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Lindsay, S, Oates, A and Bourdillon, K (2017) The detrimental impact of extracellular bacterial proteases on wound healing. *International Wound Journal*, 14 (6). pp. 1237-1247. ISSN: 1742-4801

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

2

3 **Running title:** Bacterial proteases

4

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14 **Key words:**

15 Virulence factor, bacterial protease, wound infection, point-of-care diagnostic

16

17 **Abstract**

18 In addition to clinical signs of infection (e.g. inflammation, purulence and pain), a microbial
19 count of $\geq 10^5$ colony-forming units/g has historically been used to define wound infection.
20 However, it is increasingly recognised that, rather than a high bioburden level alone being
21 detrimental to wound healing, it is the virulence of the invading microorganism and the host's
22 immune status that can affect clinical outcomes. Bacteria, such as *Pseudomonas aeruginosa*,
23 *Staphylococcus aureus* and *Staphylococcus epidermidis*, have developed a range of virulence
24 factors to help them overcome host defences and proliferate within the underlying soft tissue.
25 More specifically, bacterial proteases are one such virulence factor that has been implicated
26 in promoting the invasion and destruction of the host tissue. Because of the complexities of
27 microorganisms, the proteases can negatively impact the wound environment, leading to
28 delayed wound healing. The aim of the present paper is to describe various extracellular
29 bacterial proteases; review the impact they have on the wound environment, the host
30 immune response and biofilms; and discuss potential wound management strategies against
31 them. The evidence discussed suggests that proteases may play a profound role in wound
32 infections, contribute to the development of an inflammatory response and impede wound
33 healing.

34

35 Introduction

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

47

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

57

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

66

67 Historically, a swab or biopsy sample returning a microbial count of $>10^5$ /g tissue has been
68 associated with wound infection and delayed wound healing [11, 12]. For some bacteria, such
69 as *S. pyogenes* (β -haemolytic Streptococci; Group A Streptococci), levels far below $<10^5$ /g
70 tissue have been reported as leading to infection [13, 14]. Conversely, some wounds
71 containing less pathogenic organisms, such as enterococci or diphtheroids, have been
72 reported to heal with bioburden levels above 10^5 /g tissue [4, 15]. Whilst the quantity of
73 pathogenic bacteria in a wound has been shown to influence healing, this quantitative
74 threshold and healing rate is also affected by endogenous host factors, such as the status of
75 the immune system, underlying aetiologies and comorbidities, compounded by the type of

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

79

80 **Overview of bacterial virulence factors**

81 Virulence factors are molecules produced by microorganisms that contribute to the
82 pathogenicity of the organism. There are many types of virulence factors, including adhesins,
83 capsules, endotoxins, exotoxins, flagella, lipases, pilli and proteases. They can have a myriad
84 of functional roles, including the capacity to facilitate microbial attachment, invasion or both
85 as well as the promotion of the growth of a microbe in a host through avoidance of host
86 detection, inhibition of phagocytosis and regulation of the capacity for intracellular survival
87 [18]. Of these, proteases are discussed further in the following sections.

88

89 **Bacterial proteases**

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

99

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

107

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

120 **Impact of wound environment on production of bacterial proteases**

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

127
128 Research conducted in vitro on protease production by 95 clinical strains of *Enterococcus*
129 *faecalis*, specifically looking at Gelatinase (GelE), indicated that production of this protease is
130 influenced by carbon source availability, pH, presence of divalent cations and temperature,
131 suggesting that such conditions could affect the virulence of *E. faecalis* clinically [43]. A
132 notable observation from this study was the effect of pH on GelE production, whereby
133 protease activity peaked at around pH 8 but decreased as the pH of the culture medium was
134 lowered [43]. Additionally, it was also observed that the addition of iron, copper or zinc to the
135 culture media either completely eliminated, or dramatically reduced, GelE activity [43].
136 Interestingly, iron availability has also been shown to affect protease production in other
137 bacteria, with *P. aeruginosa* protease IV expression found to be enhanced upon iron limitation
138 [42].

