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Extracellular Bacterial Proteases in Chronic Wounds: A Potential Therapeutic Target?

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Significance: Bacterial biofilms are considered to be responsible for over 80% of persistent infections, including chronic lung infections, osteomyelitis, periodontitis, endocarditis, and chronic wounds. Over 60% of chronic wounds are colonized with bacteria that reside within a biofilm. The exaggerated proteolytic environment of chronic wounds, more specifically elevated matrix metalloproteinases, is thought to be one of the possible reasons as to why chronic wounds fail to heal. However, the role of bacterial proteases within chronic wounds is not fully understood.
Recent Advances: Recent research has shown that bacterial proteases can enable colonization and facilitate bacterial immune evasion. The inhibition of bacterial proteases such as *Pseudomonas aeruginosa* elastase B (LasB) has resulted in the disruption of the bacterial biofilm *in vitro*. *P. aeruginosa* is thought to be a key pathogen in chronic wound infection, and therefore, the disruption of these biofilms, potentially through the targeting of *P. aeruginosa* bacterial proteases, is an attractive therapeutic endeavor.
Critical Issues: Disrupting biofilm formation through the inhibition of bacterial proteases may lead to the dissemination of bacteria from the biofilm, allowing planktonic cells to colonize new sites within the wound.
Future Directions: Despite a plethora of evidence supporting the role of bacterial proteases as virulence factors in infection, there remains a distinct lack of research into the effect of bacterial proteases in chronic wounds. To assess the viability of targeting bacterial proteases, future research should aim to understand the role of these proteases in a variety of chronic wound subtypes.

Keywords: bacterial proteases, chronic wounds, infection, biofilm, *Pseudomonas aeruginosa, Staphylococcus aureus*

SCOPE AND SIGNIFICANCE

This review highlights our current understanding of bacterial proteases as facilitators of bacterial infection and immune evasion and as potential players in chronic wound pathogenesis. The therapeutic targeting of bacterial proteases and its viability as a potential treatment option in the management of chronic wounds shall be discussed.

TRANSLATIONAL RELEVANCE

Chronic wounds are characterized by delayed wound closure, persistent inflammation, and an amplified secretion of matrix metalloproteinases (MMPs). The colonization of microorganisms and the formation of a biofilm within a wound are thought to reduce wound closure and perpetuate inflammation. Bacterial proteases have been shown to target

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components of host immunity, creating a more favorable environment for the bacteria to reside. The inhibition of specific bacterial proteases may result in the disruption of biofilms and the promotion of wound closure.

CLINICAL RELEVANCE

Bacterial biofilms have been strongly associated with a number of infections, including chronic lung infections, osteomyelitis, periodontitis, and healthcare-associated infections. Biofilms are of great clinical importance primarily due to their strong association with increased resistance to antimicrobial therapies, and therefore, the effective treatment of these infections poses a great challenge. The search for alternative bacterial targets for therapeutic use is underway. The use of bacterial proteases as a diagnostic marker or as a method of biofilm disruption through their inhibition could be a welcome aid in chronic wound management.

OVERVIEW

Chronic wounds, including diabetic foot ulcers, pressure ulcers, and venous leg ulcers, pose a considerable economic burden, costing the National Health Service (NHS) an estimated £2.3–£3.1 billion per year.[1] These types of wounds are susceptible to colonization by numerous bacterial species (see Fig. 1). Investigation into the microbial profile of such wounds, as chronic venous leg ulcers, has revealed the most commonly isolated bacterial species to include *Staphylococcus aureus* (93.5%), *Enterococcus faecalis* (71.1%), *Pseudomonas aeruginosa* (52.2%), coagulase-negative Staphylococci (45.7%), *Proteus* species (43.1%), and anaerobic bacteria (39.1%).[2] To gain a more comprehensive understanding of the bacterial species within chronic wounds, such molecular techniques as pyrosequencing, denaturing gradient gel electrophoresis (DGGE), and full ribosome shotgun sequencing have allowed the identification of *Staphylococcus*, *Pseudomonas*, *Peptoniphilus*, *Enterobacter*, *Stenotrophomonas*, *Finegoldia,* and *Serratia* species in diabetic foot ulcers, venous leg ulcers, and pressure ulcers.[3]

