Such reductions in metabolic activity and the establishment of the biofilm phenotype are associated with down-regulation of a number of genes [157]. Work by Evans *et al.* on *S. epidermidis* biofilms in vitro, however, suggests that protease-encoding genes are not down-regulated in this way [158]. In this study, total protease activity was analysed using a casein assay and showed that protease activity was detected in *S. epidermidis* biofilms at levels over and above *S. epidermidis* planktonic populations. Moreover, protease activity increased as the growth rates of the biofilm and planktonic populations were increased, with protease activity of the biofilm always exceeding that detected for planktonic cultures [158]. Another study using an in vitro and in vivo *C. elegans* infection model demonstrated that secretion of *S. epidermidis* proteases inhibited the development of *S. aureus* biofilms, which was mainly due to serine protease activity [159]. It has also been reported that *S. aureus* proteases (e.g. metalloprotease aureolysin and Sp1 protease) are involved in detaching established biofilms (i.e. targeting the surface adhesions) [34].

Novel wound management strategies

Due to the detrimental impact of bacterial proteases on the host and the ubiquitous nature of these enzymes, they could be exploited for the development of a point-of-care diagnostic. It is now increasingly recognised that bioburden alone does not necessarily correlate with infection, particularly in the early stages, where clinical signs of infection may be difficult to define [160]. In addition, the clinical signs of infection (pain, swelling, heat, redness, exudate) may not be present in patients with comorbidities that suppress the immune response, such as diabetes [161]. Under such circumstances, a bacterial protease point-of-care diagnostic may help clinicians decide when bacteria present in a wound are problematic [162]. This would help guide clinicians as to when it would be most appropriate to administer prophylactic treatment.

Serena and coworkers have described a novel point-of-care diagnostic test capable of identifying a wound in a 'state of pathogenesis' even before the clinical signs of infection become apparent [163]. Using wound fluid swab samples collected from 366 chronic wounds, the authors noted that elevated levels of bacterial protease activity (BPA) was detected in 49% of wound fluid samples despite only 18% of this cohort of patients demonstrating three

or more signs of clinical infection. Using elevated BPA as a marker, early identification of wounds in a state of pathogenesis, but where infection is not obvious to the clinician, could lead to a rapid response to reduce bacterial bioburden [161]. Such prompt action could improve the clinical outcome and could have potential economic benefits [164, 165]. Identification of elevated BPA in chronic wounds also provides a novel target for the future development of bacterial protease inhibitors.

Conclusions

Although the pathogenicity of a bacterium is the combined activity of the multiple virulence factors present in its portfolio, proteases remain a central means in enabling the microorganism to overcome the host defences and proliferate. Indeed, some authors even regard proteases as the most effective virulence factor in the establishment of infection [20, 35, 84], with functions including overcoming the host immune system, tissue degradation and promoting the up-regulation of additional virulence factors. Taken together, the evidence discussed in the present review suggests that proteases play a central role in the establishment of wound infections, contribute to the development of an inflammatory response and can impede wound healing.

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1082 TABLES.
1083
1084 Table 1. Proteases from common organisms [adapted from Koziel and Potempa (2012) [34]]
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Organism	Bacterial protease
Pseudomonas aeruginosa	Las A (elastase A)
	Las B (elastase B)
	AprA (alkaline protease)
	Protease IV
Staphylococcus aureus	Aureolysin
	ScpA (staphopain A)
	SspB (staphopain B)
	SspA (staphylococcal serine protease)
Streptococcus pyogenes	SpeB (streptopain; cysteine proteinase)
	Streptlysin S
	IdeS (cysteine proteinase)
	ScpC
Enterococcus faecalis	GelE (gelatinase)
	SprE (serine protease)
Staphylococcus epidermidis	Esp (serine protease)
Finegoldia magna	SufA (subtilisin-like serine protease)
Proteus mirabilis	ZapA (metalloprotease)
Aeromonas sobria	ASP (serine protease)
Vibrio vulnificus	metalloprotease

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