139

140 **Impact of bacterial proteases on the wound environment**

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

150

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

158

159 **Impact of bacterial proteases on the host immune response**

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

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

168

169 **Induction of inflammatory reaction**

170 Wound healing is a complex series of overlapping phases (inflammation, proliferation and
171 tissue remodelling) that involves a myriad cells and mediators [60]. An inflammatory response
172 is a typical and necessary part of normal wound healing and occurs as blood vessels dilate,
173 which allows antibodies, white blood cells, enzymes and other beneficial elements into the
174 affected area [61]. In some instances, bacterial proteases can also induce a host inflammatory
175 response. For example, *P. aeruginosa* elastase A (LasA) protease enhances activity of several
176 host elastolytic proteases, including human leukocyte elastase and human neutrophil elastase
177 [62]. Whilst this may appear counterintuitive for the survival of the organism as it aids the
178 removal of bacterial organisms from the site, if this inflammatory phase is prolonged, this can
179 result in a prolonged elevation of the host's immune response, including host proteases,
180 leading to wound chronicity [19, 63]. In these cases, the host's own immune components
181 actively degrade the surrounding tissue without resolving the infection, facilitating the further
182 dissemination of the infection into the surrounding and deeper-seated tissues.

183

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

197

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

205 Reduction in phagocytosis

206

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

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

216 Inactivation of the complement system

217

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

233 Cytokine degradation

234

235 Cytokines are small proteins (8–15 kDa) that include chemokines, colony-stimulating factors
236 (CSF), interferons (IFN), interleukins (IL) and tumour necrosis factors (TNF) and are released
237 in response to tissue damage. The many functions performed by cytokines include activation
238 of phagocytic cells, antiviral and anti-parasitic activity, chemotaxis of neutrophils and T-cells,
239 growth of macrophage colonies and proliferation of B- and T-cells. As such, cytokines
240 represent an ideal target for bacteria in overcoming the host immune system, and a range of
241 bacterial proteases have been found to be able to degrade cytokines and their receptors [84].
242 *P. aeruginosa* proteases hinder a range of cytokine activities and are also able to induce
243 degradation of cytokines [59]. Examples include AprA degradation and inactivation of human
244 interferon γ (INF- γ) [90], and inactivation of human tumour necrosis factor- α (TNF- α) by LasB
245 [90, 91]. Both INF- γ and TNF- α play an important role in the host immune response, with a
246 lack of INF- γ resulting in auto-inflammatory diseases [92, 93] and TNF- α involved in systemic
247 inflammation and apoptosis [77]. The *P. aeruginosa* large extracellular protease (LepA) also
248 increases IL-8 production and secretion [50, 94], which may have a detrimental effect on the
249 host by elevating and prolonging an inflammatory response [95]. Another putative serine
250 protease of *P. aeruginosa* (PA0328, also designated AaaA) has been shown to provide the
251 bacterium with a selective advantage at establishing infection and long-term survival in a
252 chronic mouse wound model. The authors also noted that higher levels of TNF- α and IL-1 α
253 expression was detected in response to the wild-type *P. aeruginosa* strain compared with an
254 AaaA deletion mutant [96]. Bacterial proteases from other organisms such as *L. monocytogenes*,
255 *Serratia marcescens* and *S. aureus* have also been shown to elevate interleukin levels [22].

256

257 Proteases of the Gram-positive skin pathogen *S. pyogenes* can also affect cytokine activity.
258 The *S. pyogenes* protease SpeB can cleave the IL-1 precursor to produce biologically active IL-
259 1, a principle mediator of inflammation [97]. An additional protease of *S. pyogenes*,

260 Streptococcal chemokine protease (ScpC), has been found to degrade IL-8 [34]. Given that IL-
261 8 mediates neutrophil migration and activation, expression of ScpC can be detrimental to the
262 host immune response. Proteases produced by other bacteria – for example, the Gram-
263 positive skin pathogen *S. aureus* – can also interfere with IL-8 function. The serine proteases
264 of this bacterium can modulate IL-8 synthesis [98].