There is ever-emerging evidence to suggest that the bacterial species within a wound reside within a biofilm. James and colleagues examined the presence of biofilms in both acute and chronic wounds, using scanning electron microscopy. They discovered a significant difference in the presence of biofilms between chronic and acute wounds, with 60% of chronic wounds containing a biofilm compared to just 6% in acute wounds ($p < 0.001$). Peptide nucleic acid-based fluorescence *in situ* hybridization has been used to determine the structural organization of bacteria within a chronic wound, ultimately showing the aggregation of bacteria into microcolonies within an alginate matrix, with very few planktonic cells present.[4] Biofilms are a major health concern, primarily due an increased recalcitrance to antimicrobial therapies compared to bacteria within a

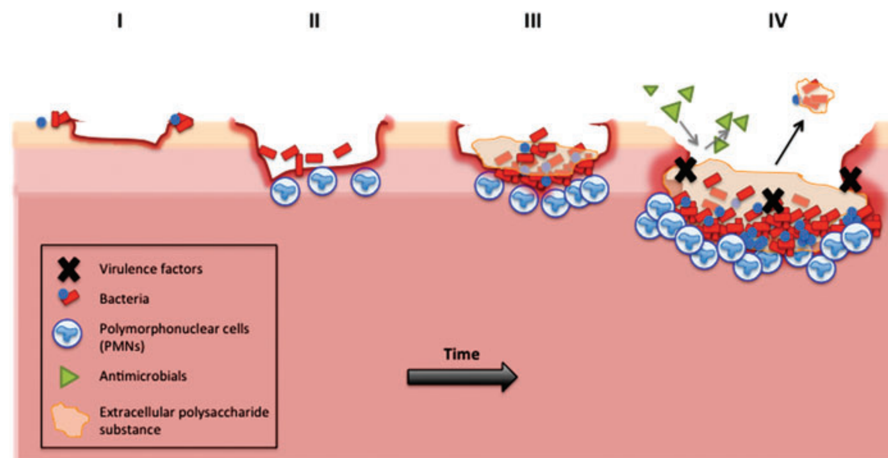

Figure 1. Schematic representation the development of a biofilm within a wound. **(I)** Bacteria, either present on the skin or contamination from an external source, reversibly attach to areas of slough or necrotic tissue. **(II)** Bacteria on the surface of the wound proliferate and become irreversibly attached through the use of bacterial appendages that anchor the bacteria to the tissue. **(III)** Colonization occurs when attached bacteria proliferate and produce extracellular polymeric substances (EPS), which is thought to protect the bacteria from external disruption. **(IV)** The mature biofilm, surrounded in EPS, is resistant to the use of antimicrobials. The release of virulence factors, including bacterial proteases, helps protect from a host immune response. Parts of the mature biofilm can break away from the main biofilm, a process known as dispersal. Ultimately, the dispersal of bacteria from the biofilm can lead to dissemination, whereby these bacteria attach and colonize new sites, perpetuating infection. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

planktonic state.[5] According to the National Institute of Health (United States), over 80% of persistent microbial infections within the body involve biofilms. Prevalent examples of biofilm-associated infections include chronic lung infections, periodontitis, endocarditis and osteomyelitis.[6–10] Bjarnsholt *et al.* hypothesized that the failure of a chronic wound to heal is due to the presence of *P. aeruginosa* biofilms.[11] Indeed, both *in vitro* and *in vivo* studies have demonstrated the deleterious effects of *P. aeruginosa* on wound closure.[12–14] The secretion of proteases from many species of bacteria is an essential process for bacterial growth and virulence. Therefore it is important to consider the impact of secreted bacterial proteases within biofilm-infected chronic wounds and whether targeting these proteases as a means of therapeutic intervention may be a fruitful venture.

DISCUSSION

Bacterial proteases

A brief introduction. Bacterial proteases embody a large and diverse group of proteases that are ubiquitously produced by all microorganisms, possessing a variety of physiological and biochemical functions.[15] The intracellular expression and extracellular secretion of proteases in both Gram-positive and Gram-negative bacteria are fundamental contributors to infection through the turnover of unfolded proteins in the host environment and the proteolysis of regulatory proteins upon environmental stimuli. Not dissimilar to mammalian proteases, bacterial proteases can be categorized into serine-, metallo-, cysteine-, and aspartic proteases. The synthesis of bacterial proteases begins within the cell and is an inactive proenzyme form, which becomes activated following extracellular autocatalytic cleavage.[16] Secreted extracellular proteases from bacterial species can act as toxins or virulence factors, and some simply play a role in the degradation of proteins (see Table 1).

Skin colonization and infection. There is emerging evidence to support the role of bacterial proteases in the attachment and penetration of skin. The Gram-positive anaerobe *Finegoldia magna*, a commensal microorganism commonly associated with skin microbiota, secretes two virulence factors that facilitate the attachment and penetration of the epidermal and dermal layers of the skin. *In vitro* studies have shown that the *F. magna* adhesion protein, Finegoldia magna adhesion factor binds with the keratinocyte marker galectin-7, while the extracellular serine protease SufA degrades collagen-IV, a major protein of the basement membrane, and collagen-V.[17] *S. aureus* staphylokinase (Sak), has been associated with the activation of plasminogen into plasmin, which in turn degrades fibrin clots and components of the extracellular matrix and activates latent MMPs.[18] Later studies by Kwiecinski *et al.* demonstrated the ability of transgenic *S. aureus* strains with high Sak expression (LS-1*spa-sak*) and moderate Sak expression (LS-1*sak*) to penetrate keratinocyte monolayers, fibrin clots, and reconstituted basal membranes *in vitro*.[19] Furthermore, immunocompromised wild-type and human plasminogen transgenic mice subcutaneously injected with *S. aureus* LS-1*spa-sak* and LS-1EP (no Sak expression) showed comparable systemic infection, demonstrating that the activation of plasminogen

Table 1. *Major secreted extracellular bacterial proteases of* Pseudomonas aeruginosa *and* Staphylococcus aureus*, their substrate specificities, and associated biological processes*

Organism	Bacterial Protease	MEROPS Family	Protease Class	Substrate	Associated Process	References
P. aeruginosa	Elastase A (LasA)	M23	Metallo-	Fibrinogen, elastin	ECM destruction	16,52
	Elastase B (Las B)	M4	Metallo-	Elastin, collagen III, collagen IV, MMP-1/MMP-9 (proenzyme), elastase B	ECM destruction, MMP proteolysis, autoproteolytic processing	16,53
	Alkaline protease (AprA)	M10	Metallo-	Fibrinogen, gelatin, casein, hemoglobin, cytokines	Complement inactivation, host immune evasion	16,54,55
	Protease IV	S1	Serine-	Plasminogen, fibrinogen, complement protein C3	Complement inactivation	16,56
S. aureus	Aureolysin	M4	Metallo-	Plasminogen, complement protein C3	Complement inactivation	16,23,57
	Staphopain A (ScpA)	C47	Cysteine-	Elastin	ECM destruction	16
	Staphopain B (SspB)	C47	Cysteine-	Fibrinogen, fibronectin, elastin	ECM destruction, complement inactivation	16,57
	Staphylococcal serine protease (SspA)	S1	Serine-	Actin, collagenase, IgG1 heavy chain, serum albumin, vimentin, casein	Host immune evasion, ECM degradation	16,57,58