265 Degradation of immunoglobulins

266
267 A further function of bacterial proteases in overcoming the host immune system is in the
268 degradation of host immunoglobulin [59]. This can be particularly detrimental to the host
269 given the role of immunoglobulins in recognising and contributing to the neutralisation of
270 invading microorganisms. Various groups have reported the impact of *P. aeruginosa*
271 proteases on the degradation of immunoglobulins and include the degradation of
272 immunoglobulin A (IgA) and immunoglobulin G (IgG) by *P. aeruginosa* protease LasB and
273 protease IV [47], respectively [99]. The *Proteus mirabilis* metalloprotease ZapA has also been
274 implicated in degrading IgA [100, 101].

275

276 **Inactivation of antimicrobial peptides**

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

282

283 The strict anaerobe and opportunistic bacterium *Fingoldia magna* associated with skin
284 infections produces a subtilisin-like serine protease SufA, which targets the human
285 cathelicidin AMP LL-37 [102]. AMP LL-37 is also targeted by other bacterial proteases
286 including SpeB of *S. pyogenes*, elastases of *P. aeruginosa*, GelE of *E. faecalis* and ZapA of *P.*
287 *mirabilis* [102]. Proteolytic degradation of AMP LL-37 prevents binding of this antimicrobial
288 peptide to the invading bacteria and, as such, destroys the bactericidal activity of the peptide
289 [84]. Interestingly, recent data indicate that inactivation of LL-37 by the *S. pyogenes* protease
290 SpeB can be found in patients with severe *S. pyogenes* soft tissue infections [103].

291 Bacterial proteases contributing to invasion

292

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

300

301 ***Pseudomonas aeruginosa***

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

307 may have a detrimental effect on wound healing because collagen controls cellular functions
308 (e.g. cell differentiation and cell migration) that are important during the phases of wound
309 healing [107]. *P. aeruginosa* elastase B and alkaline proteases have also been found to
310 degrade laminin $\alpha 3$ LG4-5, a component of the basement membrane in human skin [108].
311 Additionally, *P. aeruginosa* proteases may have a role in invasion and haemorrhagic tissue
312 necrosis in infections [77], whilst protease IV can degrade fibrinogen [109].

313
314 LasA and LasB are among the most researched *P. aeruginosa* proteases and are thought to
315 play a role in the pathogenesis of some *P. aeruginosa* strains [77, 110-113]. *P. aeruginosa*
316 elastases have been found in clinical wound fluid samples [59] and are capable of degrading
317 proteins on the surface of fibroblasts and inhibiting fibroblast growth [34]. Moreover, the *P.*
318 *aeruginosa* protease LasA is involved in host ectodomain shedding whereby cell surface
319 proteins are cleaved [114, 115], leading to epithelial disruption, tissue penetration and
320 endothelial damage [116, 117]. *P. aeruginosa* strains producing LasB have also been found to
321 inhibit fibroblast growth and degrade proteins from human wound fluid and skin biopsies [21,
322 59]. These observations suggest that *P. aeruginosa* proteases may be detrimental to wound
323 healing [59].

324
325 Quorum sensing has been shown to contribute to the virulence of *P. aeruginosa*. For example,
326 quorum sensing can regulate the expression of various virulence factors in *P. aeruginosa*,
327 including pyocyanin, rhamnolipids and proteases such as the elastases LasA and LasB [77,
328 118]. The role of quorum sensing in infection has been demonstrated using quorum sensing-
329 deficient *P. aeruginosa* strains in a range of in vivo models designed to mimic various
330 conditions, including acute and chronic lung infections, burn wound infection and microbial
331 keratitis. In these studies, the inability of quorum sensing-deficient strains to induce infection
332 was thought to be due to decreased production of proteases and rhamnolipid [119-122].
333 These observations would appear to suggest that protease production in wound infections
334 with *P. aeruginosa* increases as the density of the *P. aeruginosa* reaches a critical threshold.

335
336 ***Staphylococcus aureus***
337 *S. aureus* proteases, such as Ssp (V8, a serine protease), can mediate a phenotypic change in
338 the bacterium from adhesive to invasive by degrading its surface-associated adhesins [34].
339 The proteolysis of fibronectin-binding proteins by V8 decreases the adhesive phenotype of *S.*
340 *aureus*, allowing for the diffusion of the pathogen. Such proteases (e.g. staphopain A) can also
341 degrade host tissue, including collagen and elastin [34]. For example, the Staphopain A (ScpA)
342 protease of *S. aureus* has comparable elastinolytic activity to host neutrophil elastase. This
343 may contribute to the degradation of connective tissue in staphylococcal infections [123].
344 Additionally, similar to *P. aeruginosa* proteases, metalloprotease aureolysin and the serine
345 proteinase V8 of *S. aureus* can also cleave laminin $\alpha 3$ LG4-5 [108].

346
347 ***Staphylococcus epidermidis***
348 *Staphylococcus epidermidis*, a Gram-positive bacterium associated with the normal flora of
349 healthy skin, may be pathogenic in immunocompromised patients and has been found to be
350 responsible for surgical wound infections. Research indicates that the *S. epidermidis* cysteine
351 protease (Ecp) has a similar sequence to ScpA and SspB proteases of *S. aureus* [124].
352 Moreover, Ecp mode of action is similar to ScpA and SspB in that it has elastinolytic activity.

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

355

356 ***Streptococcus pyogenes***

357 Proteases play a pivotal role in the invasiveness of *S. pyogenes*, as indicated by *S. pyogenes*
358 protease deletion mutants that were found to be two- to threefold less invasive than the wild-
359 type strains when assessed in vitro on epithelial cells [125]. Additionally, numerous authors
360 report that SpeB (streptopain) may affect the severity and migration of *S. pyogenes* infections
361 [126-131]. SpeB has also been shown to be produced in vivo during infection in mouse and
362 primate models [132-134] and can degrade fibronectin (1993) [135]. Other *S. pyogenes*
363 proteases include Streptolysin S, which is involved in skin penetration [34], and IdeS
364 (immunoglobulin G-degrading enzyme), which inhibits opsonophagocytosis [136].

365

366 ***Fingoldia magna***

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

372

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

377

378 A further example of the influence of environmental conditions on the production of
379 proteases can be found with *S. pyogenes* [36, 145]. Using a mouse soft tissue model,
380 Loughman and Caparon identified a number of environmental factors, including growth
381 phase, pH and NaCl concentration, which altered the activity of the SpeB protease [36].
382 Consistent with other publications, the authors also found that SpeB protease activity was
383 associated with low pH [109, 146, 147]. The authors noted that as *S. pyogenes* entered
384 stationary phase, the culture medium fell from an initial pH 7.5 to pH 6, with SpeB activity
385 peaking in stationary phase. When a culture medium was buffered to maintain a constant pH
386 of around pH 6, SpeB activity was independent of growth phase, meaning that protease
387 activity could be induced in exponential phase. NaCl concentration was also shown to affect
388 the activity of SpeB, with limited protease expression detected at physiological levels of NaCl
389 (150 mM) and increasing protease activity detected as the NaCl concentration was increased
390 [36]. Such conditions may be encountered in a clinical setting, and variations in the wound
391 environment could impact bacterial protease production.

392 Protease activity in biofilms

393

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

398

399 Given the frequent isolation of biofilms from a wide range of environments, it is perhaps
400 unsurprising that they have been detected in chronic wounds, which provide ideal conditions
401 for bacterial attachment and proliferation [150]. The wound bed often contains necrotic
402 tissue and debris, aiding bacterial adherence, while exudate provides nutrients to support
403 bacterial growth [151, 152]. Additionally, chronic wounds are often associated with an
404 impaired host immune response, increasing susceptibility to infection [151-153].
405

406 A study by James *et al.* using microscopy techniques reported that 60% of chronic wound
407 specimens contained a biofilm, compared with only 6% of acute wound samples examined
408 [150]. Other research groups reported biofilms in 47–59% of chronic wounds tested,
409 correlating well with James' data [154, 155]. A further study suggests the figure could even
410 be as high as 90% [156].
411