ECM, extracellular matrix; MMP, matrix metalloproteinase.

by Sak does not cause the spread of infection. However, the bacterial-driven degradation of essential components of the basement membrane does not go unnoticed. The bacterial proteases *P. aeruginosa* elastase B (Las B), *P. aeruginosa* alkaline protease (AprA), *S. aureus* aureolysin, and *S. aureus* staphylococcal serine protease (SspA) were shown to cleave the C-terminal laminin G-domain-like modules of laminin α chains, a major glycoprotein of the basement membrane. However the cleavage of these laminin modules resulted in biologically active peptide fragments, which were shown to have antimicrobial properties and induce wound closure through the increase in keratinocyte migration and proliferation.[20]

Bacterial proteases and host immunity. In order for a pathogen to successfully invade the host and cause persistent infection, the pathogen must be able to evade host immune responses. Extracellular bacterial proteases have displayed the ability to evade host immune responses and target immune cell mediators. The first line of defense against invading microorganisms involves the infiltration of neutrophils and monocytes, which are able to effectively engulf pathogens. Despite this, the *S. aureus* cysteine protease staphopain B (SspB) is able to enhance its pathogenesis through the cleavage of CD11b, an essential component in phagocytosis, ultimately leading to phagocyte cell death through necrosis and apoptosis.[21] *S. aureus* cysteine proteases have also been implicated in the cleavage of a pulmonary surfactant protein A, which has been linked to a reduction in innate immune responses against lung infection such as neutrophil-driven phagocytosis.[22]

Bacteria can also use secreted proteases to degrade components of the complement system. Laarman *et al.* showed that the *S. aureus* metalloprotease aureolysin could effectively prevent complement-mediated phagocytosis through the cleavage of the C3 protein complex, facilitating immune evasion.[23] Furthermore, *P. aeruginosa* elastase can inactivate components of the complement system and complement-derived phagocytic factors.[24] *P. aeruginosa* is a prominent example of a microorganism that can successfully evade host immune responses. Early studies by Horvat and Parmely demonstrated the inhibitory effect of *P. aeruginosa* AprA on T-cell-derived interferon-γ (IFN- γ), reducing the antiviral capacity and immunomodulatory activity of IFN- γ.[25] *P. aeruginosa* LasB can effectively inactivate host antimicrobial peptides (AMPs), more specifically AMP LL-37, an important component of host innate immunity.[26] Specific bacterial proteases can also interfere with the host's biological communication networks through the cleavage or inactivation of host growth factors and cytokines.[27,28]

Impact of bacterial proteases in wound healing. Currently, there is no available research that investigates the impact of bacterial proteases on wound closure. Kirker *et al.* tested the effects of the conditioned medium of chronic wound-derived *S. aureus* in planktonic and biofilm form, on the *in vitro* wound closure of human epidermal keratinocytes, which resulted in a significant reduction in keratinocyte wound closure, decreased cell viability, and increased apoptosis.[13] Furthermore, later studies by the same research group showed that the presence of methicillin-resistant *S. aureus* (MRSA) planktonic-conditioned medium not only caused a significant reduction in human dermal fibroblast wound closure *in vitro* but also induced the release of a number of proinflammatory cytokines, including interleukin-6 (IL-6) and IL-8, growth factors, including vascular endothelial growth factor and transforming growth factor- beta, and the MMPs, MMP-1, and MMP-3.[12] Importantly, the treatment of human dermal fibroblasts with the MRSA biofilm-conditioned medium caused similar effects in fibroblast wound closure, however, there was a more prominent release of tumor necrosis factor-α. This simple, yet informative study emphasizes the differential effects of the soluble products of bacteria in planktonic and biofilm forms on host responses.

Elevated protease levels within chronic wounds, more specifically matrix metalloproteases (MMPs), have been well-documented.[29–31] The secretion of bacterial proteases within a wound may contribute to the exaggerated proteolytic environment of chronic wounds through the activation of MMPs (see Fig. 2). *P. aeruginosa* proteases have been shown to cleave and activate host MMPs in a model of corneal infection.[32] In addition, a mouse model of corneal infection immunized against *P. aeruginosa* AprA resulted in the reduction of host proteases, including MMP-2 and MMP-9.[33] This study highlighted the importance of *P. aeruginosa* AprA in the activation of host MMPs and the excessive tissue destruction evident in corneal infections.

Bacterial proteases as diagnostic markers in chronic wounds

Bacterial proteases are often secreted into the surrounding environment and therefore present an opportunity to utilize these proteases as diagnostic markers. There is a small amount of evidence to

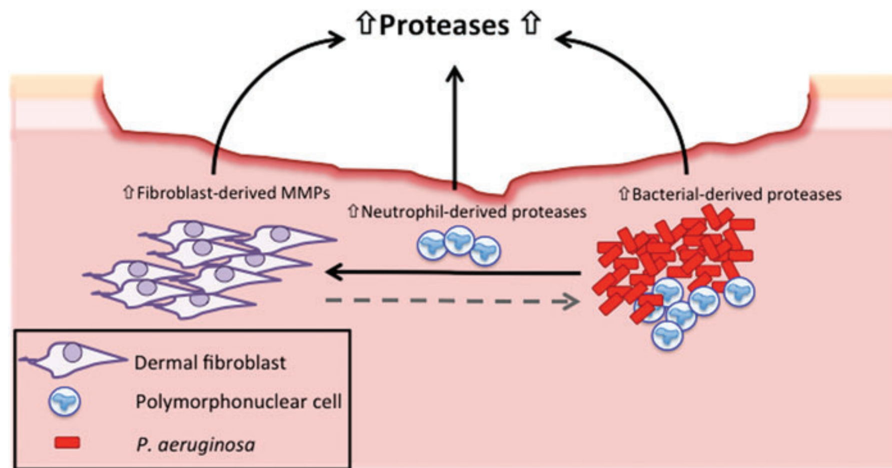

Figure 2. Schematic representation of the potential contribution of bacterial proteases to the proteolytic environment of chronic wounds. The presence of bacterial biofilms, particularly *Pseudomonas aeruginosa*, may contribute to the excessive production of proteases in chronic wound pathology, through the release of extracellular bacterial proteases. Furthermore, the presence of a *P. aeruginosa* biofilm within the wound may induce the release of matrix metalloproteinases (MMPs) from resident dermal fibroblasts. It is unknown whether fibroblast-derived MMPs effect the production of extracellular bacterial-derived proteases (indicated by *dashed grey arrow*). To see this illustration in color, the reader is referred to the web version of this article at www .liebertpub.com/wound

suggest that chronic wound-derived *P. aeruginosa* secrete bacterial proteases. One of the first research articles to acknowledge protease-producing bacteria isolated from chronic wounds was published in 2001, whereby *P. aeruginosa* from chronic leg ulcers showed varying levels of *P. aeruginosa* elastase, AprA, and an unidentified 100 kDa protease.[34] Wysocki *et al.* then went on to identify 18 different chronic wound-derived bacterial species (10 Gram-positive and 8 Gram-negative) that displayed proteolytic activity. The proteolytic activity of these bacterial isolates was not found to be consistent in any of these species after repeated isolation.[35] However, it is important to note that in this study, no quantitative data were presented to determine the varying levels of protease production between species. Another study by Wildeboer *et al.* sought to determine a correlation between the protease activity and bacterial load in chronic wounds using fluorescent-labeled peptide substrates. Although there was no correlation with most species identified in the wound and protease activity, the signal detection of two substrates strongly correlated with *P. aeruginosa* numbers.[36] This study highlights a potential use of these substrates as the basis for a diagnostic tool in the identification of *P. aeruginosa* colonization in chronic wounds. Zdzalik *et al.* aimed to identify a link between specific *S. aureus*-derived extracellular proteases and various types of infection through the investigation of prevalent *S. aureus* extracellular protease genes derived from cases of wound infection, pneumonia, sepsis, cystic fibrosis, skin infection, and bone infection to name a few.[37] In this study, the authors did not determine any correlation in gene expression patterns with specific types of infection, however, most of the *S. aureus* proteases investigated were expressed and secreted during the course of infection.