412 Upon the transition from planktonic or 'free-floating' bacteria to the establishment of a
413 biofilm, bacteria undergo a general reduction in growth rates and metabolic activity, possibly
414 contributing to a reduced susceptibility to antimicrobials [157]. Such reductions in metabolic
415 activity and the establishment of the biofilm phenotype are associated with down-regulation
416 of a number of genes [157]. Work by Evans *et al.* on *S. epidermidis* biofilms in vitro, however,
417 suggests that protease-encoding genes are not down-regulated in this way [158]. In this study,
418 total protease activity was analysed using a casein assay and showed that protease activity
419 was detected in *S. epidermidis* biofilms at levels over and above *S. epidermidis* planktonic
420 populations. Moreover, protease activity increased as the growth rates of the biofilm and
421 planktonic populations were increased, with protease activity of the biofilm always exceeding
422 that detected for planktonic cultures [158]. Another study using an in vitro and in vivo *C.*
423 *elegans* infection model demonstrated that secretion of *S. epidermidis* proteases inhibited
424 the development of *S. aureus* biofilms, which was mainly due to serine protease activity [159].
425 It has also been reported that *S. aureus* proteases (e.g. metalloprotease aureolysin and Sp1
426 protease) are involved in detaching established biofilms (i.e. targeting the surface adhesions)
427 [34].
428

429 **Novel wound management strategies**

430 Due to the detrimental impact of bacterial proteases on the host and the ubiquitous nature
431 of these enzymes, they could be exploited for the development of a point-of-care diagnostic.
432 It is now increasingly recognised that bioburden alone does not necessarily correlate with
433 infection, particularly in the early stages, where clinical signs of infection may be difficult to
434 define [160]. In addition, the clinical signs of infection (pain, swelling, heat, redness, exudate)
435 may not be present in patients with comorbidities that suppress the immune response, such
436 as diabetes [161]. Under such circumstances, a bacterial protease point-of-care diagnostic
437 may help clinicians decide when bacteria present in a wound are problematic [162]. This
438 would help guide clinicians as to when it would be most appropriate to administer
439 prophylactic treatment.
440

441 Serena and coworkers have described a novel point-of-care diagnostic test capable of
442 identifying a wound in a 'state of pathogenesis' even before the clinical signs of infection
443 become apparent [163]. Using wound fluid swab samples collected from 366 chronic wounds,
444 the authors noted that elevated levels of bacterial protease activity (BPA) was detected in
445 49% of wound fluid samples despite only 18% of this cohort of patients demonstrating three

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

452

453 **Conclusions**

454 Although the pathogenicity of a bacterium is the combined activity of the multiple virulence
455 factors present in its portfolio, proteases remain a central means in enabling the
456 microorganism to overcome the host defences and proliferate. Indeed, some authors even
457 regard proteases as the most effective virulence factor in the establishment of infection [20,
458 35, 84], with functions including overcoming the host immune system, tissue degradation and
459 promoting the up-regulation of additional virulence factors. Taken together, the evidence
460 discussed in the present review suggests that proteases play a central role in the
461 establishment of wound infections, contribute to the development of an inflammatory
462 response and can impede wound healing.

463

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1081

1082 TABLES.

1083

1084 Table 1. Proteases from common organisms [adapted from Koziel and Potempa (2012) [34]]

1085

Organism	Bacterial protease
<i>Pseudomonas aeruginosa</i>	Las A (elastase A) Las B (elastase B) AprA (alkaline protease) Protease IV
<i>Staphylococcus aureus</i>	Aureolysin ScpA (staphopain A) SspB (staphopain B) SspA (staphylococcal serine protease)
<i>Streptococcus pyogenes</i>	SpeB (streptopain; cysteine proteinase) Streptlysin S IdeS (cysteine proteinase) ScpC
<i>Enterococcus faecalis</i>	GeIE (gelatinase) SprE (serine protease)
<i>Staphylococcus epidermidis</i>	Esp (serine protease)
<i>Fingoldia magna</i>	SufA (subtilisin-like serine protease)
<i>Proteus mirabilis</i>	ZapA (metalloprotease)
<i>Aeromonas sobria</i>	ASP (serine protease)
<i>Vibrio vulnificus</i>	metalloprotease

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