Detection of bacterial proteases. The proteolytic activity of extracellular secreted proteases can be detected by the use of substrates such as collagen, gelatin, and casein, using a variety of techniques. Probably, the most common and basic method to assess the proteolytic activity in a laboratory setting is the incorporation of these substrates into microbiological agar, whereby the presence of extracellular proteases results in a zone of clearance in the agar. The use of substrates conjugated to an azo dye such as azocasein or azocoll provide a more quantitative approach, in which the presence of proteases within a sample will cleave the substrate, releasing the conjugated dye. This can then be detected by using a spectrophotometer. However, this methodology lacks specificity; therefore complex biological samples with both host- and bacterial-derived proteases will be detected. A more specific method of bacterial protease detection is the use of fluorescent- or colorimetric-labeled peptide probes as demonstrated by Wildeboer *et al.* in the detection of *P. aeruginosa* proteases.[36] The use of specific peptide substrates allows the measurement of both qualitative and quantitative data. Despite the excellent specificity of commercially available peptide

substrates, complex clinical samples consisting of numerous proteases from both host and microorganisms can still result in nonspecific proteolytic cleavage, creating concern for their potential use as diagnostic tools.

Existing and potential treatment strategies and their effectiveness against bacterial proteases

Wound dressings. The discovery of elevated levels of MMPs within chronic wounds sparked the production of wound dressings comprising superabsorptive polymers that act to effectively regulate the overproduction of proteases residing in wound exudate.[38] Likewise, the incorporation of collagen-I substrates into wound dressings has been shown to effectively sequester not only the mammalian gelatinases MMP-2 and MMP-9 but also *Clostridium histolyticum* bacterial collagenase.[39]

Photodynamic therapy. Photodynamic therapy (PDT) is the application of a photoactive dye followed by irradiation, which leads to cell death in the presence of oxygen. The application of PDT has been used in the treatment of cancerous skin lesions and cancerous tumors of the head, neck, lung, and esophagus.[40] The concept of PDT to treat nonhealing chronic wounds and eradicate bacterial biofilms has gained much attention.[41] Interestingly, an *in vitro* study by Kömerik *et al.* demonstrated the effectiveness of PDT against *P. aeruginosa* proteases, whereby there was a significant reduction in *P. aeruginosa* proteases following exposure to red light in the presence of toluidine blue.[42] PDT may be a viable option in the treatment of biofilm-infected wounds through microbial cell death, reduction in bacterial proteases, and the promotion of wound closure.[43,44]

Bacterial proteases as therapeutic targets

The developing prevalence of antibiotic-resistant microorganisms, particularly in the context of healthcare-associated infections and their management, has propelled research into the discovery of new, effective treatment strategies and novel antimicrobials. The therapeutic targeting of proteases by pharmacologically attractive compounds has been successfully used in the treatment of many diseases, including hypertension, human immunodeficiency virus, and hepatitis C virus (HCV). For instance, pharmacologically approved serine protease inhibitor boceprevir (Victrelis; Merck) reversibly binds to and inhibits the HCV nonstructural 3 active site, preventing viral replication and thus sustaining the virologic response in patients with previously untreated, chronic HCV infection.[45,46] Despite this, the pharmacological targeting of bacterial proteases in the context of bacterial infection has not been fully exploited.

Current research in biofilm-infected wounds. Many of the secreted bacterial proteases are involved in bacterial virulence or growth, and therefore, the inhibition of these proteases may disrupt biofilm formation or increase biofilm susceptibility to antimicrobials.[47] Indeed, *P. aeruginosa* proteases have been shown to regulate biofilm formation, and therefore, the inhibition of these proteases *in vitro* has resulted in the disruption of the biofilm. *P. aeruginosa* LasB has been investigated as a target of protease inhibition. A novel and potent inhibitor of LasB, *N*-mercaptoacetyl-Phe-Tyr-amide, has been developed and shown to reduce *P. aeruginosa* biofilm growth, and when combined with additional antimicrobials, such as ciprofloxacin and gentamicin, can fully eradicate the biofilm *in vitro*.[48] Similarly, the deletion of LasB in *P. aeruginosa* PA01, referred to as a Lasb deletion mutant strain, has been shown to exhibit decreased bacterial attachment and microcolony formation. However, microcolony formation in the LasB deletion mutants was restored following exogenous rhamnolipid supplementation, therefore it was hypothesized that LasB may promote biofilm formation through rhamnolipid-mediated regulation.[49]

The inhibition of other bacterial proteases, however, may not necessarily result in the disruption of the biofilm. Research by Loughran *et al.* identified that *S. aureus* aureolysin and, to a lesser extent, the proteases staphopain A and SspB actually promote the dispersal of *S. aureus* biofilms.[50]

SUMMARY

While there is a clear role for bacterial proteases in the mediation of infection, the investigation of these proteases within chronic wounds has been somewhat marginalized. Research into the extracellular secreted proteases of *P. aeruginosa*, more specifically LasB, has revealed a regulatory role of this protease in biofilm development and therefore making LasB an attractive target not only for diagnostic purposes but also as an antibacterial target. Like many of the antibiofilm strategies that have been employed in

medicine, the disruption of the bacterial biofilm simply leads to its dispersal, therefore allowing planktonic bacteria to colonize other sites. Therefore, it is important to consider the use of antimicrobials in addition to biofilm disruption to discourage new colonization sites.

While the specific targeting of bacterial proteases associated with key pathogens such as *P. aeruginosa* may help weaken bacterial virulence, it is imperative that more research into the detection of these proteases in a variety of chronic wound types is performed. Indeed, no two chronic wounds are the same, and, in the context of microbial burden, the variability of microbial profiles in varying wound types has been demonstrated.[3,51] Therefore, in addition to the varying conditions of wounds, factors such as a nutrient availability and the interactions between multiple species of microorganisms may alter specific protease production. Nevertheless, the use of bacterial proteases to control infection processes will provide interesting research in the field of microbiology and chronic wounds.

TAKE-HOME MESSAGES

- Bacteria secrete proteases that facilitate the infection process, through skin penetration and host immune evasion, creating a more favorable environment for bacteria to reside.
- As many bacterial proteases are secreted into the surrounding environment, these proteases become an ideal candidate for the creation of a diagnostic tool or an antimicrobial target.
- Some bacterial proteases play a regulatory role in biofilm formation, and *in vitro* studies have concluded that the inhibition of specific bacterial proteases, such as *P. aeruginosa* LasB, can lead to the disruption of the biofilm.
- The disruption of biofilm may lead to dissemination and colonization of new sites within the wound, and therefore, the inhibition of specific bacterial proteases must be approached with caution.
- Further research into the role of bacterial proteases in the pathogenesis of chronic wounds should be considered.

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Abbreviations and Acronyms

AprA = alkaline protease
AMP = antimicrobial peptide
DGGE = denaturing gradient gel electrophoresis
ECM = extracellular matrix
HCV = hepatitis C virus
IFN-γ = interferon-gamma
IL-6 = interleukin-6
LasA = elastase A
LasB = elastase B
MRSA = methicillin-resistant *Staphylococcus aureus*
MMPs = matrix metalloproteinases
PDT = photodynamic therapy
ScpA = staphopain A
SspA = staphylococcal serine protease
SspB = staphopain B
Sak = staphylokinase